Effect of Steroids on *Acanthamoeba* Cysts and Trophozoites

Kathy McClellan,1,2 Kevin Howard,1 Jerry Y. Niederkorn,1 and Hassan Alizadeh1

**PURPOSE.** Topical steroids are frequently used to control corneal inflammation and uveitis or is administered after surgery, to prevent corneal graft rejection. This study was undertaken to determine whether steroids could affect the pathogenicity of *Acanthamoeba castellanii*.

**METHODS.** The effect of dexamethasone phosphate on excystment, proliferation, and encystment of trophozoites and cysts of *A. castellanii* was examined in vitro. Cytolysis capacity of steroid-treated *Acanthamoeba* was quantified by a spectrophotometric assay, and plasminogen activators were measured by a fibrinolysis assay. The influence of steroid treatment on corneal infection in a Chinese hamster model of *Acanthamoeba* keratitis was examined in vivo.

**RESULTS.** Treatment of *Acanthamoeba* cysts with dexamethasone induced 4- to 10-fold increases in the number of trophozoites compared with untreated control cultures. Acceleration of trophozoite proliferation was observed when trophozoites were treated with dexamethasone. However, dexamethasone treatment did not affect encystment of *Acanthamoeba* trophozoites. Dexamethasone-treated trophozoites or cysts induced a significant cytopathic effect on corneal epithelial cells compared with untreated organisms. Supernatants collected from either dexamethasone-treated or untreated organisms failed to lyse corneal epithelial cells. Treatment of organisms with dexamethasone had no effect on production of plasminogen activators by *Acanthamoeba* trophozoites. Intramuscular injection of dexamethasone had a profound effect on the incidence, severity, and chronicity of keratitis. Keratitis in dexamethasone-treated hamsters was significantly more severe at all time points than in untreated animals (P < 0.05).

**CONCLUSIONS.** These findings indicate that exposure of *Acanthamoeba* trophozoites and cysts to dexamethasone increases the pathogenicity of the organisms. The results emphasize the importance of maintaining adequate amebicidal therapy if a topical steroid is used in the management of *Acanthamoeba* keratitis. *(Invest Ophthalmol Vis Sci. 2001;42:2885–2893)*

*A. castellanii* keratitis in humans remains a challenging early diagnosis to confirm. In this corneal disease, the chronicity, tendency for recurrent inflammation, and severe pain help to distinguish the condition from bacterial or viral keratitis. The epidemiology of *Acanthamoeba* keratitis has been explained by its prevalence in contact lens wearers. Typically, waterborne organisms gain access to the corneal stroma after initial attachment to damaged corneal epithelium.1–3

These infections have often been present for many weeks by the time the diagnosis is confirmed, and topical steroids have frequently been included in the therapy. Recrudescence of *Acanthamoeba* keratitis may occur after corneal transplantation if residual *Acanthamoeba* in the host peripheral cornea is exposed to steroids after surgery.4 Although steroids are frequently needed to control pain and inflammation in *Acanthamoeba* keratitis, their effects on trophozoites and cysts have not been investigated.5,6 It is therefore important to know whether such treatments have a direct effect on cysts and trophozoites or whether any effects of steroids on *Acanthamoeba* keratitis may be purely secondary to the resultant immunosuppression. Therefore, we wanted to determine whether steroids affect aspects of pathogenesis determined by the organism, including excystment, encystment, growth, cytolytic effects, and elaboration of plasminogen activators. We hypothesized that steroids would inhibit encystment of *Acanthamoeba*, while potentiating excystment, trophozoite growth, cytolysis, and elaboration of plasminogen activators.

**MATERIALS AND METHODS.**

* *A. castellanii* Cultured Trophozoites and Cysts*

The organism used in all experiments was a human ocular isolate of *Acanthamoeba castellanii* from the American Type Culture Collection (ATCC; 30868; Rockville, MD). Trophozoites were maintained in axenic culture at 35°C in peptone-yeast-glucose (PYG) medium in test tubes, as described previously.7,8 *Acanthamoeba* cysts were prepared by culturing trophozoites on non-nutrient agar plates without bacteria at 35°C for 1 week. At the end of 1 week, cysts were examined, and the surfaces of the agar plates were washed with 10 ml Page's saline and scraped with a tissue scraper. Double-walled cysts with a wrinkled exocyst and an average diameter of 19 μm were produced and stored in Page's saline until use.

**Excystment of Dexamethasone Phosphate–Treated *A. castellanii* Cysts In Vitro**

*A. castellanii* cysts (2 × 10⁷/ml) were incubated for 24 hours at 35°C, in 5 ml PYG medium containing 10, 100, or 400 μg/ml dexamethasone phosphate (Sigma Chemical Co., St. Louis, MO). Trophozoites were then counted by a hemocytometer. Each culture was reincubated with the original concentration of dexamethasone, and trophozoites were counted daily for an additional 6 days. The control cultures were *A. castellanii* cysts (2 × 10⁴/ml) incubated for 24 hours at 35°C in 5 ml PYG medium without dexamethasone. Experimental tubes for each dexamethasone concentration and control were set up in duplicate.

**Proliferation of Dexamethasone-Treated *A. castellanii* Trophozoites In Vitro**

*A. castellanii* trophozoites (2 × 10⁷/ml) were incubated for 24 hours at 35°C, in 5 ml PYG medium containing 10, 100, and 400 μg/ml...
dexamethasone, at concentrations ranging from 10 to 400 μg/ml. Trophozoites were then counted by a hemocytometer. Each culture was reincubated with the original concentration of dexamethasone and trophozoites were counted daily for an additional 6 days. The control cultures were A. castellanii trophozoites (2 × 10^5/ml) incubated at 35°C in 5 ml PYG medium without dexamethasone. Experimental tubes for each dexamethasone concentration and control were set up in duplicate.

Encystment of Dexamethasone-Treated A. castellanii Trophozoites In Vitro

A. castellanii trophozoites (2 × 10^5/ml) were incubated for 24 hours at 35°C, in 5 ml PYG medium containing 10, 100, and 400 μg/ml dexamethasone. Trophozoites were then counted and the tube containing 1 × 10^5 trophozoites was centrifuged at 1000g for 5 minutes and the pellet suspended in 0.2 ml Page’s saline. The trophozoite suspensions were cultured on non-nutrient agar at 35°C, as described elsewhere. Encystment was determined after incubation for 2, 4, and 24 hours, then daily for 6 days. Cysts and trophozoites were counted in each of 10 microscopic fields. Control cultures consisted of trophozoites incubated without dexamethasone. All experiments and controls were performed in duplicate. The percentage of encysted organisms in experimental and control plates was compared at each time point.

Cell Cultures

Human corneal epithelial (HCE) cells were kindly provided by Sally L. Ward (Gillette Medical Evaluation Laboratories, Gaithersburg, MD), and cultured in KGM (Clonetics, Irvine, CA) at 37°C in 5% CO2, as described previously.9

Cytopathic Effect Assay with HCE Cells

HCE cells were chosen as target cells for the cytopathic effect (CPE) assay for their demonstrated susceptibility to lysis by A. castellanii trophozoites and reliable propagation in vitro. The CPE assay was performed as described previously.10 Briefly, HCE cell suspensions were seeded into flat-bottomed 96-well microtiter plates (1 × 10^4 cells/well) and cultured until they became confluent. A. castellanii trophozoites or cysts were incubated for 24 hours at 35°C in 1 ml PYG containing 10, 100, and 400 μg/ml dexamethasone. Trophozoites were then counted by a hemocytometer and 2 × 10^5 added to the confluent cultures of HCE cells. Wells containing HCE cells alone or A. castellanii trophozoites or cysts with dexamethasone at 10, 100, and 400 μg/ml or without dexamethasone, served as control cultures. After incubation, all wells were washed three times with PYG and stained with Giemsa stain. The remaining contents were solubilized in 0.1 ml 5% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (pH 7.2; Sigma Chemical Co.) and transferred to a new 96-well plate, and the density of the residual epithelial cells was quantified by determining the optical density (OD) at 590 nm on a microplate reader (Molecular Devices Corp., Menlo Park, CA). Results were expressed as mean OD ± SD for each group. All assays were performed in triplicate. The spectrophotometric results were confirmed by inspection of the wells with compound microscopy.

Acanthamoeba Supernatants

For assay testing of cytolytic capability, supernatants were collected from A. castellanii trophozoites that had been incubated for 24 hours with dexamethasone at 10, 100, and 400 μg/ml. Supernatants were tested for cytolytic activity by adding 0.1 ml supernatant to wells containing confluent HCE cells in the 96-well plates. Control wells contained HCE cells alone or untreated A. castellanii. The CPE was quantified as described earlier.

Fibrin Clot Lysis Assay

Fibrinogen-agarose overlays were prepared by mixing 15 mg/0.1 ml fibrinogen (F-8630; fraction 1, type I5 from bovine plasma; Sigma Chemical Co.) in 0.1 M Tris-HCl (pH 7.6) with 20 mg/0.1 ml low-melting-temperature agarose (Sigma Chemical Co.), prepared in the same buffer at 40°C. After adding 1.0 IU thrombin (Sigma Chemical Co.), the mixture was poured between two glass plates separated by a 2.5-mm spacer.11 Wells 5 mm in diameter were cut into the fibrin clot and the assay performed by adding 10 μl supernatant from trophozoites treated with dexamethasone at doses of 10, 100, or 400 μg/ml or by adding untreated trophozoites to each well and incubating at 37°C. PYG medium without trophozoites was used as the control. Clots without plasminogen were included in all experiments to control for plasminogen-independent fibrinolysis.

The diameter of the clear zone in the clot was measured after incubation at 37°C for 2, 3, 5, and 8 hours. By using human tissue plasminogen activator (tPA; American Diagnostica, Greenwich, CT) as a standard, a linear relationship between the diameter of the clear zones and the tPA concentration was established as described previously.12

Animals

Chinese hamsters were purchased from Cytogen Research and Development, Inc. (West Roxbury, MA) and used at ages 4 to 6 weeks. The corneas were examined before experimentation to exclude animals with any preexisting corneal disease. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Contact Lens Preparation

Contact lenses were made 3.0 mm in diameter from dialysis membrane tubing (Spectra/Por; Spectrum Medical Industries, Los Angeles, CA) and heat sterilized. Dialysis tubing contact lenses were incubated for 24 hours at 35°C with A. castellanii in 200 μl PYG containing 3 × 10^5/well (50% trophozoites, 50% cysts) in 96-well microtiter plates (Costar, Cambridge, MA).

In Vivo Corneal Infection

Acanthamoeba keratitis was established in the cornea of the Chinese hamster, as described previously, by application of an A. castellanii-laden contact lens to an abraded corneal surface. Chinese hamsters were anesthetized with ketamine (100 mg/kg) given intramuscularly (IM; Ketaset; Ft. Dodge Laboratories, Ft. Dodge, IA) and supplemented with topical proparacaine (Alcon Inc., Hamucaco, Puerto Rico). A contact lens laden with A. castellanii was placed over the abraded cornea of one eye and the eyelids closed by central tarsorrhaphy with a 6-0 suture (Ethilon; Ethicon, Inc., Somerville, NJ). The contact lenses were removed 6 days after infection, and the corneas were observed under a dissecting microscope for clinical signs of keratitis. Animals were observed on days 7, 14, 21, and 35 after infection. Keratitis was scored on a scale of 0 to 5, according to corneal infiltration, corneal neovascularization, and corneal ulceration. Disease severity was recorded as: 0, no disease; 1, less than 10% of the cornea involved; 2, 10% to 25% involved; 3, 25% to 50%; 4, 50% to 75%; and 5, 75% to 100%, as described previously.7 Animals graded with a score of at least 1.0 for any parameter were considered infected. Acanthamoeba keratitis in Chinese hamsters resolved at approximately day 21, at which time corneal opacity, stromal necrosis, and inflammation had cleared and corneal epithelium was intact. Acanthamoeba trophozoites and cysts were not present on histologic examination of corneas in which keratitis had resolved.

Systemic Dexamethasone

Dexamethasone (0.1 mg/100 μl of saline) was administered IM daily for 5 days to 10 Chinese hamsters, beginning 24 hours after the contact lens was applied. A control group of 10 Chinese hamsters received 100 μl IM saline.
is possible that dexamethasone affects the rate of excystment rather than stimulating proliferation of the organisms. To test this possibility, cysts were exposed to different concentrations of dexamethasone, and the cysts that remained in the cultures were counted. The number of cysts recovered after 1 day of exposure to 10, 100, and 400 µg/ml dexamethasone (Fig. 2) was significantly lower than in control cultures ($P < 0.05$). Significant reduction in the number of cysts exposed to all concentrations of dexamethasone was observed in the 2-, 3-, and 4-day cultures ($P < 0.05$). These results indicate that dexamethasone induces excystment of organisms (Fig. 2).

**Proliferation of A. castellanii Trophozoites**

Steroids have been used topically to reduce corneal inflammation in patients with *Acanthamoeba* keratitis. However, it is possible that steroid treatment induces proliferation of trophozoites and contributes to the pathogenesis of the disease. It was therefore important to investigate whether dexamethasone could stimulate the proliferation of trophozoites.

Trophozoite proliferation was accelerated by dexamethasone at concentrations of 100 and 400 µg/ml after 24 hours of continuous exposure (Fig. 3). With 48 hours of continuous exposure to dexamethasone at concentrations greater than or equal to 10 µg/ml, trophozoite proliferation was significantly greater than that in untreated control cultures ($P < 0.05$). This positive effect on trophozoite growth was maintained after 72 hours of continuous dexamethasone exposure and was effective at concentrations of dexamethasone greater than or equal to 10 µg/ml ($P < 0.05$). The effect of steroid treatment of trophozoites was lost after 96 hours’ incubation, with no significant difference in number of trophozoites compared with untreated control cultures (Fig. 3).

**Excystment of A. castellanii Trophozoites**

Any delay in excystment of the trophozoites in *Acanthamoeba* keratitis also increases the trophozoite load in the corneal stroma. Topical application of steroids after corneal transplantation may increase the risk of recurrence of *Acanthamoeba* keratitis by inducing the excystment of the organisms. The effect of dexamethasone on the excystment of *A. castellanii* cysts was evaluated in vitro. The number of trophozoites in dexamethasone-treated cysts exceeded that in untreated control cultures after 24 hours’ incubation (Fig. 1). A dexamethasone concentration equal to or greater than 100 µg/ml produced the greatest number of trophozoites; above this concentration the excystment response was uniform. After 48 hours’ incubation, dexamethasone-treated cysts continued to produce three times the number of trophozoites present in untreated control cultures ($P < 0.05$). Trophozoites in treated cultures exceeded those in control at concentrations equal to or greater than 10 µg/ml dexamethasone. The number of trophozoites recovered from the dexamethasone-treated group at concentrations of 100 and 400 µg/ml was significantly higher than in control cultures after 72 hours’ incubation ($P < 0.05$). However, no significant differences were observed in the number of trophozoites between dexamethasone-treated and untreated control cultures after 96 hours’ incubation (Fig. 1).

**Rate of Excystment of A. castellanii Cysts**

Previous experiments suggest that dexamethasone may stimulate the growth and proliferation of trophozoites. However, it
ites pretreated with dexamethasone did not appear to alter cells were stained to assess the CPE (Figs. 4A, 4B). Trophozo-

HCE cells. After 48 hours’ incubation, the remaining epithelial
treated with dexamethasone for 48 hours and then applied to
dexamethasone. In some experiments, trophozoites were pre-
A. castellanii were incubated for 48 hours with
trophozoites, which are the actual mediators of CPE.

Because the pathogenesis of Acanthamoeba keratitis is depen-
dent on the parasite’s capacity to penetrate the corneal epithe-
stroma and potentially increases corneal inflammation and de-
struction. Therefore, we used encystment on non-nutrient agar
to compare dexamethasone-treated trophozoites with un-
treated trophozoites. However, there was no significant differ-
cence between the rate of encystment of trophozoites treated
with dexamethasone and that of untreated trophozoites, at any
of the time points during 7 days of culture on non-nutrient agar (Table 1).

Trophozoite-Mediated Cytolysis of HCE Cells

Initiation of infection by Acanthamoeba trophozoites is facil-
itated by the ability of the organism to bind and kill corneal
epithelial cells, removing this potential barrier to deeper cor-
neal invasion. We next determined whether steroids affect
Acanthamoeba-mediated cytolysis of HCE cells. HCE cells were incubated for 48 hours with A. castellanii trophozoites
(1 × 10^5) in medium with or without 10, 100, and 400 μg/ml
dexamethasone. In some experiments, trophozoites were pre-
treated with dexamethasone for 48 hours and then applied
to HCE cells. After 48 hours’ incubation, the remaining epithelial
cells were stained to assess the CPE (Figs. 4A, 4B). Trophozo-
ites pretreated with dexamethasone did not appear to alter
CPE. In contrast, trophozoites incubated with dexamethasone

for 48 hours induced significant cytolysis (P < 0.05). Moreover,
HCE cells incubated with 10, 100, and 400 μg/ml dexamethasone were unaffected. The results show that the presence
do not affect trophozoite-mediated cytolysis (data not shown).

**FIGURE 3.** Effect of dexamethasone on proliferation of *A. castellanii* trophozoites. *A. castellanii* trophozoites (1 × 10^5) were incubated for 24 hours at 35°C in 5 ml PYG medium containing 10, 100, or 400 μg/ml dexamethasone. Each culture was reincubated with the original concentration of dexamethasone, and trophozoites were counted daily for an additional 4 days. Results are expressed as the mean number of trophozoites per milliliter ± SD. *Significantly different from untreated control cultures (P < 0.05).

**TABLE 1.** Encystment of Dexamethasone-Treated *A. castellanii* Trophozoites In Vitro

<table>
<thead>
<tr>
<th>Concentration</th>
<th>2 Hours</th>
<th>4 Hours</th>
<th>1 Day</th>
<th>2 Days</th>
<th>4 Days</th>
<th>5 Days</th>
<th>6 Days</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYG</td>
<td>0</td>
<td>0</td>
<td>48.9</td>
<td>78.2</td>
<td>90.0</td>
<td>96.1</td>
<td>97.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10 μg/ml</td>
<td>0</td>
<td>0</td>
<td>43.7</td>
<td>80.8</td>
<td>90.6</td>
<td>95.4</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>100 μg/ml</td>
<td>0</td>
<td>0.43</td>
<td>50.5</td>
<td>73.4</td>
<td>88.3</td>
<td>93.2</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>400 μg/ml</td>
<td>0</td>
<td>0.25</td>
<td>49.8</td>
<td>71.0</td>
<td>92.3</td>
<td>99.1</td>
<td>99.5</td>
</tr>
</tbody>
</table>

Data indicate percentage of encystment of trophozoites on non-nutrient agar.

It is possible that dexamethasone induces *Acanthamoeba* trophozoites to secrete soluble cytolytic factors. To test this,
supernatants were collected from trophozoites grown in me-
dium containing different concentrations of dexamethasone
and tested directly for cytolytic activity. The results shown in
Figure 5 demonstrate that dexamethasone did not stimulate
trophozoites to release soluble factors that induce cytolysis.
Alternatively, exposure of epithelial cells to dexamethasone
may render the epithelial cells more vulnerable to trophozoite-
induced cytolysis. To test this hypothesis, HCE cells were
incubated with 10, 100, and 400 μg/ml dexamethasone at 37°C
for 24 to 72 hour. After incubation, 1 × 10^5 *A. castellanii*
trophozoites were added to each well and the CPE was deter-
mined 48 hours later. Treated and untreated HCE cells with or
without trophozoites served as the control. The results indicate
that addition of dexamethasone to epithelial cells does not
affect trophozoite-mediated cytolysis (data not shown).

**Cytolysis of HCE Cells by Dexamethasone-Treated Cysts**

Previous experiments showed that dexamethasone stimulates
encystment of the organisms. It is possible that steroid treat-
ment increases the load of organisms and contributes to the
pathogenesis of the *Acanthamoeba* infection. It was therefore
important to investigate whether cysts treated with dexameth-
ase could induce cytolysis of HCE cells.

HCE cells were incubated for 72 hours with *A. castellanii*
cysts (2.5 × 10^5) in medium with or without 10, 100, and 400
μg/ml dexamethasone. In other experiments, cysts were pre-
treated with dexamethasone for 72 hours and were then ap-
plied to HCE cells. The CPE was measured 72 hours later, as
described in the Methods section. Pretreatment of cysts with
dexamethasone did not produce significant CPE. However,
cysts incubated with dexamethasone for 72 hours induced
significant cytolysis of HCE cells (Fig. 6). Dexamethasone at
a concentration of 100 μg/ml produced more than 33% cyst-
associated cytolysis (P < 0.05). Moreover, incubation in dif-
ferent concentrations of dexamethasone alone did not affect HCE
cell viability. The results indicate that the presence of dexam-
ethasone had a profound effect on cyst-associated cytolysis.
However, the supernatant collected from cysts exposed to
different concentrations of dexamethasone did not induce sig-
ificant cytolysis (data not shown). We conclude that the
increased CPE that follows cyst treatment with steroids is due
to accelerated excystment and enhanced proliferation of tro-
phozoites, which are the actual mediators of CPE.
Figure 4. Effect of dexamethasone on CPE mediated by A. castellanii trophozoites. HCE cell monolayers were incubated for 48 hours with trophozoites (1 × 10⁵) in medium with or without 10, 100, or 400 μg/ml dexamethasone (A), or without trophozoites that were pretreated with dexamethasone for 48 hours before addition to HCE cells (B). CPE was assessed 48 hours later. Negative control cultures consisted of monolayers cultured in medium without dexamethasone or without trophozoites. Monolayers were washed and stained with Giemsa stain, and CPE was assessed spectrophotometrically. *Significantly different from untreated control cultures (P < 0.05).

Figure 5. Cytolytic activity of supernatants from A. castellanii trophozoites treated with dexamethasone. A. castellanii trophozoites were treated for 24 hours in PYG medium with or without 10, 100, or 400 μg/ml dexamethasone. After 24 hours' incubation, the culture supernatants were removed and then added to HCE cells, and the CPE was assessed 48 hours later. Negative control cultures consisted of cell monolayers cultured in medium without trophozoites. Monolayers were washed and stained with Giemsa stain, and CPE was assessed spectrophotometrically. The differences between experimental groups were insignificant (P > 0.05).

lium and enter the underlying cornéal stroma, we suspected that the organism elaborates plasminogen activators that facilitate tissue invasion and thereby contribute to the pathogenesis of the disease. A. castellanii trophozoites were treated with or without dexamethasone for 24 hours, and the supernatants were collected and assayed for plasminogen activators by radial fibrin clot assay. Ten microliters of each sample and several dilutions of tPA were applied to each well of a fibrinogen–fibrin clot assay. Ten microliters of each supernatant and several dilutions of tPA were applied to each well of a fibrinogen–fibrin clot assay. In agreement with a previous report, supernatants from A. castellanii cultures demonstrated plasminogen activator activity. A similar level of plasminogen activators was detected in the supernatants from cultures of organisms treated with dexamethasone (Fig. 7). There was no fibrinolytic activity in any culture without addition of plasminogen to fibrinogen. No fibrinolytic activity was observed in PYG medium without trophozoites.

A. castellanii Keratitis in the Chinese Hamster

The effect of systemic immunosuppression on Acanthamoeba keratitis is determined by the response of the organism to steroids and the role of the immune system in the killing of Acanthamoeba. Our in vitro studies demonstrated an increase in the numbers of trophozoites in response to dexamethasone, through accelerated excystment and trophozoite replication. Histopathology of the cornea in human Acanthamoeba keratitis showed predominantly neutrophils and macrophages, with few lymphocytes, indicating the importance of the innate immune system in the response to the presence of Acanthamoeba. Studying Acanthamoeba keratitis in an animal model enabled in vivo assessment of the effect of immunosuppression mediated by systemic steroid on Acanthamoeba keratitis. Accordingly, the incidence, severity, and duration of keratitis were evaluated in animals treated with dexamethasone IM and compared with that in untreated control animals.

Intramuscular injection of dexamethasone had a profound effect on the incidence, severity, and chronicity of keratitis. The incidence of infection in untreated animals was 89%, but increased to 100% in dexamethasone-treated animals (Fig. 8). The increased rates of infection were maintained throughout the period of observation. However, dexamethasone treatment did not accelerate the onset of keratitis.

Keratitis in dexamethasone-treated hamsters was significantly more severe at all time points than in untreated animals (P < 0.05), in some cases resulting in corneal ulceration. Severe keratitis was present in 90% of treated hamsters at day 20 and remained moderate in 55% of these animals at day 35. By contrast, 45% of untreated hamsters had only mild residual keratitis by day 20, and all infections had resolved by day 35 (Figs. 8A, 8B).

Discussion

Although topical steroids have been consistently included in therapy for Acanthamoeba keratitis, their effects on the organism have not been documented. The existence of two forms of Acanthamoeba, trophozoites and cysts, affords the organism maximal environmental adaptation and so assists in its survival. The Acanthamoeba trophozoite is capable of initiating these corneal infections if there is an epithelial defect.
thamoebae attach to corneal epithelium through a mannose-specific surface receptor. \(^{19}\) Adherent acanthamoebae secrete soluble factors that dissociate and kill corneal epithelial cells. \(^{20-22}\) Apoptosis of epithelial cells may also facilitate corneal penetration of the organism. \(^{13,23}\) Acanthamoebae secrete collagenase, as well as a series of proteases that enable it to penetrate deep into the corneal stroma. \(^{12,24-26}\)

The Acanthamoeba cyst is the dormant form of the organism, produced by inhibition of DNA synthesis and glucose starvation. \(^{27,28}\) Cysts are often found deep in the corneal stroma in established Acanthamoeba keratitis. \(^{3,5-10,11,29}\)

The susceptibility of both trophozoites and cysts to killing by polyhexamethylene biguanide, chlorhexidine, and propamidine isethionate has been confirmed, and these agents remain the mainstay of amebicidal therapy. \(^{30-35}\) Although steroids are frequently needed to control pain and inflammation in Acanthamoeba keratitis, their effect on trophozoites and cysts has not been previously investigated. \(^{5,6}\)

These experiments show for the first time that exposure of Acanthamoeba cysts to dexamethasone promotes excystment of the organism. After 24 hours of dexamethasone exposure, the number of trophozoites significantly exceeded that in untreated control cultures, at all concentrations of dexamethasone. This effect was maintained during an additional 24 hours.

**Figure 6.** Effect of dexamethasone on CPE associated with A. castellanii cysts. HCE cells were either incubated for 72 hours with A. castellanii cysts (2 × 10^5) in medium with and without 10, 100, or 400 µg/ml dexamethasone (A) or cysts were pretreated with dexamethasone for 48 hours before addition to HCE cells (B). CPE was assessed 72 hours later. Negative control cultures consisted of cell monolayers cultured in medium with dexamethasone or without cysts. The monolayers were washed and stained with Giemsa stain, and CPE was assessed spectrophotometrically. *Significantly different from untreated control cultures (P < 0.05).

**Figure 7.** Levels of Acanthamoeba plasminogen activator (APA) produced by trophozoites treated with dexamethasone. Trophozoites were incubated for 24 hours in PYG medium with or without 10, 100, or 400 µg/ml dexamethasone. After 24 hours incubation, the supernatants were collected, and APA activity was measured by radial diffusion in the fibrin agarose clot. APA activity is expressed in IU per milligram relative to a tissue plasminogen standard. The differences between trophozoites treated with dexamethasone and untreated organisms were insignificant (P > 0.05).

**Figure 8.** The effect of systemic dexamethasone on Acanthamoeba keratitis in Chinese hamsters. Animals (n = 10) were injected IM with 1 mg/ml dexamethasone in 100 µl saline. The animals were treated with dexamethasone daily for 3 days, beginning 24 hours after Acanthamoeba-laden contact lenses were applied. A control group of 10 Chinese hamsters received 100 µl saline IM. Infected lenses were removed after 6 days, and the corneas were scored for percentage of infection (A) or clinical severity (B) for the period indicated. *Significantly different from control (saline injected) animals (P < 0.05).
of exposure, and rates of excystment were similar for all concentrations of the steroid. Significant reduction in the number of cysts exposed to all concentrations of dexamethasone was observed in the culture at 2, 3, and 4 days after incubation. These results indicate that use of topical steroids at even a minimal dose promotes an increase in the number of trophozoites produced by excystment in corneal stroma while any cysts remain. This work emphasizes the clinical importance of the maintenance of an adequate dose of amebicidal drugs if steroids are considered necessary to control pain or inflammation in *Acanthamoeba* keratitis.

The greater excystment of *Acanthamoeba* at higher steroid concentrations should be considered when using steroid therapy as prophylaxis against corneal graft rejection after penetrating keratoplasty (PK). Cure of *Acanthamoeba* keratitis has not always been achieved with PK.29,36–38 Risk of recurrent *Acanthamoeba* keratitis has been considered greatest in the post-PK period.1,39 Our results indicate that residual cysts in the host peripheral cornea may undergo excystment when exposed to higher doses of steroids. Use of the minimum necessary dose of topical steroid and continuation of adequate doses of amebicidal therapy are therefore most important in the management of these corneal grafts.

Dexamethasone also stimulated the growth of trophozoites, with all steroid concentrations producing proliferation of the organism that exceeded that in control cultures, after 48 hours of exposure. This effect was maintained through 72 hours. These results mean that with continuing exposure to steroids, the number of *Acanthamoeba* trophozoites can be expected to increase significantly in the cornea by two mechanisms: excystment and accelerated multiplication of trophozoites.

Use of topical steroid, unaccompanied by amebicidal therapy, would be expected to produce proliferation of *Acanthamoeba* trophozoites. This is particularly important in patients in which the diagnosis of *Acanthamoeba* keratitis has not been made and steroids are being used in an attempt to control corneal inflammation and uveitis. The resultant proliferation of the invasive form of the organism exposes patients to the risk of significantly greater corneal destruction through an increase in organism load. This explains the observation that early diagnosis and appropriate therapy with cationic antiseptics, together with propamidine isethionate and neomycin, provides the greatest likelihood of a medical cure.40,41

It has been observed that late diagnosis of *Acanthamoeba* keratitis is a cause of failure of medical treatment, whereas use of topical steroids is not.47 However, in this study we also recognized that patients treated with topical steroids require a significantly longer duration of amebicidal therapy.15 Our study provides evidence that dexamethasone increases the number of trophozoites through excystment and proliferation. Increasing the corneal load of this invasive form of *Acanthamoeba* by use of topical steroid would be expected to prolong the infection. Clinical studies suggest that exposure to steroids does not promote resistance of the organism to amebicidal therapy.17

The effect of steroids on free-living amoebae demonstrates some species specificity. Systemic prednisolone has been shown to increase mortality rates twofold among albino mice infected intranasally with *Naegleria* spp., compared with mice inoculated with *Acanthamoeba* spp.42 The *Naegleria* spp.–infected mice had more histopathologic evidence of brain necrosis and meningeal inflammation than did those infected with *Acanthamoeba* spp.

Our results show that dexamethasone failed to influence rates of excystment of trophozoites. In the presence of an increased trophozoite load within the cornea, produced by accelerated excystment and trophozoite growth, maintenance of excystment rates of *Acanthamoeba* preserves the pathogenicity of the organism. Thus, the active trophozoites have maximum time to cause corneal damage through inflammation and epithelial and stromal lysis until excystment brings more quiescent disease.

Binding to corneal cells is the first step in the pathogenic cascade of *Acanthamoeba* keratitis and is followed by cytolysis of the corneal epithelial cells. These events are potentiated by the trophozoite load in the cornea, which plays an important role in the pathogenicity of the disease. We hypothesized that exposure of *Acanthamoeba* to dexamethasone increases *Ameoba*-mediated cytolysis. The present results suggest that pretreatment of organisms with dexamethasone did not produce any significant cytolysis. However, the presence of dexamethasone in the medium was necessary for organism-mediated cytolysis. We suspect that dexamethasone stimulates the cytolytic machinery of *acanthamoebae* by activating the organisms to release cytolytic factors. Supernatants from trophozoites treated with dexamethasone did not induce significant CPE on corneal epithelial cells, compared with untreated organisms. However, the results suggest that prolonged exposure to dexamethasone induces excystment and proliferation of organisms, which in turn potentiates trophozoite-mediated cytolysis. These results imply that under these conditions, *Acanthamoeba* trophozoites are capable of inducing more damage to corneal epithelial cells if stimulated with steroids. We and others have shown that *Acanthamoeba* spp. can destroy cells by either direct or indirect cytolysis of target cells.30,15,23,25 The exact mechanisms involved in trophozoite-mediated cytolysis are not known, but we have shown that destruction of target cells can occur by apoptosis.25

Characterization of molecules that induce excystment of *Acanthamoeba* by dexamethasone will hopefully yield further insights into the pathogenesis of *Acanthamoeba* keratitis. In the present study we examined the effect of steroids on *A. castellanii* trophozoites and cysts only. However, it would be interesting to compare the effect of steroids on morphogenesis of other *Acanthamoeba* strains.

Serine proteases play an important role in a broad array of biological processes, including tumor invasion, metastasis, and tissue invasion by metazoon parasites.45,44 We have demonstrated the close association between the elaboration of plasminogen activators in vitro and the pathogenic potential of human ocular isolates of *Acanthamoeba* spp.13 Because the pathogenesis of *Acanthamoeba* keratitis is dependent on the capacity of the organism to penetrate the corneal epithelium and enter the underlying stromal matrix, we hypothesized that dexamethasone induces trophozoites to secrete more plasminogen activators that increase tissue invasion and contribute to the pathogenesis of the disease. The results of the present study indicate that *A. castellanii* produced plasminogen activators, as previously reported. However, the results also show that dexamethasone-treated trophozoites secreted similar quantities of plasminogen activators compared with untreated trophozoites. Thus, it seems that the enhanced cytolysis of epithelial cells by dexamethasone-treated *Acanthamoeba* is not due to increased elaboration of plasminogen activators. It is possible that other enzymes such as collagenase, metalloproteinase, cysteine proteases, and elastases that are constitutively produced by pathogenic acanthamoebae are involved in dexamethasone-treated trophozoite cytolysis.24–26,45,46

Innate immune responses to acanthamoebae are considered the most important defenses against the organism. The histopathology of established *Acanthamoeba* keratitis shows macrophages, neutrophils, and few lymphocytes, indicating that adaptive immunity does not drive the cellular response to the organism. Macrophages predominate in experimental *Acanthamoeba* keratitis.7,10,12,15,30,32,47,48 It is known that steroids can suppress the host immune and inflammatory responses.
such as macrophages and neutrophils that play and important role in Acanthamoeba keratitis.

Our results show that Chinese hamsters treated with systemic dexamethasone are more likely to have Acanthamoeba keratitis and that the resultant infection is more severe and tendency to resolve. Systemic dexamethasone downregulates the innate immune system through inhibition of neutrophil chemotaxis and neutrophil and macrophage activation. Thus, the effects of steroid induced severe Acanthamoeba infection in the Chinese hamster are consistent with previous findings that macrophage depletion induces chronic Acanthamoeba keratitis.54

In our in vitro studies, exposure to dexamethasone increased the number of trophozoites through excystment and growth. Although systemic steroid may be expected to have less direct effect on the pathogenicity of acanthamoebae within the corneal stroma than topical steroids, it is possible that such steroids may reach the cornea through limbal blood vessels with increased permeability due to inflammation. Acanthamoeba infections can ultimately be cured only by elimination of the organism from the cornea. These results indicate that effective doses of amebicidal therapy should be continued while patients are receiving any concurrent topical steroid therapy to control the inflammatory complications of Acanthamoeba keratitis.

References


