Effect of Immunization with the Mannose-Induced Acanthamoeba Protein and Acanthamoeba Plasminogen Activator in Mitigating Acanthamoeba Keratitis

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PURPOSE. The mannose-induced cytopathic protein (MIP-133) and Acanthamoeba plasminogen activator (aPA) play key roles in the pathogenesis of Acanthamoeba keratitis by inducing a cytopathic effect on the corneal epithelial and stromal cells and by production of proteolytic enzymes that facilitate the invasion of trophozoites through the basement membrane. The goal of the present study was to gain insight into the pathogenicity of Acanthamoeba infection as well as to determine whether oral immunization with aPA and MIP-133 produce an additive protection against Acanthamoeba keratitis.

METHODS. MIP-133 and aPA were isolated by chromatography. The purity of the concentrated MIP-133 and aPA was confirmed by SDS-PAGE and fibrinolytic activity, respectively. aPA activity of Acanthamoeba cultures was quantitated by radial diffusion in fibrin-agarose gel. The capacity of aPA and MIP-133 to induce cytolysis of corneal epithelial cells was tested in vitro. Chinese hamsters were orally immunized with four weekly doses of aPA or MIP-133 conjugated with cholera toxin. The animals were immunized before infection to determine the prophylactic effect of oral immunization. The therapeutic effect of oral immunization with aPA and MIP-133 was determined after corneal infection had been established. The animals were then infected via Acanthamoeba castellanii-laden contact lenses.

RESULTS. aPA was characterized in pathogenic and nonpathogenic strains of Acanthamoeba spp. Oral immunization with MIP-133 before and after infection with Acanthamoeba significantly reduced the severity of corneal infection which includes infiltration and ulceration (P < 0.05) and shortened the duration of the disease. Immunization with aPA alone did not significantly affect the course of disease (P > 0.05).

CONCLUSIONS. These data suggest that once trophozoites invade the cornea, MIP-133 production is necessary to initiate corneal disease and plays an important role in the subsequent steps of the pathogenic cascade of Acanthamoeba keratitis. (Invest Ophthalmol Vis Sci. 2007;48:5597–5604) DOI:10.1167/iovs.07-0407

The sight-threatening corneal disease Acanthamoeba keratitis is caused by pathogenic free-living amoebae. Acanthamoeba spp. are ubiquitous organisms that can be isolated from a wide variety of environments. The disease is often associated with contact lens wear, which appears to be an important risk factor in infection.

The pathogenic cascade of Acanthamoeba keratitis involves a series of processes that include: (1) binding of the trophozoites to the corneal epithelial cells via lectin-glycoprotein interactions; (2) generation of cytopathic factors that destroy the corneal epithelial and stromal cells; (3) production of proteolytic enzymes that facilitate the invasion and penetration of trophozoites through the basement membrane and stroma; and (4) elaboration of collagenolytic enzymes that degrade types I and IV collagens, which constitute the corneal matrix.

It has been shown that Acanthamoeba trophozoites initiate pathogenesis by binding to corneal epithelial cells. Adhesion to the corneal epithelium is mediated by a mannose-specific lectin present on Acanthamoeba. More recently, Garate et al. have shown that immunization with mannose-binding protein provides protection against Acanthamoeba infection by inhibiting the adherence of the trophozoites to the corneal epithelial cells. We have clearly demonstrated in two animal models that it is possible to prevent the induction of Acanthamoeba keratitis by orally immunizing the host before corneal infection with parasite-laden contact lenses. However, neither oral nor parenteral immunization prevents or mitigates Acanthamoeba keratitis if the immunization is initiated after the application of parasite-laden contact lenses. Although it is not feasible to immunize contact lens wearers prophylactically to prevent corneal infection with Acanthamoeba spp., it may be feasible to use mucosal immunization as a means of mitigating the disease process once infection has been diagnosed. That is, targeting crucial molecules in the pathogenic cascade may diminish the more deleterious pathologic sequelae in Acanthamoeba keratitis. We envision the development of an “anti-disease” vaccine approach as a feasible alternative to an antimicrobial vaccine.

The mannose-induced cytopathic protein (MIP-133) has been characterized, and we have shown that MIP-133 protein can have a cytopathic effect on corneal epithelial cells and also degrade collagen in vitro. Acanthamoeba trophozoites elaborate proteolytic enzymes, especially plasminogen activators (aPAs) which may facilitate the invasion and penetration of trophozoites through the basement membrane and may contribute to the pathogenesis of Acanthamoeba. The goal of the present study was to determine the prophylactic effect of oral immunization with aPA and MIP-133 before infection with Acanthamoeba as well as the therapeutic effect of oral immunization after corneal infection had been established. In Chinese hamsters, corneal infections resolve in 14 to 21 days. However, Acanthamoeba keratitis becomes a chronic, progressive disease in these hosts if conjunctival macrophages are eliminated before corneal infection. That is, conjunctival injection of the macrophagicidal drug dichloromethylene

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diphosphonate (clodonate; CL₃MDP) encapsulated in liposomes, results in the elimination of conjunctival macrophages.

In animals treated with clodronate-containing liposomes before corneal exposure to *Acanthamoeba*-laden contact lenses, progressive, severe *Acanthamoeba* keratitis develops that clinically mimics the human counterpart. To ascertain the therapeutic effect of oral immunization with aPA and MIP-133, clodronate was administered subconjunctivally to develop a progressive and severe *Acanthamoeba* keratitis model.

**MATERIALS AND METHODS**

**Animals**

Chinese hamsters were purchased from Cytogen Research and Development (Westbury, MA). All animals used were from 4 to 6 weeks of age, and all corneas were examined before experimentation to exclude animals with preexisting corneal defects. Animals were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

**Amoebae and Cell Lines**

All *Acanthamoeba* species were originally obtained from the American Type Culture Collection (Manassas, VA). *A. castellanii* (ATCC 30868), *A. polyphaga* (ATCC 30461), and *A. rhysodes* (ATCC 50368) were isolated from human corneas. *A. culbertsoni* (ATCC 30171) was originally isolated from a diseased human kidney. *A. batchetti* (ATCC 30730), *A. astronyxis* (ATCC 30137), and *A. castellanii* nfe (ATCC 30010) were isolated from soil. The chosen species represent all three of the different subgroups of *Acanthamoeba* based on morphology, isozyme analysis, and serology. Amoebae were grown as axenic cultures in peptone-yeast extract-glucose (PYG) at 35°C, with constant agitation.

Chinese hamster corneal epithelial (HCORN) cells were immortalized with the human papillomavirus E6 and E7 genes, as previously described and were cultured in minimum essential medium (MEM; JRH Biosciences, Lenexa, KS).

**Purification of *Acanthamoeba* aPA**

The four pathogenic and three nonpathogenic strains of *Acanthamoeba* were cultured in PYG at 35°C (Table 1). The trophozoites were cultured for 7 days, and the supernatants were collected and centrifuged as described previously. The aPA was purified using the fast protein liquid chromatography system (FPLC), production of aPA was quantified byzymography assays. For zymography analysis, the FPLC-purified samples (10 µg protein) were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and overlayed on a fibrinogen-agarose slab. For preparation of fibrinogen-agarose overlays, 1 mg/mL fibrinogen (Calbiochem-Behring, La Jolla, CA) in 0.1 M Tris-HCl (pH 7.6) was mixed with 20 mg/mL of low-melting-temperature agarose prepared in the same buffer at 40°C. To this, 1.0 IU of bovine thrombin (Parke-Davis, Morris Plains, NJ) was added, and the mixture was poured between two glass plates separated by a 2.5-mm spacer. The overlaid gels were incubated at 37°C for 6 to 8 hours in a humidified chamber. Protein (1 µg) test samples were either run untreated or were pretreated with serine protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), 1 mM 1,10-phenanthroline (1,10 P; Sigma-Aldrich), and a cysteine protease inhibitor, 10 µM t-transepoxysuccinylleucylamidomethyl-(4-guardino)-butane (E6; Sigma-Aldrich) at 37°C for 30 minutes before addition to the SDS gels. Human urokinase plasminogen activator (uPA; 50 kDa) was used as a molecular weight standard. The activity of aPA was determined by radial diffusion in fibrinogen-agarose clots. Supernatants from the various *Acanthamoeba* strains (1 mg/mL) were applied into wells cut in fibrinogen-containing agarose gels. Clots lacking plasminogen were included in all experiments to control for plasminogen-independent fibrinolysis. The diameter of the clear areas in the clots was measured after incubation at 37°C for 2, 3, 5, and 8 hours. By using human tPA as a standard, a linear relationship between the diameter of clear areas and the tPA concentration was established as described elsewhere. TPA activity was expressed in units per milligram per milliliter relative to the tPA standard.

**MIP-133 Isolation**

The MIP-133 protein was purified as stated previously. Briefly, 10-fold concentrated supernatants from *A. castellanii* cultures grown with 100 mM methyl-α-α-mannopyranoside were analyzed by 4% to 15% SDS-PAGE (Ready Gels; Bio-Rad, Hercules, CA). The MIP-133 was purified using the fast protein liquid chromatography system, as described previously.

**Contact Lens Preparation**

Contact lenses were prepared from dialysis membrane tubing (Spectra/Por; Spectrum Medical Industries, Los Angeles, CA) with a 3-mm trephine before heat sterilization. The lenses were placed in sterile 96-well microtiter plates (Corning Costar, Cambridge, MA) and incubated with 3 × 10⁶ *A. castellanii* trophozoites at 35°C for 24 hours. Attachment of amoebae to the lenses was verified microscopically before infection.

**In Vivo Corneal Infections**

*Acanthamoeba* keratitis was induced as described previously. The contact lenses were removed 5 days after infection, and the corneas were visually inspected by microscopy for severity of disease. The infections were scored on a scale of 0 to 5, based on the following parameters: corneal infiltration and corneal ulceration. The disease severity was recorded according to the following scale, indicating the percentage of the cornea involved: 0, no disease; 1, <10% of the cornea involved; 2, 10%-25%; 3, 25%-50%; 4, 50%-75%; and 5, 75%-100%.

**Migration Assays**

The assays were performed in 24-well migration assay chambers (6.5-mm diameter, 3.0-µm pore size; Transwell; Corning Costar, Inc.). The top chamber membrane was coated with 100 µL synthetic basement membrane derived from murine sarcoma cells (Matrigel; Collaborative Biomedical Products, Bedford, MA) that had been diluted 1:5 in HBSS. Briefly, 1 × 10⁵ *Acanthamoeba* trophozoites were then placed in the top chamber in 100 µL PYG. Plates were incubated at 37°C for 2 hours, and the trophozoites were then counted in the bottom chamber by light microscopy (100×). Inhibition assays involved incubating *A. castellanii* with enteric washes from immunized and nonimmunized animals and serine protease inhibitors, and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), at 37°C for 30 minutes before addition to the upper chambers. All experiments were performed in triplicate.

**Assay for Cytopathic Effect**

Different concentrations of the MIP-133 and aPA proteins in PBS were added to 96-well plates with confluent monolayers of HCORN cells and
incubated for 18 hours at 35°C, as described elsewhere. After incubation, all wells were washed three times and stained with Giemsa stain (Shandon, Inc., Pittsburgh, PA). After staining, the wells were washed three times with PBS (pH 7.2) and solubilized in 0.1 mL of 5% SDS in PBS and the optical density (OD) was read at 590 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). Percent cytopathic effect (CPE) was calculated according to the following formula: % CPE = 100 − [(OD of experimental well − OD of supernatant alone/OD of control cells alone) × 100]. Assays were performed in triplicate.

Oral Immunizations

The animals received 1 mL of 0.1 M sodium carbonate (pH 9.6; Sigma-Aldrich) by lavage tube before administration of either 500 µg MIP-133 or 200 and 1000 µg aPA (in 100 µL of PBS) plus 10 µg cholera toxin/100 µg protein (Sigma-Aldrich). Immunizations were administered once a week for 4 weeks before infection with \textit{A. castellanii}. The animals were infected with contact lens-laden \textit{A. castellanii} as just described. Animals receiving PBS (nonimmunized animals) served as the control.

Collection of Chinese Hamster IgA Secretions

Enteric washes were collected as stated previously. The enteric washes were collected and centrifuged at 700g to remove sediments. Protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN) were added to the pooled enteric washes at 1 tablet per 10 mL of the liposome preparation. The washes were stored at 80°C until it was used.

Enzyme-Linked Immunosorbent Assay

The 96-well assay plates were coated with 50 µg of MIP-133 or aPA overnight in carbonate buffer. The plates were washed four times with PBS containing 0.05% Tween-20 (wash buffer: Sigma-Aldrich) and blocked with 0.5% BSA in PBS for 1 hour at room temperature. All subsequent antibodies were diluted in blocking buffer and incubated at room temperature. Enteric washes were added at a dilution of 1:2 for 1 hour and then washed. Rabbit anti-Chinese hamster IgA hyperimmune serum was then added 1:2 and incubated for 2 hours. The plates were washed, and 1:1000 goat anti-rabbit IgG-horseradish peroxidase (HRP; Santa Cruz Biotechnology, Santa Cruz, CA) was added. The plates were developed by adding 1.0 mL 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; Sigma-Aldrich) containing 0.003% H2O2 and incubated for 30 minutes at room temperature. After development, 100 µL of 10% SDS (Sigma-Aldrich) was added per well before reading on a microplate reader at 405 nm.

Preparation of Clodronate Liposomes

Multilamellar liposomes were prepared as described previously. Clodronate liposomes were tested for in vitro toxicity against macrophages before use.

Liposome-Treated Animals

Both clodronate and PBS containing liposomes were administered via subconjunctival injection on days −8, −6, −4, and −2 of infection. Briefly, 50 µL of the liposome preparation was injected in four quadrants of the eye encircling the entire conjunctiva. \textit{Acanthamoeba} infections were performed via contact lens placement, as just mentioned. Animals were orally immunized on days 5, 12, 19, and 26 after infection with 500 µg MIP-133 or 600 µg aPA protein, as described earlier.

Statistics

Statistical analyses of all data except the clinical scores were performed with unpaired Student’s \textit{t} tests. Clinical severity scores were analyzed by the Mann-Whitney test.

**RESULTS**

Expression of aPA by Pathogenic and Nonpathogenic Strains of \textit{Acanthamoeba}

We have demonstrated that ocular isolates of \textit{Acanthamoeba} produce a novel aPA with a molecular mass of approximately 40 to 45 kDa that appears as a single band of lysis on fibrin agarose zymographs. However, this enzyme was not detected in cultures from soil isolates of \textit{A. castellanii}. Thus, it is not known whether there is an association between pathogenicity (induction of ocular infection) and expression of aPA in vitro. We have shown that three \textit{Acanthamoeba} soil isolates (Table 1) did not induce severe keratitis in Chinese hamsters, and therefore they were categorized as nonpathogenic strains. However, four pathogenic strains isolated from infected patients produced severe disease in Chinese hamsters, and they were categorized as pathogenic strains. Because pathogenic strains of \textit{Acanthamoeba} produce aPA, we hypothesized that nonpathogenic strains of \textit{Acanthamoeba} are unable to produce aPA.

The four pathogenic and three nonpathogenic strains of \textit{Acanthamoeba} mentioned above (Table 1) were cultured in PYG at 35°C. aPA activity was examined by radial diffusion in fibrinogen-agarose clots. All four pathogenic strains of \textit{Acanthamoeba} produced significant amounts of aPA activity. By contrast, the aPA activity was not detectable in the culture supernatants from three soil isolates. Radial diffusion of 1 µg of purified aPA protein from \textit{A. castellanii}, displayed five to six mm diameters of lysis in fibrin agarose clots (Fig. 1). Proteolytic activity was comparable to approximately 10 units of tPA under the conditions tested. The zymography overlay using 10 µg of the purified aPA protein displayed clear lytic ability against the fibrinogen in the fibrin agarose gels (Fig. 2). Within 2 hours, lytic zones could be readily visualized. Samples pre-treated with PMSF and 1 mM 1,10-phenanthroline (1,10 PhN;
Sigma-Aldrich) displayed 100% inhibition of lysis, indicating that the protein is a serine protease. Samples pretreated with the cysteine protease inhibitor E6 produced the same lysis as the untreated protein samples on the overlay. Neither the PYG nor the PBS controls were found to be lytic against fibrinogen (data not shown). Urokinase (aPA; 50 kDa) was used as a molecular weight standard. Fibrinogen without plasminogen was used as a control. No activity was detected in the absence of plasminogen in a fibrinogen gel (data not shown).

These results indicate that the aPA activity correlates with the amoeba’s ability to cause disease. The absence of aPA in nonpathogenic strains of Acanthamoeba raises the possibility that either nonpathogenic strains do not produce aPA or constitutively produce an aPA inhibitor that inhibits the production or action of aPA. The trophozoites from pathogenic strains of Acanthamoeba were cultured with the various concentrations of cell free lysates from nonpathogenic strains, and the aPA activity was determined by radial diffusion in fibrinogen-agarose clots. The results showed that nonpathogenic strains of Acanthamoeba do not produce an aPA inhibitor (data not shown).

The Capacity of aPA to Induce CPE in Corneal Epithelial Cells

We have shown that the pathogenic Acanthamoeba and the mannanose-induced cytopathic protein MIP-133 protein are able to induce cytopathic effect on corneal epithelial cells. Therefore, we suspected that aPA not only is involved in the degradation of the corneal extracellular matrix but also may indirectly induce CPE of corneal epithelial cells.

To test the cytolytic ability of the purified aPA protein, samples were adjusted to 25 and 50 μg of protein in 25 μL of PBS (pH 7.2). Twenty-five microliters of PBS was used as a control. MIP-133 protein at concentrations of 25 and 50 μg were used as positive controls. aPA protein (25 and 50 μg) was not cytotoxic to HCORN cells, but the cells were effectively killed by 25 and 50 μg of MIP-133 (Fig. 3).

Clinical Disease after aPA Immunization

We have shown in an earlier study that oral immunizations with antigens conjugated to neutralized cholera toxin are an effective method of inducing mucosal antibody responses and the appearance of IgA antibodies in the tears and enteric washes of Chinese hamsters.16,18 Moreover, oral immunization with the MIP-133 protein conjugated to cholera toxin (CT) provides almost complete resistance to corneal infection with A. castellanii trophozoites.20 In addition, the control animals immunized with the CT alone, as well as control antigen such as lysozyme, are not protected against Acanthamoeba infections. Therefore, Chinese hamsters were immunized with either 200 or 1000 μg of aPA conjugated with neutralized CT. Animals immunized with 500 μg MIP-133 protein served as the control. The results of a typical experiment are shown in Figure 4 and demonstrated that oral immunization with different dosages of aPA did not significantly affect the course of
disease. However, immunization with 500 µg of MIP-133 protein significantly reduced the severity of corneal infection which included infiltration and ulceration \((P < 0.05)\) and shortened the duration of the disease by 5 days.

**Level of Mucosal IgA Antibody in Animals Orally Immunized with aPA and MIP-133 Proteins**

Because tears were difficult to obtain in large quantities, we used enteric washes to determine the presence of mucosal IgA antibodies specific for the MIP-133 and aPA proteins by ELISA. There was no significant difference in anti-aPA IgA levels in animals immunized with 200 µg of aPA protein. In contrast, anti-MIP IgA levels were significantly higher in animals immunized with MIP-133 compared with the control group. Animals immunized with 1000 µg aPA protein showed higher levels of antibody than animals immunized with 200 µg aPA protein (Fig. 5). Enteric washes taken from the PBS treated animals (nonimmunized) did not specifically bind to the MIP-133 and aPA proteins as measured by ELISA.

**Inhibition of Trophozoite Migration by Enteric Washes Collected from Immunized Hamsters**

The capacity of the enteric washes from animals immunized with MIP-133 or aPA to neutralize migration of *A. castellanii* trophozoites through synthetic matrix (Matrigel; Collaborative Biomedical Products, Inc.) was tested in vitro. The results of a typical experiment are shown in Figure 6 and demonstrate that enteric washes from animals immunized with MIP-133 protein significantly inhibited the migration of trophozoites \((P < 0.05)\). Enteric washes from nonimmunized (control) or aPA immunized animals did not inhibit the capacity of *A. castellanii* trophozoites to migrate through the matrix. However, *A. castellanii* trophozoites incubated with the serine protease inhibitor PMSF displayed (positive control) significant reduction in their ability to migrate through the matrix (Fig. 6).

**Effect of MIP-133 Protein and aPA as an Anti-disease Vaccine for Mitigating Acanthamoeba Keratitis**

In Chinese hamsters, corneal infections resolve in 14 to 21 days. However, *Acanthamoeba* keratitis becomes a chronic, progressive disease in these hosts if conjunctival macrophages are eliminated before corneal infection. That is, subconjunctival injection of the macrophagidal drug dichloromethylene diphosphonate (clodronate) encapsulated in liposomes, results in the elimination of conjunctival macrophages. Animals treated with CL₂MDP-LIP before corneal exposure to *Acanthamoeba*-laden contact lenses develop progressive, severe *Acanthamoeba* keratitis that clinically mimics the human counterpart. Accordingly, we wanted to determine whether mucosal immunization with the MIP-133, aPA, or a combination of the two proteins would induce immunity against the pathologic sequelae of *Acanthamoeba* keratitis. Oral immunization with MIP-133 or MIP-133 and aPA proteins produced a significant mitigation of corneal disease in animals pretreated with CL₂MDP-LIP (Fig. 7). By contrast, infected animals treated with CL₂MDP-LIP and orally immunized with aPA alone displayed clinical disease that was not significantly different from that in animals treated with CL₂MDP-LIP \((P > 0.05)\). The severity of keratitis was similar in animals treated with PBS-encapsulated liposomes compared with untreated animals (infection only).

To ensure that the antibodies were present at the mucosal site, enteric washes from hamsters orally immunized with MIP-133, aPA or MIP133+aPA were collected and examined...
for the presence of anti-MIP-133 and anti-aPA IgA by ELISA. There was no significant difference in anti-aPA IgA levels between the control (nonimmunized) and animals immunized with aPA. In contrast, anti-MIP IgA levels were significantly higher in the animals immunized with MIP-133 or MIP-133+aPA (data not shown).

**DISCUSSION**

The rationale for the present study is based on three assumptions: (1) Keratitis is the consequence of tissue damage mediated by factors (preliminary proteases) elaborated by *Acanthamoeba* trophozoites; (2) parasite-derived pathogenic molecules persist transiently, even after trophozoites encyst or die; and (3) parasite-borne pathogenic molecules are immunogenic and can be used to elicit the production of mucosal antibodies that will neutralize the pathogenic molecules and thus mitigate tissue damage and reduce corneal inflammation.

*Acanthamoeba* trophozoites express the mannose-binding receptor, which facilitates adhesion of the parasite to mucusylated proteins on corneal epithelial cells.\(^8\) It has been shown that free mannose strongly inhibits the binding of *Acanthamoeba* trophozoites to the corneal epithelial and stromal cells.\(^9\) Moreover, the presence of mannose in the medium inhibits parasite-mediated cytolysis of corneal cells in short-term in vitro assays.\(^5\) Therefore, the parasite’s binding to the mannose receptors induces the generation of cytopathic factors that destroy the corneal epithelial and stromal cells and is an important step in the pathogenicity of *Acanthamoeba* keratitis. More recently, we have shown that mucosal immunization with MIP-133 mitigates disease by neutralizing the pathogenic amoeba-borne protease, thereby reducing tissue destruction and ameliorating clinical symptoms.\(^20\)

After the binding and destruction of epithelial cells, trophozoites invade the deeper regions of the corneal epithelium and ultimately enter and reside within the stroma. Corneal invasion is facilitated by proteases secreted by the trophozoites.

Since the tissue-invasive behavior of *Acanthamoeba* trophozoites is reminiscent of invasive cancer cells, we hypothesized that *Acanthamoeba* trophozoites elaborate proteases, especially plasminogen activators that contribute to the pathogenesis of *Acanthamoeba* keratitis. It is known that proteases play an important role in a broad array of biological processes including tumor invasion, metastasis, and tissue invasion by metazoan parasites.\(^30\) It has been shown that tumor cells produce a variety of proteolytic enzymes such as plasminogen activators that are capable of degrading extracellular matrix components.\(^31\) We have purified a novel plasminogen activator from an ocular isolate of *A. castellanii*.\(^1,0\) The parasite-derived enzyme has a molecular mass of 45 to 50 kDa and produces a single band of lysis on fibrinogen-agarose zymography. *Acanthamoeba* aPA is capable of degrading human fibrinogen in the presence of plasminogen.

The present study showed that the aPA activity is detectable in four pathogenic strains of *Acanthamoeba* spp., but is conspicuously absent in three nonpathogenic soil isolates of *Acanthamoeba* spp. *A. castellanii neff* has been shown by genetic analysis to be an avirulent strain, and our study further provides evidence that the strain is deficient in both producing disease and in aPA activity.\(^32\)

Activity of the aPA enzyme is completely inhibited by serine protease inhibitors, but is not affected by cysteine protease inhibitors. These results suggest that the pathogenic potential of *Acanthamoeba* spp. is closely correlated with elaboration of the serine protease aPA. Therefore, the parasite’s elaboration of aPA may be a critical step in the pathogenic cascade of *Acanthamoeba* keratitis. Thus, inhibition of aPA activity may have a major impact on preventing the invasion of *Acanthamoeba* trophozoites into the stroma.

Because the pathogenesis of *Acanthamoeba* keratitis is dependent on the release of cytolytic molecules MIP-133 and aPA, we hypothesize that oral immunization with these protease inhibitors conjugated with cholera toxin will preferentially induce synthesis of mucosal IgA antibodies in tears. Although several different proteases may contribute to the pathogenesis of *Acanthamoeba* keratitis, we have limited our investigation to the role of the mannose-induced protein (133-kDa protein) and aPA, since recent studies in our laboratory suggest that these proteases play a major role in the pathogenicity of *Acanthamoeba* keratitis. Chinese hamsters orally immunized with MIP-133 protein have been shown to produce significantly high levels of IgA antibody that can be readily detected in both tear secretions and enteric washes.\(^20\) Because the tears continuously bathe the ocular surface, the anti-MIP-133 IgA antibodies have direct and prolonged contact with trophozoites and their products. By contrast, parenteral immunization does not elicit significant accumulation of antibodies in the tears.\(^33,34\) Repeated intramuscular immunizations with *Acanthamoeba* antigens failed to provide any evidence of protection against *Acanthamoeba* keratitis in the Chinese hamster.\(^1,7\) Tear IgA in human patients may be vital to protecting against the disease, as patients with *Acanthamoeba* keratitis have been shown to
possession significantly lower levels of Acanthamoeba-specific IgA than the normal control population.35

Before this study, it was not known whether aPA protein was immunogenic and induced protection against Acanthamoeba keratitis after oral immunization. Animals given four oral immunizations of different dosages of aPA protein before Acanthamoeba infection failed to generate protection. Furthermore, enteric washes from the animals immunized with 1000 μg of aPA protein displayed elevated levels of IgA specific for aPA protein. However, there was a lack of correlation between antibody titer and protection against Acanthamoeba keratitis. These results indicate that at very high concentrations (1000 μg), aPA is immunogenic, but did not have a detectable protective effect against Acanthamoeba keratitis. Although the enteric washes from animals immunized with MIP-133 protein significantly inhibited the migration of trophozoites, enteric washes from aPA immunized animals did not inhibit the capacity of A. castellanii trophozoites to migrate through a synthetic matrix. These results correlate with inability of immunization with aPA to induce protection against Acanthamoeba keratitis. Although oral immunization with MIP-133 before corneal exposure to Acanthamoeba trophozoites is effective in reducing disease, it is not a realistic paradigm for managing patients. Therefore, we used a more relevant model in which we tested the efficacy of oral immunization with MIP-133, MIP-133+aPA, and aPA alone, administered after the corneal infections had been established. To produce a chronic form of Acanthamoeba keratitis in Chinese hamsters, it is necessary to deplete the conjunctival macrophage population with a macrophagial drug, clodronate.31 The clodronate-treated animals developed a chronic form of Acanthamoeba keratitis. However, repeated oral immunizations with MIP alone or MIP-133+aPA conjugated with cholera toxin, produced a dramatic amelioration in corneal lesions and an eventual resolution of the disease. Moreover, immunization with MIP-133+aPA proteins did not produce an additive protection. Again, animals treated with aPA alone displayed corneal disease that was indistinguishable from the nonimmunized animals. Initially, we entertained the hypothesis that oral immunization with aPA protein mitigates Acanthamoeba keratitis in Chinese hamsters. The rationale for this hypothesis was based on the proteolytic activity of aPA that facilitates the invasion and penetration of trophozoites through the basement membrane. However, the results showed that oral immunization with aPA was not protective against Acanthamoeba keratitis. It is likely that either aPA protein is less immunogenic than MIP-133 protein and was unable to produce sufficient anti-aPA anybody to inhibit invasion of trophozoites or oral immunization with aPA was ineffectual once the parasite had invaded the cornea.

The major findings of this study are that oral immunization with MIP-133 before and after infection with Acanthamoeba significantly reduced the severity of corneal infection which included infiltration and ulceration and shortened the duration of the disease. The current results suggest that MIP-133 anti-IgA antibody elicited by immunization may inhibit the adhesion of Acanthamoeba trophozoites to the corneal epithelium and prevent the destruction of the corneal epithelial cells that are crucial in the development of corneal infection. Moreover, the protective mechanisms induced by oral immunization with MIP-133 act to prevent the initiation of disease and also play an important role in the subsequent steps of the pathogenic cascade of Acanthamoeba keratitis. Thus, MIP-133 protein may represent an important immunotherapeutic target for the treatment of Acanthamoeba keratitis.


