Susceptibility of Corneas from Various Animal Species to In Vitro Binding and Invasion by Acanthamoeba Castellani

Jerry Y. Niederkorn,* John E. Ubelaker,+ James P. McCulley,* George L. Stewar,‡ Dale R. Meyer,* Jessamee A. Mellon,* Robert E. Silvany,* YuGuong He,* Marsha Pidherney,* James H. Martin,§ and Hassan Alizadeh*

A crucial requirement for establishing corneal infection by the extracellular protozoal parasite, Acanthamoeba, is the ability of the parasite to bind to the corneal surface. In a series of in vitro studies, we examined the ability of Acanthamoeba castellani to adhere, invade, and damage normal, intact corneas of 11 mammalian and one avian species. A. castellani (80-90% trophozoites and 10-20% cysts) were incubated with corneas for 24 hours in vitro and examined by scanning electron microscopy (SEM). Results of several independent SEM experiments revealed that parasites not only failed to produce cytopathic effects but did not even bind to the corneal epithelium of mice, rats, cotton rats, horses, guinea pigs, cows, chickens, dogs, and rabbits. However, parasites adhered, invaded, and produced severe damage to human, pig, and Chinese hamster corneas during the 24-hour in vitro incubation period. Additional in vitro experiments quantified the binding of A. castellani to the corneas of selected susceptible and nonsusceptible species. In vitro binding assays revealed scant binding of parasites to mouse, rat, and rabbit (range = 5-20 parasites/7.07 mm² corneal button). In contrast, extensive binding was observed on Chinese hamster, pig, and human corneas (range = 100-200 parasites/7.07 mm² button). The results indicate that A. castellani exercises rigid host specificity at the host cell surface. Invest Ophthalmol Vis Sci 33:104-112, 1992.

Acanthamoeba keratitis is a progressive, necrotizing protozoal infection of the cornea and is intimately associated with soft contact lens wear.1 Infections can produce scarring so severe that keratoplasty is required to restore normal vision. The characteristics of Acanthamoeba would predict a high incidence of corneal infection. Acanthamoeba cysts occur in a wide variety of environmental conditions, including in fresh and salt water, under ice in northern lakes, in hot tubs, in chlorinated swimming pools, and under conditions of low pH.2 Contact lens wear, the leading risk factor for Acanthamoeba keratitis, is practiced by over 20,000,000 individuals in the United States alone. These two conditions—the ubiquitous distribution of the parasite and the large pool of individuals at risk for infection—would lead one to expect a high incidence of Acanthamoeba keratitis. However, the occurrence of disease is extraordinarily low. It seems likely, therefore, that additional risk factors are involved in the development of corneal infection. Such risk factors could be identified and characterized in an appropriate animal model. Moreover, an animal model of Acanthamoeba keratitis would permit crucial studies on the immunology, pathology, and genetics of this disease.

The present study arose from efforts to develop an animal model of Acanthamoeba keratitis. The experimental approach initially sought to produce corneal infections in the mouse because this species is amenable to various immunological and genetic analyses crucial for understanding the pathogenesis and pathophysiology of infectious diseases such as Acanthamoeba keratitis. However, the inability to establish corneal infections in various categories of immunosuppressed mice and in two species of immunocompetent rats suggested that the rodent cornea itself was
not susceptible to infection with *Acanthamoeba castellani*. Therefore, corneas from mice, rats, and several other animal species were examined for their susceptibility to in vitro infection with *A. castellani*. The results of the in vitro studies revealed that, of the 12 animal species tested, *A. castellani* bound extensively to only three species: human, pig, and Chinese hamster.

**Materials and Methods**

**Parasite Cultivation**

*A. castellani*, originally isolated from a human cornea, was obtained from American Type Culture Collection (#30868; Rockville, MD). Parasites were grown axenically in peptone-yeast-glucose (PYG) medium as previously described. Parasites were isolated, washed, and resuspended in Page's saline at a concentration of $3 \times 10^6$ parasites/ml (80–90% trophozoites; 10–20% cysts). Parasite viability was determined by trypan blue exclusion and was consistently found to be greater than 90%. This species of *Acanthamoeba* was selected for study because of its frequent association with human infection and its recent use in animal studies performed by other investigators.

**Corneas**

Adult human corneal buttons (1–4 days post mortem) were obtained from the Lions Sight and Tissue Foundation (Dallas, TX) and were stored at 4°C in 20 ml of chondroitin sulfate (Chiron Ophthalmics, Irvine, CA). Mouse (BALB/c and C57BL/6), Norway rat (Lewis), cotton rat (Sigmodon hispidus), chicken, guinea pig, Chinese hamster, and rabbit corneas were obtained from normal animals following euthanasia with metofane, carbon dioxide, or sodium pentobarbital. Cow and horse eyes were obtained from a local abattoir and the corneas were excised aseptically in the laboratory. Canine and porcine corneas were obtained from animals used as experimental subjects for cardiovascular surgical research at The University of Texas Southwestern Medical Center or Baylor University Medical Center. All of the studies were performed in accordance with the ARVO Resolution on the Use of Animals in Research. All corneas used in these studies were intact, free of discernible epithelial defects, and not abraded.

**In Vitro Binding (Electron Microscopy Studies)**

Full-thickness corneas were cut into sections (4.0 mm x 4.0 mm), placed epithelial side up, and oriented to lie flat on the bottom of individual wells of 96-well microtiter plates. Parasites were suspended in McCarey-Kaufman medium (1 x 10^6/µl), which contained gentamicin (1 µg/ml). Parasite suspension (0.2 ml) was added to each well. Corneal cultures were incubated at 35°C for 24 hr. Corneal sections were fixed in 10% Carson's formalin and processed for light and electron microscopy. Controls consisted of similar corneal specimens incubated in McCarey-Kaufman medium in the absence of parasites.

---

**Fig. 1. SEM of mouse cornea incubated with *Acanthamoeba castellani* for 24 hr. Note absence of bound parasites or evidence of cytopathic effects. Bar = 10 µm.**
Transmission Electron Microscopy (TEM)

Corneal specimens fixed in Carson's fixative were postfixed for 1 hr in 1% osmium tetroxide, dehydrated in ethanol (50-100%) and embedded in Spurr's epoxy resin overnight. Ultrathin sections were stained with uranyl acetate and Reynolds' lead citrate and viewed in a Philips 300 transmission electron microscope (Philips, Eindhoven, The Netherlands) at 40 kV. Thicker sections (1 µm) were stained with paragon stain and examined by light microscopy.

Scanning Electron Microscopy (SEM)

Specimens were fixed and postfixed as described above and dehydrated by transfer through a graded series of acetone (50-100%). Dehydrated specimens were dried in a Sorval CO2 critical point drier (Sorval Dupont, Wilmington, DE) and mounted on aluminum stubs with silver conducting paint. Specimens were coated with a thin (20 nm) film of gold-palladium using a Denton DV-502 sputter-coater (Denton Vacuum Inc., Cherry Hill, NJ) and were observed in a JEOL JSM35 CF scanning electron microscope (JEOL, Tokyo, Japan) operating at 20 kV.

In Vitro Binding Assay

An in vitro assay was developed to quantify the binding of *A. castellani* to the corneas of various mammalian species. Corneas were prepared in the same manner as previously described except a sterile 3.0 mm trephine was used to prepare uniform diameter full-thickness corneal buttons. Corneal buttons (3.0 mm in diameter) were placed endothelial side down in individual wells in 96-well microtiter plates. Parasites were labeled by a modified technique of Weisman and Korn, who demonstrated that the parasites take up polystyrene and polyvinyltoluene latex beads. *Acanthamoeba*, at a concentration of $1 \times 10^6$ organisms/ml (95% trophozoites) from log phase growth, were incubated with 1.0 µm fluorescent latex beads (Polysciences, Inc., Warrington, PA) in PYG medium at 35°C for 24 hrs. More than 95% of the *Acanthamoeba* had ingested as many as five labeled beads and were viable as determined by exclusion of trypan blue. The labeled parasites were centrifuged at 94 × g for 5 min and were washed four times in PYG medium. *Acanthamoeba* ($1 \times 10^6$ parasites/ml) labeled with fluorescent beads were incubated with 3.0
mm corneal buttons for 24 hr at 35°C. Corneal buttons were washed thoroughly with PYG and suspended epithelial side up in a depression slide (76 \times 26 mm slide with a concave well of 1.5–1.95 mm deep) in a “hanging drop” configuration. Individual corneal buttons were viewed by compound microscopy using light and fluorescent illumination (Leitz Orthoplan compound microscope; Ernst Leitz Wetzlar GMBH, Midland, Ontario, Canada). The entire epithelial surface (3.0 mm in diameter; 7.07 mm² surface area) was inspected, and the number of labeled parasites binding to the corneal epithelium was recorded.

Statistical Analysis

An unpaired, one-tailed Student’s t-test was used for statistical analysis of the in vitro binding assays.

Results

SEM Analysis of Parasite Binding to Corneal Buttons

The present studies were precipitated by our inability to establish clinically and histopathologically convincing *Acanthamoeba* keratitis in rats and mice. Because there are many examples of exquisite host specificity among protozoal parasites, rats and mice may belong to a long list of conventional laboratory animals that are refractory to corneal infection with *A. castellani*. Therefore, it was important to determine whether the corneas of various laboratory animals were equally susceptible to in vitro binding and cytopathic effects of *Acanthamoeba castellani* and to compare these corneal buttons with human corneas that are known to be vulnerable to infection.

Corneas from various mammalian and one avian species (ie, the chicken) were trimmed to individual square sections approximately 3 mm on each side. Individual corneal sections (2–5/experimental group) were placed into microtiter wells and incubated for 24 hr at 35°C with $1 \times 10^5$ parasites in 200 μl of Page’s saline, incubated for 24 hr, and examined by SEM and TEM.

Corneas from rabbits, mice, rats, cotton rats, guinea pigs, rabbits, horse, cow, chicken, and dog
were remarkably free of adherent parasites or evidence of corneal lesions. Corneas incubated for 24 hr in medium not containing parasites were indistinguishable from those exposed to parasites. An example of a mouse cornea incubated in the presence of parasites is shown in Figure 1.

We recently described the morphological features of in vitro infection and penetration of human corneal epithelium by *A. castellani*. The results of the present study parallel the previous findings. Briefly, human corneal buttons contained abundant numbers of trophozoites (Fig. 2) and cysts adhering to the epithelial surface. The surface epithelium often was discontinuous and the cells were frequently observed in the process of sluffing. However, parasites were not observed binding to sluffing cells. In peripheral regions of the cornea, the epithelium displayed deep pits containing numerous *Acanthamoeba* trophozoites. More importantly, trophozoites penetrated various levels of the otherwise intact corneal epithelium. In contrast, human corneas incubated in the absence of parasites retained their normal epithelial architecture and appeared intact (data not shown).

Examination of pig and Chinese hamster (Fig. 3) corneas infected in vitro revealed numerous trophozoites and cysts adhering to the surface of the cornea. Closer examination of the infected pig corneas revealed trophozoites binding to and penetrating between epithelial cells at the corneal surface (Fig. 4). The trophozoites of *A. castellani* were readily identifiable by their characteristic morphology and the presence of typical acanthopodia (Fig. 4). TEM evaluation of the affected pig corneas confirmed that the trophozoites were bound firmly to the epithelium (Fig. 5), and in many cases, were in the process of invading the basal layers of the epithelium. The overall appearances of the infected Chinese hamster and pig corneas were virtually identical to infected human corneal buttons and distinctly different from the other species examined.

**Quantitative Analysis of Parasite Binding to Corneal Buttons**

Results from the SEM studies indicated that the corneas could be readily categorized as highly susceptible or nonsusceptible to in vitro binding. To quantify this disparity in binding, an in vitro binding assay was
developed. Full-thickness corneal buttons were cut with a sterile 3.0 mm trephine. Prior to incubation with the 3.0 mm corneal buttons, parasites (80–90% trophozoites; 10–20% cysts) were fed 1.0 μm fluorescein isothiocyanate (FITC)-labeled latex beads. Parasites rapidly ingested the FITC-labeled beads, which were readily visible within the parasite's cytoplasm (Fig. 6). Each corneal button (3.0 mm in diameter) was incubated with $3 \times 10^6$ parasites/ml for 24 hr. Each button was washed thoroughly and examined by conventional and fluorescent microscopy in a “hanging drop” suspension. The internalized FITC-labeled beads permitted easy and unambiguous identification of parasites bound to corneal epithelium (Fig. 7). Using this microscopic technique, it was possible to accurately quantify parasite binding and to confirm the results obtained by SEM. The results summarized in Figure 8 indicate extensive parasite binding to human, Chinese hamster, and pig corneas. Binding ranged from 100 to 500 parasites per 7.07 mm² corneal button. By contrast, only small numbers of parasites (i.e., < 20 parasites/7.07 mm²) were bound to mouse, rat, and rabbit corneas.

**Discussion**

The original rationale for this study was the notion that *Acanthamoeba* keratitis could be induced in labo-
ratory rodents by merely injecting viable *A. castellani* directly into the cornea or by exposing an abraded cornea to a heavy inoculum of infectious parasites. However, in preliminary studies, we have been consistently unable to produce lesions that resemble the human counterpart (unpublished findings). In fact, the corneas of mice inoculated in this manner returned to near normal clarity in a surprisingly short period of time. Our inability to produce a progressive keratitis in mice and rats parallels the findings of Badenoch et al., who reported that Acanthamoebae could produce keratitis in rats only if the parasites

---

**Fig. 6.** Photomicrograph demonstrating internalized FITC-labeled latex beads in *A. castellani* trophozoites using both fluorescent and transmitted illumination simultaneously. Bar = 20 μm.

**Fig. 7.** Fluorescent photomicrograph of a FITC-labeled *A. castellani* trophozoite binding to the epithelium of a human corneal button after 24 hr incubation. Note internalized FITC-labeled latex beads within the trophozoites (arrows). Bar = 20 μm.
No. 1 SUSCEPTIBILITY OF CORNEAS TO ACANTHAMOEBA INFECTION / Niederkorn et al 111

Fig. 8. Binding of *A. castellani* trophozoites to 3.0-mm corneal buttons of nonsusceptible and susceptible species as determined by the in vitro binding assay. Each bar represents the mean and standard error. Nonsusceptible species of hosts included rat (*n* = 9), rabbit (*n* = 6), and mouse (*n* = 4). Susceptible species included human (*n* = 5), Chinese hamster (*n* = 11), and pig (*n* = 6). Asterisk indicates difference between that group was significantly different (*P* < 0.05) from each of the nonsusceptible experimental groups (ie, mouse, rat, and rabbit).

were coinjected with *Corynebacterium xerosis*. The role of the corynebacteria in the pathogenesis of *Acanthamoeba* keratitis in rats, however, is unclear. The present in vitro results provide compelling evidence that *A. castellani* displays rigid host specificity for binding to the corneal epithelial surface. Moreover, the facile manner in which *A. castellani* trophozoites bound, invaded, and eroded human, Chinese hamster, and pig corneas in vitro indicates a striking and fundamental difference between the corneas of these hosts and rodent corneas in terms of susceptibility to infection with this protozoal species. Larkin and Easty reported that keratitis of short clinical duration can be induced in Wistar rats by intrastromal inoculation of *Acanthamoeba polyphaga*, and they concluded that *Acanthamoeba* was less infective for the rat cornea than human cornea. These results further emphasize that corynebacteria are not necessary for corneal infection, at least not in vitro. It is conceivable that the bacteria might act as an adjuvant in vivo as suggested by Badenoch et al and play a role in determining the severity of infection.

If the pathological sequelae of *Acanthamoeba* keratitis were due to cytopathic effects produced directly by the parasite's interaction with the target organ, as suspected by most investigators, one would expect to see such lesions produced in tissues infected in vitro. This was the case with human, Chinese hamster, and pig corneas. Parasites were found in intimate association with corneal epithelial cells. The progressive and invasive nature of the parasite-host interaction was striking. Parasites produced deep pits in the corneal epithelium, and exfoliated corneal epithelial cells were numerous. In contrast, none of these processes was evident in dog, horse, cow, mouse, rat, chicken, rabbit, or guinea pig corneas similarly exposed to *Acanthamoeba* in vitro.

The stark absence of parasite binding to corneas from species other than Chinese hamster, pig, or human is noteworthy and emphasizes an important fundamental characteristic of *A. castellani* and perhaps other species of *Acanthamoeba* as well—that is, host specificity at the cellular level. The intimate and specific binding of *Acanthamoeba* to human, Chinese hamster, and pig corneas is an exciting observation that suggests the presence of parasite recognition by species-specific surface molecules on the corneal epithelium. This in turn, raises interesting questions regarding the biology of the host-parasite interaction and offers hints for designing novel therapeutic strategies for preventing corneal infections in contact lens wearers.

**Key words:** *Acanthamoeba*; keratitis; cornea; host-specificity

**Acknowledgments**

The excellent photographic assistance of Ms. Julie Wolff is greatly appreciated. We also thank Mr. Thomas Cunningham for providing valuable technical assistance.

**References**


