

Experimental *Acanthamoeba* keratitis:

I. Preliminary findings

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Abstract

Experimental *Acanthamoeba* keratitis was induced in Wistar rats by intrastromal inoculation of *Acanthamoeba polyphaga* cysts. Keratitis developed in all corneas injected and was characterised by a diffuse granular opacity. The animals were examined for 70 days and a gradual reduction of keratitis was observed. Pathological sections showed liquefactive stromal necrosis and *Acanthamoeba* localised in deep stroma persisting to 70 days. A brisk inflammatory infiltrate of neutrophils and macrophages was seen in the early days of infection. Severity of keratitis was found to correlate with inflammatory activity in pathological sections. The uses of an animal model of *Acanthamoeba* keratitis are described.

Acanthamoeba is a genus of free-living protozoon with a ubiquitous distribution in nature. It has a life cycle of active trophozoite and dormant cyst stages. First isolated and described in 1913,¹ it was not considered to have pathogenic potential until reported to cause fatal meningoencephalitis in animal inoculation experiments in 1959.² Some years later, when suspected amoebic encephalitis was reported in humans, the similarity to *Acanthamoeba* infection induced experimentally was recognised and suggested the aetiological role of *Acanthamoeba*. The pathological features of human encephalitis are so similar to those of experimental *Acanthamoeba* encephalitis in mice that the use of animal models for studying other forms of *Acanthamoeba* infection is clearly indicated.³

After initial reports^{4,5} *Acanthamoeba* infection of the cornea remained a rare disease until an association with contact lens wear was first recognised.⁶ Reports of cases have exponentially increased since that time, and a recent analysis identified 85% of affected patients as contact lens wearers.⁷ *Acanthamoeba* keratitis typically has an indolent but progressive course, with intense discomfort and stromal infiltration. Therapy of *Acanthamoeba* keratitis is difficult in all cases, with frequent failures of medical and surgical treatment and loss of the eye in some of these.

Even though the keratitis has become more common than the encephalitis, few laboratory in-vivo studies have been reported. Rabbit models have been developed, first by Font and colleagues.^{8,9} After subconjunctival administration of corticosteroid for four days the direct intrastromal injection of *A polyphaga* induced stromal keratitis and corneal ulceration. Some corneas were culture-positive at intervals, and encysted amoebae were observed in tissue on microscopy. A further rabbit model was reported

by Lin and colleagues in which the effect of steroid administration was analysed.¹⁰ Badenoch *et al* reported a model in Porton rats in which induction of *Acanthamoeba* keratitis was dependent on coinoculation with bacteria.¹¹

In this study the Wistar rat was used as the experimental animal. The initial objective of the study was to induce reproducible disease similar to the human disease, for use in evaluation of immunopathology and therapeutic agents. So that the specific immune response to *Acanthamoeba* could be studied coinoculation with bacteria was avoided.

Materials and methods

Animals. Male Wistar rats weighing approximately 125 g were used. The corneas were examined prior to inoculation to exclude abnormality (Fig 1A). There were 24 rats in the infected group and six in the control group. All procedures were performed on the right eye.

Amoeba. All studies were performed with the Shinh strain of *A polyphaga*, a human corneal isolate (kindly provided by Mr S Kilvington, Public Health Laboratory, Bath). This had been frozen in liquid nitrogen and passaged on a Vero cell monolayer three times. An axenic suspension of 1×10^6 amoebae/ml (90% cysts) was prepared in Page's amoeba saline.¹²

Anaesthesia. Diazepam 0.25 mg and fentanyl 0.01 mg/fluanisone 0.3 mg were given by intramuscular injection, and one drop of preservative-free benoxinate was applied to the right eye.

Inoculation technique. The procedure was performed under an operating microscope (OPMI 1 FC, Carl Zeiss Ltd). Initially a half thickness linear blade incision was made approximately 2 mm from the centre of the cornea. With a microlitre syringe (Hamilton Bonaduz AG) and a sterile 30 G needle (Becton-Dickinson & Co.), the needle was advanced from the incision through the lamellae of the stroma to the centre of the cornea. 1 μ l was injected into the stroma. Control animals received a mock inoculum of 1 μ l of Page's saline; infection animals received a suspension of 1×10^6 amoebae/ml (90% cysts) in Page's amoeba saline.

Clinical evaluation Under fentanyl general anaesthesia the rats were examined with a slit-lamp microscope on days 1, 3, 5 and 7 following inoculation and weekly thereafter until 70 days. Examination was by retroillumination, resulting in transillumination of the albino iris and the cornea. The oblique slit beam was used to illuminate lesser degrees of opacity. A clinical grading system was used to describe corneal opacity:

Grade 3: opacity visible on retroillumination, obscuring iris vessel detail.

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Accepted for publication
29 March 1990

Figure 1A Normal rat cornea with transillumination of albino iris seen on retroillumination.

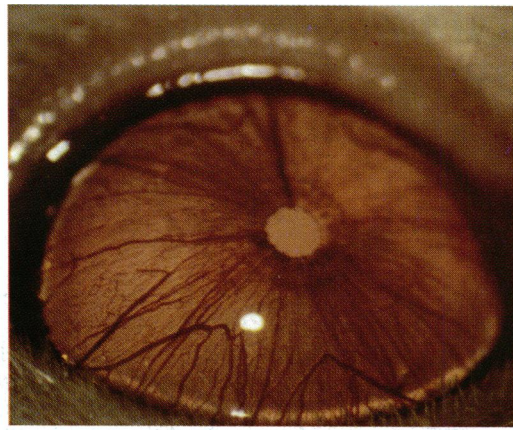


Figure 1B Day 5: infected rat cornea showing diffuse granular central corneal opacity.

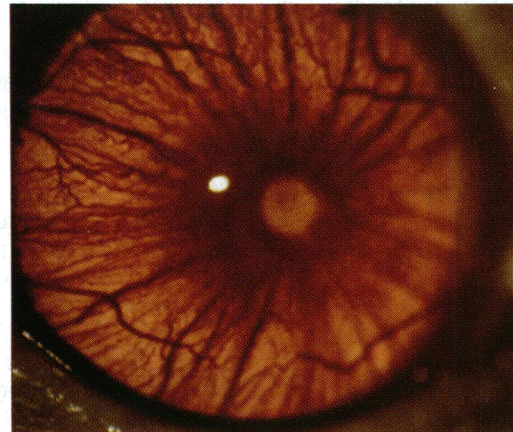


Figure 1C Day 14: clearance of much of the corneal oedema and maximal opacity at the inoculation site (arrow).

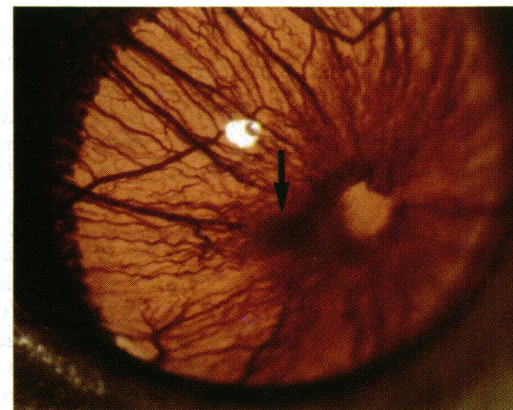
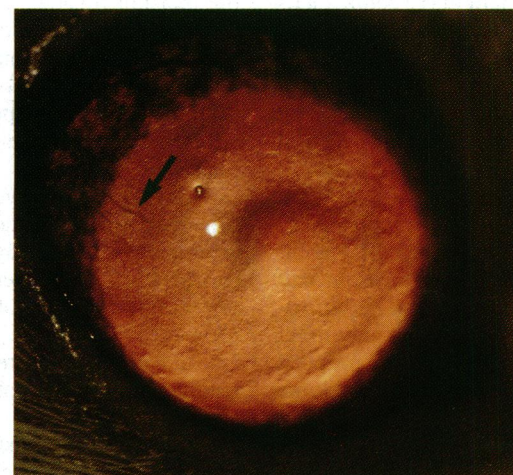


Figure 1D Day 35: paracentral ring-shaped granular opacity and fine superficial peripheral vascularisation (arrow), photographed following mydriatic administration.



Grade 2: opacity visible on retroillumination, but not sufficient to obscure iris vessels.

Grade 1: opacity visible only by oblique slit beam.

Grade 0: normal.

A mean opacity grade was calculated for the infected corneas on each examination day. Photographs were taken with the operating microscope with retroillumination of the cornea, 160 ASA tungsten film (Kodak Ektachrome) and automatic exposure.

On days 1, 3, 7, 14, 21, 28, 49, and 70 after inoculation two infected rats were killed by intraperitoneal injection of 60 mg pentobarbitone. Two control rats were killed on days 1, 3, and 7.

Tissue processing. The eye was enucleated and the cornea dissected from the globe and hemisected. One half of the cornea was fixed for 24 h in neutral buffered formaldehyde, embedded in wax, and 6 μ m thickness sections cut with a microtome; the other half was either immediately frozen in liquid nitrogen or homogenised with a glass grinder for culture.

Preparation of specific antibody. Rabbit antiserum was prepared to the experimental strain of *A. polyphaga* according to the method described by Warhurst.¹³ Briefly, a 2.5 kg New Zealand White rabbit was injected intramuscularly with 30 ml *A. polyphaga*/Freund's complete adjuvant and this was repeated at a three-week interval. This was followed by two 0.6 ml intravenous injections of formalinised *A. polyphaga* cysts at four and five weeks. After venepuncture at six weeks the antiserum titre was measured and found to be 1/1024. The serum was then affinity purified on a chromatography column using protein A-Sepharose (Sepharose CL-4B, Pharmacia, Uppsala, Sweden) to yield the IgG fraction.¹⁴

Staining of wax sections. Sections were stained with haematoxylin and eosin or with rabbit anti-*Acanthamoeba* antibody. For the latter procedure sections were first incubated at room temperature with 1:5 normal swine serum (Dakopatts a/s) to block non-specific background staining; they were then incubated in sequence with rabbit anti-*Acanthamoeba* antibody at dilution 1:10 for 30 minutes, and peroxidase-conjugated swine anti-rabbit immunoglobulin (Dakopatts a/s) at dilution 1:50 for 30 minutes. Phosphate-buffered saline (PBS) was used for all dilutions, and sections were washed with PBS following each of these incubations. 3,3'-Diaminobenzidine (Sigma Chemical Co.) was the chromagen used to identify antibody binding, mixed with H₂O₂ in 0.1 M trometamol (Tris) buffer, giving a brown end-product: sections were incubated for three minutes and then washed in tap water for five minutes. Sections were counterstained with haematoxylin.

Culture of corneas for Acanthamoeba. Non-nutrient agar was set in each well of 25-well Petri plates. A lawn of live *Escherichia coli* was added to the agar surface in each well, and after 8 hours' incubation at 37°C the plates were ready for inoculation with tissue. Hemicorneas were homogenised in 150 μ l of Page's saline with a glass grinder, 10 μ l of the undiluted homogenate added to each well, and the plate sealed. Corneas

from control and infected eyes were cultured and observed by microscopy for seven days. If no growth was seen by seven days, the culture was deemed negative.

Results

CLINICAL EVALUATION

The cloudy opacification observed immediately after injection of all corneas cleared completely in control rats before the first examination day 1. Reepithelialisation of the linear incision had also occurred by this time.

All rats inoculated with *Acanthamoeba* developed keratitis. Certain clinical features were consistently observed. Opacity was granular in character, with corneal thinning superficial to infiltrates apparent in animals with more long-standing keratitis. Minimal, if any, vascularisation and no corneal perforation was seen (Figs 1B, 1C, 1D). No epithelial disease and no signs of anterior uveitis such as nodular infiltrates or hypopyon were observed.

On days 1, 3, and 5 following inoculation grade 3 opacity was observed in almost all rats, maximal at the inoculation site. On day 7 corneal opacity was grade 3 in most rats but had diminished to grade 2 in some. On subsequent examinations a gradual reduction in mean grade of corneal opacity was found (Table I).

EXAMINATION OF PATHOLOGICAL SECTIONS

Haematoxylin and eosin (H-E) staining of infected corneas identified a marked inflammatory response of neutrophils and macrophages at the inoculation site on day 1 (Fig 2A). A distinction between *Acanthamoeba* and inflammatory cells was not possible by H-E stain but was facilitated by immunoperoxidase staining with anti-*Acanthamoeba* antibody (Fig 2B). Excystment of many amoebae (Fig 2C) and

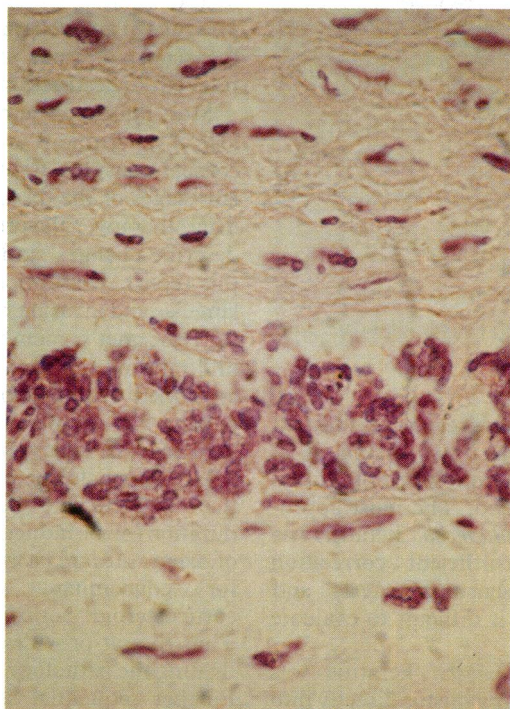


Figure 2A Day 1: non-specific staining by amoebae and inflammatory cells at the inoculation site in an infected cornea. (H-E, ×540.) They are very difficult to distinguish.

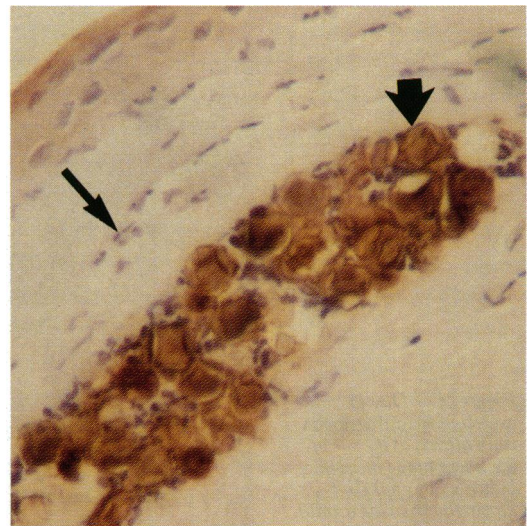


Figure 2B Day 1: Acanthamoeba cysts (large arrow) and migrating polymorphs (small arrow). Little excystment has taken place. (Anti-*Acanthamoeba* antibody × 300).

trophozoite migration from the inoculation site was seen by day 3. A more subdued and mixed inflammatory response was evident by this time, with lymphocytes and monocytes appearing among the neutrophils and macrophages seen on day 1. By day 7 trophozoites were seen throughout the cornea, though large numbers of cysts remained at the inoculation site. Marked stromal thinning was seen overlying the inoculation site in some corneas at day 14 (Fig 2E). Examination of corneas late in disease, from days 21 to 70, consistently revealed amoebae and inflammatory cells to be localised to the inoculation site, surrounded by eosinophilic material.

A gradually diminishing inflammatory response was seen in control corneas at 1, 3 and 7 days after mock inoculation (Fig 2F).

CULTURE OF CORNEA

Positive *Acanthamoeba* cultures resulted from infected corneas of all animals killed at days 1–49 inclusive. The homogenised corneas of the rat killed at day 70 and all control rats were culture-negative.

Discussion

The value of an animal model of *Acanthamoeba* keratitis is attested to by the gravity and increasing prevalence of clinical disease in humans. Study of *Acanthamoeba* keratitis in an animal model allows control of many of the variable factors involved in human disease. In

Table 1 Mean grade of keratitis observed following injection of *Acanthamoeba*

Day	Number of rats examined	Mean grade of keratitis (SD)
1	24	2.72 (0.60)
3	22	2.76 (0.61)
5	20	2.77 (0.59)
7	18	2.42 (0.79)
10	16	2.08 (0.81)
14	14	1.75 (0.65)
21	12	1.71 (0.76)
28	10	1.93 (0.83)
42	8	1.29 (0.49)
70	6	1.50 (0.58)

Figure 2C Day 1: excysting *Acanthamoeba* (thin arrow) at the site of the linear blade incision, indicated by epithelial downgrowth (wide arrow). (Anti-*Acanthamoeba* antibody, $\times 300$.)

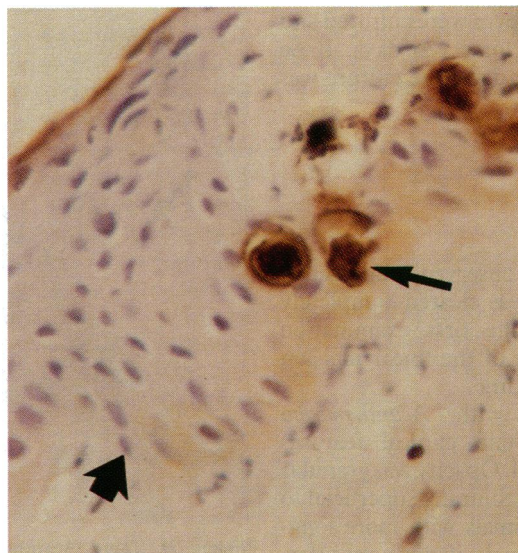


Figure 2D Day 7: cyst (large arrow) and trophozite (small arrow), each being killed by several inflammatory cells (Anti-*Acanthamoeba* antibody, $\times 760$.)

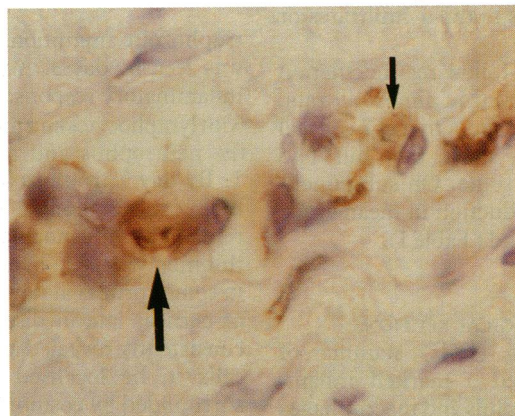


Figure 2E Day 14: marked thinning of corneal stroma overlying *Acanthamoeba* cysts which are in deep stroma. These have not migrated and rest on Descemet's membrane. (Anti-*Acanthamoeba* antibody, $\times 140$.)



particular, the clinical features of established stromal infection may be analysed, pathological study of tissue undertaken, and, in an established model, treatments evaluated. The most appropriate treatment for *Acanthamoeba* keratitis has yet to be decided: insufficient correlation between in-vitro drug sensitivity tests and clinical response renders it difficult to evaluate new drugs.

In this study *Acanthamoeba* keratitis was induced by inoculation of organisms directly into

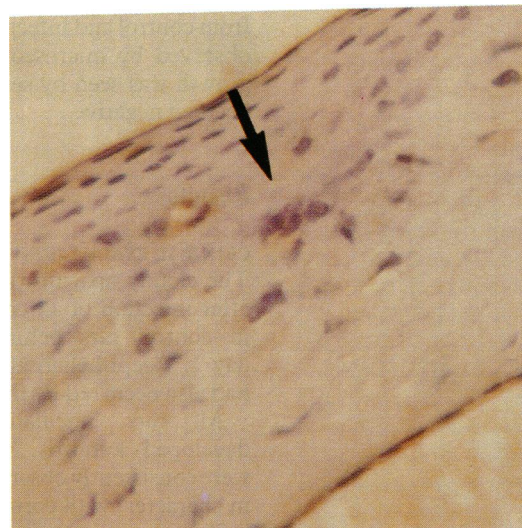


Figure 2F Day 3: control cornea showing low-grade inflammation at inoculation site (arrow). (Anti-*Acanthamoeba* antibody, $\times 190$.)

the corneal stroma. Therefore this model does not address questions on the means by which infection arises, but what follows once a large number of cysts reach the stroma. Reproducible keratitis developed in all rat corneas inoculated with 1 μ l of a suspension of *Acanthamoeba* at a concentration of 1×10^6 /ml. Because in-clinical practice such an inoculum of cysts would be very unlikely, this may indicate that *Acanthamoeba* are less infective for the rat cornea than the human. Certain clinical features mimic those observed in human infection, such as minimal corneal vascularisation and diffuse granular opacity formation rather than formation of an abscess. Although acute fulminating infection was not induced, the clinical course of the experimental keratitis is shorter than the human disease, which may remain active for several months. This might be due to differences in rat and human susceptibility to infection. In general, clinical inflammation correlated with histopathological indices of inflammation rather than presence of amoebae as such. In accordance with the report of Font *et al.*⁹ cysts seemed more resistant to destruction than trophozoites.

Most human specimens on which histopathological descriptions are available originate from corneal transplantation and accordingly describe late stages 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 of deep stroma to Descemet's membrane are observed with infiltrates of neutrophils; in some cases there is an added lymphocyte and plasma cell component.⁶⁻¹⁵⁻¹⁷ However, *Acanthamoeba* can be seen in tissue relatively free of inflammation.¹⁷⁻¹⁸

Initial conclusions from this study indicate that reproducible keratitis of short clinical duration can be induced by intrastromal injection of axenic *Acanthamoeba* cysts without the need for concomitant immunosuppression by corticosteroid⁸⁻¹⁰ or coinoculation with bacteria.¹¹ Thus the rat provides a useful model for investigating the pathology of *Acanthamoeba* keratitis. Further sequential studies are in progress to

characterise the cellular composition of the immune response to *Acanthamoeba*. This model will provide improved understanding of *Acanthamoeba*-host interactions and ultimately will allow evaluation of specific antimicrobial drugs with and without corticosteroid.

This study was supported in part by grants from Alcon Laboratories Ltd, the British Council for the Prevention of Blindness, and the South-Western Regional Health Authority.

The authors are indebted to Simon Kilvington, BSc, for initially providing *Acanthamoeba* cysts and assisting with preparation an anti-*Acanthamoeba* antibody; and to Ian Williams for assistance with the preparation of tissue sections.

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