Growth of Acanthamoeba on Human Corneal Epithelial Cells and Keratocytes In Vitro

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Acanthamoebic keratitis, a potentially devastating infection usually associated with contact lens wear, has been recognized with increasing frequency in recent years. Once the Acanthamoeba organisms gain access to the human cornea, it is not clear which constituents of the corneal milieu provide a substrate for their growth. The growth of *Acanthamoeba polyphaga* was investigated on cultured monolayers of human corneal epithelial cells, stromal keratocytes, and stromal homogenate suspensions. Growth was determined through organism counts and observation of cytopathic effects on tissue culture dishes. Compared with tissue culture media controls, acanthamoebic growth was supported by cultured epithelial cells and keratocytes but not stromal homogenates. These results suggest that in acanthamoebic keratitis the organisms depend on the cellular components of the cornea as substrates for growth. This in vitro model may also provide further information on the pathogenesis of keratitis and a system for drug sensitivity testing. Invest Ophthalmol Vis Sci 32:354-359, 1991

Acanthamoebic keratitis was first documented by Jones et al. in 1973. In recent years, this organism has been recognized with increasing frequency as a potentially devastating cause of keratitis that is usually associated with contact lens wear. Acanthamoebae are free-living protozoa found in air, soil, fresh water, salt water, drinking water, chlorinated swimming pools, and hot tubs. They exist as motile trophozoites under favorable environmental conditions and encyst under hostile conditions.

Contact lens wear has been associated with acanthamoebic keratitis in up to 80% of cases. Soft contact lenses are most often implicated; however, hard lenses and rigid gas-permeable lenses may also be involved. Therapy involves long-term toxic medications and multiple, difficult surgical procedures. Even with the best therapies currently available, there are treatment failures.

The mechanism of pathogenesis of acanthamoebic keratitis is believed to begin with minor trauma to the corneal epithelium followed by inoculation of organisms through a contaminated contact lens or other foreign body. Studying which human corneal constituents act as substrates for acanthamoebic growth may lead to a better understanding of the pathogenesis and possible treatment of this potentially blinding infection. We examined the ability of *Acanthamoeba polyphaga* to grow on cultured human corneal epithelial cells, stromal keratocytes, and stromal homogenate suspensions.

Materials and Methods

Preparation of Cell Cultures

Primary cultures of human corneal epithelial cells and stromal keratocytes were grown to confluent monolayers on 35-mm petri dishes (Falcon 3001; Becton Dickinson, Lincoln Park, NJ) using techniques described by Ebato et al. Briefly, rims of peripheral cornea remaining after removal of the corneal button from a corneoscleral preparation for penetrating keratoplasty (Medical Eye Bank of Western Pennsylvania) were used as the source of human corneal epithelial cells. The limbal cornea was split through the midstroma, and the anterior portion was divided into 2 x 2-mm blocks. Seven of these explants were placed epithelial-side up in 35-mm petri dishes and left uncovered for approximately 10 min. Then 2.0 ml of modified SHEM medium was added to the dishes which were incubated at 37°C in 5% CO₂-95% air for 7 days with the medium changed twice a week. The explants were removed at day 8, before keratocyte outgrowth had begun, and the cell cultures were left to reach confluency.
For corneal stromal keratocyte cultures, the limbal corneal explants removed on day 8 were placed in 35 mm-dishes with medium consisting of Dulbecco’s modified Eagle’s (Gibco, Grand Island, NY) containing 15% fetal bovine serum (Gibco) and 40 μg/ml of gentamicin sulfate (ESI, Cherry Hill, NJ). Keratocyte outgrowths were allowed to reach confluency. For subculturing, a cell suspension was produced using 0.05% trypsin solution (Sigma, St. Louis, MO) and (0.02%) ethylenediaminetetraacetic acid (EDTA) (Sigma). Media were changed once a week. Passages 2–6 were the source of the kerocytes.

To prepare stromal homogenates, epithelium and endothelium were scraped from human corneas obtained from the Medical Eye Bank of Western Pennsylvania. The corneal stromas were then frozen in liquid nitrogen and pulverized with a stainless steel mortar and pestle. The finely powdered stromas were placed in modified SHEM medium, and 2.0 ml of the suspension was placed in 35-mm petri dishes so that the equivalent of one half of a corneal button was contained in each dish.

*A. polyphaga* (#30461; American Type Culture Collection, Rockville, MD) were grown axenically in proteose-yeast-glucose (PYG) growth media (American Type Culture Collection). Each petri dish containing 2.0 ml of modified SHEM medium and epithelial cells, keratocytes, or stromal homogenate suspension was inoculated with 2 × 10^3 acanthamoebae in 20 μl of PYG medium which were 85% trophozoites and 15% cysts. As controls, the acanthamoebae were inoculated into dishes containing only 2.0 ml of either modified SHEM or PYG media. All dishes were incubated at 37°C in a humidified 5% CO2; 95% air atmosphere.

**Determination of Acanthamoebic Growth**

Acanthamoebic growth on cultured epithelial cells and keratocytes was determined qualitatively by direct observation of cytopathic effect (CPE) with phase-contrast microscopy.25 This was defined as areas grossly devoid of cells that had been confluent initially.

To quantitate acanthamoebic growth from each group, the organisms were stained with fluorescein-conjugated concanavalin A (Vector, Burlingame, CA) in a modification of the technique of Robin et al.26 The specific, strong staining by fluorescein-conjugated concanavalin A of Acanthamoeba cysts and trophozoites and nonspecific, weak staining of corneal epithelial cells and fibrocytes was confirmed by examining pure cultures of organisms or cells with phase-contrast and fluorescent microscopy (data not shown). The culture medium was aspirated from each dish, and the cells and *acanthamoeba* were trypsinized done using 1.0 ml of 0.25% trypsin and 0.4% EDTA at 37°C for 20 min. The enzyme was neutralized by 0.5 ml of modified SHEM medium. The suspension was transferred to a centrifuge tube containing 0.4 ml of fluorescein-conjugated concanavalin A (0.02 mg/ml) in phosphate-buffered saline (PBS) (pH 7.2) (Difco, Detroit, MI). The tubes were then incubated for 20 min in a humid chamber at room temperature and then centrifuged for 8 min at 850 × g. The pellets were resuspended in 0.2 ml PBS, and the organisms were counted using a hemocytometer under fluorescent microscopy (Fig. 1). The counts were done in duplicate on three dishes from each treatment and control group on days 1, 3, 5, 8, 10, and 12.

**Statistical Analysis**

Acanthamoebic doubling time represents the length of time necessary for the organisms to double (a positive doubling time) or halve (a negative doubling time) their numbers. This was determined from growth-curve slopes during the log phase of growth using the following formula:24

\[
\text{Doubling Time (hr)} = \frac{(\text{Day}_2 - \text{Day}_1) \times 24 \times \log 2}{\log (\text{Acanthamoeba Count}_2) - \log (\text{Acanthamoeba Count}_1)}
\]

where a positive value represents a culture in which growth is occurring and a negative value represents a culture in which the number of organisms is decreasing, ie, growth is not supported.

Counts on day 0 were determined by direct counts of PYG-suspended acanthamoebae using a hemocytometer. Because it was an early time point and determined using a method different from the remainder of the counts, the period from day 0–1 was not used in any doubling-time determinations.

These data were analyzed using one-way analysis of variance with *P* < 0.05 considered significant.

**Results**

**Phase-Contrast Microscopy**

The cultures were examined daily by phase-contrast microscopy. Trophozoites were observed in all treatment and control dishes, and no cysts were ever noted on epithelial cell, keratocyte, or PYG-containing dishes. However, rare cysts were found on stromal homogenate and modified SHEM medium controls after day 5. Definite CPE was noted on all dishes of epithelial cells (Fig. 2) and keratocytes (Fig. 3) by day 8.
Fig. 1. Staining of two *Acanthamoeba polyphaga* trophozoites and one cyst (arrow) with fluorescein-conjugated concanavalin-A visualized under fluorescent microscopy. Corneal fibrocytes in field do not fluoresce (bar = 100 μm).

**Acanthamoebic Growth Curves**

Acanthamoebic growth curves were generated for each treatment and control group (Fig. 4). The doubling time (±standard deviation) of the organisms on epithelial cells (58.1 ± 5.2 hr) and keratocytes (53.5 ± 8.1 hr) was statistically different from that in modified SHEM medium controls (−137.7 ± 66.9 hr; *P* < 0.008) or on stromal homogenates (−95.1 ± 43.1 hr; *P* < 0.004) (Table 1). There was no statistically significant difference between growth on stromal homogenates compared with modified SHEM medium controls, nor was there any difference in the organisms' growth on epithelial cells versus keratocytes. The PYG culture media provided optimal growth in our system with a doubling time of 22.5 ± 2.0 hr and showed a statistically significant difference over all other substrates (*P* < 0.004). After day 8, a decreasing number of organisms were recovered from epithelial cell and kerocyte dishes that was coincidental with gross CPE, ie, large areas of the monolayer devoid of cultured cells.

**Discussion**

Acanthamoebic keratitis today is most often associated with contact lens wearers who use inadequate disinfection techniques. It is generally accepted that antecedent trauma to the epithelium occurs before the onset of keratitis. The initial insult to the epithelium may come in the form of chemical, organic matter, insect, or microtrauma related to contact lens...

Fig. 2. Cultured human epithelial cells exhibiting marked CPE from *Acanthamoeba polyphaga* at day 8. Note area devoid of once confluent cells (asterisk) (bar = 100 μm).
Fig. 3. Cultured human corneal fibrocytes exhibiting marked CPE from *Acanthamoeba polyphaga* at day 8. Note areas devoid of once confluent cells (asterisk) and clusters of *Acanthamoeba* trophozoites (arrows) (bar = 100 μm).

The organism is inoculated by trauma, contaminated water, or perhaps is preexisting in the adnexal flora. The substrate for its survival and growth, once it has gained access to the human cornea, has not been clearly identified. It is well known that *acanthamoeba* grow on living or dead bacteria. This forms the basis for the standard culture technique of growing *acanthamoebae* on a lawn of *Escherichia coli* or *E. aerogenes* over nonnutrient agar. Jones et al. were able to induce infection in rabbits only when intrastromal injection of *Acanthamoeba* was accompanied by *Staphylococcus aureus* or *E. coli*. Although it might be tempting to suggest that bacteria provide a food source for *Acanthamoeba* during keratitis, most

![Acanthamoeba Counts/35mm Dish](image)

Fig. 4. *Acanthamoeba* growth curves on different substrates over 12 days; cultured human corneal epithelial cells (solid line), cultured human corneal keratocytes (diamonds), stromal homogenates (circles), modified SHEM medium (complex cell culture medium) (triangles), and proteose-yeast-glucose *Acanthamoeba* growth medium (PYG) (squares).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Doubling time, hr ± SD*</th>
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<tbody>
<tr>
<td>Epithelial cells</td>
<td>58.1 ± 5.2</td>
</tr>
<tr>
<td>Keratocytes</td>
<td>53.5 ± 8.1</td>
</tr>
<tr>
<td>PYG medium</td>
<td>22.5 ± 2.0</td>
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<tr>
<td>Stromal homogenates</td>
<td>-95.1 ± 43.1</td>
</tr>
<tr>
<td>Modified SHEM medium</td>
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*A positive value represents a culture in which growth is occurring and a negative value represents a culture in which the number of *Acanthamoeba* is decreasing, ie, growth is not supported.*
reported cases of acanthamoebic keratitis did not have a documented coexisting infection at the time of diagnosis. Also, it is unlikely that preexisting flora could support the growth of numerous acanthamoebae.32

The organism have been shown to grow and produce CPE on various cultured cell lines such as VERO, Hep-2, MDBK, HEL, HEK, pig kidney, HeLa, PMI, and mouse melanoma.33 In animal models, damaged host cells were believed to act as a food supply for Acanthamoeba after subcutaneous injection.34 In our study we demonstrated the ability of axenic A. polyphaga to use confluent monolayers of living, cultured human corneal epithelial cells and stromal keratocytes as a food source in vitro. Modified SHEM medium, a complex cell culture medium, however, was not able to sustain acanthamoebic growth. This was also the case with homogenized stroma which contains mainly type I collagen, glycosaminoglycans, and relatively little cellular material. This may reflect the obligatory nutritional need of acanthamoeba for more complex macromolecules found most commonly in living cells or its relative preference for intact cells. The PYG medium is widely used for Acanthamoeba culture and is considered an optimal axenic growth media containing all the nutrients required for growth and propagation. This was confirmed by our results. The decreasing recovery of these organisms after day 8 from epithelial cell and keratocyte dishes was probably related to extensive CPE and therefore depletion of the food source after this time.

The organisms that cause infectious keratitis may depend on either the cellular or noncellular components of the cornea for nutrition. Viruses are obligatory intracellular parasites using live host cells for propagation. By releasing enzymes, bacteria and fungi metabolize and consume adjacent tissue, whether cellular or extracellular matrix.35,36 Acanthamoeba appears to require the cellular elements of the cornea for sustenance. It can use host cells by both releasing phospholipase and direct phagocytosis.33

These findings have led us to postulate a new mechanism for the pathogenesis of acanthamoebic keratitis (Fig. 5). First, organisms bind to the epithelial surface which may be intact, damaged, or denuded.37 We speculate that by using cultured human epithelial cells or stromal fibrocytes, substrates more characteristic of the in vivo situation, the acanthamoebae will use metabolic pathways similar to those in vivo. Therefore, it is possible that using our system for in

rate of progression and degree of destruction from the disease.

Our findings also have implications for the treatment of acanthamoebic keratitis. Although there are reports of successful outcomes with medical and/or surgical modalities, in many cases the results are unsatisfactory.21-23 An approach to therapy, in addition to conventional medical treatment, may include elimination of the food source for the organism by complete epithelial debridement followed by aggressive cryotherapy to destroy stromal keratocytes.39 In the very earliest stages, epithelial debridement may be particularly effective in the resolution of keratitis as described in a case report by Holland et al.38 Such debridement would eliminate both the organisms and their food source.

Cryotherapy has been shown to eliminate trophozoites, but not cysts, in vitro and has had mixed results in isolated clinical reports.40 Binder41 reported five cases in which cryotherapy to the host cornea was used in combination with medical and surgical treatment. Viable organisms were eliminated in all cases. We postulate that cryotherapy may be effective by killing the stromal keratocytes, therefore eliminating the major food supply for the organisms, which are themselves relatively tolerant to freezing.

Finally, in vitro drug testing for antiamoebic agents has been disappointing because of inconsistent results.32 This may be due to different virulence and resistance characteristics between species and strains or differences in drug sensitivity-testing techniques. We speculate that by using cultured human epithelial cells or stromal fibrocytes, substrates more characteristic of the in vivo situation, the acanthamoebae will use metabolic pathways similar to those in vivo.
vitro drug testing may yield more representative and useful results.

Key words: Acanthamoeba, in vitro model, corneal epithelial cells, corneal keratocytes, infectious keratitis

References


