THE PATHOGENIC CASCADE OF *ACANTHAMOEBA* KERATITIS

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DEDICATION

To my beautiful wife, Marcie.

THE PATHOGENIC CASCADE OF *ACANTHAMOEBA* KERATITIS

by

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Acanthamoeba keratitis is a blinding infection of the cornea caused by the ubiquitous, free-living amoeba, *Acanthamoeba*. The pathogenic cascade of *Acanthamoeba* keratitis is a sequential process that begins with adherence of trophozoites to the corneal epithelium and culminates in the destruction of the corneal epithelium and the dissolution of the corneal stroma. This work examined the pathophysiology and immunobiology of *Acanthamoeba* keratitis.

First, we explored possible mechanisms to explain why *A. castellanii* remains restricted to the cornea and rarely produces intraocular infections. One hypothesis proposed that trophozoites cannot penetrate Descemet's membrane and the corneal endothelium to

enter the anterior chamber. However, amoebae utilized a mannose-induced serine protease to penetrate Descemet's membrane within 24 hr of *in vitro* culture.

The second hypothesis proposed that the trophozoites can enter the anterior chamber; however, the aqueous humor contains factors that either induce encystment or kill the amoebae. Injection of amoebae into the anterior chamber induced a robust neutrophil infiltrate, which was associated with complete clearance by day 15 post anterior chamber injection. This indicates that neutrophils of the innate immune apparatus are important in preventing *Acanthamoeba* keratitis from progressing to become an intraocular infection.

Previous reports have shown that intracorneal instillation of sterile latex beads results in resistance to *Acanthamoeba* keratitis and mitigation of corneal inflammation. This study examined the mechanisms that could be responsible for the latex bead protective effect. Latex bead treatment induced a significant increase in the infiltration of macrophages into the corneas that peaked at day 4 of infection. Additionally, depletion of conjunctival macrophages with the macrophagicidal drug, clodronate, eliminated the latex bead protective effect, providing further evidence that macrophages are crucial in resistance to *Acanthamoeba* keratitis.

With the exception of mucosal IgA antibody, the adaptive immune apparatus is not typically effective against *Acanthamoeba* keratitis. The results presented here provide evidence that neutrophils and macrophages of the innate immune response are crucial in resistance to *Acanthamoeba* keratitis. Collectively, these results suggest that recruitment and/or activation of the innate immune apparatus may lead to resolution of disease in *Acanthamoeba* keratitis patients.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Review Articles:

Clarke D.W. and Niederkorn J.Y. (2005) Pathophysiology of *Acanthamoeba* keratitis. *Trends in Parasitology*. In press.

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Clarke D.W., Alizadeh H., and Niederkorn J.Y. Intracorneal instillation of latex beads induces macrophage-dependent protection against *Acanthamoeba* keratitis. Manuscript submitted to *Infection and Immunity*.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AC	Anterior chamber
AH	Aqueous humor
AIDS	Acquired immune deficiency syndrome
aPA	*Acanthamoeba* plasminogen activator
APC	Antigen presenting cell
BSA	Bovine serum albumin
CNS	Central nervous system
CPE	Cytopathic effect
Cpm	Counts per minute
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAE	Granulomatous amebic encephalitis
HBSS	Hanks' balanced salt solution
HCN	Human corneal endothelial cells
HCORN	Chinese hamster corneal epithelial cells
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICB	Iris ciliary body cells

Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneally
ISG	IFN-stimulated genes
kDa	Kilodalton
LC	Langerhans cells
MIP-133	Mannose-induced protein 133
MIP-2	Macrophage inflammatory protein 2
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NHK	Normal human keratocytes
NK	Natural killer cells
OD	Optical density
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PHMB	Polyhexamethylene biguanide
PMSF	Phenylmethylsulfonyl fluoride
proMMP	pro-matrix metalloproteinase
PYG	Peptone-yeast-glucose medium
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction

SDS	Sodium dodecyl sulfate
^{35}S	^{35}Sulfur
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factor
^{3}H	^{3}Hydrogen (tritium)
°C	Degrees Celsius

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

THE EYE

The function of the eye is to transmit external visual stimuli to the brain. Light enters through the cornea, which provides approximately 70% of the refractive power of the eye [1]. The light rays are directed by the cornea through the fluid in the anterior chamber (AC), termed aqueous humor (AH), through the pupil (Figure 1). The iris controls the amount of light entering the eye by adjusting the diameter of the pupil. By modifying its form and thickness through a process called accommodation, the lens further focuses the light and projects the image through the semi-viscous vitreous humor of the posterior segment onto the retina. The image formed on the retina is then sent to the brain via the optic nerve.

The cornea is the most external part of the eye and is comprised of several layers (Figure 2). The corneal epithelium, approximately 6 cells deep, is the eye's first barrier to the external environment and potential pathogens [1]. Bowman's membrane, composed of type IV collagen, separates the corneal epithelium from the anterior stroma. The stroma consists of several layers of keratocytes that develop and maintain a collagenous extracellular matrix, composed primarily of type I collagen. Beneath the stroma lies Descemet's membrane, which is composed of basement membrane glycoproteins and collagen types IV and VIII [1]. The corneal endothelium is a single layer of cells between Descemet's membrane and the AC. As mentioned previously, the AC is filled with AH and is bound posteriorly by the iris.

Figure 1. Sagittal section of the eye. The anterior segment of the eye includes the cornea, anterior chamber (filled with aqueous humor), and iris ciliary body. The posterior segment of the eye is composed of vitreous humor and is bound posteriorly by the retina.

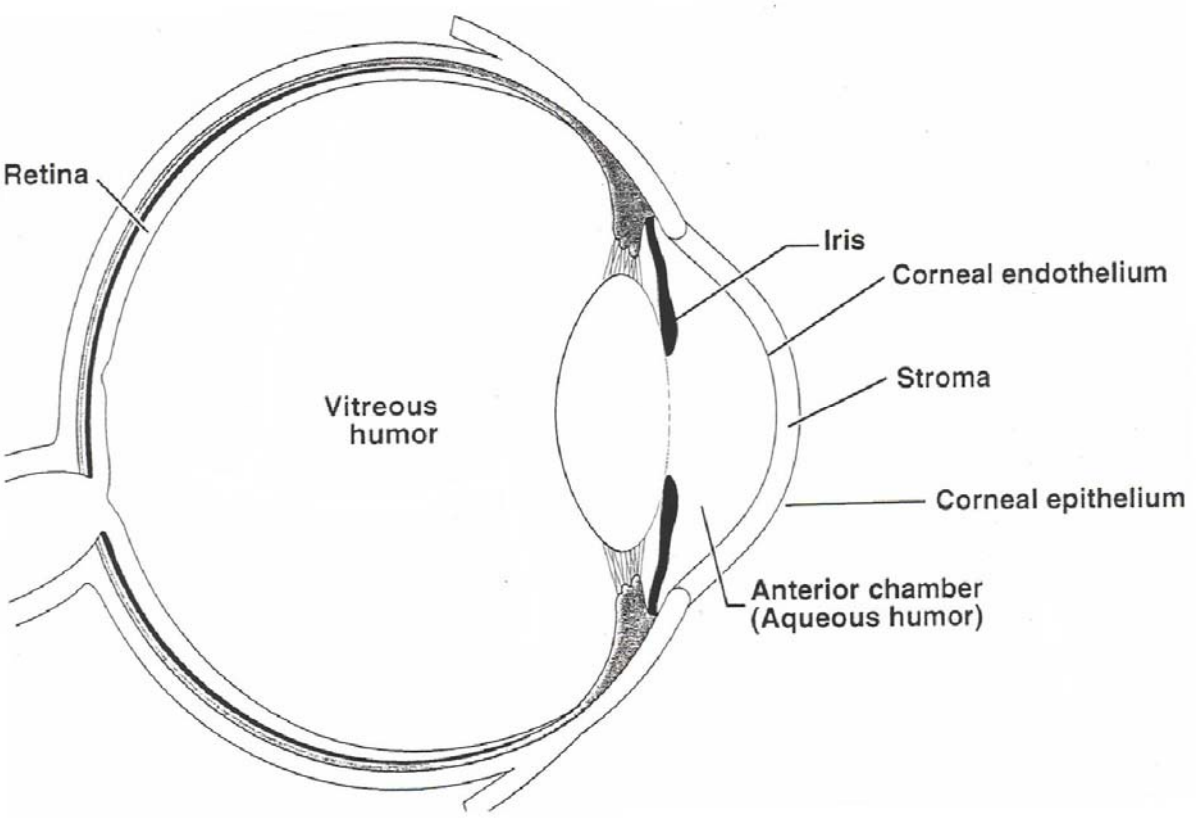
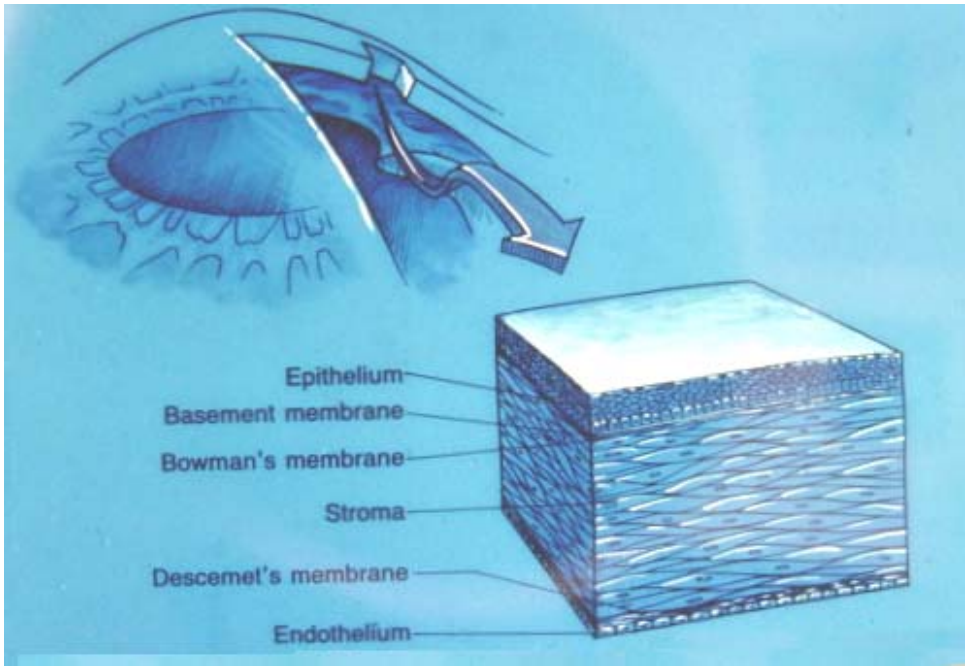


Figure 2. Cross section of the cornea. The cornea is composed of the following components: the epithelium, Bowman's membrane, stroma, Descemet's membrane, and the corneal endothelium. Reprinted with permission from [2].



In addition to focusing incoming light onto the retina, the cornea also functions to protect the ocular surface from foreign material, including environmental pathogens. The corneal epithelium is continuously bathed in tears, which contain antimicrobial proteins, including lysozyme, lactoferrin, defensins, and immunoglobulin (Ig) that eliminate many potential pathogens from the ocular mucosal epithelium [3]. However, when both immune and non-immune mechanisms are unable to remove pathogens from the ocular surface, the resulting corneal infections may lead to severe visual impairment or blindness.

ACANTHAMOEBA TAXONOMY

Acanthamoeba spp. were first described by Castellani in 1930 as contaminants of yeast cultures [4]. After a brief period under the genus *Hartmanella*, Volkonsky re-classified the amoebae under the genus *Acanthamoeba*, based on its double-walled cyst and mitosis characteristics [5,6]. During the 1960s and 1970s, consistent classification by morphological criteria was problematic due to the variant nature of the morphology of the organism [7,8]. Since then, immunological, biochemical, and physiological criteria have also been used to identify different species of *Acanthamoeba* [9]. However, these approaches were also unsuccessful due to the widely-vacillating organism profiles when isolates are grown under different conditions [10]. Today, deoxyribonucleic acid (DNA)-based approaches combined with the morphological groups established by Pussard and Pons have identified over 20 unique species under the genus *Acanthamoeba* [8,11].

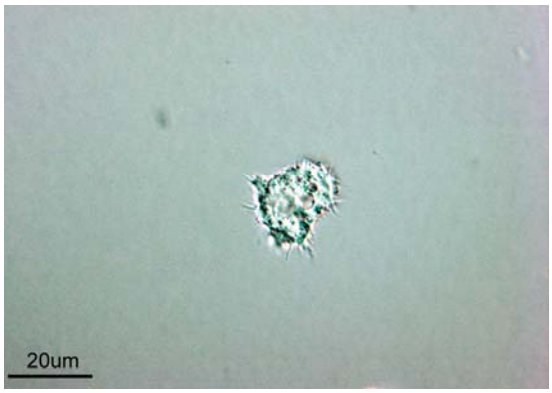
ACANTHAMOEBA BIOLOGY

Acanthamoeba spp. are ubiquitous free-living protozoa that have been isolated from a variety of habitats including soil, air, salt water, fresh water, bottled water, hot tubs, swimming pools, and even eyewash stations [12]. The life cycle of this environmental amoeba consists of two stages: the motile trophozoite (10-25 μm) and the dormant cyst (8-12 μm) (Figure 3). The trophozoite is characterized by a large central nucleus and spine-like pseudopodia. *Acanthamoeba* trophozoites exist under hospitable conditions and normally feed on bacteria and yeast. *Acanthamoeba* spp. can adapt to extreme environmental conditions such as hyperosmolarity, glucose starvation, desiccation, extreme temperatures, or extreme pH by encysting [13]. Moreover, a monoclonal antibody to a 40-kD trophozoite protein induces the encystment of trophozoites and prevents the excystment of cysts, suggesting that cell surface receptors also control *Acanthamoeba* differentiation [14]. The cyst has a bilaminated cellulose wall that tolerates repeated freeze-thawing and very high doses of UV and gamma irradiation [15]. The highly resistant nature of the cyst may enable the organism to survive during harsh environmental conditions, including immunological attack.

ACANTHAMOEBA SPP. AS OPPORTUNISTIC PATHOGENS

Acanthamoeba spp. cause both opportunistic and non-opportunistic infections. The opportunistic infections caused by *Acanthamoeba* spp. include granulomatous amebic

Figure 3. The dimorphism of *Acanthamoeba* spp. Trophozoites are the motile form, while cysts are the dormant form of the organism. Photomicrographs were taken at 60X.



Trophozoite

Cyst

meningoencephalitis (GAE) and cutaneous acanthamebiasis. GAE is a progressive, potentially fatal infection of the central nervous system (CNS), characterized by severe necrosis of the brain [16,17]. GAE is generally associated with immunocompromised individuals, such as those with human immunodeficiency virus (HIV) [18-20]. Inhalation of infectious trophozoites is the primary route of infection; however, GAE can develop if the organism is introduced through skin lesions [21]. Symptoms of CNS infection include headache, confusion, nausea, and stiff neck [20,22]. Unless diagnosed early, GAE usually culminates in the death of the patient.

Acanthamoeba spp. also cause cutaneous infections characterized by erythematous nodules and skin ulcers [23-26]. Although cutaneous acanthamebiasis is most common in patients with acquired immune deficiency syndrome (AIDS), the disease has also been described in patients with amebic encephalitis, in patients undergoing immunosuppressive therapy, and in patients with immunological disorders [19,27-31]. Cutaneous acanthamebiasis without CNS involvement results in the death of 73% of patients, while cutaneous infection with CNS involvement results in a 100% fatality rate [26].

ACANTHAMOEBA SPP. AS NONOPPORTUNISTIC PATHOGENS

Although *Acanthamoeba* spp. can cause GAE and cutaneous acanthamebiasis in immunocompromised patients, it most commonly infects the eyes of immunocompetent individuals to produce *Acanthamoeba* keratitis, a progressive, sight-threatening corneal infection, caused by several species of pathogenic amoebae [12,32]. The first case of

Acanthamoeba keratitis, reported in 1974, occurred when a rancher washed his traumatized eye using water contaminated with the amoebae [33]. Few additional cases were reported until the late 1980s when the incidence of reported *Acanthamoeba* keratitis rose due to increased diagnostic acumen [34]. Since then, the number of reported *Acanthamoeba* keratitis cases has decreased due to awareness of the leading risk factors, contact lens wear and corneal trauma [35]. Animal studies have confirmed this clinical impression as corneal abrasion prior to the application of contact lenses is required for the development of disease in experimental animals [36,37]. Although greater than 80% of the cases of *Acanthamoeba* keratitis occur in contact lens wearers, the incidence of disease is relatively rare, with less than one in 10, 000 contact lens wearers developing *Acanthamoeba* keratitis [35].

Characteristic symptoms of *Acanthamoeba* keratitis include eyelid ptosis, conjunctival hyperemia, epithelial ulcers, lack of discharge, a ring-like stromal infiltrate, and resistance to many antimicrobial agents [12,35,38,39]. One puzzling manifestation of *Acanthamoeba* keratitis is radial neuritis and severe pain, which is not commensurate with the amount of tissue damage [12]. Since *Acanthamoeba* spp. are resistant to many antimicrobial drugs, treatment can be problematic and recurrence of infection occurs. Diagnosis can be difficult, but once identified, effective therapy typically consists of hourly, topical applications of brolene, chlorhexidine, or polyhexamethylene biguanide (PHMB) alone or in combination for several weeks. Mechanistically, PHMB displaces divalent cations that provide structural integrity to the cytoplasmic membrane [40]. Disruption of the outer membrane causes leakage of cellular components and inhibition of essential respiratory enzymes [41,42]. In this manner, PHMB effectively kills both trophozoites and cysts at the

ocular surface. However, even with such therapies, many patients may need corneal transplants to restore vision. The corneal transplants can themselves become infected due to the activation of dormant cysts that can reside in the graft bed [12]. Furthermore, the corticosteroids that are used to prevent immune rejection of corneal transplants are known to induce excystment and increase the cytopathogenicity of the emerging trophozoites [43].

Topical steroids are often used to control the extreme pain and inflammation associated with *Acanthamoeba* keratitis; however, the role of steroids in the treatment of *Acanthamoeba* keratitis is controversial [12]. As mentioned above, corticosteroids exacerbate *Acanthamoeba* keratitis by inducing excystment and stimulating the proliferation and cytopathic activity of trophozoites [43]. Dexamethasone stimulates *Acanthamoeba*-mediated cytopathic effects (CPE) on corneal epithelial cells *in vitro* and increases the incidence, severity, and duration of disease in experimental animals, presumably by inhibition of macrophage and neutrophil activation [43]. Clearly, a better understanding of the pathogenic cascade of *Acanthamoeba* keratitis is crucial in order to develop new treatment strategies for patients.

THE PATHOGENIC CASCADE OF *ACANTHAMOEBA* KERATITIS

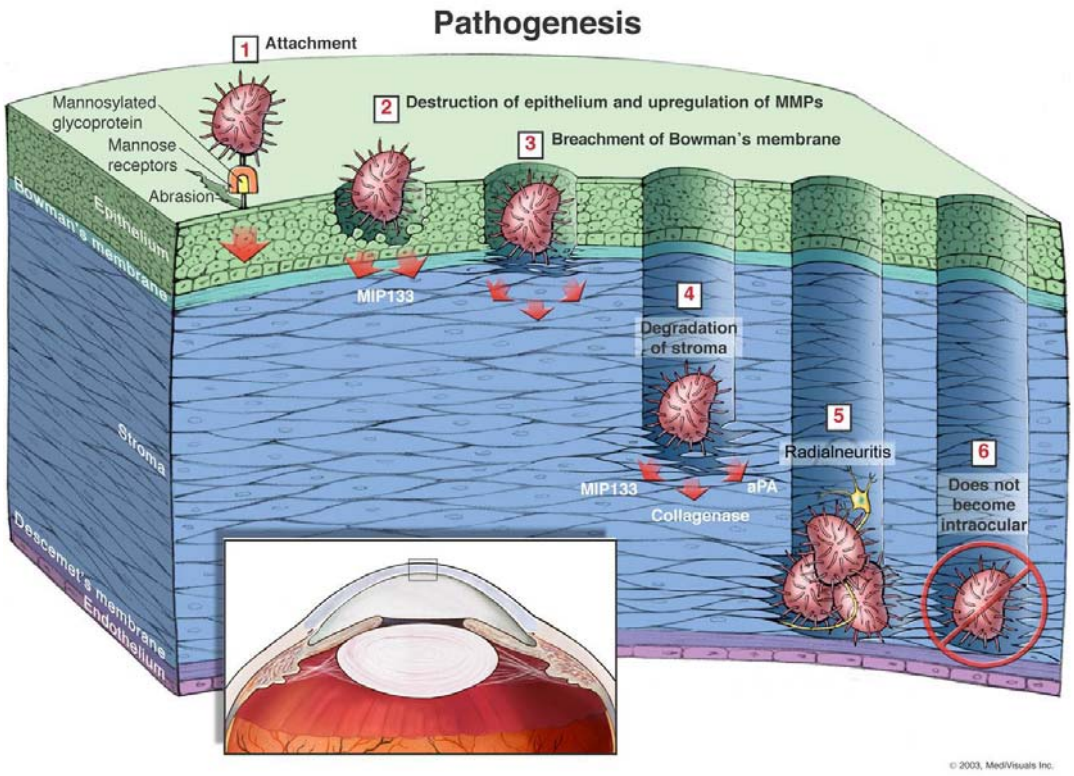
Trophozoite adherence to the corneal epithelium

The pathogenic cascade of *Acanthamoeba* keratitis occurs in a sequential manner beginning with trophozoite adherence to mannosylated glycoproteins on the corneal epithelium, the most external part of the eye, via a 136-kDa mannose-binding protein on the

trophozoite membrane (Figure 4) [44,45]. *In vitro* adherence assays using corneal buttons from 11 different mammalian species revealed that the extent of trophozoite binding to the corneal buttons *in vitro* correlated closely with the susceptibility of each mammalian species to corneal infection *in vivo* [46]. Trophozoite adhesion to the corneal surface is a crucial prerequisite for the establishment of infection in humans, Chinese hamsters, and pigs, which are the most susceptible species to corneal infection with *Acanthamoeba* trophozoites [46]. Thus, the exquisite host specificity seen in *Acanthamoeba* infections is likely the result of species-specific adherence of trophozoites to the corneal epithelium. Recent evidence suggests that the *Leishmania*-sand fly interactions responsible for the species-specificity associated with disease are caused by surface lipophosphoglycan, which mediates promastigote adherence to the mid-gut epithelium [47]. Thus, it is possible that the species-specificity associated with *Acanthamoeba* keratitis is due to the expression of one or more proteins on the corneal epithelium of susceptible hosts.

As mentioned previously, contact lens wear and corneal trauma are the leading risk factors of *Acanthamoeba* keratitis [35]. Moreover, corneal abrasion, prior to the application of *Acanthamoeba*-laden contact lenses, is required for the establishment of infection in animals [36,37]. Experimental animal models have demonstrated that both corneal abrasion and contact lens wear enhance trophozoite binding to the ocular surface, resulting in increased incidence of disease [37,48]. Recent findings suggest that both contact lens wear and corneal trauma upregulate mannose glycoproteins on the corneal epithelium, to which the amoebae adhere with high affinity [48,49]. Thus, contact lenses not only serve as vectors for introducing infectious trophozoites to the cornea, but they also facilitate trophozoite

Figure 4. Schematic of the pathogenic cascade of *Acanthamoeba* keratitis. Reprinted with permission from [50].



adherence to mannosylated glycoproteins on the corneal surface [48]. Mannose, but not other sugars, competitively inhibits trophozoite binding to corneal epithelial cells and blocks invasion of the cornea [45,51].

Trophozoite-mediated cytopathic effects

After binding to the corneal epithelium, the pathogenesis of *Acanthamoeba* keratitis proceeds with trophozoite-mediated destruction of the corneal epithelium and penetration of the underlying Bowman's membrane (Figure 4). *Acanthamoeba* trophozoites implement contact-dependent and contact-independent killing of a wide variety of target cells including corneal epithelial cells, corneal endothelial cells, corneal fibroblasts, iris ciliary body cells, monkey kidney cells, rat glial cells, melanoma, neuroblastoma, and fibrosarcoma *in vitro* [9,35,52,53]. *Acanthamoeba* trophozoites utilize direct cytolysis, phagocytosis, and apoptosis to induce CPE in target cells [12]. Although recent work suggests that *Acanthamoeba* spp. produce amebostome-like structures when co-cultured with hamster corneas or corneal epithelial cells, there is no evidence that *Acanthamoeba*-mediated CPE is due to trogocytosis, the piecemeal ingestion of target cells by amebostomes, as exhibited by *Naegleria fowleri* [54-57]. Instead, *Acanthamoeba*-mediated CPE is dependent on cytoskeletal elements and calcium channel activity [50,54].

Acanthamoeba trophozoites also utilize contact-independent mechanisms that facilitate the destruction of the corneal epithelium. Exposure to mannose on the corneal epithelium induces *Acanthamoeba* trophozoites to release a 133-kDa mannose-induced serine protease (MIP-133), which is highly cytolytic to corneal epithelial cells *in vitro* [58,59]. The

importance of mannose in the establishment and progression of *Acanthamoeba* keratitis was exemplified in experiments in which mannose-coated, *Acanthamoeba*-laden contact lenses created more severe corneal infections than untreated contact lenses, presumably due to the ability of the mannose to induce trophozoites to produce increased amounts of MIP-133 [48]. In addition to promoting the apoptosis of corneal epithelial cells in a caspase 3-dependent pathway *in vitro*, MIP-133 also induces the apoptosis of keratocytes, iris ciliary body cells, retinal pigment epithelial cells (JY Niederkorn *et al*., unpublished), and corneal endothelial cells [59]. Clinical isolates, but not nonpathogenic soil isolates, of *Acanthamoeba* spp. produce MIP-133 and are capable of producing disease in animals, indicating there may be a correlation between MIP-133 production and pathogenic potential [59].

Recent evidence suggests that mannose expressed on the cell wall of *Corynebacterium xerosis*, a constituent of the ocular bacterial flora, may also increase the pathogenicity of *Acanthamoeba* trophozoites [48]. Alizadeh and coworkers demonstrated that *C. xerosi*s induces the release of MIP-133, which promotes increased pathogenicity of trophozoites, both *in vitro* and *in vivo* [48]. Trophozoite-mediated MIP-133 production and subsequent increase in pathogenic potential is specifically due to the high mannose content in the cell wall of *C. xerosis* as exposure to bacteria containing low amounts of mannose in their cell wall does not affect the pathogenicity of the amoebae. Collectively, these results demonstrate that mannose, whether it is on the contact lens, corneal epithelium, or the bacterial flora, increases the pathogenic potential of *Acanthamoeba* trophozoites.

Degradation of the collagenous stroma

After dissolution of the corneal epithelium and breachment of the underlying Bowman's membrane, *Acanthamoeba* trophozoites continue their path of destruction by penetrating and degrading the collagenous stroma (Figure 4) [50]. Pathogenic *Acanthamoeba* spp. produce multiple proteases that assist trophozoites in the degradation of the extracellular matrix and invasion of the corneal stroma including three serine proteases, a cysteine protease, an elastase, a metalloproteinase, and a novel plasminogen activator [9,60-62]. Further support for the notion that *Acanthamoeba* spp. employ enzymes with nonspecific collagenolytic activity was demonstrated in experiments in which intrastromal injections of *Acanthamoeba*-conditioned medium into rat corneas resulted in the generation of ring-like stromal infiltrates and corneal lesions that were characteristic of *Acanthamoeba* keratitis [63]. Moreover, ocular isolates, but not nonpathogenic soil isolates of *Acanthamoeba* spp. constitutively elaborate a novel 40-kDa plasminogen activator, termed *Acanthamoeba* plasminogen activator (aPA) [64]. Plasminogen activators catalyze the cleavage of host plasminogen to form plasmin, which has broad substrate specificity. Specifically, aPA may facilitate the degradation of the extracellular matrix in the stroma by activating pro-matrix metalloproteinases (proMMPs) in the extracellular space [65].

Absence of endophthalmitis

After binding to and desquamating the corneal epithelium, penetrating Bowman's membrane and invading the stroma, the pathogenic cascade of *Acanthamoeba* keratitis appears to stop before invasion of the corneal endothelium and the AC of the eye (Figure 4).

Trophozoites exhibit strong chemotactic responses to corneal endothelial cell extracts and kill corneal endothelial cells *in vitro*; however, unlike other ocular infections, such as *Pseudomonas aeruginosa* keratitis, *Acanthamoeba* keratitis rarely progresses beyond the corneal endothelium to produce intraocular infection, an infection of the ocular tissues that lie beneath the cornea, including the AC (Figure 1) [52,66].

Only a handful of cases of *Acanthamoeba* endophthalmitis have been reported in which *Acanthamoeba* has been shown to be the causative agent of infection and even fewer cases were confirmed to be the result of the natural progression of trophozoite penetration of Descemet's membrane and the corneal endothelium [67-71]. Additionally, *Acanthamoeba* trophozoites are not found in the AC in Chinese hamsters with experimental *Acanthamoeba* keratitis (JY Niederkorn *et al*.; unpublished). In cases reported in 1988 and 1992, two different patients presented with *Acanthamoeba* keratitis and inflamed intraocular tissues; however, there was no demonstration that *Acanthamoeba* trophozoites were present in the intraocular tissues in either case [70,71]. In 1996, a case was reported in which an AIDS patient presented with disseminated cutaneous acanthamebiasis and intraocular infection; however, given the absence of *Acanthamoeba* keratitis, it is likely that the intraocular infection was due to disseminated infection [68]. In 2001, a patient who had received four corneal transplants due to *Acanthamoeba* keratitis presented with intraocular infection [69]. It is possible that this case of endophthalmitis was due to the introduction of trophozoites into the eye at the time of corneal transplantation and not due to the natural progression of the trophozoite beyond the corneal endothelium. To date, it is not known why *Acanthamoeba* keratitis rarely progresses beyond the cornea to produce intraocular infections.

CORNEAL IMMUNITY

Both specific and nonspecific immunity protect the ocular surface against potential pathogens in the external environment. Nonspecific immunity of the cornea includes anatomical, mechanical, physiological, and immunological barriers that protect against infection. In many animals, the eye is recessed and surrounded by bone, reducing the probability of injury. Blinking of the eyelids serves not only to remove foreign materials from the ocular surface, but also to coat the cornea with tears, which contain a variety of antimicrobial factors. The corneal epithelium contains several layers of stratified squamous epithelial cells, which further protect the cornea from injury. Moreover, the lacrimal gland, located in the superolateral corner behind the orbital margin, contains both lymphocytes and plasma cells that respond to corneal infection [72]. The conjunctiva, a mucus membrane that lines the inner surface of the eyelid, also provides protection against pathogens [1]. T-cells, natural killer (NK) cells, and antigen presenting cells (APCs) such as macrophages and Langerhans cells (LC) are housed in the conjunctiva [73-75]. Although the cornea is normally devoid of blood vessels, the cornea becomes vascularized during infection and allows recruitment of these immune cells from the conjunctiva to the central cornea. Furthermore, the conjunctiva contains meibomian glands, which produce the lipid layer of the tear film [76]. Constituents of the ocular bacterial flora present in the conjunctiva may also prevent the establishment of corneal infection by other pathogens [77,78].

The tear film is crucial in maintenance and defense of the ocular surface and contains both specific and non-specific immune elements. Tears moisten the ocular surface,

preventing desiccation of the corneal tissues. As mentioned above, meibomian glands produce cholesterol and lipids to form the outer most layer of the tear film, which is responsible for preventing desiccation of the tear film and ocular surface [79]. The middle, aqueous layer of the tear film is produced by the lacrimal apparatus and contains a variety of anti-microbial proteins including lysozyme, lactoferrin, lactoperoxidase metabolites, β-lysin, interferon, prostaglandins, and complement [74,80-84]. The plasma cells residing in the lacrimal glands also produce antigen-specific secretory IgA antibody [80]. The inner most tear film layer, mucin, is produced by globlet cells of the conjunctiva [76]. This layer attaches the aqueous tear layer to the corneal epithelium, further preventing evaporation of the tear film and dehydration of the cornea.

Due to their exposure to the external environment, the mucosal surfaces of the body are particularly vulnerable to infection. These include the eye, nose, mouth, throat, lungs, and gut [85]. The mucosal immune system is comprised of collections of lymphoid cells in the epithelia and lamina propria of the body's mucosal surfaces and is referred to as the mucosal associated lymphoid tissue (MALT) [86]. Other elements of the mucosal immune system include the bronchial-associated lymphoid tissue (BALT), the gut associated lymphoid tissue (GALT), and the conjunctiva-associated lymphoid tissue (CALT) [87-90]. The MALT's primary function is to trap and concentrate foreign antigen, which is presented to T-cells and B-cells [91]. This results in either tolerance or the generation of antigen-specific T lymphocytes and IgA antibodies [86,92]. Mucosal surfaces preferentially produce secretory IgA antibody locally [80]. Secretory IgA antibody is the most abundant immunoglobulin present in the human body and is the major antimicrobial component in

tears [81,93,94]. Moreover, secretory IgA antibody is the predominant mediator of mucosal defense against microbial pathogens. However, secretory IgA antibody does not effectively fix complement or augment complement-mediated lysis [95]. Protection is typically derived from IgA-mediated inhibition of pathogen adhesion to the mucosal surfaces, including the corneal epithelium [96]. In this manner, secretory IgA prevents the establishment of infection by potential pathogens [97-100].

IMMUNOBIOLOGY OF *ACANTHAMOEBA* KERATITIS

Acanthamoeba spp. are ubiquitous, free-living protozoa that have been isolated from the nasopharyngeal cavities and contact lens cases of asymptomatic individuals [101-104]. Furthermore, serological surveys have shown that as many as 50-100% of asymptomatic individuals possess serum antibodies to *Acanthamoeba*-specific antigens [105-107]. Despite frequent environmental exposure and the prevalence of contact lens wear, the leading risk factor, the incidence of disease is relatively rare with less than one in 10,000 contact lens wearers developing *Acanthamoeba* keratitis [34,108]. The low incidence of disease is puzzling considering the ubiquity of the organism and the millions of contact lens wearers who are at risk for developing infection. This suggests that other risk factors, such as genetic or immune elements, influence the incidence and severity of *Acanthamoeba* infections.

Acanthamoeba keratitis is a self-limiting disease in both the pig and Chinese hamster models of infection [36,37]. However, reinfection and recrudescence can occur, suggesting that elements of innate immunity, not adaptive immunity, may be responsible for resolution

of disease in experimental animals. Experimental evidence supports the notion that elements of innate immunity, such as macrophages and neutrophils, are crucial in the resolution of *Acanthamoeba* keratitis [109,110]. *In vitro* experiments have demonstrated that activated macrophages are chemotactically attracted to *Acanthamoeba* trophozoites and cyst lysate [111,112]. Moreover, activated macrophages are capable of killing trophozoites and cysts *in vitro* [111-113]. Depletion of conjunctival macrophages with the macrophagicidal drug, dichloro-methylene diphosphonate (clodronate), profoundly exacerbated the incidence, severity, and chronicity of infection in experimental animals [109]. Taken together, these results support the hypothesis that macrophages are crucial in the resolution of disease, presumably by direct killing of trophozoites at the ocular surface.

Although clodronate has poor cell membrane permeability, clodronate liposomes are readily phagocytosed by macrophages [114]. In this manner, clodronate-containing liposomes selectively deplete macrophages via phospholipase-mediated disruption of internalized liposomes which leads to the intracellular release of clodronate [115,116]. The exact mechanism of selective clodronate-mediated depletion of macrophages is unknown; however, it is believed that after exceeding an intracellular threshold concentration of clodronate, the macrophage dies by apoptosis [117,118]. Numerous reports have demonstrated that clodronate liposomes selectively deplete macrophages and are not toxic to non-phagocytic cells [116,119,120]. Although neutrophils are phagocytic, clodronate liposomes are not normally phagocytosed by neutrophils and do not deplete neutrophils *in vivo* [119,121-123]. Moreover, neutrophils display normal phagocytic and chemotactic abilities in clodronate-treated BALB/c mice [121,123]. Further, subconjunctival

administration of clodronate liposomes does not deplete corneal LC or lymph node dendritic cells *in vivo* [124].

Histological evaluation of *Acanthamoeba*-infected corneas reveals an abundance of neutrophils in both humans and experimental animals, indicating that neutrophils may be involved in the resolution of *Acanthamoeba* keratitis [38,105,110,125]. Neutrophils are attracted to cyst lysate and are capable of killing both *Acanthamoeba* cysts and trophozoites *in vitro* [113,126,127]. Animal studies have further implicated neutrophils in resistance to *Acanthamoeba* keratitis [110]. Recruitment of neutrophils into the cornea via intrastromal injections with macrophage inflammatory protein 2 (MIP-2), a potent chemotactic factor for neutrophils, resulted in decreased severity and rapid resolution of corneal *Acanthamoeba* infections [110]. By contrast, inhibition of neutrophil migration with subconjunctival injections with neutralizing MIP-2 antibody resulted in increased severity and duration of infection compared to untreated control animals [110]. Likewise, depletion of neutrophils by intraperitoneal injection of anti-Chinese hamster neutrophil antibody significantly exacerbated disease in experimental animals [110].

Recrudescence and reinfection can occur in *Acanthamoeba* patients and experimental animals, indicating that corneal infection does not induce protective immunity [12,128,129]. Corneal infection with *Acanthamoeba* spp. does not elicit either delayed-type hypersensitivity (DTH) or IgG antibody against *Acanthamoeba* antigens in animal models, which is referable to the immune-privileged nature of the eye [130,131]. Although systemic immunization with *Acanthamoeba* antigens elicits DTH and serum IgG antibodies, neither cell-mediated nor serum antibody protects against subsequent ocular challenge with

Acanthamoeba trophozoites, indicating that T cell-mediated immunity and serum antibody do not directly protect against infection [130].

Oral immunization with *Acanthamoeba* antigens promotes the generation of *Acanthamoeba*-specific IgA in mucosal secretions, including tears, and protects against infection in the rabbit, pig, and Chinese hamster models of *Acanthamoeba* keratitis [128,129,132]. Anti-*Acanthamoeba* IgA antibody inhibits trophozoite adherence to corneal epithelial cells *in vitro*, indicating that protection is most likely due to IgA-mediated inhibition of trophozoite adhesion to the corneal epithelium [128,129]. Additionally, oral immunization with the serine protease, MIP-133, promoted the generation of anti-MIP-133 IgA antibody in tears, which mitigated corneal infections in the Chinese hamster model of *Acanthamoeba* keratitis [59]. An alternate mechanism of IgA-mediated protection, provided by Said and coworkers, proposes that IgA reaches the stroma and interacts with the Fcα receptors of neutrophils, thereby augmenting inflammation and clearance of the pathogen in the rabbit model of *Acanthamoeba* keratitis [132]. Collectively, these results suggest that at least one component of the adaptive immune apparatus, IgA antibody, can protect against disease by preventing trophozoite adhesion to the corneal epithelium, by augmenting neutrophil-mediated lysis of trophozoites, and by inhibiting MIP-133-mediated lysis of the corneal epithelial cells and stromal cells.

IMMUNE-MEDIATED DISEASES OF THE CORNEA

Immune responses, including DTH, often cause nonspecific damage to normal tissues, such as the cornea. Immune-mediated diseases are those that elicit immune responses that inadvertently cause nonspecific damage to normal tissues, often exacerbating disease. In order to maintain corneal transparency and prevent blindness, immune-mediated damage to nonregenerating ocular tissues must be minimized. Thus, immune responses in the eye are tightly regulated. Unlike other epithelial surfaces such as the skin, the corneal epithelium is uniquely devoid of mature antigen presenting cells (APC) [133,134]. The absence of APCs in the central corneal epithelium impairs the induction of DTH by antigens and pathogens that reach the ocular surface [135,136]. Although this reduces the risk of DTH-mediated injury to the corneal surface, it also creates an immunological "blind spot" that prevents the full induction and expression of T_H1 immune defense mechanisms. In this manner, the absence of APCs in the central cornea alters the immune response in the eye in order to maintain corneal clarity and prevent blindness [137].

LC are bone marrow-derived APCs that induce T_H1 immune responses such as DTH [138]. Although LC are typically found in epithelial surfaces such as the skin, they are normally absent from the central corneal epithelium and are found only in the corneal periphery (the limbus) [134,138]. It is likely that the absence of antigen-presenting LC in the healthy central cornea reduces the possibility of eliciting a DTH reaction in the cornea, thereby preserving normal corneal function and clarity. However, infections, phagocytic

stimuli, or injection of proinflammatory cytokines such as interleukin-1 (IL-1) can induce the migration of LC from the corneal periphery to the central cornea [139].

Herpes simplex virus keratitis, (HSV) the leading cause of infectious blindness in North America, and trachoma, the leading cause of infectious blindness in developing countries, are immune-mediated diseases [140,141]. In fact, *P. aeruginosa* keratitis, a common bacterial infection of the cornea, and onchocerciasis, a helminth corneal infection, are also immune-mediated diseases [142,143]. In these diseases, the DTH response, which is elicited via T_H1 cells, contributes to the development of blindness [140-143]. Therefore, it is not surprising that induction of LC migration into the central cornea before exposure to HSV exacerbates HSV keratitis by promoting a more robust DTH response [135]. By contrast, the depletion of LC via ultraviolet irradiation results in a milder DTH response and less severe HSV keratitis [135]. Moreover, recent evidence from Hazlett and coworkers demonstrated that induction of LC migration into the central cornea prior to infection resulted in enhanced DTH, inflammation with a predominant macrophage infiltrate, increased mRNA levels for IFN-γ, and exacerbation of *P. aeruginosa* keratitis [136].

The immune response to corneal infection with *Acanthamoeba* spp. does not normally involve DTH or serum IgG antibody against *Acanthamoeba* antigens [130,131]. It was hypothesized that a robust DTH response, triggered by LC in the central cornea, could exacerbate *Acanthamoeba* keratitis resulting in an immune-mediated disease analogous to HSV keratitis or *P. aeruginosa* keratitis [37]. This hypothesis was examined in the Chinese hamster model of *Acanthamoeba* keratitis. Van Klink *et al*. induced the centripetal migration of LC into the central cornea via intracorneal placement of sterile latex beads (a phagocytic

stimulus) [37]. Subsequently, *Acanthamoeba*-laden contact lenses were applied to produce corneal infection [37]. Surprisingly, latex bead treatment reduced both the incidence and severity of *Acanthamoeba* keratitis [37]. Intriguingly, a similar level of protection was observed in animals that received intracorneal injections of IL-1 prior to infection with *A. castellanii* [37]. The mechanism by which corneal latex bead treatment protects against *Acanthamoeba* keratitis remains unknown, but it is speculated that neutrophils and macrophages may be involved, as they have been shown to play an important role in *Acanthamoeba* keratitis infection [109,110].

ANIMAL MODEL

Although a mouse model of *Acanthamoeba* keratitis would allow both immunological and genetic analysis, *Acanthamoeba* keratitis could not be produced in various immunocompetent or immunosuppressed mice [46]. *Acanthamoeba* keratitis has been putatively produced in rabbits and rats; however, infection was produced by injecting trophozoites directly into the corneal stroma and not by the natural route of infection, adherence of trophozoites to the corneal epithelium [132,144-146]. Moreover, co-treatment with corticosteroids or co-injection with *C. xerosis* was required in the rabbit and rat model, respectively. After examining the ability of *Acanthamoeba* trophozoites to adhere to corneal buttons from 11 different mammalian species *in vitro*, Niederkorn and coworkers demonstrated that *Acanthamoeba* trophozoites preferentially bind to human, pig, and Chinese hamster corneas, which are the most susceptible to corneal infection [46]. Contact lens wear

and corneal trauma, the leading risk factors of disease, were necessary to produce keratitis in the Chinese hamster model of *Acanthamoeba* keratitis [37]. The clinical symptoms and pathology of Chinese hamster *Acanthamoeba* keratitis closely resemble those of human *Acanthamoeba* keratitis [37]. However, unlike human infections, which do not resolve without medical intervention, *Acanthamoeba* keratitis resolves spontaneously within 3 weeks in normal Chinese hamsters [37].

OBJECTIVES AND RATIONALE FOR RESEARCH

The pathogenic cascade of *Acanthamoeba* keratitis begins with trophozoite adherence to mannosylated glycoproteins on the corneal epithelium via the amoeba's mannose-binding receptor [44,45,49,147]. *Acanthamoeba* trophozoites penetrate the corneal epithelium and underlying Bowman's membrane by utilizing MIP-133, which is highly cytolytic to corneal epithelial cells *in vitro* [58,59]. Once in the corneal stroma, *Acanthamoeba* trophozoites continue to elaborate a variety of proteases, including MIP-133, that facilitate invasion and dissolution of the corneal stroma [59,63,64,148]. However, there have been few reports of intraocular infection in the literature and it is questionable that each case was due to the natural progression of the trophozoite beyond Descemet's membrane and the corneal endothelium [67-71]. It is unknown why *Acanthamoeba* keratitis rarely progresses beyond the corneal endothelium to produce intraocular infection and endophthalmitis [69].

Although the central cornea has no resident APCs, previous studies have shown that intracorneal instillation of sterile latex beads induces the migration of LC into the central

cornea [149]. In other infectious diseases of the cornea, such as HSV keratitis and *P. aeruginosa* keratitis, the induction of LC into the central cornea exacerbates disease by promoting a more severe DTH response via T_H1 cells [135,136]. However, induction of LC migration into the central cornea prior to infection as a means of promoting DTH responses to *Acanthamoeba* antigens did not exacerbate *Acanthamoeba* keratitis as anticipated, but instead, resulted in a remarkable resistance to infection and a mitigation of corneal inflammation [37]. The mechanism by which corneal latex bead treatment protects against *Acanthamoeba* keratitis remains unknown. The adaptive immune apparatus is not usually effective against *Acanthamoeba* keratitis, as neither T cell-mediated nor humoral immunity protects against disease [130]. Furthermore, the latex bead protective effect is observed within four days of exposure to *Acanthamoeba* trophozoites. Thus, if the immune apparatus is involved in the latex bead protective effect, it is likely the innate immune apparatus, and not the adaptive immune apparatus that mediates protection.

This dissertation addressed two specific aims. The first specific aim was to determine the mechanism of the latex bead protective effect associated with *Acanthamoeba* keratitis. One hypothesis proposed that latex bead treatment decreased binding of trophozoites to the corneal epithelium. The second hypothesis proposed that latex beads protected corneal epithelial cells or corneal stromal cells against trophozoite-mediated cytolysis. A final hypothesis proposed that latex beads provide protection by recruiting and/or activating cells of the innate immune system, including neutrophils and macrophages, which can kill trophozoites [111,112,126,127].

The second specific aim was to determine why *A. castellanii* does not progress beyond the cornea to produce intraocular infections. The first hypothesis proposed that trophozoites cannot penetrate Descemet's membrane and the corneal endothelium to enter the AC. The second hypothesis proposed that the amoebae can enter the AC; however, the AH contains factors that either induce encystment or kill the amoebae.

Although the incidence of *Acanthamoeba* keratitis is extraordinarily low, invasive amoebae affect 50 million people world-wide and it has been estimated that over 40,000 die each year from *Entamoeba histolytica* infections [150]. In many ways, the pathogenic cascade of *E. histolytica* parallels that of *Acanthamoeba* keratitis; this is, infection is initiated by lectin-mediated adhesion to glycoproteins expressed on the intestinal epithelial cells and invasion is facilitated by amoeba-borne proteases [150]. Thus, a greater understanding of the pathogenic cascade of *Acanthamoeba* keratitis could lead to improved clinical treatment of this and other amoebic infections, such as those caused by *E. histolytica*.

CHAPTER TWO
MATERIALS AND METHODS

Animals

Chinese hamsters (17A strain) of mixed gender were purchased from Cytogen Research and Development, Inc. (West Roxbury, MA) and used at 4 to 6 weeks of age. All corneas were examined before experimentation to exclude animals with preexisting corneal defects. Animals were handled in accordance with the Association of Research in Vision and Ophthalmology "Statement on the Use of Animals in Ophthalmic and Vision Research."

Amoebae and cell lines

Acanthamoeba castellanii ATCC 30868, originally isolated from a human cornea, was obtained from the American Type Culture Collection (Manassas, VA). Trophozoites were grown as axenic cultures in peptone-yeast-glucose (PYG) medium at 35°C as described previously [37,151]. To confirm that the pathogenicity of *Acanthamoeba castellanii* ATCC 30868 did not decrease during growth as axenic cultures, we examined the ability of the trophozoites to produce MIP-133 approximately once every three months. Additionally, we examined the ability of *Acanthamoeba castellanii* ATCC 30868 to produce disease in Chinese hamsters approximately once every three months. In the event that the pathogenicity of *Acanthamoeba castellanii* ATCC 30868 decreased, the original isolate was obtained from freeze.

Human corneal endothelial cells (HCN) were isolated from a corneal explant and immortalized with human papillomavirus E6 and E7 genes, as described previously [152].

HCN were cultured in complete Eagle's minimal essential medium (EMEM; BioWhittaker, Walkersville, Maryland) containing 1% L-glutamine (BioWhittaker), 1% penicillin, streptomycin, amphotericin B (Fungizone; BioWhittaker), 1% sodium pyruvate (BioWhittaker), and 10% fetal calf serum (FCS; HyClone Laboratories , Logan UT).

Primary human iris/ciliary body cells (ICB) were harvested from a corneal scleral rim and cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) containing 1% L-glutamine, 1% penicillin, streptomycin, amphotericin B, 1% sodium pyruvate, and 10% human serum type AB (Sigma-Aldrich, St. Luis, MO).

Chinese hamster corneal epithelial cells (HCORN) were immortalized with human papillomavirus E6 and E7 genes, as previously described [153] and cultured in complete Eagle's minimum essential medium (EMEM; BioWhittaker, Walkersville, MD) containing 10 mM HEPES buffer solution, 1% nonessential amino acids solution, 1% L-glutamine (BioWhittaker), 1% penicillin, streptomycin, amphotericin B (Fungizone; BioWhittaker), 1% sodium pyruvate (BioWhittaker), and 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT).

Normal human keratocytes (NHK) were a generous gift from Dr. James Jester at The University of California at Irvine (Irvine, California). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) containing 10 mM HEPES buffer solution, 1% nonessential amino acids solution, 1% L-glutamine, 1% penicillin, streptomycin, amphotericin B, 1% sodium pyruvate, and 10% fetal calf serum.

The monocyte-macrophage cell line originally derived in BALB/c mice, RAW 264.7 cells (TIB 71) were obtained from American Type Culture Collection (ATCC), Rockville

MD. Cells were cultured in complete RPMI-1640 (BioWhittaker) containing 10 mM HEPES buffer solution, 1% nonessential amino acids solution, 2 mM L-glutamine, 1mM sodium pyruvate solution, 1% penicillin, streptomycin, amphotericin B, and 10% fetal calf serum.

BALB/c corneal epithelial cells and BALB/c keratocytes were established as described previously [154]. Briefly, corneal cells were isolated from corneal explants and immortalized with the human papillomavirus E6 and E7 genes [152]. Cells were cultured in complete RPMI-1640 (BioWhittaker) containing 10 mM HEPES buffer solution, 1% nonessential amino acids solution, 2 mM L-glutamine, 1mM sodium pyruvate solution, 1% penicillin, streptomycin, amphotericin B, and 10% fetal calf serum.

Intracorneal instillation of latex beads

Latex beads (1.0 µm; Polysciences, Inc., Warrington, PA) were deposited into shallow corneal epithelial incisions as a reproducible method to induce centripetal LC migration into the center of the corneal epithelium [37,139]. Briefly, hamsters were deeply anesthetized as described previously [37]. Next, 10 µl of sterile 1.0 µm polystyrene beads suspended in phosphate-buffered saline (PBS) were deposited into shallow incisions in the center of the corneal epithelium 10 days prior to infection.

Adherence assay

The ability of *Acanthamoeba* trophozoites to adhere to latex bead-treated corneas was examined using an assay modified from van Klink et al. [37]. Briefly, hamsters were sacrificed 10 days post latex bead treatment and eyes were enucleated. The corneal

epithelium was abraded immediately prior to enucleation in all groups. Normal, abraded, but otherwise untreated eyes served as controls. Eyes were sterilized in 10% iodine in water for 1 min and rinsed in saline. Subsequently, the eyes were placed into 200 µl pipette-tips (eye-cups), cornea side up. The eyes became wedged in the eye-cups such that the corneal surface served as the bottom of the cup. *Acanthamoeba castellanii* trophozoites were metabolically labeled as described previously [153]. Briefly, trophozoites (5×10^6/ml PYG) were cultured with 100 µCi of [^{35}S] methionine (ICN Biomedicals, Inc., Irvine, CA.) overnight at 35°C. Radiolabelled trophozoites were washed three times in PYG and added to each eye-cup at 10^5 trophozoites/eye. Controls consisted of trophozoites incubated in the presence of 100mM mannose on untreated corneas. Adherence of trophozoites to corneas was determined after 2 hr of incubation at 35°C. The eye-cups were washed three times with PYG to remove unbound amoebae. The eyes were then transferred to scintillation vials containing 2 ml of Ready-Gel scintillation cocktail (Becton Dickson, Fullerton, CA), and counts were measured in a Beckman LS3801 liquid scintillation counter. The results are expressed as percent adherence with 100% corresponding to the number of trophozoites that bound to untreated corneas.

The ability of *Acanthamoeba* trophozoites to adhere to latex bead-treated HCORN cells was examined using a modified assay [153]. Briefly, HCORN cells were grown to confluency in a 96-well plate. Latex beads were added to the indicated wells at a concentration of 1,000 beads/cell and incubated for 24 hr. *Acanthamoeba* trophozoites were radiolabelled with 100 µCi of [^{35}S] methionine overnight at 35°C. Radiolabelled trophozoites were washed three times in PYG and added to wells at 10^5 trophozoites/ well.

Adherence of trophozoites to HCORN cells was determined after 2 hr of incubation at 35°C. The wells were washed three times with PYG and solubilized in 100 ul of 5% SDS. The fluid was transferred to scintillation vials containing 2 ml of Ready-Gel scintillation cocktail and counts were measured on the scintillation counter.

Assay for cytopathic effects

The CPE of trophozoites on corneal cells was determined with a previously described photometric assay [58]. Briefly, latex beads were added to confluent monolayers of HCORN or NHK cells to obtain a concentration of 10 beads/cell. Latex beads were not added to control wells. Trophozoites (5×10^3, 5×10^4, or 5×10^5) were then added to the confluent monolayers and incubated for 48 hr at 37°C. Wells were washed in their respective growth medium and stained with the Hema 3 Manual Staining System (Fisher Scientific, Pittsburgh, PA). After staining, the wells were washed three times with PBS (pH 7.2) and solubilized in 0.1 ml of 5% sodium dodecyl sulfate (SDS) in PBS. Solubilized cells were transferred to a new 96-well plate, and the optical density (OD) was read at 590 nm in a microplate reader (Molecular Devices, Menlo Park, CA). The OD of trophozoites alone was subtracted from the OD of experimental wells to determine the OD of live cells. In addition, trophozoites (2.5×10^4) were added to 96-well plates, with or without confluent monolayers of HCN or ICB and incubated for 48 hr at 37°C. Wells were Giemsa stained (Shandon, Inc., Pittsburgh, PA) and the OD was read at 590 nm in a Microplate Reader. The OD of trophozoites alone was subtracted from the OD of experimental wells to determine the OD of live cells. Percent

of cells alive was calculated according to the following formula: Cells alive (%) = (OD of experimental well/OD of control well) X 100.

Contact lens preparation

Miniature contact lenses were prepared from Spectra/Por dialysis membrane tubing (Medical Industries, Los Angeles, CA) using a 3-mm trephine [37]. After heat sterilization, lenses were placed in sterile 96-well microtiter plates (Costar, Cambridge, MA) and incubated with 3 x 10^4 *A. castellanii* trophozoites at 35°C for 24 hr in 200 µl of PYG. Attachment of amoebae to the lenses was verified microscopically before the lenses were applied to the Chinese hamster corneal surface.

***In vivo* corneal infections**

Acanthamoeba keratitis was induced in Chinese hamsters as described previously [37]. Briefly, Chinese hamsters were anesthetized with ketamine (100mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) injected peritoneally and corneas were anesthetized by topical application of Alcain (Alcon Laboratories, Fort Worth, TX). Approximately 25% of the cornea was abraded using a sterile cotton applicator, and then amoeba-laden lenses were placed onto the center of the cornea. The eyelids were then closed by tarsorrhaphy using 6-0 Ethicon sutures (Ethicon, Somerville, NJ). The contact lenses were removed 4 days post-infection, and the corneas were visually inspected utilizing a dissecting microscope. Visual inspection results were recorded daily during the times indicated and infections were scored based on the degree of corneal infiltration, corneal neovascularization, and corneal ulceration

[110]. The pathology was recorded as follows, as described previously [110]: 0, no pathology; 1, <10% of the cornea involved; 2, 10 to 25% of the cornea involved; 3, 25 to 50% of the cornea involved; 4, 50 to 75% of the cornea involved; and 5, 75 to 100% of the cornea involved. The results are expressed as clinical severity of keratitis (%). In Chinese hamsters, *Acanthamoeba* keratitis resolves in approximately 3 weeks. By this time, the cornea is devoid of edema, epithelial defects and stromal necrosis and inflammation.

Production of anti-Chinese hamster neutrophil antiserum

Anti-Chinese hamster neutrophil antiserum was produced as described previously [110]. Briefly, Chinese hamsters were injected with 3 ml of 3.0% thioglycollate intraperitoneally (IP). Four hours post-injection, hamster peritoneal exudate was collected by peritoneal lavage with 10 ml of HBSS. The peritoneal exudate was layered onto 3 ml of Histopaque (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1, 500 X *g* for 30 min. The neutrophilic band was collected using a Pasteur pipette and the cells were washed three times in HBSS and resuspended in PBS. Cells were stained with the Hema 3 system (Fisher Scientific, Pittsburgh, PA) to confirm neutrophil purity of >95%. Trypan blue exclusion test confirmed that >95% of the cells were viable.

Neutrophils (10^6) suspended in phosphate buffered saline (PBS) were mixed 1:1 with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected intramuscularly into a New Zealand White rabbit. Additional injections were performed without adjuvant approximately once a week for 8 weeks. Blood was collected by cardiac puncture and allowed to clot overnight at 4°C. Serum was removed from the clot and centrifuged at 2,000

X *g* for 10 min at 4°C. Blood was also collected from a naïve rabbit and processed as stated above. Sera were stored at -20°C.

Serum absorption

Anti-Chinese hamster antiserum was absorbed as described previously [110]. Briefly, Chinese hamster spleens were harvested and ground between two glass slides. Erythrocytes were lysed and remaining cells were strained through a sterile Falcon cell strainer (Becton Dickinson and Company, Franklin Lakes, NJ), and suspended in HBSS supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) at 10^7 cells/ml. Antiserum was absorbed for 2 hr at 4°C with Chinese hamster spleen cells to remove antibodies against Chinese hamster histocompatibility and lymphoid antigens. The absorbed serum was then centrifuged at 1,700 x *g* for 10 min and the antiserum was removed and tested for cytotoxicity to neutrophils *in vivo*.

***In vivo* cytotoxicity assay**

Chinese hamsters were injected with 1.0 ml of anti-Chinese hamster antiserum or normal rabbit serum IP. Blood was collected from tail veins at 0, 2, and 4 hr post-injection and streaked on glass slides. Slides were stained with the Hema 3 Manual Staining System for histological examination. Neutrophils and lymphocytes were counted in 10 random fields per slide. The results are expressed as mean number of cells per five high power fields.

Complement-mediated cytotoxicity assay

The efficacy of the anti-neutrophil antibody was examined in a cytotoxicity assay as described previously [110]. Antiserum was heat-inactivated at 56°C for 1 hr and diluted 1:50 and 1:100 in HBSS. Neutrophils or splenocytes were added to a 96-well plate at $2x10^5$ cells/well in a total volume of 200 µl. Neutrophils and splenocytes were incubated for 1 hr at 37°C in either antiserum or normal serum. The plates were centrifuged at 380 X *g* for 15 min, washed twice in HBSS-BSA and resuspended in 200 µl of HBSS-BSA. Low-Tox M rabbit complement (Accurate Chemical Co., Westbury, NY) diluted 1:10 in HBSS-BSA was added to each well and the plates were incubated for 30 min at 37°C. The cells were then washed three times in HBSS-BSA and resuspended in 100 µl of HBSS-BSA, and cell viability was determined by trypan blue exclusion.

Anti-Chinese hamster neutrophil antibody treatment

Hamsters were administered anti-Chinese hamster neutrophil antiserum or normal rabbit serum as described previously [110]. Briefly, hamsters were injected IP with 0.5 ml of absorbed serum twice daily for seven days beginning 3 days prior to infection. The hamsters were treated with topical applications of bacitracin to prevent bacterial infection for 14 days post-infection.

Preparation of clodronate liposomes

Multilamellar liposomes were prepared as described previously [109,116]. Briefly, 8 mg of cholesterol (Sigma-Aldrich) and 86 mg of phosphatidylcholine (Sigma-Aldrich) were

dissolved in 10 ml of chloroform in a round-bottomed flask. After low-vacuum rotary evaporation at 37°C, a thin film formed on the inner surface of the flask. This film was then dispersed by gentle rotation overnight at 37°C in a mixture containing 2.5 g of dichloro-methylene diphosphonate (clodronate; Roche Diagnostics, Alameda, C A) dissolved in 10 ml of PBS. To remove free clodronate, liposomes were then washed twice in PBS by centrifugation at 10, 000 x *g* for 30 min and resuspended in 4 ml of PBS. PBS containing liposomes were prepared similarly. Clodronate liposomes and PBS liposomes were stored at 4°C and used within 14 days of preparation. Clodronate liposomes were tested for *in vitro* toxicity against macrophages prior to use. Clodronate liposomes selectively deplete macrophages, and are not toxic to other phagocytic cells [116,120]. Clodronate was not toxic to Langerhans cells *in vitro* (data not shown). Liposomes ranged from 100 nm to 3 µm in diameter.

Clodronate liposome treatment

Clodronate-containing liposomes (50 µl) were administered via subconjunctival injection on days -9, -7, -5, and -3 prior to infection with *Acanthamoeba*-laden lenses [109]. Clodronate liposomes were injected subconjunctivally into four quadrants of the eye encircling the entire conjunctiva. This protocol successfully depletes conjunctival macrophages in the Chinese hamster [109]. Van Klink and coworkers showed that injection of PBS-containing liposomes did not deplete resident macrophages or alter the severity of disease [37].

Quantification of NO synthesis

Macrophage activation was quantified by measuring nitric oxide (NO) production [113]. RAW cells were plated at $5x10^4$ cells/well in a 24 well plate and allowed to grow to confluency. As a positive control for activation, the indicated wells were stimulated with 2 µg/ml LPS (Sigma-Aldrich) and100 ng/ml IFN-γ (BD Biosciences Pharmingen) as described previously [111]. To determine if latex beads induced macrophages to produce NO, 1.0 µm latex beads were added to the indicated wells to obtain concentrations of 100, 1,000, or 5,000 beads/cell. After 24 and 48 hr of incubation at 37°C, 100 µl of supernatant was removed and assayed for nitrite, a stable reaction product of NO. Supernatants were then incubated with 100 µl of Griess reagent (Sigma-Aldrich) in a 96-well flat bottom plate for 10 min, and the OD was read at 590 nm in a microplate reader as described previously [113]. To determine NO concentration, a standard curve of NO production was generated by incubating serial dilutions of sodium nitrite (Sigma-Aldrich) with Griess reagent. Results are expressed as µM of NO.

To determine if latex beads induced corneal epithelial cells to produce a factor that activates macrophages, BALB/c corneal epithelial cells were grown to confluency in a 24-well plate. Latex beads (1.0 µm) were added to the indicated wells to obtain concentrations of 10, 100, and 1,000 beads/cell. After 24 hr of incubation at 37°C, the supernatant was collected and added to RAW cells in a 24-well plate. As a positive control for activation, the indicated wells of RAW cells were stimulated with 2 µg/ml LPS (Sigma-Aldrich) and100 ng/ml IFN-γ. After 24 and 48 hr, supernatant was removed and assayed for NO activity as

described above. The same method was utilized to determine if latex bead treatment induced BALB/c corneal keratocytes to produce a factor that activates macrophages.

As both IFN-γ and LPS are required to induce macrophages to produce NO, the macrophage activation assays were also performed with corneal cell supernatant in the presence of LPS. Briefly, 2 µg/ml LPS was added to macrophages at the time of addition of latex bead-treated corneal cell supernatant. Both BALB/c corneal epithelial cell supernatant and BALB/c keratocyte supernatant was examined in this manner. The macrophage cell supernatant was then collected at 24 and 48 hrs and assayed for NO activity as described above.

Macrophage-mediated lysis of *A. castellanii* trophozoites

The ability of latex bead-treated macrophages to lyse trophozoites was determined using a modified [^{3}H] uridine (Amersham biosciences, Piscataway, NJ) release assay [112]. Briefly, 10^5 trophozoites in 1 ml PYG were labeled with 5µCi [^{3}H] uridine for 24 hr at 35°C. RAW cells were plated in a 24-well plate at 10^5/well. Various dilutions of latex beads were incubated with RAW cells for 24 hr. As a positive control for macrophage-mediated killing, 100 ng IFN-γ and 2 µg LPS were added to control wells. Radiolabeled trophozoites were washed three times in PYG before addition (10^4 trophozoites/well) to each well of macrophages in the 24-well plate. The final volume was 200 µl PYG and 300 µl RPMI. The plates were incubated at 37°C for 24 hr and then centrifuged for 10 minutes at 380 X *g*. The supernatant was collected from each well and was transferred to scintillation vials containing 2 ml of Ready-Gel scintillation cocktail (Becton Dickson, Fullerton, CA). Counts per minute

(cpm) were measured in a Beckman LS3801 liquid scintillation counter. Spontaneous release and total release were included as controls. % killing = cpm (supernatant) – cpm (spontaneous release) / [cpm (total release) – cpm (spontaneous release)] X 100%.

To determine if latex beads induced corneal epithelial cells to produce a factor that increases the ability of macrophages to kill trophozoites, BALB/c corneal epithelial cells were grown to confluency in 24-well plates. Latex beads (1uM) were added to the indicated wells to obtain concentrations of 10, 100, and 1,000 beads/cell. After 24 hr of incubation at 37°C, the supernatant was collected and added to RAW cells in a 24-well plate. As a positive control for macrophage-mediated killing, wells of RAW cells were stimulated with 2 µg/ml LPS (Sigma-Aldrich) and 100 ng/ml murine IFN-γ. Radiolabeled trophozoites were added to each well and the plates were incubated for 24 hr as described above. After centrifugation, the supernatant was collected and the cpm were determined in a scintillation counter. The same method was utilized to determine if latex bead treatment induced BALB/c corneal keratocytes to produce a factor that activates macrophages.

Because both IFN-γ and LPS are required to induce macrophages to kill trophozoites, the macrophage-mediated killing assays were also performed with corneal cell supernatant in the presence of LPS. Briefly, 2 µg/ml LPS was added to macrophages at the time of addition of latex bead-treated corneal cell supernatant. Both BALB/c corneal epithelial cell supernatant and BALB/c keratocyte supernatant was examined in this manner. Radiolabeled trophozoites were added to each well and macrophage-mediated killing was determined as described above.

Analysis of IFN-γ gene and protein expression

To determine if latex beads induced corneal epithelial cells to produce IFN-γ, BALB/c corneal epithelial cells were grown to confluency in a 24-well plate. Latex beads (1.0 μm) were added to the indicated wells to obtain concentrations of 1, 10, and 100 beads/cell. After 24 hr, 48 hr, and 72 hr of incubation at 37°C, corneal cell messenger RNA (mRNA) was analyzed using reverse transcriptase–polymerase chain reaction (RT-PCR) for IFN-γ expression modified from a previous report [155]. Briefly, mRNA from bead-treated BALB/c corneal epithelial cells were isolated using Oligotex Direct mRNA Mini Kit (QIAGEN Inc, CA). The samples were treated with DNase I (0.2 U/μl; Ambion, Austin, TX) to remove possible DNA contamination. RT-PCR was performed using SuperScript One-Step RT-PCR with Platium Tag (Invitrogen, CA) according to the manufacturer's instructions. Relatively equal amounts of mRNA (50ng) were used for this reaction. Cycle parameters were as follows: 1 cycle of 45-55°C for 15-30 min and 94°C for 2 min for cDNA synthesis and predenaturation; 35 cycles of: 94°C for 30 s, 60-64°C for 30 s, 68-72°C for 1 min for PCR amplification; and 1 cycle of 72°C for 7 min for final extension. The PCR products were visualized in 2.0% agarose gels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. The IFN-γ and GAPDH primers were obtained from R&D systems, Inc. (Minneapolis, MN). The same method was utilized to determine if latex bead treatment induced BALB/c corneal keratocytes to produce IFN-γ. T cells stimulated with 5 μg/ml of phytohemagglutinin (PHA) for 72 hr served as positive control for the production of IFN-γ mRNA. T cells were harvested from BALB/c mice as

previously described [156]. Briefly, spleens were harvested from BALB/c mice and erythrocytes were lysed. T cells were collected by incubating the cell suspension on scrubbed nylon wool (Fenwal Laboratories, Deerfield, IL) at 37°C for 1 hr. Subsequently, the T cells were eluted with 30 ml of HBSS. T cell IFN-γ mRNA was quantified as described above.

In order to confirm that latex bead treatment does not induce corneal cells to produce IFN-γ *in vitro*, latex beads (1.0 μm) were added to BALB/c corneal epithelial cells to obtain concentrations of 1, 10, and 100 beads/cell. Latex bead-treated corneal cell supernatant was collected at 24 hr, 48 hr, and 72 hr and analyzed for IFN-γ protein. Enzyme-linked immunosorbent assays (ELISA) for mouse IFN-γ were performed on culture supernatants according to the manufacturer's instructions (Quantikine ELISA kits; R&D Systems, Minneapolis, MN). The same method was utilized to determine if latex bead treatment induced BALB/c corneal keratocytes to produce IFN-γ.

Acid phosphatase staining

To determine if there were more macrophages present in latex bead-treated, *Acanthamoeba*-infected corneas, compared to untreated, infected corneas, eyes were processed and stained for macrophage-endogenous acid phosphatase activity as described previously [109,124,136]. Reports have indicated that osteoclasts, macrophages, and other cells of monohistiocytic lineage express acid phosphatase [157,158]. Importantly, neutrophils and other leukocytes that may be present in the cornea do not stain positively with acid phosphatase [109,124,136]. Thus, the acid phosphatase stain can be used to

specifically stain macrophages in the cornea. Briefly, Chinese hamster corneas were infected with *Acanthamoeba castellanii* with or without prior latex bead treatment as described above. Infected eyes of bead-treated and untreated Chinese hamsters (n=5/group) were enucleated 3, 4, and 5 days post-infection. The enucleated eyes were washed once in PBS and embedded in optimal cutting temperature media (OCT compound; Miles Scientific, Naperville, IL) in a plastic dish. Subsequently, the eyes were snap-frozen in isopentane cooled with liquid nitrogen, and stored at -80°C until use. Meridional cryostat sections (8 μm) were made in the middle of the eye at -20°C and collected on poly-L-lysine-coated slides (Polysciences). Sections were air dried for 10 min, fixed in cold acetone for 10 min, and air dried for 10 min. To demonstrate endogenous acid phosphatase activity, slides were incubated with naphthol AS-BI phosphate and pararosaniline for 35 min at 37°C [159]. Frozen spleen sections were used as a positive control for acid phosphatase staining. All slides were counterstained with 0.5% methyl green in 0.1 M sodium acetate for 1 min. Positively reacting cells appeared pink or red. Acid phosphatase-positive cells were counted via light microscopy by three masked observers.

Chicken anti-MIP-133 antiserum

Chicken anti-MIP-133 antiserum was generated by Aves Labs, Inc. (Aves Labs, Inc.; Tigard, OR). The specificity of the chicken anti-MIP-133 IgY (Aves Labs Inc., Tigard, Oreg.) was confirmed via western blotting and ELISA as described previously [59]. Anti-MIP-133 antiserum inhibits the CPE of purified MIP-133 against human and Chinese

hamster corneal epithelial cells *in vitro* and effectively blocks trophozoite migration through Matrigel collagenous matrices (Collaborative Biomedical Products, Bedford, Mass) [59].

Migration assays

To determine if *Acanthamoeba* trophozoites can penetrate Descemet's membrane, a modified transwell migration assay was used. First, approximately 200 µl of PYG was placed in the bottom chamber of a Boyden chamber (Costar Corp., Cambridge, MA). Corneas were removed from pig eyeballs (Owen's, Dallas, TX) and placed in 5% Dispase (Gibco Invitrogen Corp., Carlsbad, CA) in PBS (pH 7.2) and incubated at 37°C for 30 min. Descemet's membrane was peeled from pig corneas, washed, and placed on a nitrocellulose membrane (13 mm diameter, 3.0 µm pore size; Millipore Corp., Bedford, MA) in the Boyden chamber. *Acanthamoeba* trophozoites (10^5) were placed in the top chamber in 200 µl PYG and incubated at 37°C for 24 hr. Subsequently, the contents of the top chamber were removed along with Descemet's membrane. The bottom chamber was vigorously washed with PYG to be certain that trophozoites did not adhere to the sides of the Boyden chamber. *Acanthamoebae* in the bottom chamber were then counted using a hemocytometer. Trophozoite viability was ascertained by trypan blue exclusion. Inhibition assays involved incubating *Acanthamoeba* trophozoites with the serine protease inhibitors, 1.0 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 1.0 mM 1, 10-phenanthroline (1, 10 P.; Sigma-Aldrich). A cysteine protease inhibitor, cystatin (10 µM Cystatin; Sigma-Aldrich), was also used. In similar assays, a 1:100 dilution of chicken anti-MIP-133 antiserum (0.2 mg/ml) [59] or a 1:100 dilution of chicken preimmune serum (0.2 mg/ml) was added to trophozoites 30 min prior to their addition to the upper chambers.

To ensure that the protease inhibitors were nontoxic to trophozoites, 10^6 trophozoites were incubated in 1.0 ml of PYG with either 1.0 mM PMSF and 1.0 mM 1, 10 phenanthroline, a 1:100 dilution of chicken anti-MIP133 antiserum (0.2 mg/ml), or a 1:100 dilution of chicken preimmune serum control (0.2 mg/ml) at 37°C for 24 hr. The number of viable trophozoites was determined using trypan blue exclusion and direct trophozoite counts on a hemocytometer.

To determine if the protease inhibitors would impede trophozoite motility, trophozoites (10^5) were incubated in 1.0 ml of PYG with either: a) 1.0 mM PMSF and 1.0 mM 1, 10 Phenanthroline; b) a 1:100 dilution of chicken anti-MIP-133 antiserum (0.2 mg/ml); c) a 1:100 dilution of chicken preimmune serum control (0.2 mg/ml); or d) 50 µg/ml cytochalasin D at 37°C for 30 min prior to their addition to the upper chamber of a transwell (6.5 mm diameter, 3.0 µm pore size: Costar, Corning, N.Y). The trophozoites were then placed in the top chamber in 200 µl PYG and incubated at 37°C for 24 hr. Trophozoites were counted in the bottom chamber via light microscopy (magnification X 100).

The effect of aqueous humor on trophozoites

Trophozoites (10^6) were incubated in either PYG, PBS, or porcine AH and viability of organisms was determined via trypan blue exclusion at 24 hr, 48 hr, and 7 days.

AH was concentrated 10X using microcentrifugal concentrators with membranes having a molecular mass cutoff of approximately 5 kDa (Millipore Corp., Bedford, MA). Trophozoites (10^6) were incubated in PYG, AH, 10X AH, or PBS for 7 days. Serial dilutions

of the trophozoites, ranging from 10^5 to 10^1, were made and placed on a lawn of *E. coli* on non-nutrient agar as described previously [32]. Subsequently, the plates were observed for the presence of *Acanthamoeba*-produced trails at 24, 48, and 72 hr.

Trophozoites (2.5 x 10^5/well) were incubated in a 24-well plate in PYG, AH, or in AH on a confluent monolayer of ICB cells. Since trophozoites kill monolayers of ICB cells within 48 hr, it was necessary to transfer trophozoites to new wells containing fresh PYG, fresh AH, or fresh AH on new monolayers of ICB cells at 24, 48, and 72 hr. The contents of each well were suspended in the respective medium and viability of cysts and trophozoites was determined at 24, 48, 72, and 96 hr using trypan blue exclusion. Trophozoites and cysts were easily differentiated from ICB cells by morphology.

Anterior chamber injections

Trophozoites were injected into the AC of the Chinese hamster eye using a previously described method for transplanting precise numbers of tumor cells into the AC of the mouse eye [160]. Briefly, hamsters were deeply anesthetized with ketamine (100mg/kg of body weight; Fort Dodge Laboratories, Fort Dodge, Iowa) given IP. Trophozoites (10^6/5 µl of PYG) were inoculated into the AC using a 0.1-ml Hamilton syringe (Hamilton Co., Reno, NV) fitted with a 35-gauge glass needle. Control experiments involved injecting PYG alone (5 µl) into the AC of the eye. Eyes were examined 3 times per week for 3 weeks for the appearance of clinical disease.

Immunoperoxidase staining

Four micron eye cross-sections were labeled with rabbit anti-*Acanthamoeba* antiserum using the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA) modified from a previous report [161]. Briefly, *Acanthamoeba castellanii*-containing eyes were harvested from euthanized mice, fixed in formalin, and cut into 4 μm sections. After rehydration, sections were heated in a microwave for 3.5 min in 10 mM trisodium citric acid to expose the antigen sites. Subsequently, sections were incubated in 0.1% trypsin in 10mM trisodium citric acid for 1 hr at 37°C. Sections were bleached by incubating slides in 1% sodium phosphate with 1% hydrogen peroxide (Sigma Aldrich, St. Louis, MO) overnight at room temperature. Slides were washed twice in PBS and incubated in blocking serum (Vectastain Elite ABC kit, Vector Laboratories) for 1.0 hr. Sections were then incubated overnight at 4°C in either rabbit anti-*Acanthamoeba* antiserum or pre-immune rabbit serum control. Slides were washed in PBS/0.01% tween 20 twice and incubated in biotinylated anti-rabbit IgG (Vectastain Elite ABC kit). Slides were washed as before and incubated in Vectastain ABC reagent for 30 min at 37°C. Slides were washed and developed in peroxidase substrate (DAB Peroxidase Substrate kit, Vector Laboratories) for 2-10 min at room temperature. Slides were washed in distilled water and counter stained in 0.5% methyl green in 0.1 M sodium acetate.

Histological examination

Eyes were removed at 30 min and on days 1, 4, and 15-post inoculation of trophozoites into the AC and stored in 10% Carson's formalin for 24 hr. Specimens were

embedded in paraffin, cut into 4-μM sections using a rotary microtome (Shandon; Thermo Shandon, Pittsburgh, PA) and placed on positively charged glass slides (VWR Scientific, Suwanee GA). Slides were then bleached overnight in 3% hydrogen peroxide in 1% sodium phosphate buffered saline. Sections were stained with hematoxylin and eosin, cover-slipped, and examined by light microscopy.

Statistics

Statistical analyses of all data except clinical scores were performed by using unpaired Student's t tests. Clinical severity scores were analyzed by the Mann-Whitney test.

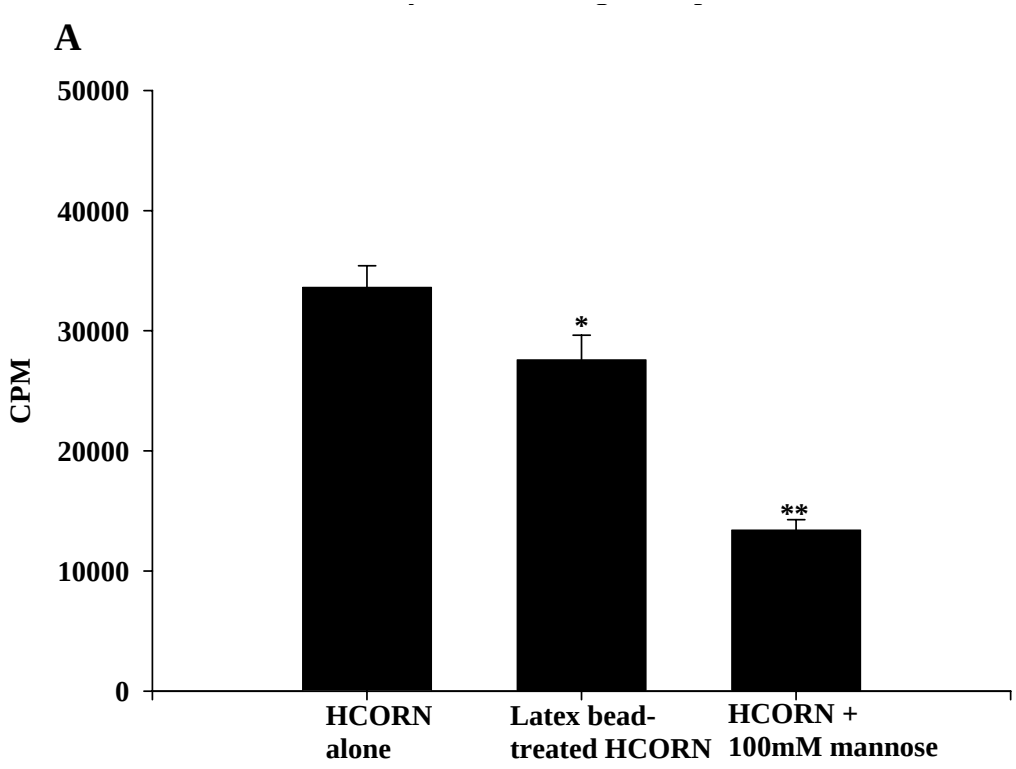
CHAPTER THREE
RESULTS

THE ROLE OF NON-IMMUNE MECHANSIMS IN THE LATEX BEAD PROTECTIVE EFFECT

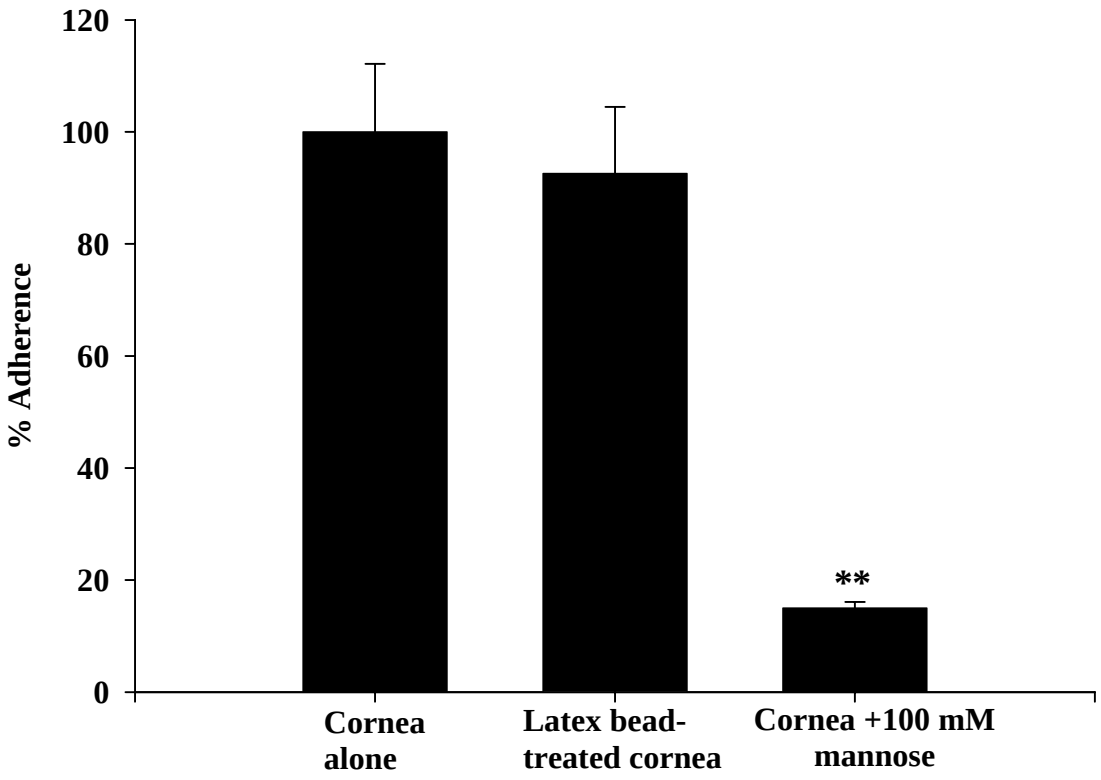
The effect of latex beads on trophozoite adherence to the corneal epithelium

Previous studies have shown that trophozoite adherence to mannose glycoproteins on the corneal epithelium is a crucial prerequisite for the establishment of infection [45,46,147,162]. The extent of trophozoite binding to the corneal epithelium of various mammalian species correlates closely with the susceptibility of each mammalian species to corneal infection *in vivo* [46]. Moreover, reports have shown that free-mannose, but not other sugars, inhibits trophozoite adherence to corneal epithelial cells *in vitro* [45,51,153]. To ascertain the mechanism of the latex bead protective effect, it was first necessary to determine if latex bead treatment inhibited the binding of trophozoites to the corneal epithelium, thereby preventing infection. Accordingly, the ability of trophozoites to adhere to latex bead-treated HCORN cells was examined *in vitro*. Latex bead treatment decreased trophozoite adherence to HCORN cells by approximately 15% (Figure 5A). However, in this system, latex beads may interfere with trophozoite binding to corneal epithelial cells by steric hindrance. To more closely resemble the binding of *Acanthamoeba* trophozoites to the cornea *in vivo*, the ability of trophozoites to adhere to corneas was examined in an organ culture system. Corneal latex bead treatment 10 days prior to the binding assay did not alter the adherence of *A. castellanii* trophozoites to the corneal epithelium (Figure 5B). By contrast, binding of trophozoites to hamster corneas in the presence of 100 mM mannose was reduced by 85% compared to the untreated control.

Figure 5. Effect of latex bead treatment on trophozoite adherence to corneal cells. (**A**) Effect of latex bead treatment on binding to HCORN cells. Adherence of radiolabeled trophozoites to HCORN cells with or without prior latex bead treatment was examined after 2 hr of incubation. As a control, the adherence assay was also performed in the presence of 100mM mannose. Data are the mean +/- SE of results of triplicate experiments. * ($P=0.038$). Attachment to corneas in the presence of 100mM mannose was reduced by 50%. ** ($P<0.01$). (**B**) Effect of latex bead treatment on binding of trophozoites to hamster corneas. Sterile latex beads suspended in PBS were deposited into shallow incisions in the center of the corneal epithelium of Chinese hamsters. Ten days later, the corneas were abraded immediately prior to enucleation as described in the Materials and Methods. Adherence of radiolabeled trophozoites to hamster corneas with or without prior latex bead treatment was examined after 2 hr of incubation in an organ culture system. As a control, the adherence assay was also performed in the presence of 100mM mannose. Data are the mean +/- SE of five hamster corneas. There was no significant difference between latex bead-treated and normal corneas ($P>0.05$). Attachment to corneas in the presence of 100mM mannose was reduced by 85%. ** ($P<0.01$). Each experiment was performed in triplicate.


A
50000
40000
30000
20000
10000
0
CPM
*
**
HCORN alone
Latex bead-treated HCORN
HCORN + 100mM mannose

B

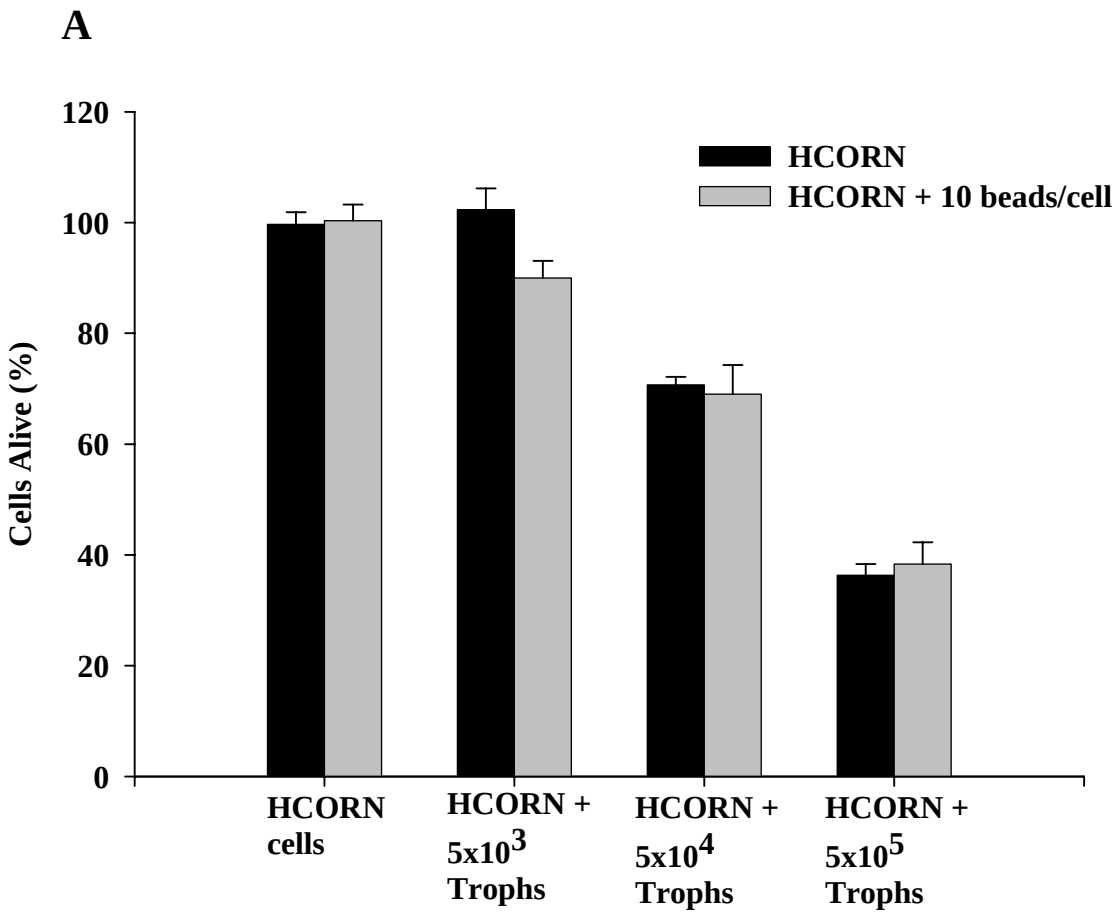

120
100
80
60
40
20
0
% Adherence
**
Cornea alone
Latex bead-treated cornea
Cornea +100 mM mannose

The effect of latex bead treatment on trophozoite-mediated cytolysis of corneal epithelial and corneal stromal cells

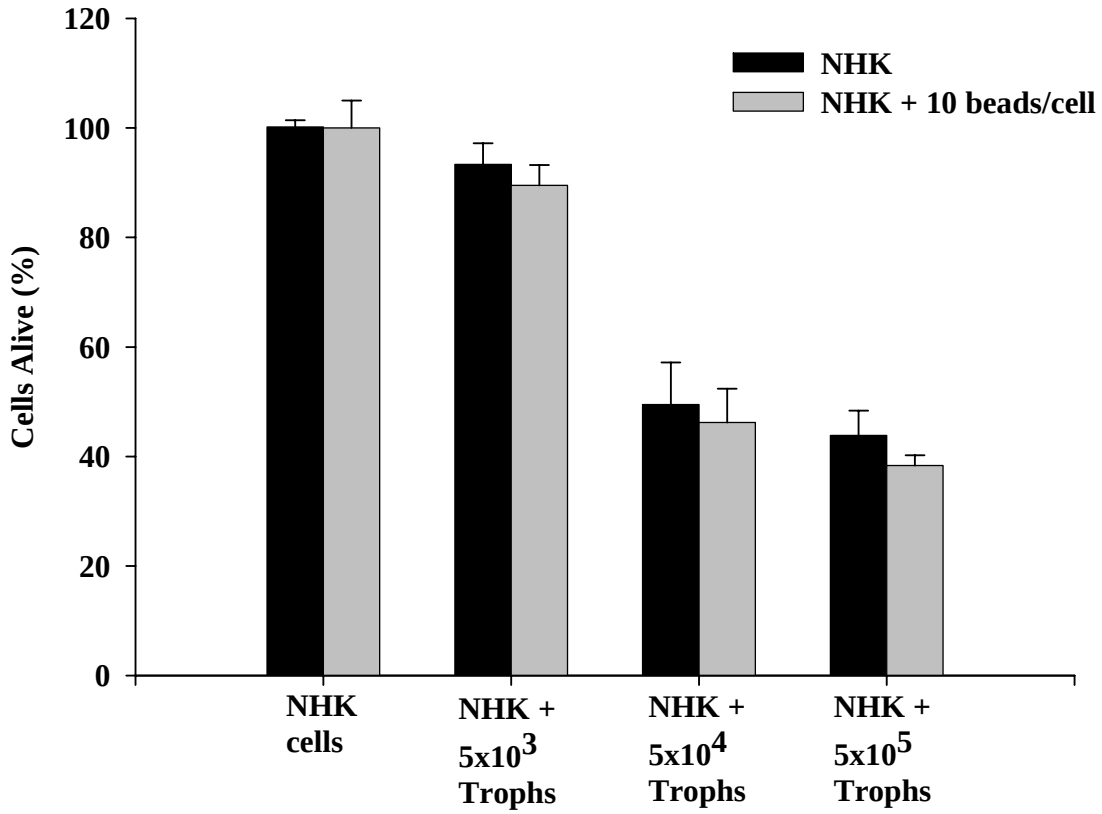
In the pathogenic cascade of *Acanthamoeba* keratitis, adhesion to the corneal epithelium is followed by trophozoite-mediated destruction of the corneal epithelium and penetration of the underlying Bowman's membrane. It is possible that latex bead treatment protects the corneal epithelium from trophozoite-mediated cytolysis. Therefore, we next examined the ability of trophozoites to kill HCORN cells that were pretreated with latex beads *in vitro*. However, latex beads (10/cell) did not protect HCORN cells from trophozoite-mediated killing (Figure 6A). Trophozoites killed as many as 60% of latex bead-treated and untreated HCORN cells after 48 hr. Similar results were obtained with HCORN cells that were pretreated with 100 and 1,000 beads/cell (data not shown).

After desquamation of the corneal epithelium, the pathogenesis of *Acanthamoeba* keratitis continues with penetration and dissolution of the underlying stroma. It is possible that latex bead treatment provides protection against *Acanthamoeba* keratitis by protecting the corneal stroma from trophozoite-mediated cytolysis. However, latex beads did not protect normal human corneal stromal cells (NHK) cells from trophozoite-mediated killing (Figure 6B). Similar results were obtained with NHK cells that were pretreated with 100 and 1,000 beads/cell (data not shown).

Figure 6. Effect of latex bead treatment on trophozoite-mediated cytolysis of corneal cells. Latex beads were added to HCORN (**A**) or NHK (**B**) cells and incubated for 24 hr at 37°C. Trophozoites were then added to the indicated wells and incubated for 48 hr. CPE was assessed spectrophotometrically. Data are the mean +/- SE of triplicate counts. CPE on latex bead-treated cells was not significantly different from untreated cells. ($P>0.05$). Abbreviation: Trophs, trophozoites; HCORN, Chinese hamster corneal epithelial cells; NHK, normal human keratocytes. Each experiment was performed in triplicate.



B


120
100
80
60
40
20
0
Cells Alive (%)
NHK
NHK + 10 beads/cell
NHK cells
NHK + 5x10^3 Trophs
NHK + 5x10^4 Trophs
NHK + 5x10^5 Trophs

THE ROLE OF IMMUNE MECHANISMS IN THE LATEX BEAD PROTECTIVE EFFECT

The role of neutrophils in the latex bead protective effect

Neutrophils are capable of killing *Acanthamoeba* trophozoites and cysts *in vitro* [113,126,127]. Additionally, *in vivo* depletion of neutrophils by intraperitoneal injection of anti-Chinese hamster neutrophil antibody results in a more severe infection compared to untreated controls [110]. Therefore, the possibility that neutrophils are involved in the latex bead protective effect was explored. To determine the role of neutrophils in the latex bead protective effect, rabbit anti-Chinese hamster neutrophil antiserum was generated to deplete neutrophils in latex bead-treated animals. First, the efficacy of the rabbit anti-Chinese hamster neutrophil antibody was examined *in vivo*. Antiserum injections resulted in approximately an 85% decrease in peripheral blood neutrophils after 2 hr and a 95% decrease after 4 hr (Figure 7). Lymphocyte and monocyte counts were not significantly decreased. Furthermore, anti-Chinese hamster neutrophil antibody (1:100) lysed 83% of neutrophils in the presence of complement without significantly depleting splenocytes *in vitro* (data not shown). As a control, normal rabbit serum was not toxic to neutrophils *in vivo* or *in vitro*. Successful depletion of neutrophils with anti-neutrophil antiserum resulted in a more severe disease than untreated control animals. As reported previously, latex bead treatment reduced the incidence, severity, and duration of disease [37]. However, anti-Chinese hamster neutrophil antibody failed to exacerbate disease in latex bead-treated animals (Figure 8).

Figure 7. Cytotoxic activity of absorbed anti-Chinese hamster neutrophil antiserum on neutrophils and lymphocytes *in vivo*. Chinese hamsters were injected with 1 ml of anti-Chinese hamster neutrophil antiserum or normal rabbit serum IP. Blood was collected from tail veins at 1, 2, and 4 hr post-injection and streaked on glass slides. Neutrophils and lymphocytes were counted in 10 random fields per slide. Data represent the mean +/- SE of 10 separate counts. **P<0.01.

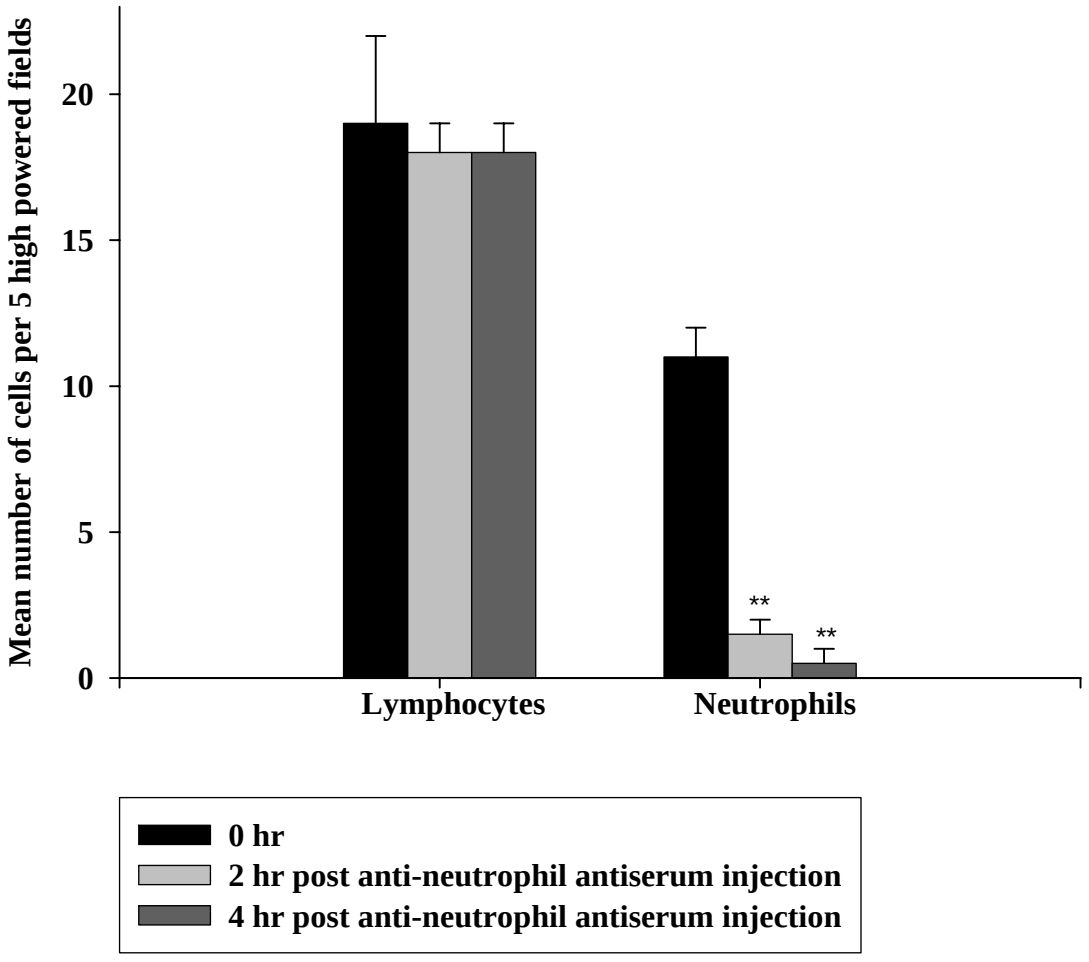
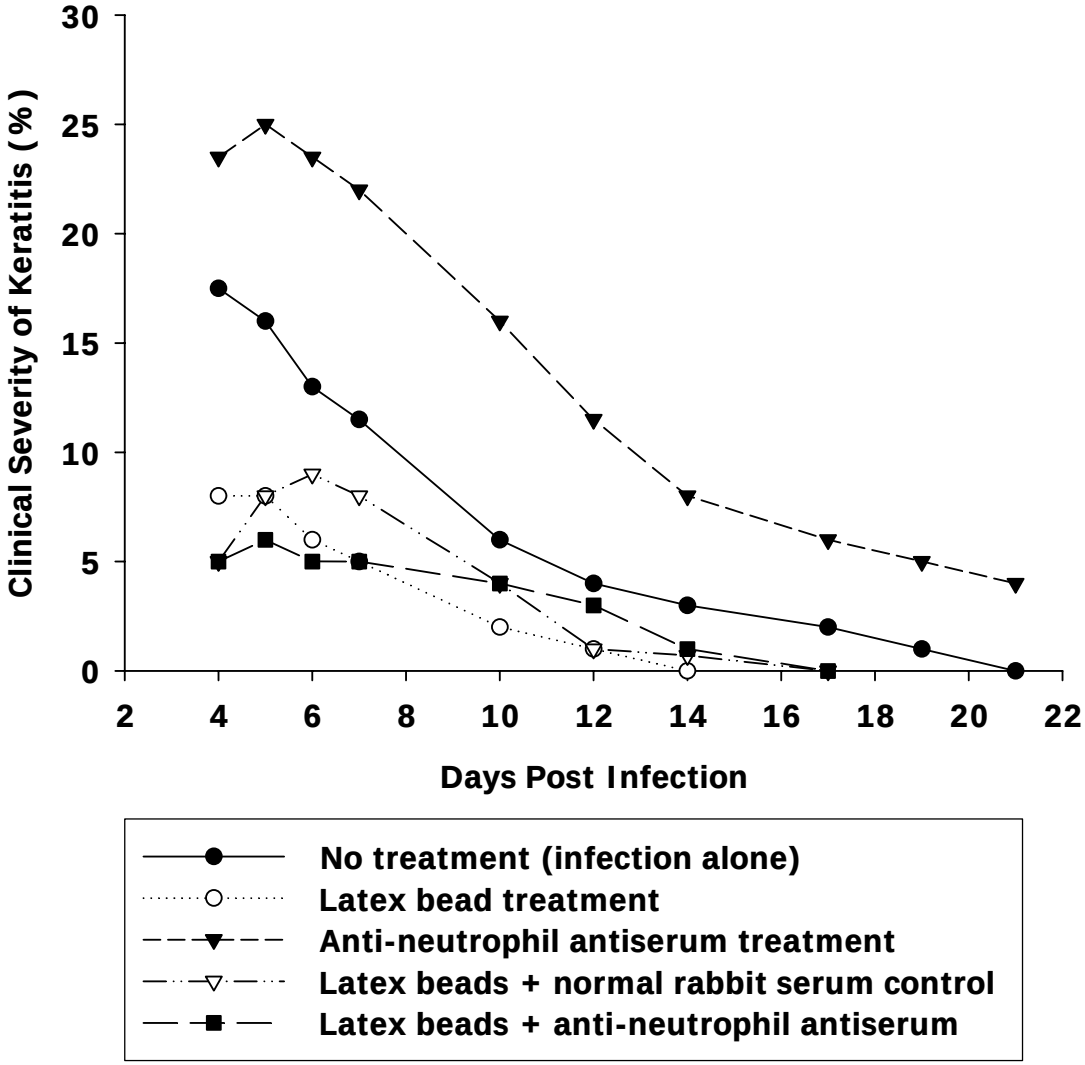


Figure 8. The role of neutrophils in the latex bead protective effect. Sterile latex beads suspended in PBS were instilled into shallow corneal epithelial incisions in Chinese hamster corneas 10 days prior to infection in the indicated groups as described in the Materials and Methods section. Hamsters were injected IP with 0.5 ml of either anti-neutrophil antiserum or naïve rabbit serum twice daily for seven days beginning 3 days before corneal infection. Corneas were observed for signs of clinical disease beginning at day 4 post-infection and on days 5, 6, 7, 10, 12, 14, 17, 19, and 21 as described in the Materials and Methods section. Latex bead treatment reduced the severity of disease at days 4, 5, 6, and 14 post-infection compared to the untreated (infection alone) group. ($P<0.05$). Anti-neutrophil antiserum treatment increased the severity of disease at days 4, 5, 6, 12, 14, 19, and 21 post infection. ($P<0.05$). Anti-neutrophil antiserum treatment did not exacerbate disease in latex bead-treated corneas at any of the time points examined. ($P>0.05$). (n=7/group). This experiment was performed twice with similar results.


Clinical Severity of Keratitis (%)
30
25
20
15
10
5
0
2
4
6
8
10
12
14
16
18
20
22
Days Post Infection
No treatment (infection alone)
Latex bead treatment
Anti-neutrophil antiserum treatment
Latex beads + normal rabbit serum control
Latex beads + anti-neutrophil antiserum

The role of macrophages in the latex bead protective effect

The role of macrophages in resistance to *Acanthamoeba* keratitis is well-established. *In vivo* depletion of conjunctival macrophages with the macrophagicidal drug, dichloro-methylene diphosphonate (clodronate), results in a chronic, exacerbated form of *Acanthamoeba* keratitis [109]. Moreover, macrophages can kill *Acanthamoeba* trophozoites and cysts *in vitro* [111,112]. We hypothesized that corneal latex bead treatment induces the migration and/or activation of macrophages, which mitigates disease. To ascertain the role of macrophages in the latex bead protective effect, clodronate was administered subconjunctivally to remove corneal macrophages in latex bead-treated animals. The results of this experiment showed that clodronate treatment eliminated the latex bead protective effect, exacerbating disease similar to the clodronate alone group (Figure 9). Furthermore, the latex beads plus clodronate group was not significantly different from the clodronate treatment alone group.

We next proposed that latex bead treatment may induce the activation of macrophages, which could kill trophozoites. To determine if latex bead treatment activated macrophages, latex beads were added to the RAW macrophage cells *in vitro*. Macrophage activation was determined by measuring nitrite generation, a stable reaction product of NO. Latex beads did not increase the production of NO at 24 or 48 hr (Figure 10A). However, treatment with IFN-γ and LPS significantly activated macrophages compared to the untreated control group. Alternatively, it is possible that latex beads induce corneal epithelial cells or corneal stromal cells to produce a factor that activates macrophages. To test this, BALB/c corneal epithelial cells were incubated with latex beads for 24, 48, or 72 hrs. Subsequently, latex bead-treated BALB/c epithelial cell supernatant was added to RAW macrophages. Supernatant from corneal epithelial cells treated with latex beads for 24 hr did not increase RAW cell production of NO at 24 or 48 hr (Figure

Figure 9. The role of macrophages in the latex bead protective effect. Latex beads were administered to hamster corneas 10 days prior to infection in the indicated groups. Clodronate-containing liposomes were administered via subconjunctival injection 9, 7, 5, and 3 days prior to infection with *Acanthamoeba*-laden lenses. Corneas were observed for signs of clinical disease beginning at day 4 post-infection, as described in Materials and Methods. Latex bead treatment reduced the severity of disease at days 4 and 5 post-infection compared to untreated (infection alone) group. ($P<0.05$). Clodronate treatment increased the severity of disease at all times examined. ($P<0.05$). Clodronate treatment exacerbated disease in latex bead-treated corneas at all of the times examined. ($P<0.05$). The clodronate treatment alone group was not significantly different from the latex bead-treated, clodronate-treated group. ($P>0.05$). The results are representative of three separate experiments (n=7 hamsters/group).

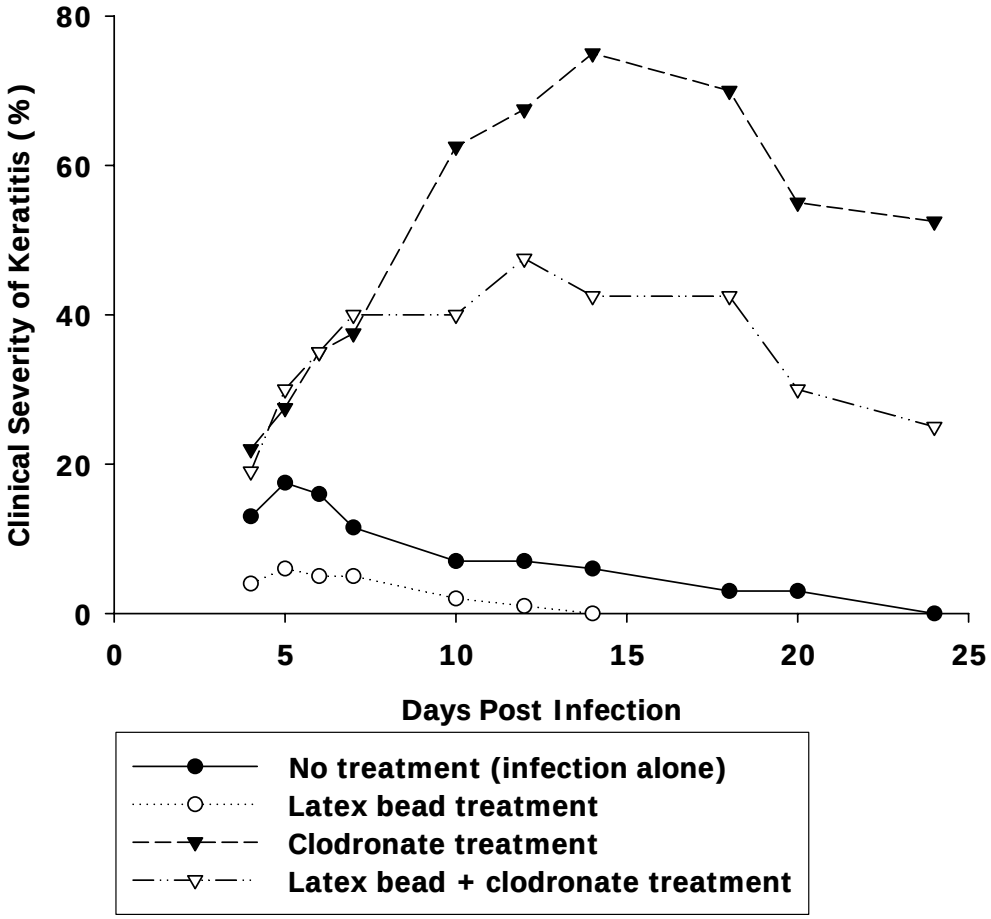
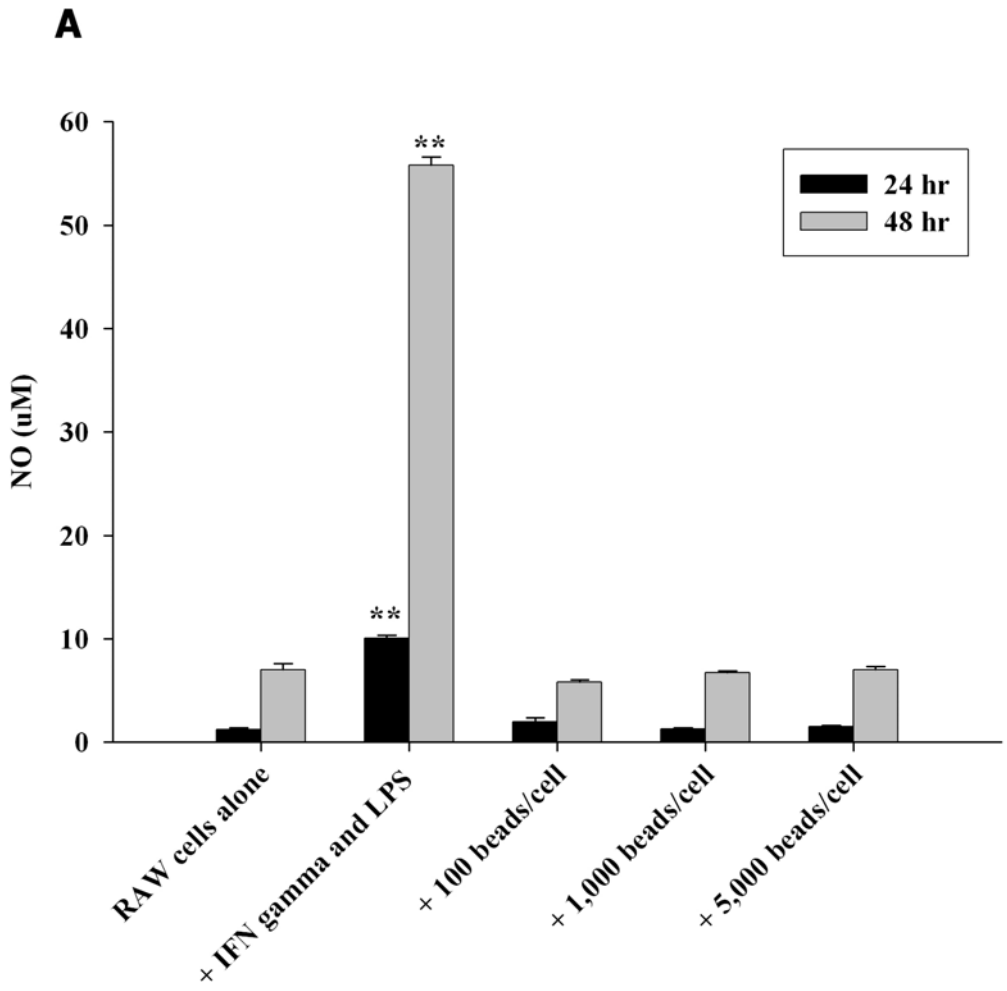
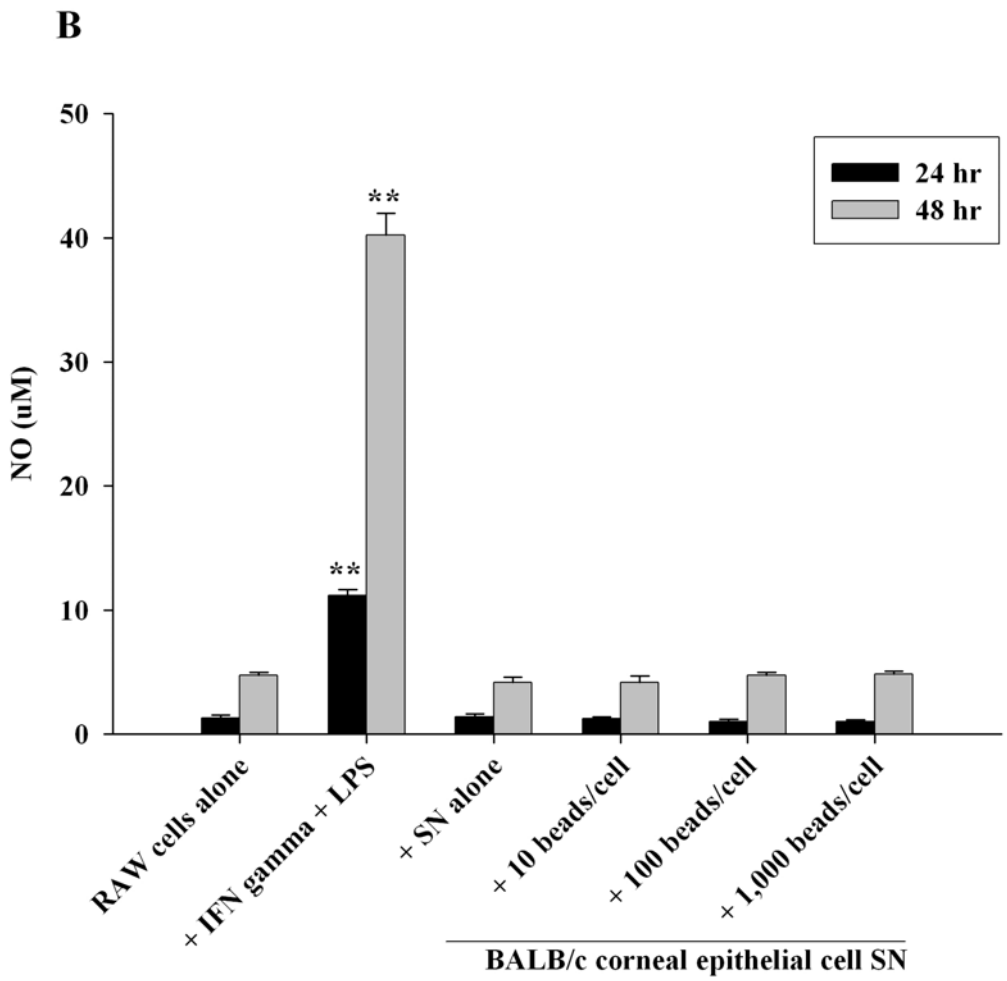
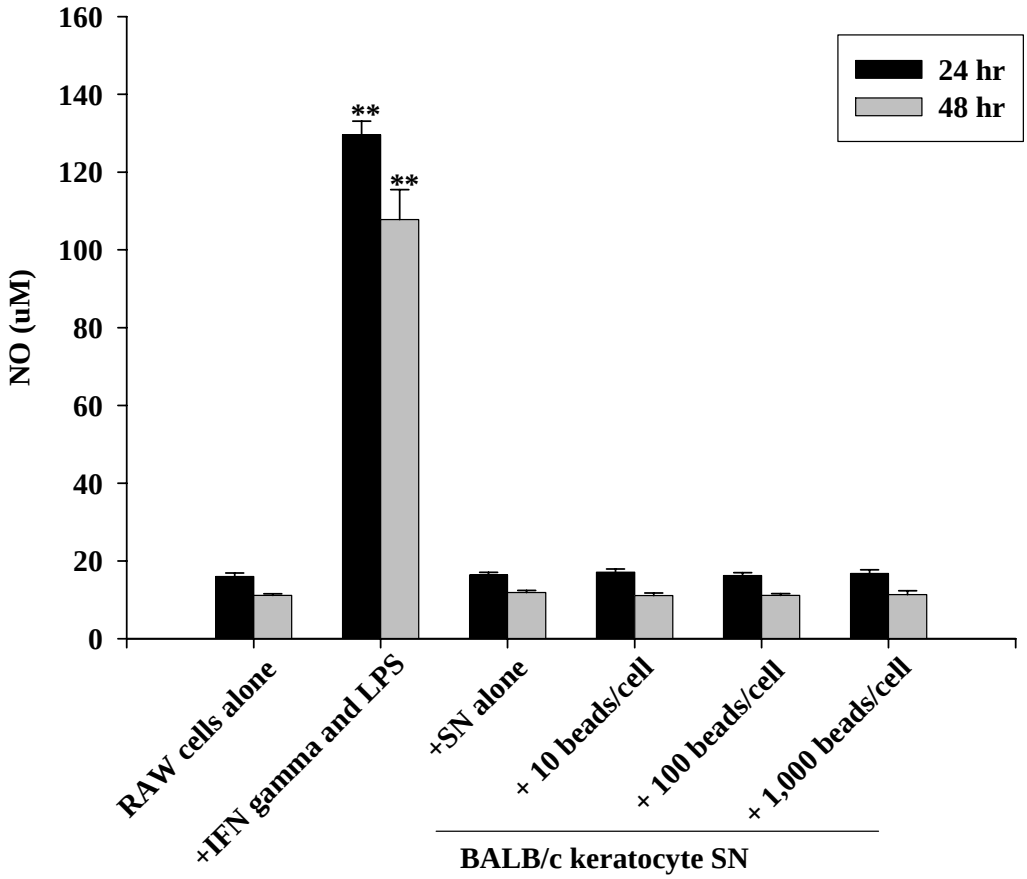

Clinical Severity of Keratitis (%)
80
60
40
20
0
0
5
10
15
20
25
Days Post Infection
No treatment (infection alone)
Latex bead treatment
Clodronate treatment
Latex bead + clodronate treatment

Figure 10. Effect of latex beads on macrophage activation. (**A**) RAW cells were treated with various concentrations of latex beads and incubated at 37°C. As a measure of macrophage activation, NO production was determined at 24 and 48 hr as described in Materials and Methods. IFN-γ (100 ng/ml) and LPS (2 μg/ml) treatment served as a positive control for macrophage activation. IFN-γ and LPS treatment resulted in increased production of NO at 24 and 48 hr. ** ($P<0.05$). Latex bead treatment did not increase NO production at 24 or 48 hr. (**B**) BALB/c corneal epithelial cells were treated with various concentrations of latex beads and incubated at 37°C for 24 hr. The supernatant was collected and added to RAW cells in a 24-well plate. NO production was determined at 24 and 48 hr. BALB/c corneal epithelial supernatant did not increase NO production at 24 or 48 hr. (**C**) BALB/c corneal keratocytes were treated with various concentrations of latex beads and incubated at 37°C for 24 hr. The supernatant was collected and added to RAW cells in a 24-well plate and NO production was determined at 24 and 48 hr. The results are representative of three experiments. SN; supernatant.


A
60
50
40
30
20
10
0
NO (uM)
**
**
24 hr
48 hr
RAW cells alone
+ IFN gamma and LPS
+ 100 beads/cell
+ 1,000 beads/cell
+ 5,000 beads/cell


B
50
40
30
20
10
0
NO (uM)
24 hr
48 hr
**
**
RAW cells alone
+ IFN gamma + LPS
+ SN alone
+ 10 beads/cell
+ 100 beads/cell
+ 1,000 beads/cell
BALB/c corneal epithelial cell SN

C


160
140
120
100
80
60
40
20
0
NO (uM)
24 hr
48 hr
**
**
RAW cells alone
+IFN gamma and LPS
+SN alone
+ 10 beads/cell
+ 100 beads/cell
+ 1,000 beads/cell
BALB/c keratocyte SN

10B). Supernatant from corneal epithelial cells treated with latex beads for 48 and 72 hr did not increase RAW cell production of NO (data not shown). Similarly, 24 hr supernatant from latex bead-treated corneal stromal cells did not increase RAW cell production of NO at 24 or 48 hr (Figure 10C). Supernatant from corneal stromal cells treated with latex beads for 48 and 72 hr did not increase RAW cell production of NO (data not shown).

As both IFN-γ and LPS are required to induce macrophages to produce NO, the macrophage activation assays were also performed with corneal cell supernatant in the presence of LPS. Briefly, 2 μg/ml LPS was added to macrophages at the time of addition of latex bead-treated corneal cell supernatant. The macrophage cell supernatant was then collected at 24 and 48 hrs and assayed for NO activity as described above. The addition of LPS did not affect the ability of bead-treated corneal epithelial cell supernatant to activate macrophages (data not shown). Further, the addition of LPS did not affect the ability of bead-treated stromal cell supernatant to activate macrophages (data not shown). The results obtained from experiments performed in the presence of LPS were similar to the results reported above that were obtained in the absence of LPS.

It is possible that latex bead treatment increases the capacity of macrophages to kill trophozoites. In order to test this, various dilutions of latex beads were added to macrophages in a 24-well plate. Radiolabelled trophozoites were added to each well and [^{3}H] uridine release was determined after 24 hr to quantify macrophage-mediated trophozoite lysis. Latex bead-treated macrophages did not kill more trophozoites than the untreated macrophage control (~20% killing) (Figure 11A). Macrophages pretreated with IFNγ and LPS lysed approximately 50% of trophozoites in 24 hr. Our next hypothesis proposed that latex beads induce corneal cells to produce a factor that increases the ability of macrophages to kill trophozoites. However, neither

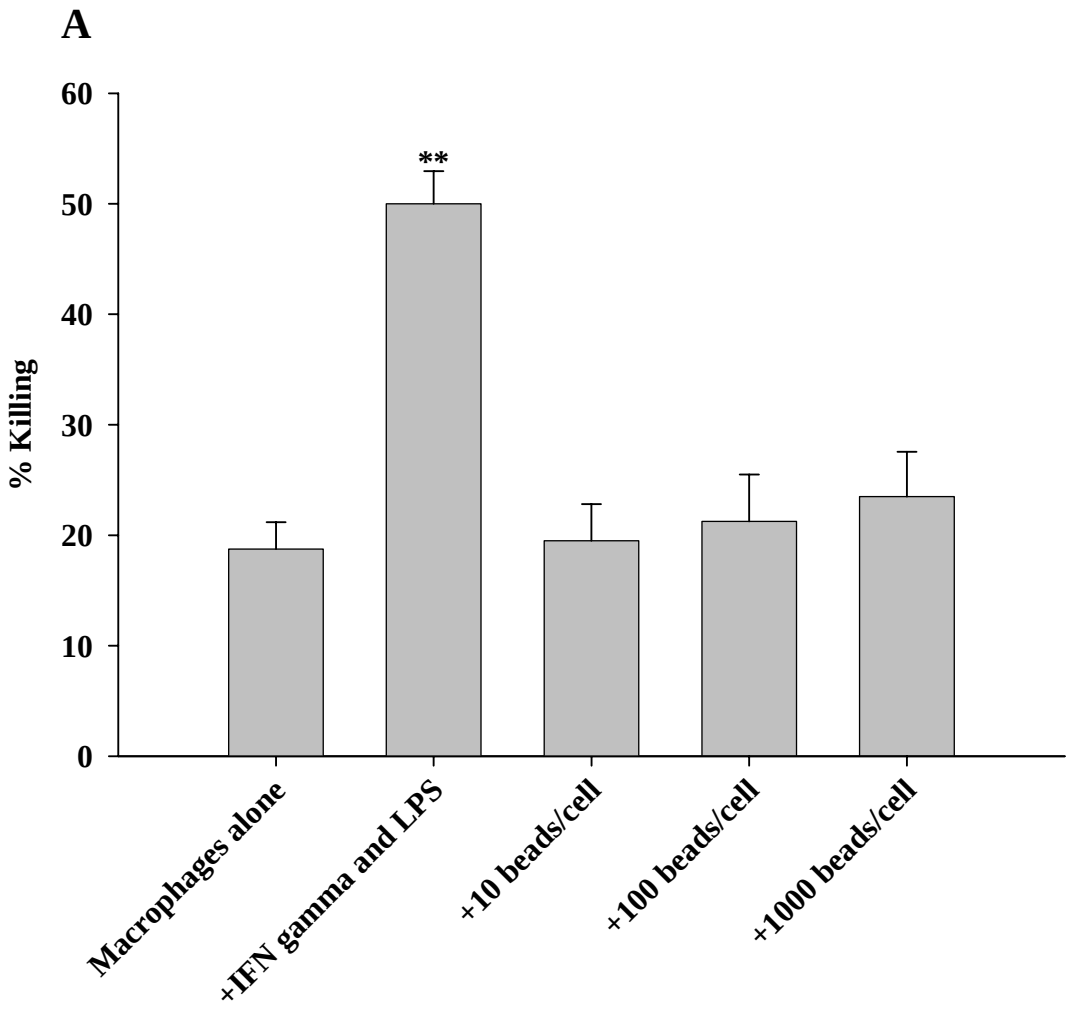
latex bead-treated corneal epithelial cell supernatant nor latex bead-treated corneal stromal cell supernatant increased the ability of macrophages to kill trophozoites (Figure 11B, 11C).

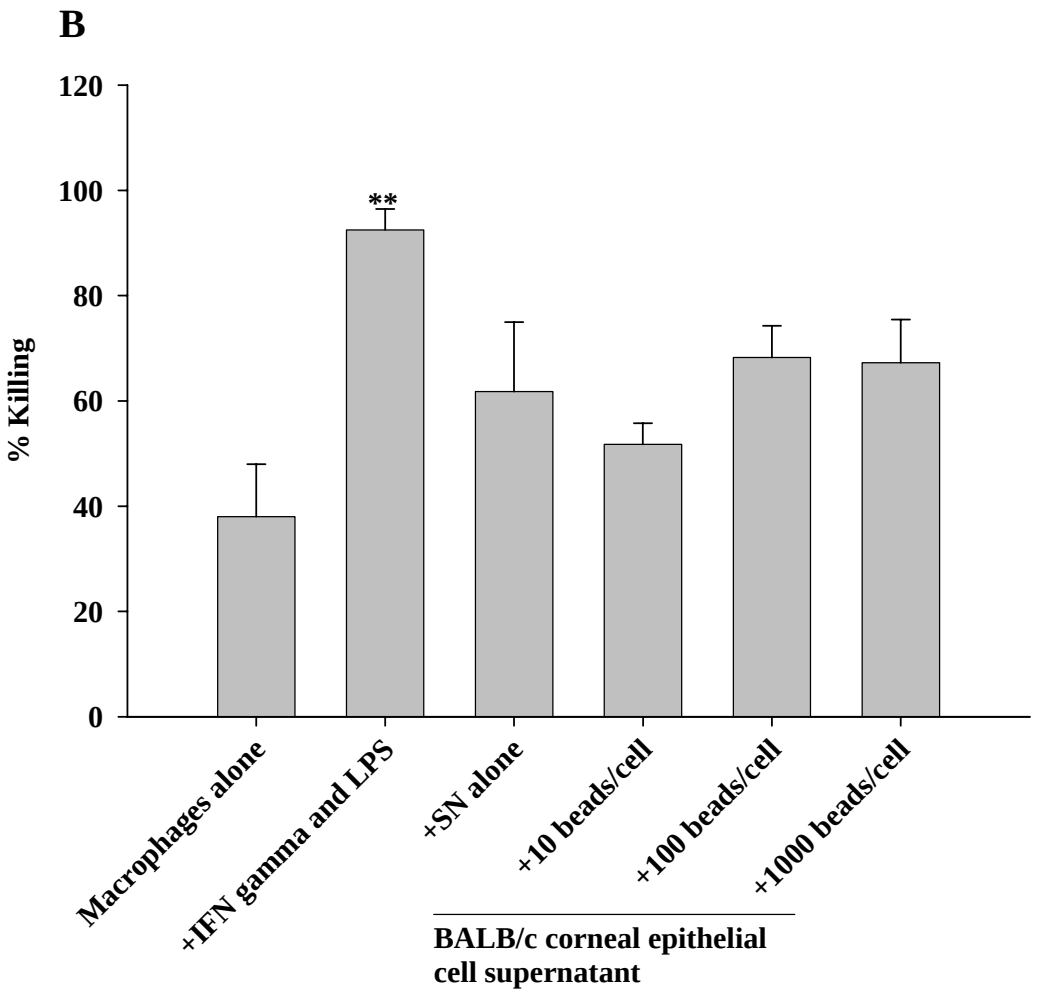
A recent report from a conference proceeding has indicated that both human and murine corneal epithelial cells produce IFN-γ [163]. Moreover, it is well established that IFN-γ is one of the most potent activators of macrophages [85]. Thus, it is possible that latex beads induce corneal epithelial cells to produce IFN-γ, which activates macrophages *in vivo*. To test this, latex beads (1.0 μm) were added to BALB/c corneal epithelial cells to obtain concentrations of 1, 10, and 100 beads/cell. Latex bead-treated cells were collected at 24 hr, 48 hr, and 72 hr and corneal cell mRNA was analyzed for IFN-γ expression. However, the results show that latex bead treatment did not induce corneal epithelial cells or keratocytes to produce increased amounts of IFN-γ mRNA by 72 hr (Figure 12A). Similar results were obtained for the 24 hr and 48 hr time points (data not shown). As a positive control for the production of IFN-γ mRNA, T cells were incubated with the mitogen, PHA, for 72 hr. Importantly, PHA induced T cells to produce increased amounts of IFN-γ (Figure 12A). GAPDH mRNA levels were used as an internal control (Figure 12A).

In order to confirm that latex bead treatment does not induce corneal cells to produce IFN-γ *in vitro*, latex beads (1.0 μm) were added to BALB/c corneal epithelial cells to obtain concentrations of 1, 10, and 100 beads/cell. Latex bead-treated corneal cell supernatant was collected at 24 hr, 48 hr, and 72 hr and analyzed for IFN-γ protein. ELISAs for mouse IFN-γ were performed on culture supernatants according to the manufacturer's instructions. ELISAs for mouse IFN-γ revealed that latex bead treatment did not induce corneal epithelial cells to produce increased amounts of IFN-γ protein at any of the permutations examined (Figure 12B). Similar results were obtained for bead-treated keratocytes (Figure 12C).

We next hypothesized that latex bead treatment prior to corneal infection may induce the migration of macrophages to the central cornea, which kill trophozoites resulting in a mitigation of corneal disease. Previous work by Hazlett and coworkers demonstrated that intracorneal instillation of latex beads prior to corneal infection with *P. aeruginosa* induced the corneal migration of macrophages [136]. Acid phosphatase staining of corneal macrophages in latex bead-treated, *Acanthamoeba*-infected corneas revealed that latex bead treatment induced a significant increase in the infiltration of macrophages at day 4 of corneal infection (Figure 13).

Figure 11. Effect of latex beads on macrophage-mediated lysis of trophozoites. Macrophage-mediated lysis of [^{3}H] uridine-labeled trophozoites was determined after 24 hr. As a positive control, the [^{3}H] uridine release assay was also performed in the presence of IFN-γ and LPS. Data are the mean +/- SE of triplicate experiments. Data are expressed as % killing of trophozoites. (**A**) There was no significant difference between latex bead-treated and untreated macrophages ($P>0.05$). Macrophages pretreated with IFN-γ and LPS killed 30% more trophozoites ** ($P<0.01$). (**B**) Bead-treated corneal epithelial cell supernatant did not induce macrophages to kill more trophozoites than untreated control macrophages. (**C**) Bead-treated corneal stromal cell supernatant did not induce macrophages to kill more trophozoites than untreated control macrophages. Each experiment was performed in triplicate. SN; supernatant.


A
60
50
40
30
20
10
0
% Killing
**
Macrophages alone
+IFN gamma and LPS
+10 beads/cell
+100 beads/cell
+1000 beads/cell


B
120
100
80
60
40
20
0
% Killing
**
Macrophages alone
+IFN gamma and LPS
+SN alone
+10 beads/cell
+100 beads/cell
+1000 beads/cell
BALB/c corneal epithelial cell supernatant

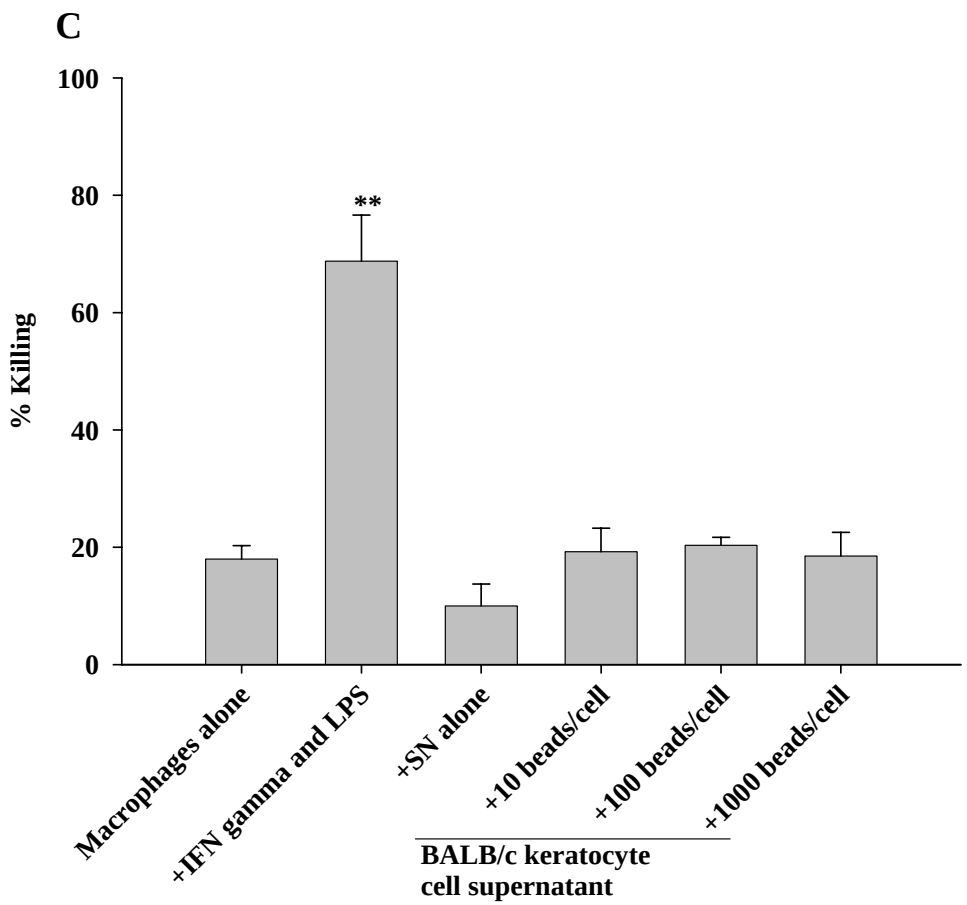
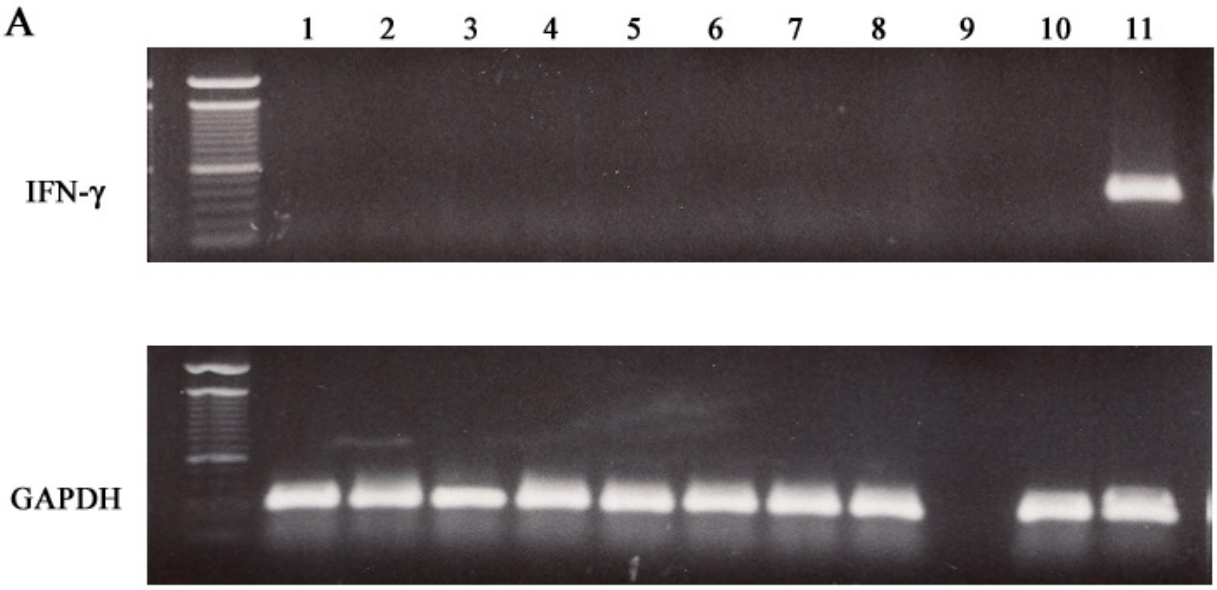
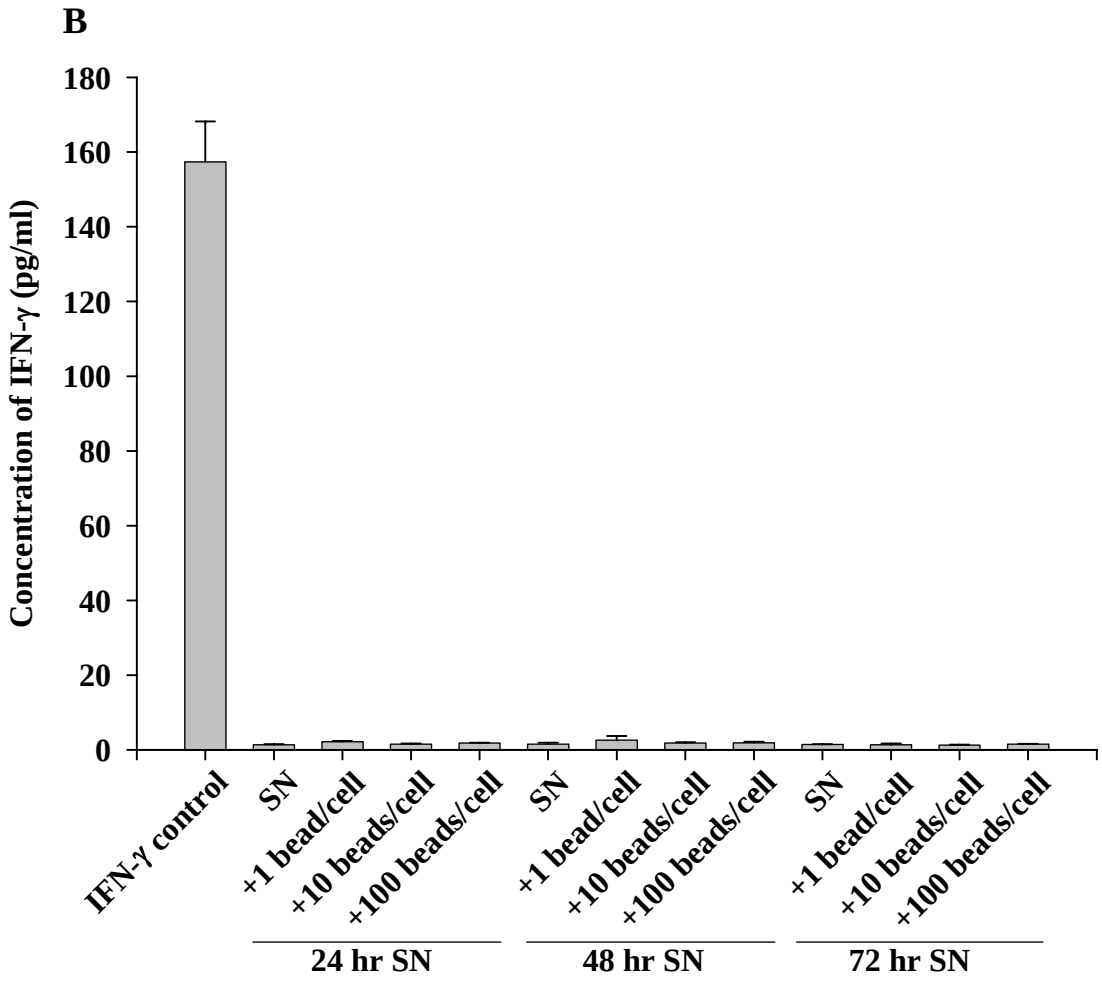

C
100
80
60
40
20
0
% Killing
**
Macrophages alone
+IFN gamma and LPS
+SN alone
+10 beads/cell
+100 beads/cell
+1000 beads/cell
BALB/c keratocyte
cell supernatant

Figure 12. Effect of latex beads on corneal cell-mediated production of IFN-γ *in vitro*. Latex beads (1.0 μm) were added to BALB/c corneal cells to obtain concentrations of 1, 10, and 100 beads/cell. (**A**) Latex bead-treated corneal cells were collected at 72 hr and corneal cell mRNA was analyzed for IFN-γ expression. GAPDH mRNA levels were used as an internal control. Lanes: **1.** Corneal epithelial cells alone. **2.** Corneal epithelial cells + 1 bead/cell. **3.** Corneal epithelial cells + 10 beads/cell. **4.** Corneal epithelial cells + 100 beads/cell. **5.** Keratocytes alone. **6.** Keratocytes + 1 bead/cell. **7.** Keratocytes + 10 beads/cell. **8.** Keratocytes + 100 beads/cell. **9.** Water. **10.** T cells alone. **11.** T cells + PHA. (**B**) Latex bead-treated corneal epithelial cell supernatant was collected at 24 hr, 48 hr, and 72 hr and analyzed for IFN-γ protein. ELISAs for mouse IFN-γ were performed on culture supernatants according to the manufacturer's instructions. (**C**) Latex bead-treated corneal keratocyte cell supernatant was collected at 24 hr, 48 hr, and 72 hr and analyzed for IFN-γ protein. ELISAs for mouse IFN-γ were performed on culture supernatants according to the manufacturer's instructions.


A
1
2
3
4
5
6
7
8
9
10
11
IFN-γ
GAPDH


B
Concentration of IFN-γ (pg/ml)
180
160
140
120
100
80
60
40
20
0
IFN-γ control
SN
+1 bead/cell
+10 beads/cell
+100 beads/cell
SN
+1 bead/cell
+10 beads/cell
+100 beads/cell
SN
+1 bead/cell
+10 beads/cell
+100 beads/cell
24 hr SN
48 hr SN
72 hr SN

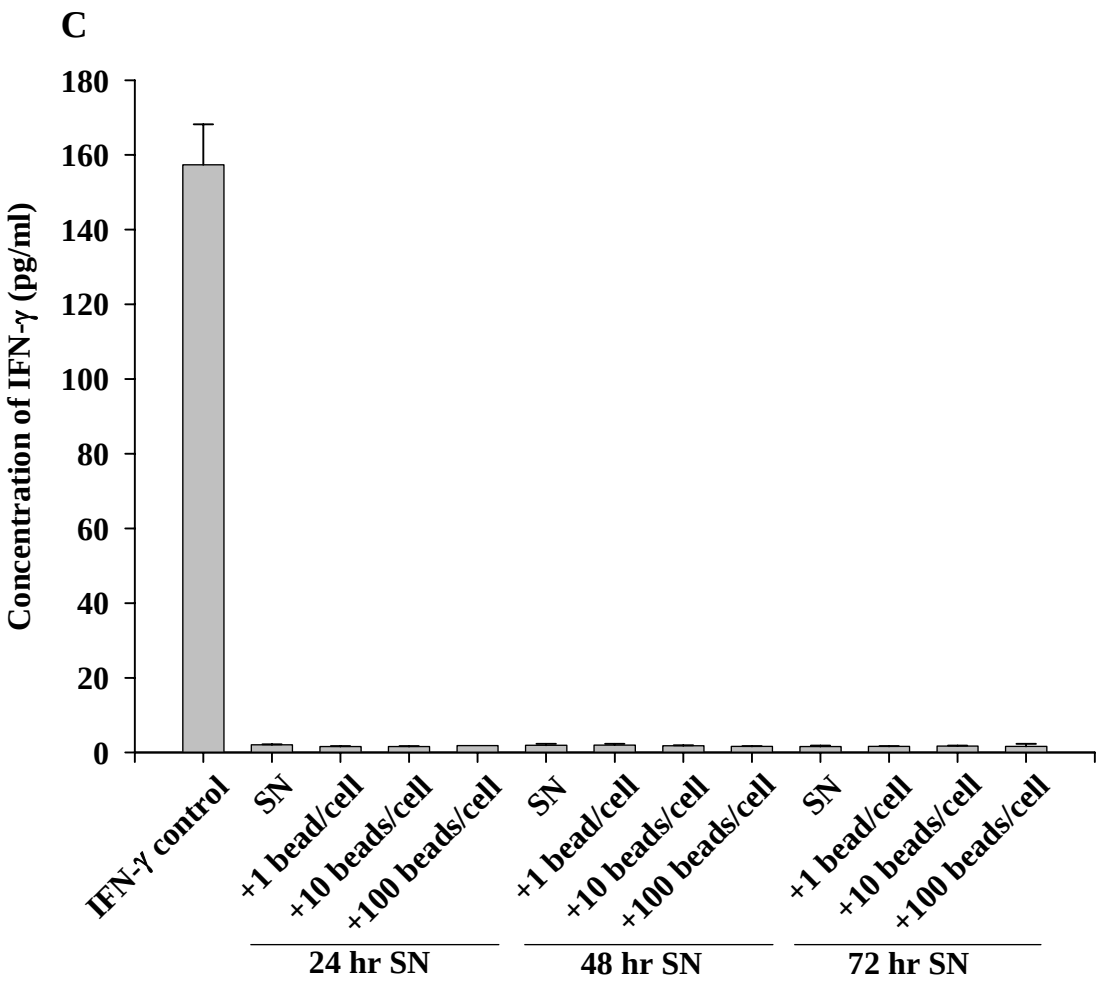
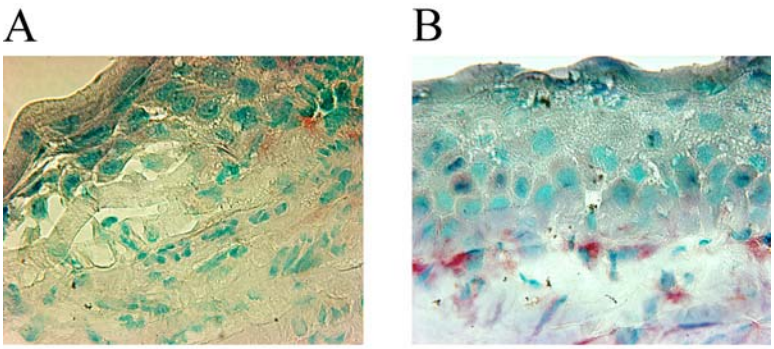
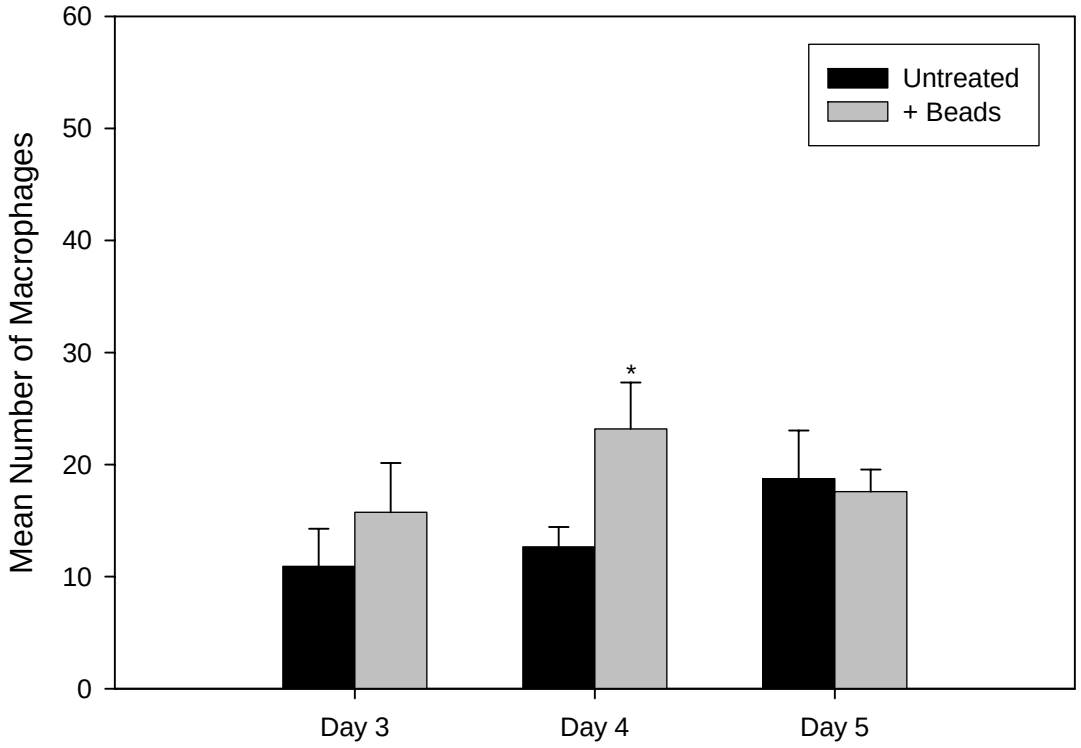

C
Concentration of IFN-γ (pg/ml)
180
160
140
120
100
80
60
40
20
0
IFN-γ control
SN
+1 bead/cell
+10 beads/cell
+100 beads/cell
SN
+1 bead/cell
+10 beads/cell
+100 beads/cell
SN
+1 bead/cell
+10 beads/cell
+100 beads/cell
24 hr SN
48 hr SN
72 hr SN

Figure 13. Effect of corneal latex bead treatment on the migration of macrophages. To determine if there were more macrophages present in latex bead-treated, *Acanthamoeba*-infected corneas, compared to untreated, infected corneas, eyes were processed and stained for macrophage-endogenous acid phosphatase activity as described in the Materials and Methods section. Briefly, Chinese hamster corneas were infected with *Acanthamoeba castellanii* with or without prior latex bead treatment as described in the Materials and Methods section. Infected eyes of bead-treated and untreated Chinese hamsters (n=5/group) were enucleated 3, 4, and 5 days post-infection. To demonstrate endogenous acid phosphatase activity, corneal sections were incubated with naphthol AS-BI phosphate and pararosaniline for 35 min at 37°C [159]. Positively reacting cells appeared pink or red. Acid phosphatase-positive cells were counted via light microscopy by three masked observers. The results are representative of the three observers' counts. (**A**) Untreated cornea day 4 post-infection. (**B**) Latex bead-treated cornea day 4 post-infection. (**C**) Acid phosphatase-positive cell counts in untreated and bead-treated corneas. Acid phosphatase-positive cells were counted in the entire cornea (conjunctiva to conjunctiva) in four corneal sections per eye in each of three separate experimental eyes per group. *($P<0.05$).



C


Mean Number of Macrophages
60
50
40
30
20
10
0
Untreated
+ Beads
*
Day 3
Day 4
Day 5

MECHANISMS AND POTENTIAL BARRIERS THAT PREVENT CORNEAL INFECTIONS FROM PROGRESSING TO INTRAOCULAR INFECTIONS

Trophozoite penetration of Descemet's membrane and the corneal endothelium

It is possible that Descemet's membrane and the corneal endothelium serve as a physical barrier to *Acanthamoeba* trophozoites, preventing entry of trophozoites into the AC and subsequent intraocular infection. Accordingly, the ability of *Acanthamoeba* trophozoites to penetrate Descemet's membrane was examined *in vitro* (Figure 14). Over one-third of the trophozoites were capable of penetrating Descemet's membrane and the corneal endothelium within 24 hr of incubation (Fig 15A). Serine protease inhibitors, PMSF and 1, 10 P., inhibited the penetration of Descemet's membrane by approximately 75%, while the cysteine protease inhibitor, cystatin, had no significant effect. Previous studies have shown that *A. castellanii* trophozoites elaborate a 133-kDa serine protease, MIP-133, that is cytolytic against corneal epithelial cells and degrades collagen *in vitro* [58]. Therefore, the ability of anti-MIP-133-antiserum to prevent the penetration of Descemet's membrane was examined. Anti-MIP-133-antiserum treatment decreased the penetration of Descemet's membrane by approximately 50%, while the preimmune normal serum control had no significant effect (Fig. 15B).

In the context of the eye, Descemet's membrane and the corneal endothelium are bound posteriorly by the AH of the AC. To mimic the natural environment of the eye, the migration assay was performed with AH in the bottom well of the Boyden chamber. There was no statistically significant difference in the ability of trophozoites to penetrate Descemet's membrane with PYG or AH in the bottom chamber (Figure 16).

Figure 14. Boyden chamber design. Descemet's membrane was isolated from pig corneas and placed on a nitrocellulose membrane in a Boyden chamber. *Acanthamoeba* trophozoites were placed in the top chamber. After 24 hr, the bottom chamber was vigorously washed with the endogenous PYG to be certain that trophozoites did not adhere to the sides of the Boyden chamber. *Acanthamoebae* were counted in the bottom chamber via trypan blue exclusion.

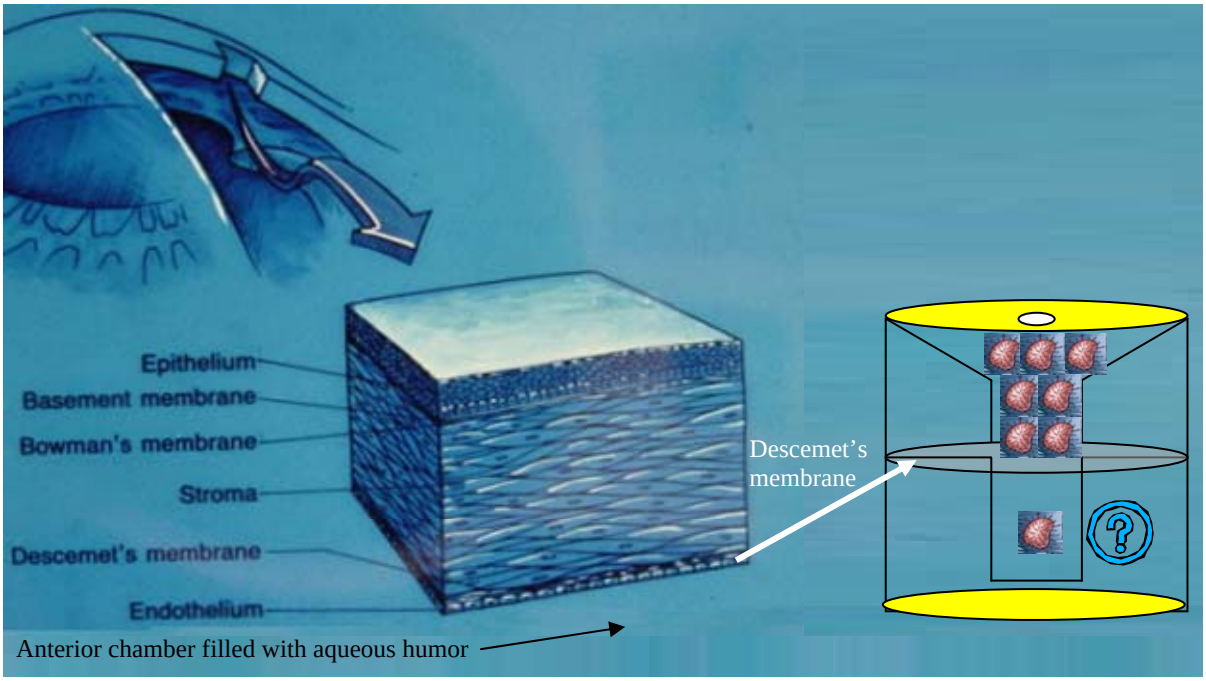
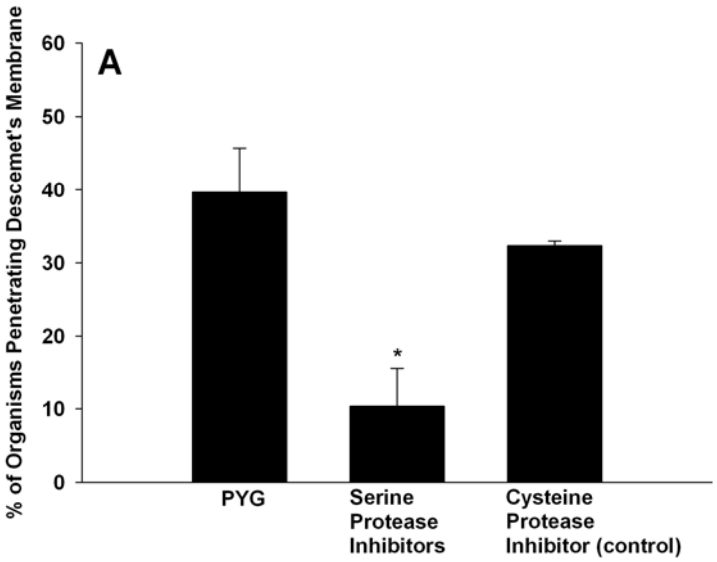


Figure 15. Effect of protease inhibitors and anti-MIP-133 antiserum on trophozoite penetration of Descemet's membrane *in vitro*. *Acanthamoeba* trophozoites (10^5/200µl PYG) were placed on Descemet's membrane in the top chamber of a Boyden chamber and incubated at 37ºC for 24 hr. (**A**) Inhibition assays involved incubating trophozoites with serine protease inhibitors (1mM PMSF + 1mM 1, 10 phenanthroline) or a cysteine protease inhibitor control (10µM cystatin) at 37°C for 30 min prior to their addition to the upper chamber. (**B**) Inhibition assays involved incubating trophozoites with chicken anti-MIP-133 antiserum (0.2 mg/ml; ImS) or chicken preimmune normal serum control (0.2 mg/ml; NS) at 37°C for 30 min prior to their addition to the upper chamber. Data are represented as percent organisms penetrating Descemet's membrane. Bars and error bars represent the means +/- SE of 12 wells. Each experiment was performed in triplicate. *$p<0.05$.


A
% of Organisms Penetrating Descemet's Membrane
60
50
40
30
20
10
0
*
PYG
Serine Protease Inhibitors
Cysteine Protease Inhibitor (control)

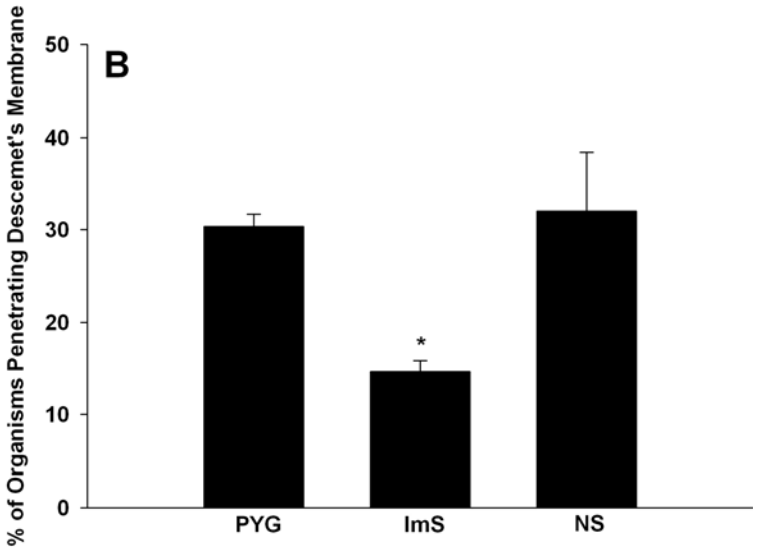
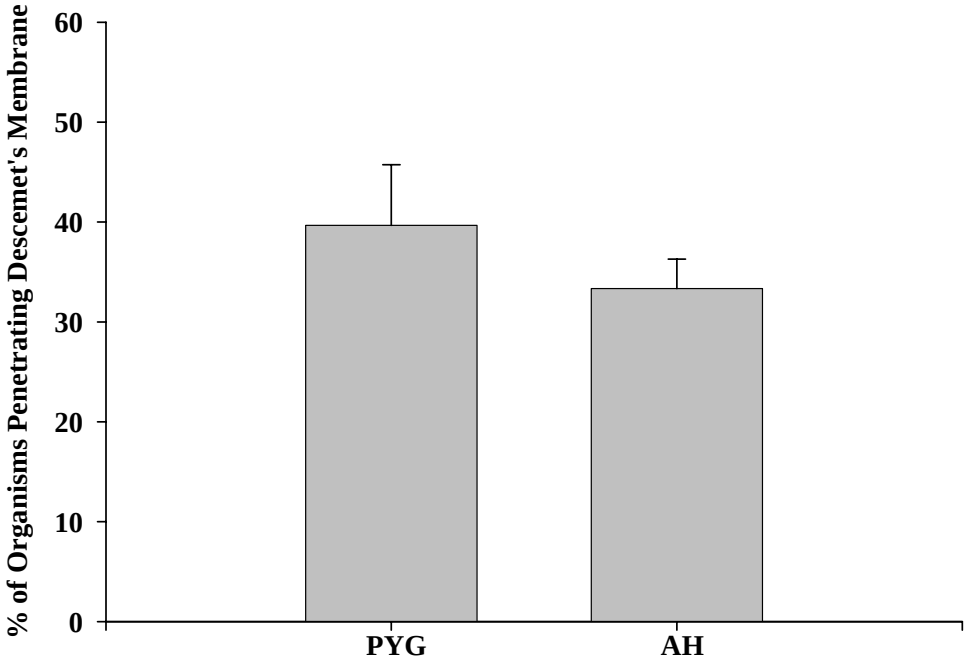

B
% of Organisms Penetrating Descemet's Membrane
50
40
30
20
10
0
*
PYG
ImS
NS

Figure 16. The effect of AH on trophozoite penetration of Descemet's membrane. *Acanthamoeba* trophozoites (10^5/200 μl PYG) were placed on Descemet's membrane in the top chamber of a Boyden chamber and incubated at 37ºC for 24 hr. The bottom chamber contained either PYG or AH. Data are represented as percent organisms penetrating Descemet's membrane. Error bars represent the means +/- SE of 12 wells. This experiment was performed in triplicate.



Importantly, the inhibition of trophozoites by protease inhibitors was not due to adverse effects on trophozoite viability or motility (Fig 17A and B). However, cytochalasin D inhibited trophozoite migration through a transwell membrane by 70% without evidence of toxicity to trophozoites (Fig 17B).

Trophozoite-mediated CPE of human corneal endothelial cells *in vitro*

Previous studies have shown that trophozoites are capable of killing a variety of target cells *in vitro* [66,164,165]. In order to ascertain if *A. castellanii* rarely progresses beyond the corneal endothelium to produce intraocular infections, it was important to determine if trophozoites could either disrupt or kill human corneal endothelial cells (HCN) *in vitro* as these cells are the last barrier between the cornea and the interior of the eye. *A. castellanii* trophozoites produced extensive CPE on HCN cells *in vitro*, killing approximately 90% of cells within 48 hr compared to untreated control cells (Figure 18). Trophozoite-mediated cytolysis of HCN cells was dose dependent (data not shown).

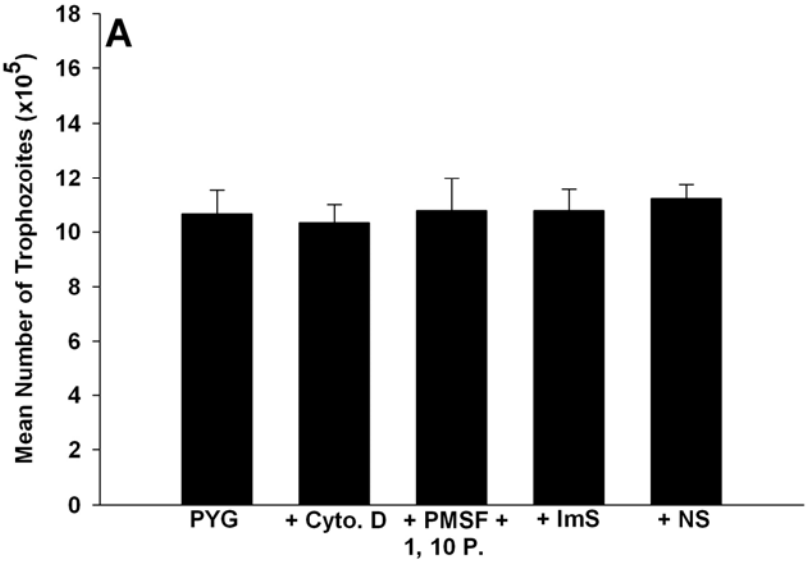
Effect of aqueous humor on trophozoite viability

AH contains several factors that are known to be toxic to microorganisms, including complement and immunoglobulin [1]. Figure 19 shows that approximately 37% of trophozoites encysted in AH after 48 hr, while only 10% of trophozoites encysted in PYG during the same time frame.

To determine if the cysts were still viable after incubation in AH, ten-fold dilutions of cysts were made ranging from 10^5 to 10^1 cysts after a 7-day incubation in AH, PBS, or PYG. Each dilution of cysts was plated on a lawn of *Escherichia coli* on non-nutrient agar to determine cyst viability.

By 72 hr, only the dilution containing 10 cysts in the AH group did not produce trails on the lawn of *E. coli*, indicating that at least 99.999% of the cysts were viable after the 7-day incubation in AH (Table 1). These results were similar to the PBS control group. Trophozoites were incubated in 10X AH for seven days to determine if AH contains a factor that is toxic to trophozoites when present at higher concentrations. Serial dilutions of cysts were made ranging from 10^5 to 10^1 cysts after the 7-day incubation in 10X AH and plated on lawns of *E. coli* on non-nutrient agar to assess cyst viability. By 72 hr, as few as 10 cysts produced trails, suggesting that virtually 100% of the cysts were still viable after incubation in 10X AH for 7 days (Table 1).

Figure 17. Effects of protease inhibitors on trophozoite viability and motility. (**A**) Effect of serine protease inhibitors and a cytoskeleton inhibitor on trophozoite viability. *Acanthamoeba* trophozoites (10^6) were incubated in 1 ml of PYG with either cytochalasin D (50 μg/ml), 1mM PMSF + 1, 10 phenanthroline, anti-MIP-133 antiserum (0.2 mg/ml; ImS), or preimmune normal serum control (0.2 mg/ml: NS) at 37°C for 24 hr. The number of viable trophozoites was determined using trypan blue exclusion. Data are the mean +/- SE of triplicate counts. The results are not significantly different from the PYG control. (**B**) Effect of protease inhibitors on trophozoite migration through a transwell. *Acanthamoeba* trophozoites (10^5/200μl PYG) were incubated with serine protease inhibitors, cysteine protease inhibitor, anti-MIP-133 antiserum (ImS), preimmune normal serum (NS), or cytochalasin D control at 37°C for 30 min prior to their addition to the upper chamber of a two chamber transwell culture system. A 3.0 μm pore size membrane separated the upper and bottom chambers. After a 24 hr incubation at 37ºC, *Acanthamoebae* were counted in the bottom chamber. Data are the mean +/- SE of triplicate counts. Each experiment was performed in triplicate. **$p<0.01$.


A
Mean Number of Trophozoites (x10^5)
18
16
14
12
10
8
6
4
2
0
PYG
+ Cyto. D
+ PMSF + 1, 10 P.
+ ImS
+ NS

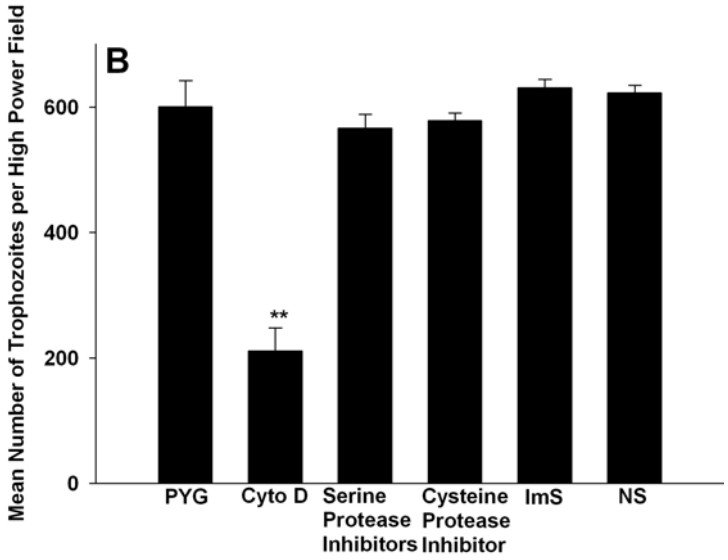

B
Mean Number of Trophozoites per High Power Field
600
400
200
0
**
PYG
Cyto D
Serine Protease Inhibitors
Cysteine Protease Inhibitor
ImS
NS

Figure 18. Trophozoites kill human corneal endothelial cells *in vitro*. Trophozoites (2.5×10^4) were added to 96-well plates, with or without confluent monolayers of HCN cells and incubated for 48 hr at 37°C. Wells were Giemsa-stained and the optical density (OD) was read at 590 nm in a Microplate Reader. The OD of trophozoites alone was subtracted from the OD of experimental wells to determine the OD of live cells. Percent of cells alive was calculated according to the following formula: Cells alive (%) = (OD of experimental well/OD of control well) X 100. The results are representative of three separate experiments.

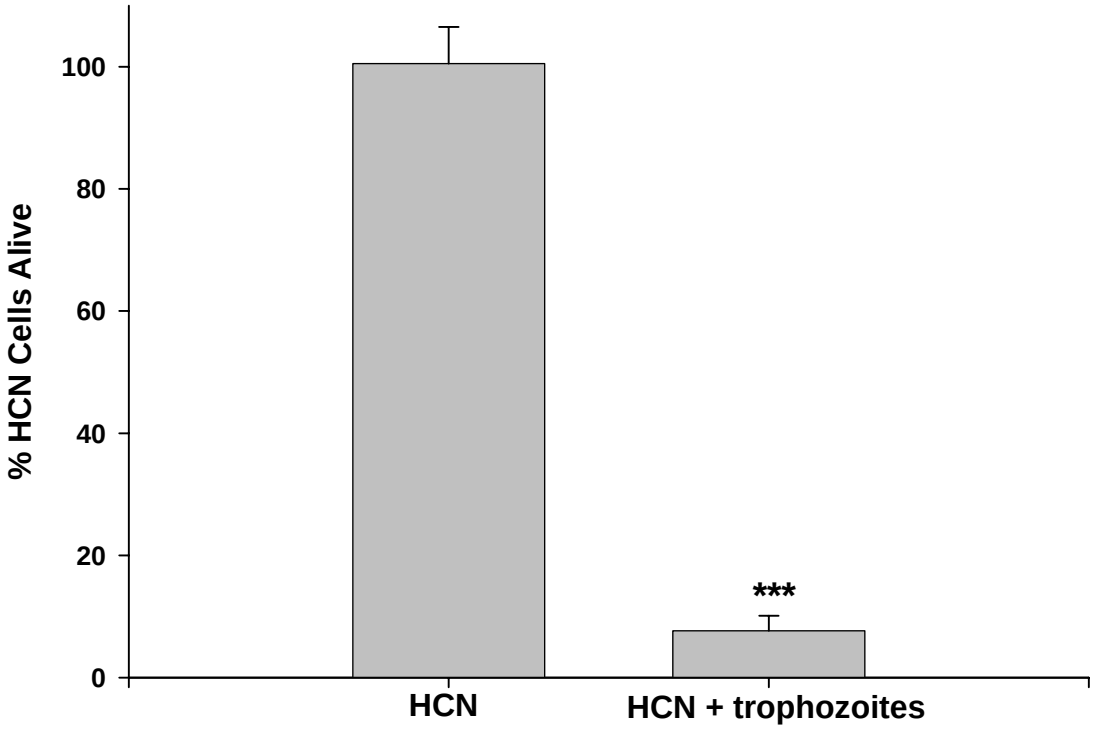


Figure 19. Trophozoite encystment in AH *in vitro*. Trophozoites (10^6) were incubated in 1ml of either PYG (negative control), aqueous humor (AH), or phosphate buffered saline (PBS; positive control for encystment). Trophozoites and cysts were counted at 24 and 48hr and viability was determined by trypan blue exclusion. Data are presented as percent cysts. **p<0.01. This experiment was performed in triplicate.

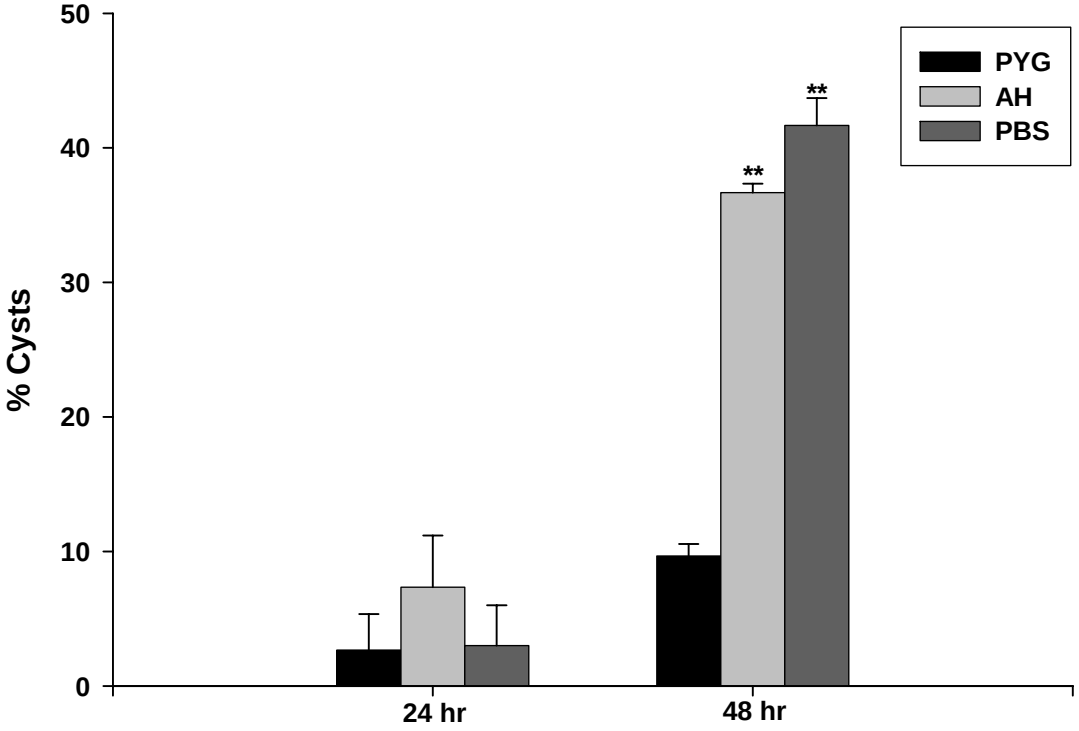


Table 1. Viability of cysts after incubation in AH. Trophozoites (10^6) were incubated in PYG, aqueous humor (AH), 10X AH, or PBS for 7 days. After the incubation, serial dilutions of the trophozoites ranging from 10^5-10^1 were made and placed on a lawn of *E. coli* on non-nutrient agar. Subsequently, the plates were observed for the presence of *Acanthamoeba*-produced trails at 24, 48, and 72 h. The results are expressed in terms of trails (+/-), an indicator of amoeba viability. This experiment was performed in triplicate.

24h	**PYG**	**1X AH**	**10XAH**	**PBS**
10^5	-	-	-	-
10^4	-	-	-	-
10^3	-	-	-	-
10^2	-	-	-	-
10^1	-	-	-	-
48h				
10^5	+	+	+	+
10^4	+	+	+	+
10^3	-	+	-	+
10^2	-	+	-	-
10^1	-	-	-	-
72h				
10^5	+	+	+	+
10^4	+	+	+	+
10^3	+	+	+	+
10^2	+	+	+	+
10^1	+	-	+	-

Trophozoites do not encyst in the anterior chamber of the eye

It is possible that trophozoites can enter the AC; however, the AH that fills this compartment of the eye contains factors that either induce encystment or kill the amoebae *in vivo*. To determine if trophozoites can survive in the AH in the AC, 10^6 trophozoites were injected into the AC and the eyes were enucleated for histology 30 min later and at days 1, 4, and 15 (Fig. 20). By 30 min post inoculation, trophozoites were observed around the iris. By day 1, trophozoites had infiltrated the iris, but did not encyst in the AC. Additionally, neutrophils had migrated into the AC and circumscribed the trophozoites. The number of trophozoites in the AC decreased by day 4 post inoculation. By day 15, both trophozoites and inflammatory cells were not detected anywhere in the eye. The presence of trophozoites in the AC was confirmed with immunoperoxidase staining (Figure 21). Trophozoites stained positively with rabbit anti-*Acanthamoeba* antiserum, but did not stain with preimmune serum control.

Trophozoites kill human iris ciliary body cells

Trophozoites rapidly encysted when incubated in AH *in vitro*, yet there was no evidence of encystment of trophozoites following injection into the AC of Chinese hamsters. We believe the encystment induced by AH *in vitro* was the consequence of nutrient deprivation. The extensive accumulation of trophozoites on the iris and the conspicuous absence of trophozoite encystment, suggested that iris/ciliary body cells might serve as a source of nutrition for trophozoites that enter the AC. Accordingly, the capacity of trophozoites to kill ICB cells was examined *in vitro*. Within 48 hr of *in vitro* incubation, trophozoites lysed approximately 80% of the ICB cells compared to untreated control cells (Figure 22).

Figure 20. Inflammation and clearance of *Acanthamoeba* trophozoites injected into the AC of Chinese hamsters. Trophozoites ($1x10^6$ in 5 µl) were injected into the AC of Chinese hamsters on day 0. Eyes were enucleated and examined after 30 min, 1 day, 4 days, and 15 days. (**A**) Normal Chinese hamster AC and iris (I) (100X). (**B**) AC containing trophozoites within 30 min post AC injection (100X). (**C**) Trophozoites (arrows) localized on the iris surface (I) within 30 min post AC injection (600X). (**D**) Trophozoites and inflammatory cells in the AC within 1 day post AC injection (100X). (**E**) Neutrophils (arrows) circumscribed trophozoites (T) within 1 day post AC injection (600X). Note the trophozoite (T) nestled in the iris (I). (**F**) Abundance of inflammatory cells in the AC 4 days post AC injection (100X). (**G**) Paucity of trophozoites in the AC within 4 days post AC injection (600X). (**H**) Absence of both trophozoites and inflammatory cells in the AC 15 days post AC injection (100X). (**I**) Normal iris architecture 15 days post AC injection (600X). Note the absence of inflammatory cells. Control experiments involving the injection of PYG alone (5 µl) into the AC of the eye did not result in inflammation (data not shown). This experiment was performed three times with similar results.

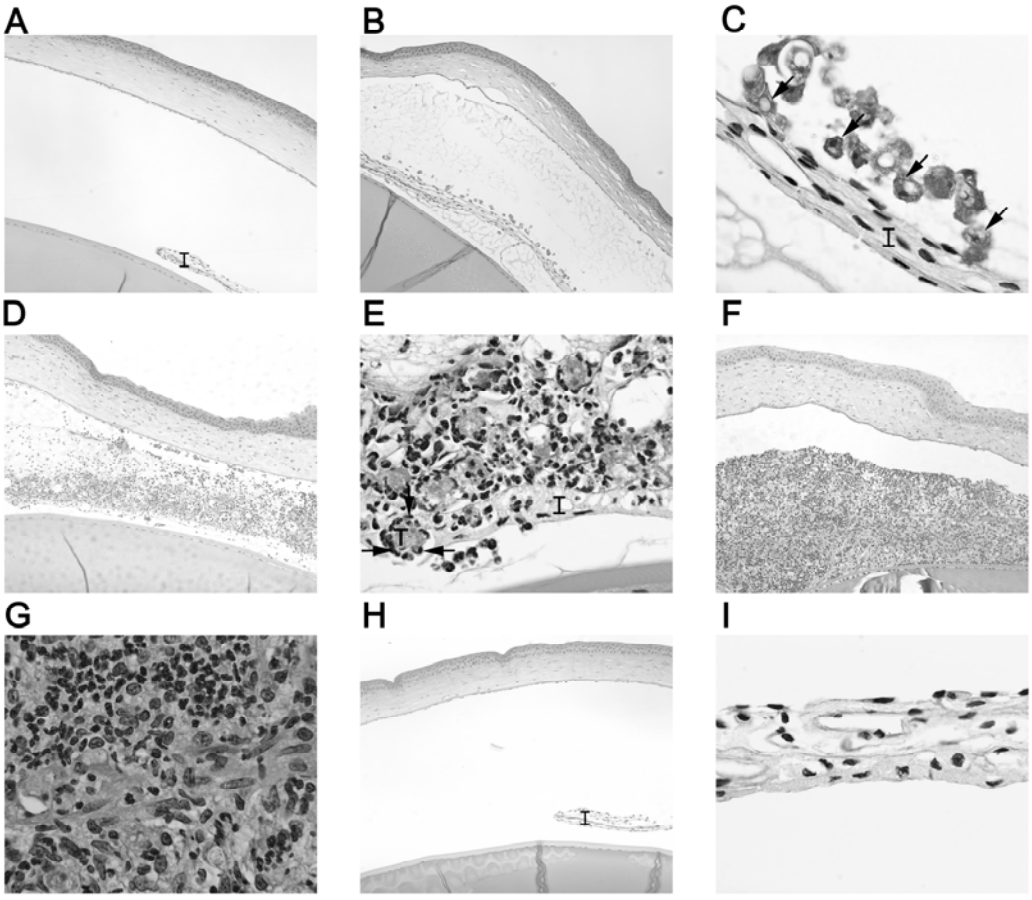

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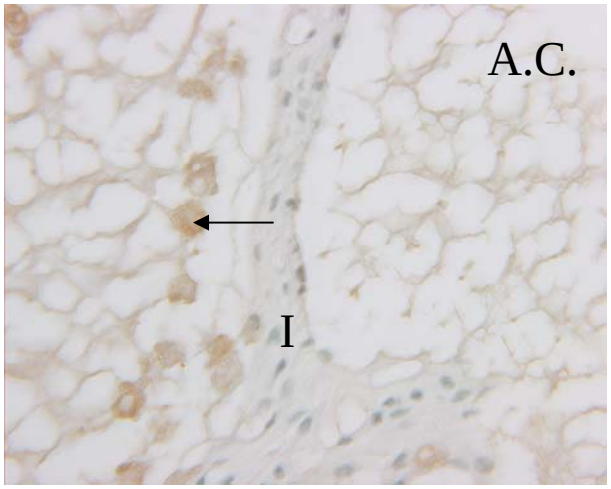
Figure 21. Immuno-staining of trophozoites in the AC. The presence of *Acanthamoeba* trophozoites in the A.C. 30 min post inoculation was confirmed via immunohistochemistry using anti-*Acanthamoeba* antiserum. Trophozoites (black arrows) in the A.C. near the iris (I) stained positively with anti-*Acanthamoeba* antiserum, but did not stain with the preimmune serum control.

Anti-*Acanthamoeba* antiserum



Preimmune control serum

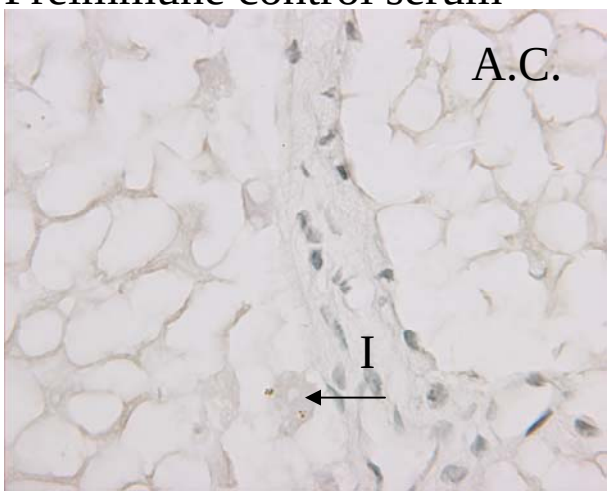
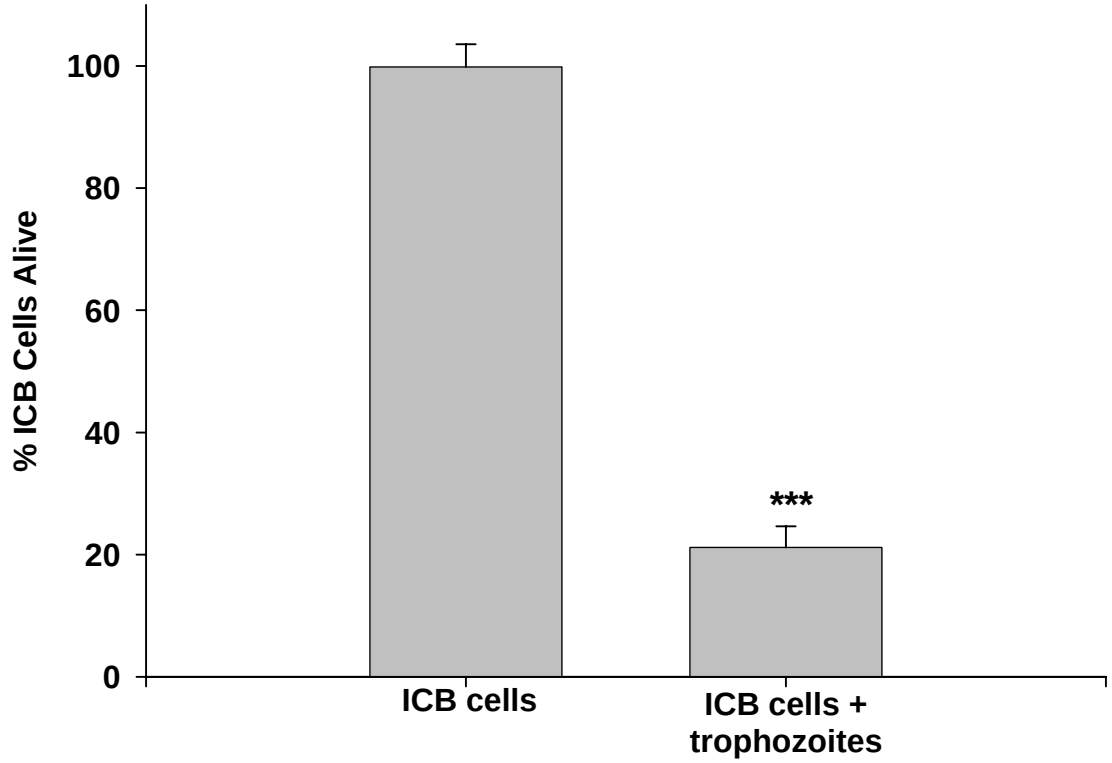


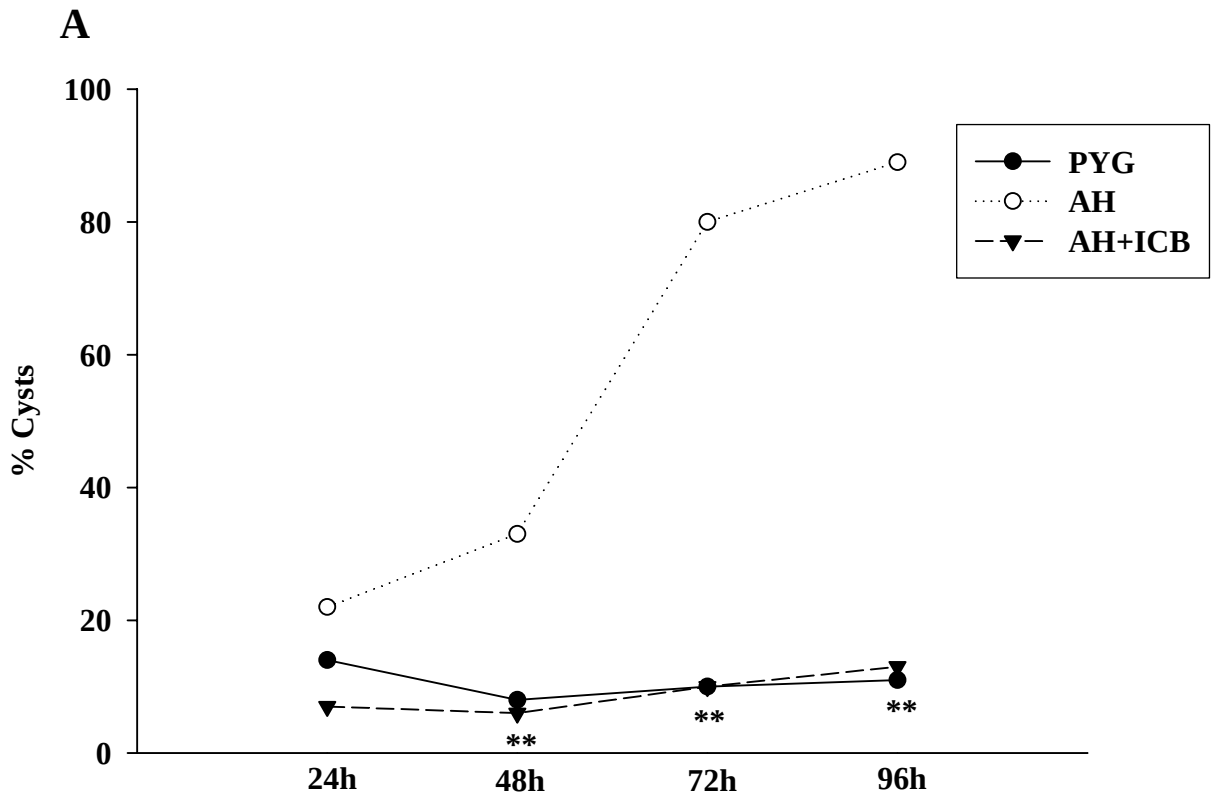
Figure 22. Trophozoites kill iris ciliary body cells *in vitro*. Trophozoites (2.5×10^4) were added to 96-well plates, with or without confluent monolayers of ICB cells and incubated for 48 hr at 37°C. Wells were Giemsa stained and the OD was read at 590 nm in a Microplate Reader. The OD of trophozoites alone was subtracted from the OD of experimental wells to determine the OD of live cells. Percent of cells alive was calculated according to the following formula: Cells alive (%) = (OD of experimental well/OD of control well) X 100. This experiment was performed in triplicate.

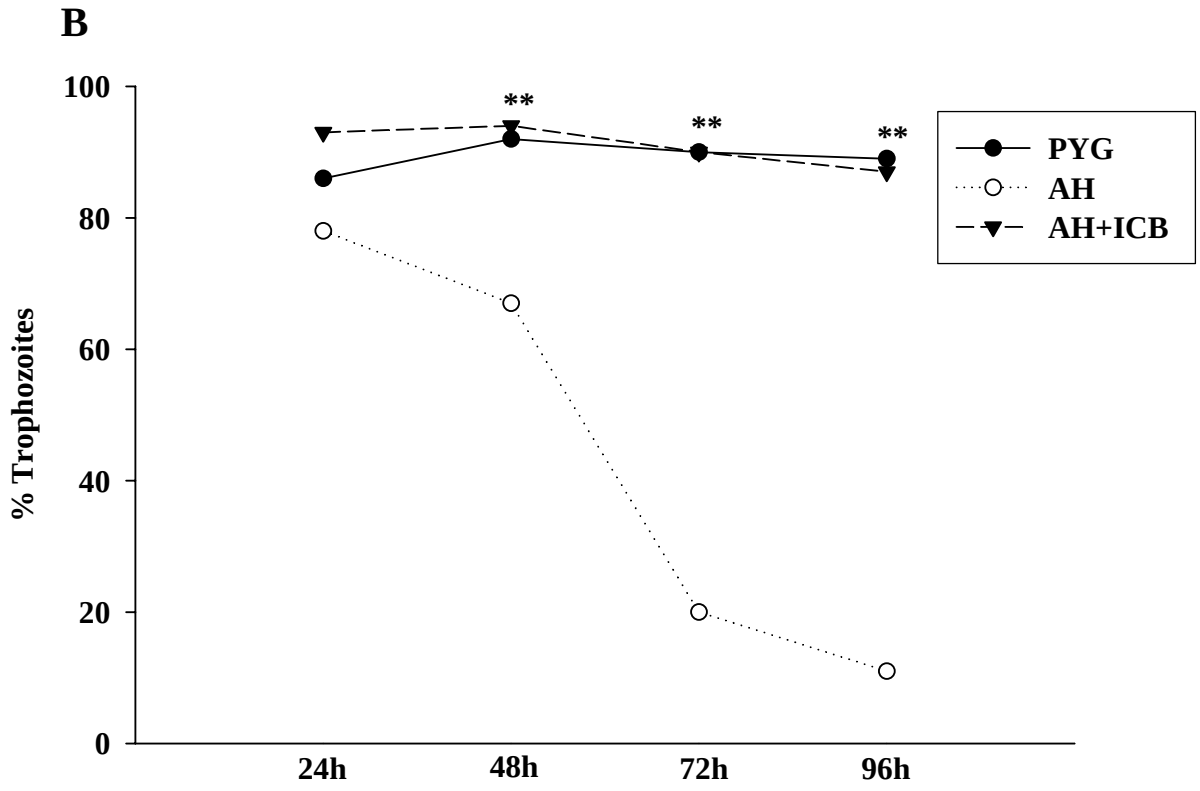


Effect of aqueous humor on encystment of trophozoites incubated with iris ciliary body cells

Additional experiments were performed to determine if ICB cells could serve as a source of nutrition and offset encystment associated with AH. Trophozoites were incubated in either PYG, AH, or in AH on a confluent monolayer of ICB cells, and cultures were examined for evidence of encystment. Figure 23 shows that AH did not induce encystment if trophozoites were incubated on a confluent monolayer of ICB cells. By 48 hr, 40% of the trophozoites incubated in AH alone had encysted. By contrast, only 6% of the trophozoites incubated in AH on a confluent monolayer of ICB cells encysted. Almost 100% of the trophozoites incubated in AH alone had encysted by 96 hr. However, only 13% of the trophozoites incubated in AH on a confluent monolayer of ICB cells had encysted by 96 hr. The capacity of ICB cell cultures to prevent AH-induced encystment was comparable to that found with PYG culture medium control and supports the notion that ICB cells prevent encystment by preventing nutrient deprivation.

Figure 23. Effect of AH on encystment of trophozoites incubated with iris ciliary body cells. Trophozoites (2.5×10^4) were added to 24-well plates in either PYG (negative control), AH (positive control for encystment), or AH on a confluent monolayer of ICB cells. Trophozoites were transferred to either fresh PYG, fresh AH, or fresh AH on a fresh confluent monolayer of ICB cells at 24, 48, and 72 hr. Cysts and trophozoites were counted at 24, 48, 72, and 96 hr. Data are represented as percent cysts (**A**) and percent trophozoites (**B**). **p<0.01. Each experiment was performed in triplicate.

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CHAPTER FOUR
DISCUSSION AND FUTURE RESEARCH

THE ROLE OF NON-IMMUNE MECHANSIMS IN THE LATEX BEAD PROTECTIVE EFFECT

The findings reported here support previous results demonstrating that intracorneal instillation of sterile latex beads prior to infection reduces the incidence, severity, and duration of *Acanthamoeba* keratitis [37]. By contrast, intracorneal instillation of latex beads prior to infection with *P. aeruginosa* exacerbates keratitis by promoting a more robust DTH response via T_H1 cells [136]. Furthermore, increased levels of mRNA transcripts for IFN-γ, a T_H1 type cytokine, were detected in bead-treated, *P. aeruginosa*-infected corneas [136]. Intriguingly, intracorneal instillation of latex beads prior to infection with *A. castellanii* also promotes the development of *Acanthamoeba*-specific DTH [137]. However, neither cell-mediated immunity nor serum antibody protects against *Acanthamoeba* keratitis, indicating that the adaptive immune apparatus is not effective against *Acanthamoeba* keratitis [130,131]. Additionally, it is unlikely that the adaptive immune apparatus can generate an effective immune response within four days of infection, as is observed in the latex bead protective effect.

Perhaps the simplest explanation of the latex bead protective effect associated with *Acanthamoeba* keratitis is that latex beads induce the encystment of trophozoites and prevent the establishment of infection. However, previous results from our lab indicate that trophozoite-mediated phagocytosis of latex beads does not induce the encystment of trophozoites [37]. Moreover, as clodronate eliminates the latex bead protective effect, it is unlikely that latex bead-induced encystment of trophozoites is solely responsible for the latex bead protective effect.

Latex beads may provide protection at any step in the pathogenic cascade of *Acanthamoeba* keratitis. The pathogenesis of *Acanthamoeba* keratitis begins when trophozoites adhere to mannosylated glycoproteins on the corneal surface via a 136-kDa mannose binding protein on the trophozoite membrane [44]. It is possible that phagocytosis of latex beads might induce corneal epithelial cells to downregulate surface mannosylated glycoproteins, resulting in decreased adherence of trophozoites to the corneal epithelium. However, the data show that latex bead treatment did not significantly affect the ability of trophozoites to bind to corneas in an organ culture system, indicating that latex beads must provide protection via an alternate mechanism.

After trophozoite adherence to mannosylated glycoproteins on the corneal surface, the pathogenic cascade of *Acanthamoeba* keratitis continues with trophozoite-mediated destruction of corneal epithelial cells and penetration and dissolution of the corneal stroma. Trophozoites elaborate a variety of proteases, including MIP-133, that assist trophozoites in invasion of the cornea [59]. Previous reports have shown that corneal epithelial cells produce increased amounts of IL-1 after phagocytosis of latex beads [139]. Thus, it is not unreasonable to predict that phagocytosis of latex beads may induce corneal epithelial cells to elaborate protease inhibitors that may prevent trophozoite-mediated destruction of the corneal surface. Alternatively, latex beads may induce corneal epithelial cells to produce antimicrobial factors such as IL-12, IL-18, or tumor necrosis factor-alpha (TNF-alpha) that may be toxic to trophozoites, reduce trophozoite-mediated destruction of the cornea and mitigate disease. However, the data reported here suggest that latex beads do not protect corneal cells from trophozoite-mediated killing at any of the permutations examined.

After binding to and degrading the corneal epithelium, trophozoites may phagocytose the latex beads that have been released by killed corneal cells, which may result in a decrease in the pathogenicity of the trophozoites. For example, phagocytosis of latex beads may inhibit trophozoite-mediated production of the serine protease, MIP-133, resulting in mitigation of corneal infection. In the experiment described above, trophozoites are free to phagocytose latex beads that have been released by killed corneal cells. However, given the absence of decreased trophozoite-mediated killing in this system, it is unlikely that trophozoite-mediated phagocytosis of latex beads decreases the pathogenicity of the amoebae.

THE ROLE OF IMMUNE MECHANISMS IN THE LATEX BEAD PROTECTIVE EFFECT

With the exception of mucosal IgA antibody, the adaptive immune apparatus is not typically effective against *Acanthamoeba* keratitis. Although serum IgG antibody and DTH can be generated by systemic immunization with *Acanthamoeba* antigens, they fail to protect against subsequent ocular challenge with *Acanthamoeba* trophozoites, indicating that T cell-mediated immunity and serum antibody do not directly protect against infection [130]. Mucosal surfaces, such as the conjunctiva, are shielded from microbial invasion by a variety of humoral factors, including secretory IgA antibody, which is the most abundant immunoglobulin present in the human body and is a major antimicrobial component in tears [81,93,94]. Secretory IgA prevents the establishment of infection by inhibiting pathogen adhesion to the mucosal surfaces, including the corneal epithelium [96-100]. Thus, it is no surprise that oral immunization with *Acanthamoeba* antigens promotes the generation of *Acanthamoeba*-specific IgA in mucosal secretions, including tears, and protects against infection in the rabbit, pig, and Chinese hamster models of *Acanthamoeba* keratitis [128,129,132]. Anti-*Acanthamoeba* IgA antibody does not affect the viability of the trophozoites, but inhibits their adherence to corneal epithelial cells *in vitro*, indicating that protection is most likely due to IgA-mediated inhibition of trophozoite adhesion to the corneal epithelium [128,129]. As discussed previously, IgA antibody can protect against disease by preventing trophozoite adhesion to the corneal epithelium, by augmenting neutrophil-mediated lysis of trophozoites, and by inhibiting MIP-133-mediated lysis of the corneal epithelial and stromal cells.

The latex bead protective effect is observed within four days of exposure to *Acanthamoeba* trophozoites [37]. Thus, if the immune apparatus is involved in the latex bead protective effect, it is likely the innate immune apparatus, and not the adaptive immune apparatus mediates protection. Elements of the innate immune system are clearly involved in resistance to *Acanthamoeba* infections [166]. Both neutrophils and macrophages kill *Acanthamoeba* trophozoites and cysts *in vitro* [110-113,126,127]. The results demonstrated here indicate that neutrophils are also involved in the removal of trophozoites from the AC. As shown here and elsewhere, *in vivo* depletion of neutrophils with anti-Chinese hamster neutrophil antibody resulted in an exacerbation of clinical infection [110]. However, the results demonstrate that depletion of neutrophils did not abrogate the latex bead protective effect, indicating that neutrophils are not the primary mediator of the latex bead protective effect.

Numerous studies with experimental animals have indicated that macrophages are critical for protection against *Acanthamoeba* keratitis [109]. Clodronate-mediated depletion of macrophages in the Chinese hamster model of *Acanthamoeba* keratitis results in a more severe, chronic infection compared to untreated control animals [109]. Thus, we hypothesized that macrophages may play a role in the latex bead protective effect. Previous investigators have observed increased numbers of macrophages in latex bead-treated, *P. aeruginosa*-infected mouse corneas [136]. Moreover, elevated levels of mRNA for macrophage-activating IFN-γ were also detected in these corneas [136]. Results reported here show that *in vivo* depletion of macrophages in latex bead-treated corneas abrogated the latex bead protective effect associated with *Acanthamoeba* keratitis, indicating that macrophages play a role in this effect.

There is evidence that macrophages have a maximum life span of several months, and are replaced at the rate of ~1%/day [167]. Furthermore, monocyte chemotactic protein-1 (MCP-1)

can prolong the life span of alveolar macrophages, presumably by inhibition of apoptosis of macrophages [168]. It is possible that latex beads could induce the production of MCP-1 and prolong the life span of corneal macrophages, resulting in an increase in macrophage-mediated killing of trophozoites and a mitigation of *Acanthamoeba* keratitis. However, since the latex bead protective effect is observed within 4 days of exposure to *Acanthamoeba* trophozoites, it is unlikely that latex bead-induced elongation of the macrophage lifespan is responsible for the latex bead protective effect.

Previous reports have indicated that macrophages produce increased levels of IL-1 after phagocytosis of latex beads [169]. As mentioned above, IFN-γ activates macrophages and dramatically increases their ability to kill *Acanthamoeba* trophozoites *in vitro* [112]. Therefore, we hypothesized that phagocytosis of latex beads induces the production of IFN-γ, priming the cornea to respond to infection with *Acanthamoeba* spp. However, the results show that latex beads did not directly activate macrophages *in vitro*. This result is not surprising as phagocytosis alone does not normally induce the activation of macrophages. Furthermore, latex bead treatment did not induce macrophages to kill trophozoites, indicating that it is unlikely that latex beads induce macrophage-mediated destruction of trophozoites at the corneal surface.

Since corneal epithelial cells produce increased amounts of IL-1 after phagocytosis of latex beads, we predicted that latex beads may induce corneal epithelial cells or stromal cells to produce a factor that activates macrophages [139]. However, the data show that latex bead-treated corneal epithelial cell supernatant did not activate macrophages at the various permutations examined, indicating that latex beads do not induce corneal cells to produce a macrophage-activating factor, such as IFN-γ. Moreover, latex bead-treated corneal cell supernatant did not induce macrophage-mediated cytolysis of trophozoites *in vitro*.

It is well established that IFN plays an important role in protecting the cornea from infection [170-172]. Previous results have demonstrated that corneal epithelial cells and keratocytes can produce IFN and IFN-γ is detectible in HSV- and *P. aeruginosa*-infected corneas [136,172,173]. Moreover, recent evidence indicates that IFN-stimulated genes (ISG), such as ISG-15, augment T cell and NK cell proliferation and function during viral infection [174-176]. The ubiquitin-like protein, ISG-15, is transcriptionally regulated by IFN-α or IFN-β via the upstream IFN stimulatory response element [177-181]. Monocytes, lymphocytes, and corneal cells produce ISG-15, which induces the production of IFN-γ [176,182,183]. It is possible that latex beads may induce corneal cells to produce ISGs, such as ISG-15, which then induces the production of IFN-γ. Alternatively, latex bead treatment may induce corneal epithelial cells to produce IFN-γ directly. Latex bead-induced IFN-γ may activate macrophages *in vivo*, resulting in mitigation of corneal disease. However, the results show that mRNA for IFN-γ in latex bead treated corneal cells is similar to IFN-γ mRNA levels in untreated control corneal cells.

An increased number of macrophages in latex bead-treated, *Acanthamoeba*-infected corneas compared to untreated, infected corneas would indicate that latex bead treatment induces the chemotaxis of macrophages. Latex bead treatment might induce corneal cells to produce a chemoattractant for macrophages, such as MCP-1, macrophage inflammatory protein-1α (MIP-1α), IL-1β, or IL-8, all of which are produced by corneal epithelial cells [184-188]. Previous results have demonstrated that that intracorneal instillation of latex beads prior to corneal infection with *P. aeruginosa* induced the corneal migration of macrophages [136]. Acid phosphatase staining of latex bead-treated, *Acanthamoeba*-infected corneas revealed that latex beads induced the migration of macrophages at day 4 post-infection compared to untreated

controls. Thus, it is likely that the latex bead protective effect is mediated by the corneal migration of macrophages, which kill trophozoites at the ocular surface.

THE ABSENCE OF INTRAOCULAR INFECTION ASSOCIATED WITH *ACANTHAMOEBA* KERATITIS

Endophthalmitis is defined as any inflammation of the internal ocular spaces, including the AC. Bacterial keratitis caused by *P. aeruginosa* can progress beyond the cornea to produce endophthalmitis [189]. By contrast, few cases of intraocular infection with *Acanthamoeba* spp. have been reported in the literature [68-71]. It is unlikely that any of these cases were due to the natural progression of the trophozoite beyond Descemet's membrane and the corneal endothelium into the AC of the eye. For example, one case of intraocular infection with *Acanthamoeba* spp. involved an immunocompromised patient with disseminated cutaneous and pulmonary acanthamebiasis [68]. However, given the absence of *Acanthamoeba* keratitis in this patient, it is unlikely that the intraocular infection was due to the natural progression of the trophozoite beyond the corneal endothelium.

The failure of *Acanthamoeba* spp. to produce intraocular infections is puzzling. Pidherney and coworkers demonstrated that *Acanthamoeba* trophozoites exhibit positive chemotactic responses to corneal endothelial cell extracts [66]. Additionally, *Acanthamoeba* spp. elaborate multiple proteases that are capable of killing a variety of corneal cells *in vitro* [9,35,52,53]. Specifically, MIP-133 kills corneal endothelial cells, indicating that *Acanthamoeba* trophozoites may utilize this protease to invade the AC (Niederkorn *et al*., unpublished). Two hypotheses were considered to explain the absence of intraocular infection associated with *Acanthamoeba* trophozoites. The first hypothesis proposed that trophozoites cannot penetrate Descemet's membrane and the corneal endothelium to enter the AC. However, *Acanthamoeba* trophozoites were capable of killing corneal endothelial cells and penetrating Descemet's

membrane *in vitro*. To more accurately reflect the environment of the eye, the invasion assay was performed with AH in the bottom well of the Boyden chamber. It was predicted that AH may alter the physical properties of Descemet's membrane such that *Acanthamoeba* trophozoites could no longer penetrate the membrane. Importantly, the addition of AH to the bottom well of the Boyden chamber did not significantly alter the ability of *Acanthamoeba* trophozoites to penetrate Descemet's membrane. Collectively, these results indicate the *Acanthamoeba* trophozoites can degrade the corneal endothelium and penetrate Descemet's membrane to invade the AC *in vivo*.

In addition to the ability of MIP-133 to kill a variety of mammalian cells, it is also responsible for trophozoite-mediated penetration of the collagenous matrix, Matrigel, which was used as a model of the corneal basement membrane [59]. Likewise, since serine protease inhibitors, including anti-MIP-133 antiserum, prevented the penetration of Descemet's membrane, we concluded that trophozoites utilize serine proteases, such as MIP-133, to degrade corneal endothelial cells and penetrate Descemet's membrane *in vivo*. These results are not surprising as Descemet's membrane contains type IV collagen, which can be degraded by MIP-133 [1,59]. The serine protease inhibitors were not toxic to the amoebae, nor did they inhibit trophozoite migration. Thus, it is likely that the inhibitors prevented the serine proteases responsible for the penetration of Descemet's membrane, which supports the notion that trophozoites can employ the serine protease MIP-133 to penetrate the cornea and enter the AC.

An alternate hypothesis to explain the absence of intraocular infection associated with *Acanthamoeba* keratitis suggests that trophozoites can invade the AC; however, the AH that fills this compartment of the eye contains factors that either induce encystment or kill the amoebae. AH contains several factors that are known to be toxic to microorganisms, including lysozyme,

lactoferrin, transferrin, complement, immunoglobulin, and β-lysin [1]. Moreover, AH is toxic to both *Staphylococcus aureas* and *P. aeruginosa in vitro* [190]. However, our results show that trophozoites encyst in AH *in vitro*, but are still viable after a 7-day incubation in 10X AH, suggesting that it is unlikely that AH contains a factor that is toxic to trophozoites. We speculate that AH-induced encystment of trophozoites *in vitro* may be due to nutrient deprivation.

Although AH induced the encystment of *Acanthamoeba* trophozoites *in vitro,* injection of trophozoites into the AC did not induce their encystment or death. Instead, trophozoites localized on the iris surface and induced a robust neutrophilic infiltrate. As mentioned above, previous experiments have shown that neutrophils are critical in the innate immune response to *Acanthamoeba* keratitis, presumably due to their ability to kill trophozoites and cysts [110,126,127]. Moreover, recruitment of neutrophils into the cornea via intrastromal injections of recombinant MIP-2, a chemotactic factor for neutrophils, results in transient infiltration of neutrophils followed by rapid resolution of corneal infection [110]. Taken together, these findings suggest that neutrophils are responsible for the elimination of trophozoites from the AC. Given the intense neutrophil infiltrate associated with trophozoite clearance from the AC, it is interesting that the damage to the non-regenerating ocular tissues was minimal. This may be due to the abundant antioxidants present in the AH, such as ascorbic acid, which inactivate superoxide radicals produced by neutrophils and other inflammatory cells, thereby preserving the integrity of the AC [191,192].

As mentioned previously, corticosteroids are often utilized to control the pain and inflammation associated with *Acanthamoeba* keratitis [12]. However, recent experiments have demonstrated that dexamethasone treatment exacerbates *Acanthamoeba* keratitis in the Chinese hamster model of infection, presumably by inhibition of macrophage and neutrophil activation

[43]. Furthermore, dexamethasone treatment induces excystment and proliferation of trophozoites and also stimulates the *Acanthamoeba*-mediated CPE of corneal epithelial cells *in vitro* [43]. Consistent with previous findings, the present results suggest that steroid treatment may increase the corneal load of trophozoites and exacerbate ocular disease in patients with *Acanthamoeba* keratitis.

The failure of trophozoites to encyst in the AH of the AC suggests that inadequate nutrients present in AH were responsible for encystment of the amoebae *in vitro*. Support for this hypothesis was demonstrated in experiments showing that trophozoites did not encyst in AH when grown in the presence of iris ciliary body cells as a source of nutrition *in vitro*. Thus, it is likely that trophozoites feed on iris cells *in vivo*, thereby preventing their encystment in the AC. An alternative hypothesis proposed that trophozoites live on soluble factors produced by iris cells in the AC, thus preventing their encystment. However, this scenario is unlikely. Importantly, trophozoites are capable of killing ICB cells *in vitro*. Furthermore, trophozoites encyst in AH after depletion of ICB cells *in vitro*, indicating that trophozoites do not depend on ICB-derived factors to prevent their encystment in the AC. This evidence provides further support for the notion that AH-induced encystment of trophozoites *in vitro* is due to nutrient deprivation.

FUTURE RESEARCH

Although resting macrophages can succumb to trophozoite-mediated killing, the presence of IFN-γ and *Acanthamoeba*-specific antibody dramatically increases the capacity of macrophages to kill trophozoites *in vitro*, suggesting that activated macrophages eliminate trophozoites *in vivo* [112]. Our results show that macrophages are involved in the latex bead protective effect. However, it is currently unclear how latex bead-treatment induces macrophage-mediated protection against *Acanthamoeba* keratitis. Corneal infiltration of macrophages and increased transcript levels of IFN-γ have been detected in latex bead-treated, *P. aeruginosa*-infected corneas [136]. The investigators of this study concluded that latex beads may prime the cells of the innate immune system to respond more rapidly to corneal insult, presumably by inducing macrophage chemotaxis and activation.

As corneal epithelial cells produce increased amounts of IL-1 after phagocytosis of latex beads, it is possible that latex beads may induce corneal cells to produce a factor that induces the chemotaxis or activation of macrophages [139]. Corneal latex bead treatment may induce corneal cells to produce a factor, such as MCP-1, MIP-1α, IL-1β, or IL-8 that induces the chemotaxis of macrophages to the latex bead-treated, *Acanthamoeba*-infected cornea. Experimental approaches to test this hypothesis include RT-PCR and real-time RT-PCR to identify and quantify mRNA expression of potential macrophage chemotactic proteins in latex bead-treated, *Acanthamoeba*-infected corneas. Elevated levels of a macrophage chemotactic factor in latex bead-treated, *Acanthamoeba*-infected corneas would indicate that latex bead treatment protects against *Acanthamoeba* keratitis by inducing the migration of macrophages to the corneal surface.

Moreover, it is possible that latex bead treatment induces corneal epithelial cells or stromal cells to produce increased amounts of IFN-γ, which may activate macrophages *in vivo*, increasing their ability to kill *Acanthamoeba* trophozoites. RT-PCR and real-time RT-PCR could be utilized to detect and quantify IFN-γ mRNA expression in latex bead-treated, *Acanthamoeba*-infected corneas. Detection of IFN-γ mRNA in latex bead-treated, *Acanthamoeba*-infected corneas may demonstrate increased levels of this T_H1 type cytokine compared to untreated control animals and support the hypothesis that latex bead treatment induces the activation of macrophages *in vivo*.

Elucidation of the role of macrophages in the latex bead protective effect may provide new treatment options for *Acanthamoeba* keratitis. The finding that macrophages are involved in the latex bead protective effect provides further support for the role of macrophages in resistance to *Acanthamoeba* keratitis. Moreover, liposome-mediated delivery of IFN-γ to the conjunctiva of Chinese hamsters prior to infection with *A. castellanii* results in mitigation of disease, presumably due to the activation of macrophages (J.Y. Niederkorn *et al.*; unpublished). Collectively, these results suggest that activation of ocular macrophages may lead to the resolution of corneal disease in patients with *Acanthamoeba* keratitis. It is predicted that phagocytosis of IFN-γ-containing liposomes will activate macrophages and increase their capacity to kill trophozoites in patients with *Acanthamoeba* keratitis.

Our results suggest that *Acanthamoeba* trophozoites utilize MIP-133, a serine protease, to penetrate Descemet's membrane and the corneal endothelium. However, once in the AC, trophozoites induce a robust neutrophil infiltrate, which is associated with eventual clearance of trophozoites from the AC. Additional experimental approaches may confirm the role of neutrophils in preventing the development of intraocular infection with *Acanthamoeba* spp. We

would predict that *in vivo* depletion of neutrophils with anti-Chinese hamster neutrophil antiserum prior to injection of trophozoites into the AC would result in fulminant intraocular infection. Alternatively, administration of the corticosteroid, depomedrol, to cause systemic immunosuppression could confirm that trophozoites are being eliminated from the AC by the immune process. We would hypothesize that depletion of neutrophils by intraperitoneal injection of anti-Chinese hamster neutrophil antiserum prior to infection with *Acanthamoeba* trophozoites would result in the progression of *Acanthamoeba* keratitis beyond Descemet's membrane and the corneal endothelium to produce an intraocular infection. Results obtained from this experiment could further support the role of neutrophils in preventing the progression of *Acanthamoeba* keratitis into the AC.

Although *Acanthamoeba* keratitis is a self-limiting disease in Chinese hamsters, it is a chronic infection in humans [12]. *Acanthamoeba* keratitis rarely progresses to become an intraocular infection in both Chinese hamsters and humans [69]. It is interesting that the innate immune apparatus effectively eliminates both intraocular and extraocular trophozoites in experimental animals [37]. It is uncertain why the innate immune system is effective at preventing intraocular infection, but is incapable of resolving chronic *Acanthamoeba* infections in the human cornea. Perhaps intraocular neutrophils are efficiently activated in response to *Acanthamoeba* trophozoites, while neutrophils at the ocular surface are not adequately activated. Future studies are necessary to determine why neutrophils are not effective at resolving infection at the ocular surface.

The results indicate that neutrophils are involved in clearance of trophozoites from the AC. Similarly, previous reports have shown that neutrophils are crucial in resistance to *Acanthamoeba* infection at the ocular surface [110]. As mentioned above, intracorneal

administration of rMIP-2 induces the migration of neutrophils to the corneal surface and results in the rapid resolution of *Acanthamoeba* keratitis in experimental animals [110]. Taken together, these results suggest that intracorneal administration of rMIP-2 may lead to the resolution of corneal disease in patients with *Acanthamoeba* keratitis.

The serine protease, MIP-133, plays a role in trophozoite-mediated desquamation of the corneal epithelium and penetration of the underlying stroma. The results presented here indicate that trophozoites also utilize MIP-133 to penetrate the Descemet's membrane and the corneal endothelium. Previous reports have shown that mucosal immunization with MIP-133 promoted the generation of anti-MIP-133 IgA antibody in tears, which mitigated corneal infections in the Chinese hamster model of *Acanthamoeba* keratitis [59]. Additionally, in contrast to previous findings that mucosal immunization with *Acanthamoeba* cell membrane antigens once infection has been established was not effective at mitigating disease, mucosal immunization with MIP-133 post-infection resulted in amelioration of clinical symptoms. Collectively, these results indicate that topical application of anti-MIP-133 IgA antibody may mitigate disease in patients with *Acanthamoeba* keratitis.

It is interesting that trophozoites encyst in AH, but do not encyst in AH while grown in the presence of iris ciliary body cells. It is well known that trophozoites encyst under a variety of environmental conditions, including hyperosmolarity, glucose starvation, desiccation, extreme temperatures, or extreme pH [35]. One possible avenue of future research is to determine how *Acanthamoeba* trophozoites sense their external environment. It is predicated that cell-surface receptors control *Acanthamoeba* differentiation as a monoclonal antibody to a 40-kD trophozoite protein induces the encystment of trophozoites and prevents the excystment of cysts [14].

In the final analysis, neutrophils and macrophages of the innate immune system elaborate a variety of products, such as defensins and NO, that have direct antimicrobial activity on a variety of protozoan pathogens. The results reported here provide further support for the role of the innate immune system in resistance to *Acanthamoeba* keratitis and indicate that recruitment and/or activation of elements of the innate immune system may ameliorate clinical symptoms in patients with protozoan infections.

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VITA

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