JOURNAL OF CLINICAL MICROBIOLOGY, May 1993, p. 1122–1126
0095-1137/93/051122-05$02.00/0

Vol. 31, No. 5

Occurrence of Bacterial Endosymbionts in *Acanthamoeba* spp. Isolated from Corneal and Environmental Specimens and Contact Lenses

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Received 30 October 1992/Accepted 2 February 1993

Free-living and parasitic protozoa are known to harbor a variety of endosymbiotic bacteria, although the roles such endosymbionts play in host survival, infectivity, and invasiveness are unclear. We have identified the presence of intracellular bacteria in 14 of 57 (24%) axenically grown *Acanthamoeba* isolates examined. These organisms are gram negative and non-acid fast, and they cannot be cultured by routine methodologies, although electron microscopy reveals evidence for multiplication within the amoebic cytoplasm. Examination for *Legionella* spp. with culture and nucleic acid probes has proven unsuccessful. We conclude that these bacteria are endosymbionts which have an obligate need to multiply within their amoebic hosts. Rod-shaped bacteria were identified in 5 of 23 clinical *Acanthamoeba* isolates (3 of 19 corneal isolates and 2 of 4 contact lens isolates), 4 of 25 environmental *Acanthamoeba* isolates, and 2 of 9 American Type Culture Collection *Acanthamoeba* isolates (ATCC 30868 and ATCC 30871) previously unrecognized as having endosymbionts. Coccus-shaped bacteria were present in one clinical (corneal) isolate and two environmental isolates. There was no statistical difference ($P > 0.8$) between the numbers of endosymbiont strains originating from clinical (26% positive) and environmental (24% positive) amoebic isolates, suggesting that the presence alone of these bacteria does not enhance amoebic infectivity. Rods and cocci were found in both clinical and environmental isolates from different geographical areas (Seattle, Wash., and Portland, Oreg.), demonstrating their widespread occurrence in nature. Our findings suggest that endosymbiosis occurs commonly among members of the family Acanthamoebidae and that the endosymbionts comprise a diverse taxonomic assemblage. The role such endosymbionts may play in pathogenesis remains unknown, although a variety of exogenous bacteria have been implicated in the development of amoebic keratitis, warranting further evaluation.

Amoebic keratitis is a progressive ocular disease seen most commonly in wearers of soft contact lenses, and it is caused exclusively by free-living amoebae of the genus *Acanthamoeba* (22, 27). The disorder is painful and may progress to corneal perforation with subsequent loss of vision. Reliable medical intervention is lacking, and keratoplasty is usually required to save the affected eye (2).

Mechanisms of pathogenesis of the infection are poorly understood, although the ubiquitous nature of the *Acanthamoeba* organism in the environment and the relative rarity of human cases of amoebic keratitis suggest a low inherent virulence potential (24). Biochemical studies have demonstrated the presence of adhesin-like factors and the production of membrane-associated or secreted hydrolytic enzymes which may be associated with invasion and phagocytosis (16, 17).

Normal eye flora has also been implicated in the development of amoebic keratitis by presumably playing a nutritional role (3, 13). Enhanced growth of *Acanthamoeba* organisms in the presence of certain bacterial cocultivants has recently been demonstrated, and when such growth occurs in improperly disinfected contact lens cases, it may serve as a prelude to corneal infection (6). This finding may not be surprising considering the prominent environmental role *Acanthamoeba* organisms play in complex interactions with other aquatic and soil microorganisms (24).

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While pursuing laboratory studies on cloned and axenically grown isolates of *Acanthamoeba* recovered from corneal specimens submitted by local ophthalmologists, we have observed the presence of intracellular bacteria in a number of isolates. These organisms were not present as contaminants in the growth media, which suggested to us the possibility of their being endosymbionts. We subsequently undertook an investigation to elucidate the relationship of these bacteria to their hosts and to determine the frequency with which they occur in clinical and environmental isolates of *Acanthamoeba*.

MATERIALS AND METHODS

Isolation and maintenance of *Acanthamoeba* spp. Acanthamoebae were isolated from clinical and environmental specimens by standard techniques (12). Briefly, 1.5% nonnutrient agar plates were overlaid with a suspension of live *Escherichia coli*. Corneal scrapings or other inocula were applied directly to the centers of the plates, which were then incubated at an ambient temperature for up to 10 days. Upon growth of the *Acanthamoeba* organisms, a single double-walled cyst was transferred with a micromanipulator apparatus to a fresh *E. coli*-seeded nonnutrient agar plate. Clonal cultures were subsequently grown on nonnutrient agar plates containing heat-killed (60°C for 1 h) *E. coli* and adapted to axenic growth at 27°C in Trypticase-soy-yeast extract broth.

Clinical isolates. A total of 19 strains of *Acanthamoeba* isolated from corneal specimens and 4 isolated from contact

lenses or lens cases were examined during the course of this study. Of the 23 strains examined, 14 isolates originated from the clinical laboratories of the University of Washington-affiliated hospitals in Seattle; 6 were obtained from the clinical laboratories at Oregon Health Sciences University, Portland; 2 were obtained from the Provincial Laboratory of Public Health, University of Alberta, Edmonton, Canada; and 1 was obtained from Virginia Mason Medical Center in Seattle, Wash.

Environmental isolates. A total of 25 isolates of *Acanthamoeba* originating from a variety of environmental habitats (soil, forest detritus, lake and stream sediments, pond water, tree bark, potting soil, etc.) from the Seattle environs and western Washington State were examined.

ATCC isolates. Isolates obtained from the American Type Culture Collection (ATCC) for use in these studies included *Acanthamoeba culbertsoni* ATCC 30866, *Acanthamoeba castellanii* ATCC 30011, *A. castellanii* ATCC 30868, *Acanthamoeba rhysodes* ATCC 30973, *Acanthamoeba polyphaga* ATCC 30871, *A. polyphaga* ATCC 30461, *Acanthamoeba astronyxis* ATCC 30137, *Acanthamoeba hatchetti* ATCC 30730, *Acanthamoeba palestinensis* ATCC 30870, and *Acanthamoeba* sp. strain ATCC 30173. All were maintained axenically in Trypticase-soy-yeast extract broth. Isolate ATCC 30173 was included as an endosymbiont-positive control strain and has been described previously (11). Endosymbionts from the remaining nine isolates had not been described.

Stains. Air-dried smears of amoebic trophozoites were methanol fixed and stained with Hemacolor (Harleco; Gibbstown, N.J.), a modified Wright stain, to examine for intracellular bacteria. Other stains utilized included Gram, Ziehl-Neelsen, and auramine O.

Culture techniques and nucleic acid probes. Bacterial preparations for culture experiments included both intact bacterium-containing amoebae and bacteria isolated from freeze-thawed lysates of amoebae which had been filtered through a 5-μm-pore-size syringe filter. Standard culture techniques were used in our attempts to grow the bacteria (5). Briefly, the bacterial preparations were planted on sheep blood, brucella, and chocolate agars, and incubations were performed aerobically, anaerobically, or with increased (5%) CO_2. Broth media inoculated included Trypticase-soy-yeast extract, brain heart infusion, and BACTEC 26 PLUS (Becton Dickinson, Towson, Md.). Culture for *Legionella* spp. was performed with buffered charcoal-yeast extract agar with added α-ketoglutarate in 5% CO_2. All incubations were performed for extended periods (>3 weeks) at both an ambient temperature and 37°C. All isolates containing bacteria were examined with nucleic acid probes for the presence of *Legionella* spp. by using the Gen-Probe Rapid Diagnostic System for *Legionella* (Gen-Probe Inc., San Diego, Calif.).

Electron microscopy. Amoebae were prepared for transmission electron microscopy by using a variation of published methods (11). Briefly, aliquots of amoebae grown in broth were fixed with 2% glutaraldehyde in 0.1 M cacodylate and subsequently pelleted in agar and embedded. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM-10 electron microscope.

RESULTS

During the course of this study, 57 axenically growing isolates of *Acanthamoeba* were examined for the presence of intracellular bacteria, and 14 (24%) were found to be positive (Table 1). Differences in percentages of clinical (26%) and environmental (24%) isolates harboring such organisms were not significant ($\chi^2 = 0.028$; $P > 0.8$). Two (22%) of the ATCC isolates examined were also positive for intracellular bacteria, as was ATCC 30173, which is known to harbor endosymbionts (11) and which served as our positive control.

TABLE 1. Frequency of occurrence of bacterial endosymbionts found in clinical *Acanthamoeba* isolates (from corneas and contact lenses) and environmental specimens

Isolate source	No. (%) of isolates			
	Tested	Positive for:		
		GNR[a]	GNC[b]	All endosymbionts
Clinical specimens	23	5 (22)	1 (4)	6 (26)
Environmental specimens	25	4 (16)	2 (8)	6 (24)
ATCC	9	2 (22)	0 (0)	2 (22)
Total	57	11 (19)	3 (5)	14 (24)

[a] GNR, gram-negative rods.
[b] GNC, gram-negative cocci.

The bacteria recognized are all gram negative, non-acid fast, and nonmotile, and when stained with Hemacolor, they appear within the cytoplasm as straight or slightly curved rods measuring approximately 0.3 by 0.8 to 2.3 μm or as cocci measuring 0.8 μm in diameter (Fig. 1 and 2). Rods were present in four clinical amoebic isolates (three from corneas and one from a contact lens case) and four environmental amoebic isolates from Seattle, one clinical isolate (from a contact lens case) from Portland, and two ATCC isolates (ATCC 30868 from a human cornea and ATCC 30871 from fresh water). Cocci were present in one clinical (corneal) isolate from Portland and two environmental isolates from Seattle. The finding of rods and cocci in both clinical and environmental isolates from two separate geographic areas suggests that this phenomenon occurs widely in na-

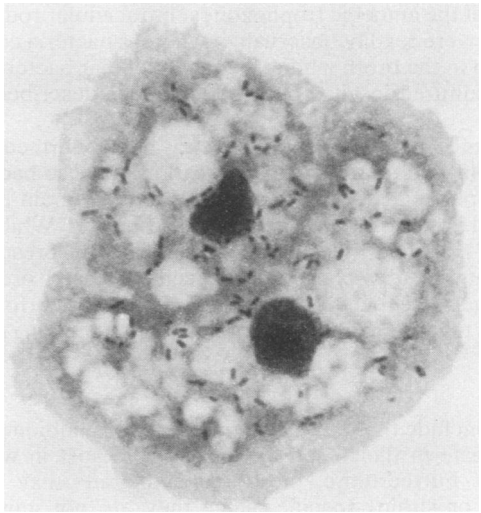

FIG. 1. Gram-negative rods in axenically cultured *Acanthamoeba* spp. isolated from corneal scrapings. Hemacolor stain; magnification, ×1,250.

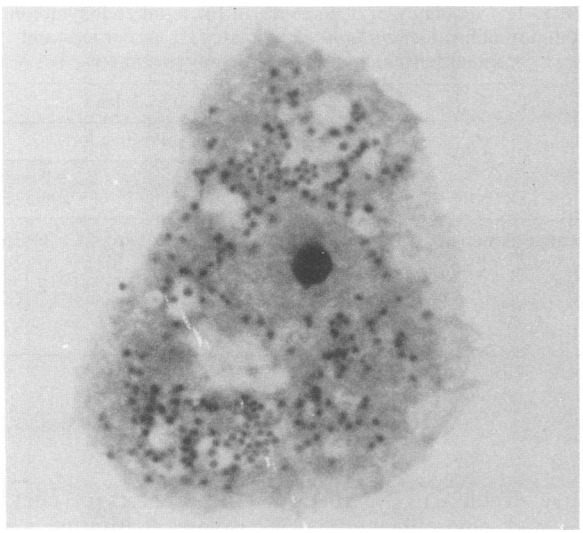

FIG. 2. Gram-negative cocci in axenically cultured *Acanthamoeba* spp. isolated from soil. Hemacolor stain; magnification, ×1,250.

ture. Mixtures of both morphotypes in a single amoebic strain were not detected.

Multiple attempts to culture these bacteria on a variety of solid and broth media under aerobic, anaerobic, and increased CO_2 conditions at both an ambient temperature and 37°C for extended periods of incubation were unsuccessful. Examination for *Legionella* spp. by culture and DNA probe analysis was likewise unsuccessful.

A second culture of ATCC 30868 was requested to confirm the presence of endosymbionts in this *Acanthamoeba* isolate, and this culture was planted directly into Trypticase-soy-yeast extract broth without the addition of exogenous bacteria. Antibiotics (penicillin [100 U/ml] and streptomycin [100 μg/ml]) were added to prevent growth of *E. coli* organisms, which are included in the ATCC freeze-dried cyst preparations of this particular isolate. These procedures were performed in separate laboratory facilities with media from separate sources. Upon both initial and subsequent growth of the amoebic trophozoites, intracellular rod-shaped bacteria were readily observable, whereas bacteria could not be found in the broth media. The intracellular bacteria could not be cultured in vitro by the techniques described previously.

Transmission electron microscopy has confirmed the intracytoplasmic (extravacuolar) location of these bacteria in both trophozoites and cysts and the typical gram-negative cell wall structure of both rods and cocci. While some bacteria may be found in vacuoles, these are presumed to represent recently phagocytosed cells released previously from other amoebae (8). The presence of septum formation suggests that intracellular multiplication is occurring (Fig. 3).

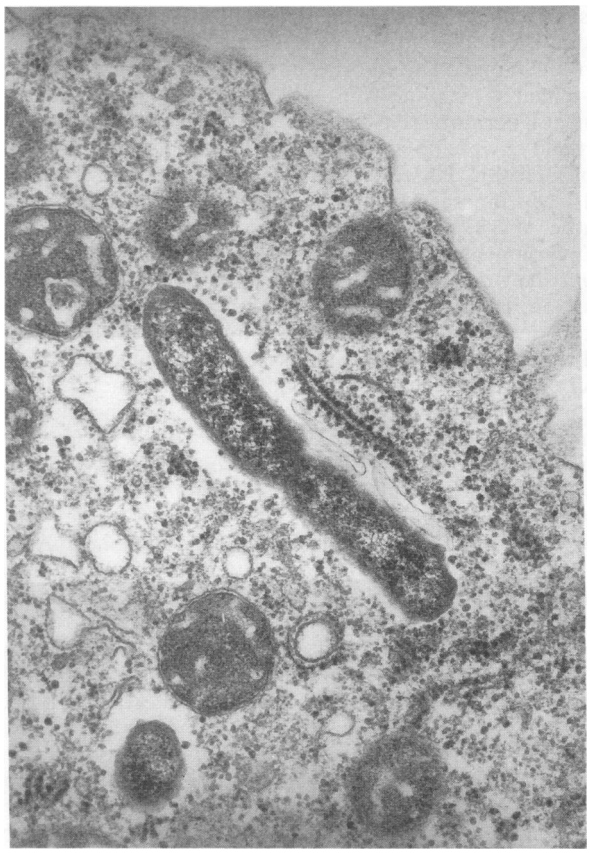

FIG. 3. Bacterial rod demonstrating binary fission within an *Acanthamoeba* organism, surrounded by several mitochondria. Magnification, ×21,000.

DISCUSSION

We conclude that these bacteria are endosymbionts which are obligate in their need for an amoebic host in which to multiply. Furthermore, these endosymbionts may be host (species or strain) specific, since they are not universally present in *Acanthamoeba* spp. The actual mechanisms of transmission are not well understood. Some endosymbionts are thought to persist and spread by cellular heredity alone, whereas for others, horizontal transmission has been experimentally demonstrated (18).

The presence of bacterial endosymbionts in other free-living protozoa, such as the kappa particle in *Paramecium aurelia*, omicron in *Euplotes aediculatus*, and X-bacteria in *Amoeba proteus*, is well known. The effects these organisms have on their hosts are varied. Omicron and X-bacteria are necessary for the survival of their hosts. *Paramecium* spp. may be killed by multiplying kappa particles when placed in a bacterium-free medium which limits their own growth (18). One report has demonstrated that a trypanosomatid (*Blastocrithidia culicis*) containing a bacterial symbiont was found to be less fastidious in growth requirements than similar isolates lacking symbionts (7). Such relationships have been postulated to be important sources of evolutionary innovation, although this issue remains in contention among biologists (15). The inability to culture most bacterial endosymbionts free of their protozoal hosts has limited further characterization (18).

Facultative intracellular bacteria from *Acanthamoeba* spp. isolated from environmental sources have been described on several occasions. Amoebae of this and other genera (*Naegleria*, *Echinamoeba*, and *Hartmanella*) are known to serve as effective hosts for the amplification of pathogenic *Listeria* and *Legionella* spp., and in the latter case, they may play an important role in the epidemiology of Legionnaires' disease (1, 9, 21, 25). The presence of obligate intracellular bacteria in *Acanthamoeba* spp. has been de-

scribed twice for axenic amoebic cultures originating from environmental sources (8, 19) and once for cultures originating from a human nasal swab (11). In all cases, these bacteria were described as gram-negative rods which multiplied within the cytoplasm of their host cells. None could be successfully cultured free of their hosts, indicating a high degree of adaptation to their intracellular habitat.

The finding of obligate endosymbionts in 26% of our clinical *Acanthamoeba* isolates raised suspicions as to their role, if any, in amoebic infectivity and virulence. If infectivity is enhanced by the presence of these bacteria, endosymbiont-bearing amoebic strains may be expected to constitute a larger portion of clinical *Acanthamoeba* isolates than of nonclinical isolates. When a number of clinical *Acanthamoeba* isolates were compared with a similar number of environmental *Acanthamoeba* isolates, no significant difference ($P > 0.8$) was detected between the numbers of endosymbiont-harboring isolates in each group, suggesting that the presence alone of endosymbionts does not enhance infectivity.

The presence of exogenous bacteria, however, has been implicated in the establishment of corneal infection (13). Coinoculation of a corynebacterium and acanthamoebae into rat corneas has been shown to produce suppurative keratitis, whereas inoculation of either a corynebacterium or acanthamoebae alone does not (3). Enhanced growth rates of acanthamoebae have also been demonstrated when cocultivation is performed with such commonly found environmental flora as *Xanthomonas maltophilia*, *Flavobacterium breve*, and *Pseudomonas paucimobilis*. Such interactions have been proposed as a prelude to invasion and establishment of acanthamoebae in the corneal stroma (6). If these bacterial species assume a nutritional role in the growth and development of acanthamoebae, as has been postulated, endosymbionts may also be expected to enhance the physiologic economy of their hosts. Many advantages to such symbiotic associations have been presented (15).

The creation of genetically identical strains of *Acanthamoeba* with and without endosymbionts would be a useful step in the experimental investigation of the role these organisms play in amoebic pathogenesis, and this is a goal we are pursuing (10). Other stable symbioses have been created with the transmission of endosymbionts to uninfected cell lines (15, 18). The recently described rat keratitis and tissue culture models for assessing amoebic pathogenicity would be particularly appropriate for studying such paired isolates (4, 14, 23).

Our preliminary findings suggest that bacterial endosymbiosis is a common theme among members of the family Acanthamoebidae and that it occurs with nearly equal frequency in clinical and environmental isolates. The morphologic variety of the endosymbionts we have described, including the newly recognized gram-negative coccus, suggests a diverse taxonomic assemblage, and their presence in *Acanthamoeba* isolates from distinct geographic localities demonstrates wide dispersion. The paucity of information available on the frequency with which *Acanthamoeba* organisms are hosts to endosymbionts may be a reflection of the techniques commonly used for primary isolation and maintenance of these organisms. Unless amoebic isolates are rendered axenic, the presence of endosymbionts may be expected to remain undetected when cocultivation with coliform bacilli or other bacteria is performed.

Whether endosymbiont-containing strains of *Acanthamoeba* are responsible for more serious ocular disease than endosymbiont-free strains is presently unknown. The role that exogenous bacteria appear to play in synergy with acanthamoebae in the development of corneal disease (3, 6) makes this a promising topic for further investigation. Our inability to cultivate these organisms free from their hosts, however, limits the extent to which our studies can proceed. Recent developments utilizing the polymerase chain reaction and analysis of the 16S rRNA gene have proven useful in studying the phylogenetic and evolutionary relationships of both prokaryotes and eukaryotes (28) and in identifying noncultivatable disease-associated microorganisms (20, 26). These techniques may be of particular help in studying phylogenetic relationships among bacterial endosymbionts of protozoa as well.

ACKNOWLEDGMENTS

We are especially grateful for the isolates submitted to us by Sharon Poor and Abdel Rashad at Oregon Health Sciences University, Iris Perry and Kinga Kowalewska-Grochowska at the Provincial Laboratory of Public Health for Northern Alberta, and Stanford E. Sumida at Virginia Mason Medical Center. We also thank Daniel E. Possin of the University of Washington and R. S. Dwivedi of Harvard University for assistance with the thin-section preparations and electron microscopy, and we thank Judy Condon for expert technical assistance.

This work was supported in part by Public Health Service grant EY08179 from the National Eye Institute and by the Graduate School Research Fund and a Biomedical Research Support grant, both from the University of Washington.

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