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ORIGINAL PAPER

Clinical and histologic evaluations of experimental *Acanthamoeba* keratitis

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Abstract Amoebic keratitis, a sight-threatening, progressive corneal disease, is commonly caused by ubiquitous, pathogenic, free-living *Acanthamoeba* spp., which are widely distributed in the environment. We investigated clinical findings and histology of *Acanthamoeba* keratitis in a rat cornea model. Experimental *Acanthamoeba* keratitis was induced in Wistar rats by intrastromal inoculation of *Acanthamoeba castellanii* trophozoites. The clinic features of *Acanthamoeba* keratitis by day 70 are observed. All rats inoculated with *Acanthamoeba* developed keratitis. Histologically, the eyes displayed blood vessels, edema, and amoebae in stroma. A mixed cellular response, including neutrophils, mononuclear cells, and spindle-shaped cells, was seen. In conclusion, progressive, suppurative *Acanthamoeba* keratitis can be induced in the rat cornea model. This rat cornea model assists researchers who study the pathogenesis of *Acanthamoeba* keratitis and devise treatment for this difficult condition.

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Introduction

Acanthamoeba is a free-living amoeba that has been isolated from soil, air, fresh water, and sea water environments (Marciano-Cabral and Cabral 2003; Illingworth and Cook 1998). *Acanthamoeba* exists in two stages as both the mobile trophozoites and the dormant cysts. The mobile trophozoites (10–25 μm) are characterized by a large central nucleus and prominent nucleolus and normally subsist on bacteria and yeast (Khan 2001). The dormant cyst (8–12 μm) is protected by a bilaminated cellulose wall and resists repeated freeze–thawing cycles, as well as extraordinarily high doses of UV and gamma irradiations (Aksozek et al. 2002).

Human infection with *Acanthamoeba* is rare and opportunistic. It involves the skin and central nervous system in immunosuppressed patients and patients with acquired immune deficiency syndrome and occasionally involves the cornea in relatively healthy patients by causing *Acanthamoeba* keratitis (Awwad et al. 2007). *Acanthamoeba* keratitis is largely restricted to contact lens wearers who have experienced corneal trauma, implicating contact lens wear (Niederkorn et al. 1999a, b). The disease is characterized by severe pain because of radial neuritis and inflammation with redness and photophobia, but this condition is also noted for its wide variety of clinical presentations (Bacon et al. 1993; Illingworth and Cook 1998; Martinez 2001). It is characterized with chronic stromal inflammation as pathology (Kremer et al. 1994; Larkin and Easty 1991; Niederkorn et al. 1999a, b).

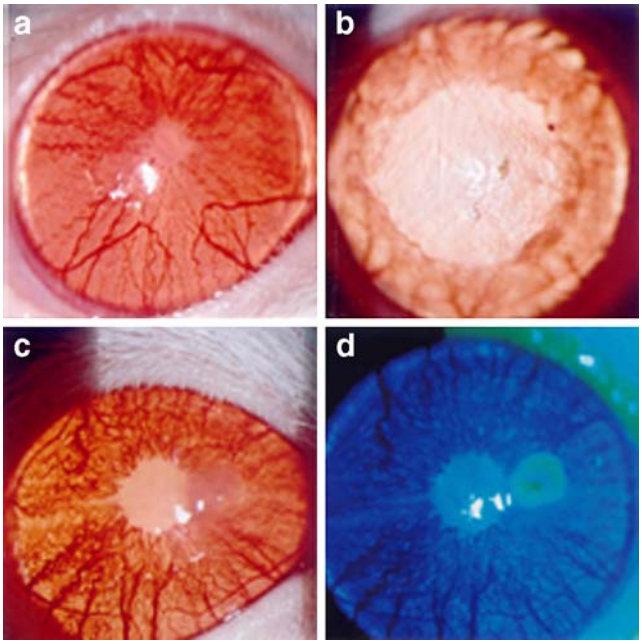

Fig. 1 **a** Normal rat cornea with transillumination of albino iris seen on retro-illumination. Epitelial vesicular (**b**) and bullous (**c**) edema in cornea. **d** Epithelial defect formation fluorescein staining of the cornea on day 3 (250×)

The aim of present study was to evaluate clinical findings and histology of *Acanthamoeba* keratitis in a rat cornea model.

Materials and methods

Animals Thirty-three male Wistar rats weighing approximately 125 g were used for the present study. All corneas were examined before inoculation to exclude any abnormality (Fig. 1a). In the first study set for clinical evaluation, 36 eyes of 18 rats were used: 24 eyes of 12 rats for *Acanthamoeba* infection and 12 eyes of six rats as control. In the second study set for histological evaluation, 30 eyes of 15 rats were used: 20 eyes of ten rats for *Acanthamoeba* infection and ten eyes of five rats as control.

Amoeba All studies were performed with *Acanthamoeba castellanii* strain 1BU, a human corneal isolate (Walochnick et al. 2000).Vegetative forms were obtained from axenic cultures in 25-cm^2 corning flasks containing 10 ml protease peptone, yeast extract, and glucose medium (Schuster 2002) and kept at 37°C. Trophozoites in the stage of exponential growth (72 to 96 h) were concentrated by centrifugation at 500×*g* for 10 min. The amoebae were washed twice in sterile Neff's saline solution (1.2 g NaCl, 0.4 g $MgSO_4 \cdot H_2O$, 0.4 g $CaCl_2 \cdot 2H_2O$, 1.42 g Na_2HPO_4, 1.36 g $KHPO_4$ in 100 ml distilled water), counted in a hemacytometer, adjusted to a final concentration in Neff's saline solution at a density of 1×10^6 amoebae/ml (95.0% trophozoites), and used immediately for testing.

Anesthesia Rompun 10 mg/kg and ketamine HCl mg/kg were given by intramuscular injection, and one drop of preservative-free benoxinate was applied to the right and left eye.

Inoculation technique The procedure was performed under an operation microscope (Leica-M841). Initially, a half-thickness linear blade incision was made approximately 2 mm from the center of the cornea. With a microliter syringe and 30 G needle, the needle was advanced from the incision through the lamellar of the stroma to the center of the cornea. One microliter of the solution including 1×10^6 amoeba/ml was injected into the stroma. Control animals received a mock inoculum of 1 μl Neff's saline.

Clinical evaluation Under general anesthesia, the rats were examined with a slit-lamp microscope on days 1, 3, 5, and 7 after inoculation and weekly thereafter until 70 days. Examination was by retro-illumination, resulting in transillumination of the albino iris and the cornea. The oblique slit beam was used to illuminate lesser degrees of opacity. The following grading scheme, which has been previously described (Larkin and Easty 1990), was used: grade 0=normal, grade 1=opacity visible only by oblique slit beam, grade 2=opacity visible on retro-illumination but not sufficient to obscure iris vessels,

Table 1 The clinic features of *Acanthamoeba* keratitis by day 70

Clinic features	Times (day)							
	1	3	5	7	14–28	35	42	49–70
Epithelial edema (%)	20.8	62.5	29.1	16.6	0	0	0	0
Punctate epitheliopathy (%)	0	37.5	66.6	4.1	0	0	0	0
Grade 1 opacity (%)	0	0	4.1	62.5	8.2	0	0	0
Grade 2 opacity (%)	0	0	0	16.6	36.0	16.6	4.1	4.1
Grade 3 opacity (%)	0	0	0	0	55.5	83.3	95.8	95.8
Stromal vascularization (%)	0	0	0	0	33.3	45.8	45.8	48.6
Stromal infiltration (%)	0	0	0	0	0	0	29.1	46.8
Pannus (%)	0	0	0	0	0	0	0	51.0

grade 3=opacity visible on retro-illumination, obscuring iris vessel detail. We evaluated clinical findings according to the presence of epithelial edema, punctate epitheliopathy, stromal vascularization, stromal infiltration, and pannus tissue and opacitiy grade (1–3).

Histological evaluation Under general anesthesia, the rats were examined with a slit-lamp microscope weekly thereafter until 70 days. Then, the eye was enucleated, and the cornea was dissected from the globe and hemisected. The cornea was fixed for 24 h in neutral-buffered formaldehyde, embedded in wax, and 6-μm-thick sections were cut with microtome. All of the tissue sections were stained by hematoxylin–eosin. Eight or more sections were viewed for each eye. We evaluated histological changes according to the presence of stromal edema and neovascularization and trophozoite, neutrophil, eosinophile, and spindle-shaped cells.

Results

Clinical evaluation

The clinic features of *Acanthamoeba* keratitis at days 1, 3, 5, 7, 14–28, 35, 42, and 49–70 are shown in Table 1. All rats inoculated with *Acanthamoeba* developed keratitis. In the control eyes, no infection was developed. Slit-lamp examination on day 3 showed corneal edema in 62.5% of the infected eyes (Fig. 1b and c). Fluorescein staining of the cornea on day 3 showed the formation of epithelial defect (Fig. 1d). Opacity was granular in character, with corneal thinning superficial to infiltrates apparent in eyes with more long-standing keratitis. On day 7 after inoculation, grade 1 opacity was observed in 66.6% of the infected eyes (Fig. 2c). On days 14–28 after inoculation, grade 2 opacity was observed in 36.0% of the infected eyes (Fig. 2b). On day 42 after inoculation, grade 3 opacity was observed in 95.8% of the infected eyes (Fig. 2a). Corneal neovascularization was developed in 45.8% of the infected eyes by day 35 (Fig. 3a). In the chronic stage of experimental keratitis, stromal infiltration and pannus developed by day 49 (Fig. 3b and c).

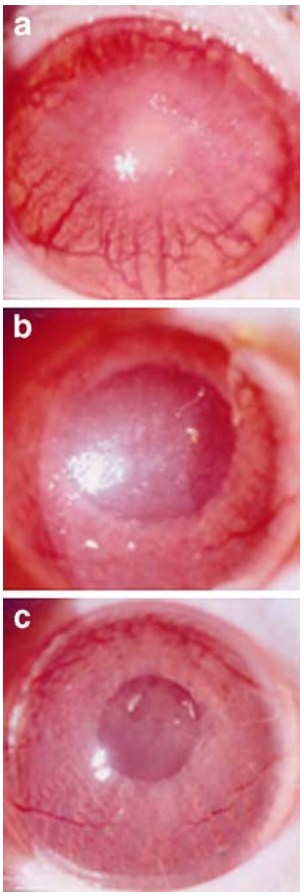

Fig. 2 Grades 3 (**a**), 2 (**b**), and 1 (**c**) corneal opacity (250×)

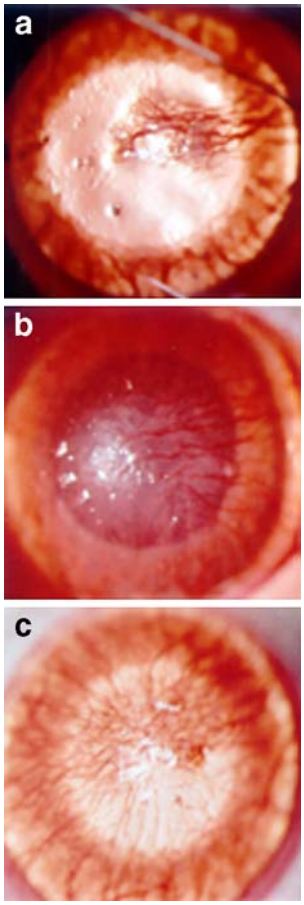

Fig. 3 Stromal vascularization (**a**), stromal infiltration (**b**), neovascularization (**b**), and pannus (**c**) in cornea (250×)

Histological evaluation

The histological features of *Acanthamoeba* keratitis by weeks 1–10 are shown in Table 2.

Histological features of normal rat cornea are shown in Fig. 4a. In all examinations, the corneal epithelium was

Table 2 The histologic features of *Acanthamoeba* keratitis by day 70

Histologic features	Times (week)									
	1	2	3	4	5	6	7	8	9	10
Stromal edema	+	+	+	+	+	+	+	−	−	−
Neavascularisation	+	+	+	+	+	+	+	+	+	+
Trophozoite	+	+	+	+	−	−	−	−	−	−
Neutrophile	+	+	+	+	+	+	+	+	+	+
Eosinophile	+	+	+	+	+	+	+	+	+	+
Spindle-shaped cells	+	+	+	+	+	+	+	+	+	+

present, but the squamous cells were convoluted and spaced compared with those of the normal rat eye. This was interpreted as being due to edema (Fig. 4b and c). The endothelium was also present, but it was infiltrated and supplanted by neutrophils and mononuclear cells in the central region (Fig. 4c). Hypopyon was observed in some eyes. Amoebae were confined to the stroma (Fig. 4b and c). Monocytes, macrophages, spindle-shaped cells, eosinophils, and blood vessels were also present in the stroma (Fig. 4b and c). Many corneas were seen to have microabscesses, most of which were present in the posterior stroma. Although trophozoites were not shown in the stroma on 5–10 weeks, inflammatory cells are present in the stroma.

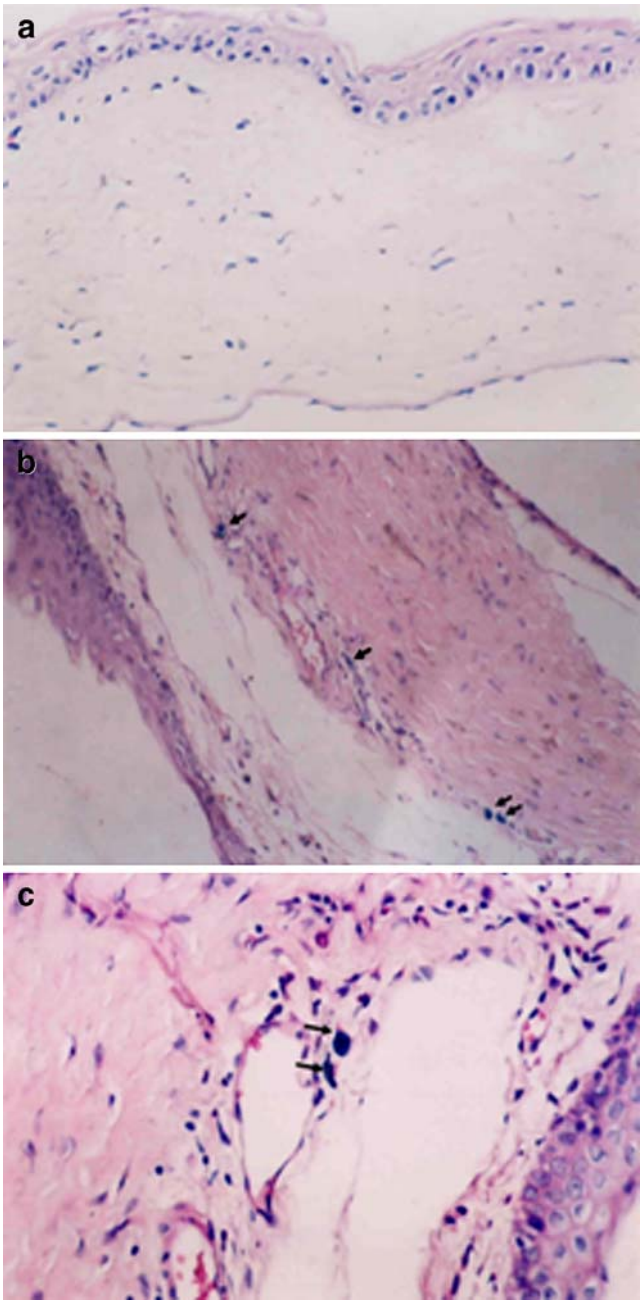

Fig. 4 **a** Histopathological feature of normal rat cornea (200×). **b**, **c** Edema, trophozoites (*arrows*), and inflammatory cells in stroma (100×, 200×)

Discussion

In this study, the development of *Acanthamoeba* keratitis was monitored by clinical corneal and histological examination for 70 days postinfection. Epithelial edema was observed on eyes (20.8, 62.5, 29.1, and 16.6%) on days 1, 2, 5, and 7, respectively. After progress of infection from epithelial to stromal tissue, the ratio of epithelial edema gradually decreased to 0 on day 14. Grades 1, 2, and 3 corneal opacity was found on days 5, 7, and 14. Corneal neovascularization was developed in 45.8% of the infected eyes by day 35. In the chronic stage of experimental keratitis, stromal infiltration and pannus developed by day 49.

Acanthamoeba keratitis typically has an indolent but progressive course, with intense discomfort and stromal infiltration (Jones 1986). The disease is often initially misdiagnosed as herpes simplex keratitis or adenoviral keratitis (Tabin et al. 2001). Early signs of *Acanthamoeba* keratitis clinically include epithelial irregularities, opacities, microerosions, microcystic edema, and patchy anterior stromal infiltrates (Berger et al. 1990; Moore and McCulley 1989). Late during the disease course (prolonged infection), Limbal hyperemia, edema, and ring infiltrate can develop. Ring infiltrate was shown to be present in 18% of *Acanthamoeba* keratitis cases in the first month and in 83% after 2 months (Bacon et al. 1993). Trophozoites are transformed into cysts (Khunkitti et al. 1998).

The development of an animal model of *Acanthamoeba* keratitis is essential to the detailed study of this condition and for controlled in vivo testing of new pharmacologic agents. We used rats because they are inexpensive, they are easy to keep in large numbers, and the size of their eyes

allows controlled surgical procedures. The inocula were prepared so that most of the amoebae injected were in the trophozoite form. It was thought that inoculation with active amoebae would give the best change of establishing an infection. To our knowledge, the present study is the first model that evaluated clinically and histologically in the rat cornea by 70 days without the need for concomitant immunosuppression by corticosteroid or coinoculation with bacteria. Badenoch et al. (1990) reported that chronic corneal infection was achieved using coinfection of *Acanthamoeba* and *Corynebacterium*, a limitation for the evaluation of antiamoebal agents. The anti-inflammatory and immunosuppressive activities of betamethasone may partially control local responses to amoeba-induced necrosis and parasite cytolytic factor release (Mathers et al. 1987). In human cases, local cordicosteroids was also found to promote inhibition of cyst–trophzoite transformation and result in severe corneal lesion (Lindquist 1998).

Most human specimens on which histopathological descriptions are available originate from corneal transplantation and accordingly describe the late stage of the disease, probably modified by drug therapy. Nevertheless, available reports describe changes similar to those described in this report. Disruption and necrosis of stromal lamellae and invasion by amoebae in stroma are observed.

In conclusion, progressive *Acanthamoeba* keratitis can be induced in rats with these laboratory settings. This *Acanthamoeba* keratitis rat model will greatly assist researchers who study the pathogenesis and devise treatment of this difficult condition.

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