Impact of Oral Immunization with *Acanthamoeba* Antigens on Parasite Adhesion and Corneal Infection

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**Purpose.** To determine whether oral immunization mitigates ongoing *Acanthamoeba castellanii* corneal infections in pigs.

**Methods.** Pigs were orally immunized with aqueous *Acanthamoeba* antigen mixed with cholera toxin (Ac-CT) or with saline, before or after ocular infection with *A. castellanii*. Mucosal secretions (i.e., tears and enteric wash) were tested for *Acanthamoeba*-specific IgA by enzyme-linked immunosorbent assay. Enteric washes were used as a source of IgA in assays measuring the binding of trophozoites to Chinese hamster corneal epithelial (CHCE) cells.

**Results.** Pigs immunized with Ac-CT before ocular challenge with *A. castellanii* had significant anti-*Acanthamoeba* IgA antibody titers in their tears and enteric washes and were protected against ocular infection. Enteric washes from orally immunized pigs inhibited trophozoite binding to CHCE cells in vitro by more than 75%. By contrast, pigs immunized after corneal infections had been established displayed keratitis of the same severity and duration as that in control pigs. However, 80% of the orally immunized animals were resistant to rechallenge with *Acanthamoeba*-laden contact lenses, whereas none of the control animals was resistant.

**Conclusions.** Oral immunization with Ac-CT protects against *Acanthamoeba* keratitis when administered before corneal challenge. However, delaying oral immunization until after corneal disease is established fails to mitigate keratitis. The appearance of parasite-specific tear IgA correlates with protection and may act by preventing the parasite's binding to the corneal epithelium. (Invest Ophthalmol Vis Sci. 1998;39:2337-2343)

*Acanthamoeba* are ubiquitous protozoa commonly encountered in the environment. 1,2 The first report of ocular infection with *Acanthamoeba castellanii* was recorded in 1973, and the prevalence increased greatly in the 1980s with increasing contact lens wear. 3 However, improved diagnostic acumen and effective chemotherapy have greatly improved the prognosis of patients with *Acanthamoeba* keratitis. 4,5

Pathogenesis usually stems from contaminated contact lenses associated with an antecedent corneal injury. 3,6 The parasites are thought to bind to the injured cornea and secrete enzymes that facilitate penetration of the epithelial cells and degradation of the collagenous stroma. 7-9 *Acanthamoeba* preferentially migrate toward neuronal tissue and kill cells of neuroectodermal origin, which may explain the disproportionate pain reported with the disease. 10,11 Although hourly around-the-clock treatment can bring the disease under control, patients commonly sustain therapy for months to years and recrudescence may occur. 12,13 Clearly, patients would benefit from less exhaustive therapies.

Infectious diseases of the cornea are exposed to mucosal immune responses. The exterior part of the cornea, lacrimal gland, tears, and conjunctiva are a part of the common mucosal immune system. 14 Tears contain high quantities of IgA, the primary antibody produced in the mucosal tract and the most abundant immunoglobulin in the body. 15,16 In fact, more IgA is produced each day than all the other immunoglobulins combined. 17,18 Because IgA does not activate the classical complement cascade, it is generally thought that IgA exerts its protective effects by preventing the binding of pathogens to mucosal surfaces. 19 This mechanism appears to be highly effective in preventing pathogens, such as *Entamoeba bistolytica* and *Salmonella typhimurium*, from binding to mucosal surfaces. 20,21

We have shown in pigs and Chinese hamsters that preferential activation of the mucosal immune system protects against *Acanthamoeba* keratitis. 22,23 That is, animals orally immunized with aqueous *Acanthamoeba* antigen plus cholera toxin show strong protection against ocular challenge with *A. castellanii*. 22,23

In the present study, we considered the hypothesis that the protection produced by oral immunization with *Acanthamoeba* antigens is mediated by IgA antibodies that prevent the binding of the parasite to the corneal epithelium. Furthermore, we hypothesized that if tear IgA acted solely to prevent parasite binding, oral immunization would not affect ongoing corneal infections with *A. castellanii*. These hypotheses were
tested in the pig model of *Acanthamoeba* keratitis, which closely paralleled the human counterpart, clinically and histopathologically.\(^{24}\)

**METHODS**

**Animals and Cell Lines**

Young domestic piglets (7 kg) were purchased from Kabar Live Stock (Sabinia, TX). Pigs were used in accordance with the institutional guidelines for animal research and conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. An ocular human isolate of *A. castellanii* was acquired from American Type Culture Collection (ATCC 30868 Rockville, MD) and was maintained in axenic culture at 35°C in peptone-yeast-glucose, as previously described.\(^{24}\) Complete minimum essential medium contained the following: minimum essential medium (RJ Biosciences, Lenexa, KS) supplemented with 1% glutamine (BioWhittaker, Walkersville, MD); 1% sodium pyruvate (BioWhittaker); 1% minimum essential medium vitamins (BioWhittaker); and 1% penicillin, streptomycin, and fungizone (BioWhittaker) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Chinese hamster corneal epithelial cells (CHCE) were immortalized from a corneal explant using E6/E7 virus, as previously described.\(^{26}\) CHCE cells were cultured in complete minimum essential medium containing 500 µg/ml geneticin (Sigma, St. Louis, MO).

**Specimen Collection**

Nonreflex tears were collected at weekly intervals using 2-µl micropipettes (Drummond Scientific, Broomall, PA). Tears were placed into sterile microfuge tubes and snap frozen in liquid nitrogen before storage at —80°C until used. Enteric wash was collected after intracardiac injection of 10 ml pentobarbital (180 mg/ml; Sigma). Approximately 20 cm of small intestine were removed. One protease inhibitor cocktail tablet (Boehringer-Mannheim, Indianapolis, IN) dissolved in 10 ml phosphate-buffered saline (PBS) was injected through the intestinal section by using a syringe with an 18-gauge needle. The enteric wash was collected and centrifuged at 700g to remove sediments. The wash was stored at —80°C until used.

**Oral Immunization**

*Acanthamoeba* antigen (Ac-ag) was generated by disruption of *A. castellanii* trophozoites mixed with an equal volume of sterile 10-µm glass beads. After 10 to 12 rounds of mixing by vortex, the pellet was centrifuged at 2000g for 15 minutes. The wash was collected after intracardiac injection of 10 ml pentobarbital, the wash was mixed with 10 µg cholera toxin (Ac-CT) in a 2-ml volume, followed by the administration of 20 ml PBS through the feeding tube. Control pigs received PBS instead of Ac-CT. The pigs were anesthetized systemically with isoflurane (Mallinckrodt Stock (Sabinia, TX)) and was maintained in axenic culture at 35°C in peptone-yeast-glucose, as previously described.\(^{24}\) Complete minimum essential medium contained the following: minimum essential medium (RJ Biosciences, Lenexa, KS) supplemented with 1% glutamine (BioWhittaker, Walkersville, MD); 1% sodium pyruvate (BioWhittaker); 1% minimum essential medium vitamins (BioWhittaker); and 1% penicillin, streptomycin, and fungizone (BioWhittaker) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Complete minimum essential medium contained the following: minimum essential medium (RJ Biosciences, Lenexa, KS) supplemented with 1% glutamine (BioWhittaker, Walkersville, MD); 1% sodium pyruvate (BioWhittaker); 1% minimum essential medium vitamins (BioWhittaker); and 1% penicillin, streptomycin, and fungizone (BioWhittaker) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Chinese hamster corneal epithelial cells (CHCE) were immortalized from a corneal explant using E6/E7 virus, as previously described.\(^{26}\) CHCE cells were cultured in complete minimum essential medium containing 500 µg/ml geneticin (Sigma, St. Louis, MO).

**Enzyme-Linked Immunosorbent Assay**

Enzyme-linked immunosorbent assay plates were coated with 10 µg/ml Ac-ag overnight in 0.1 M carbonate buffer (pH 9.6; Sigma). Plates were washed four times with PBS containing 0.05% Tween-20 (wash buffer; Sigma), then blocked with 5% bovine serum albumin (Sigma) in PBS (blocking buffer) for 2 hours at 37°C. All subsequent antibodies were diluted in blocking buffer. Enteric washes were added undiluted or diluted 1:10. Tears were serially diluted from 1:100 to 1:3200. Samples were incubated for 0.5 hour at 37°C and washed. IgA, IgM, and IgG were detected using the following antibodies; 1:10,000 rabbit anti-p IgG (Accurate Chemical, San Diego, CA); 1:10,000 goat anti-p IgM-biotin (Accurate); and 1:10,000 rabbit anti-p IgG horseradish peroxidase (Sigma). The antibodies were diluted in blocking buffer, added to the wells, and incubated at 37°C for 0.5 hour. Secondary antibodies were goat anti-rabbit horseradish peroxidase (Accurate, 1:10,000) or, for plates receiving biotinylated antibodies, 1:2000 streptavidin-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), and were added for 0.5 hours at 37°C. Plates were washed and developed using 2,2' azinobis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma) containing 0.03% H₂O₂. Plates were allowed to develop and 0.1% 10% sodium dodecyl sulfate (Sigma) was added to stop the reaction before reading the optical density on a microplate reader (Molecular Devices, Menlo, CA) at 405 nm. Background optical density values for saline controls varied between experiments; therefore, the results are expressed as net optical density value after subtracting the background optical density value.

**Adhesion Assay**

Adhesion of *Acanthamoeba* trophozoites to CHCE cells in vitro was evaluated by radiolabeling 2 × 10⁶ trophozoites/ml
overnight in 0.1 μCi 35S-methionine–cysteine (New England Nuclear, Boston, MA). The labeled parasites were washed three times in Hanks' balanced salt solution (HBSS, BioWhitaker) and resuspended at 1 X 10^5 trophozoites/ml in HBSS containing 1:400 or 1:800 dilutions of each enteric wash. One hundred microliters of this solution was added to each well of a 96-well plate containing confluent monolayers of CHCE cells. Incubation was carried out at 35°C for 45 minutes, after which the wells were washed three times with HBSS. One hundred microliters 10% sodium dodecyl sulfate was added, and the plates were incubated 15 minutes at room temperature. Supernatants were transferred to scintillation vials containing 2 ml scintillation fluid (Budget-Solve; Research Products, Mount Prospect, IL) and counts were measured on a scintillation counter (LS3801; Beckman, Irvine, CA). The percentage of adhesion was calculated relative to the maximum number of counts placed in the well. Adherence assays conducted after removal of IgA were performed as follows: 1 ml protein A beads (Pierce Chemical, Rockford, IL) was incubated at room temperature for 1.5 hours with 50 μl rabbit anti-pig IgA (Accurate) or 50 μl naive rabbit serum mixed 1:1 in binding buffer (Pierce). The beads were washed according to manufacturer's specifications and incubated at 4°C overnight with enteric washes mixed 1:1 in binding buffer. The beads were centrifuged at 12,000 rpm for 5 minutes (Eppendorf microfuge, Brinkman Instruments, Westbury, NY) and the supernatant collected. Statistical analyses were carried out using Student's t-test and are noted in figure legends.

RESULTS

Domestic pigs were orally immunized with Ac-ag mixed with cholera toxin (Ac-CT) or with PBS only. After 4 weekly immunizations, the animals were infected with A. castellani. All pigs that received PBS were infected, producing an average severity score of 4.90 (Fig. 1). Only one of the three pigs orally immunized with Ac-CT was infected, with a resultant severity score of 1.67. Moreover, the corneal infection in the single infected animal resolved within 14 days. Comparison of the two groups showed that the severity scores were significantly different at all time points (P < 0.05; Student's t-test).

Having verified the protective effects of oral immunization, we sought to show the presence of anti-Acanthamoeba IgA at the site of infection. By the third week of oral immunization, animals receiving Ac-CT showed significantly higher quantities of anti-Acanthamoeba IgA than did enteric washes from pigs immunized with PBS. These same enteric washes were negative for anti-Acanthamoeba IgG and anti-Acanthamoeba IgM (data not shown).

Because tears were difficult to obtain in large quantities, we used enteric washes to investigate the mechanism of protection in orally immunized animals. Because IgA is known to inhibit binding of pathogens to epithelial surfaces, we sought to determine whether mucosal antibody from orally immunized hosts would affect parasite binding to the corneal epithelium. Trophozoites preincubated in enteric wash from pigs immunized orally with Ac-CT showed a fivefold decrease in binding to corneal epithelial cells (Fig. 3). To verify that the inhibition was a result of IgA in the enteric wash, the wash was treated with protein A-conjugated beads bound with anti-pig IgA antibodies. Pretreatment of enteric wash from Ac-CT-immunized animals with anti-IgA resulted in two times more trophozoites binding to epithelial cells than occurred with the same enteric wash treated with beads charged with normal rabbit serum (Fig. 4). Thus, removal of IgA resulted in increased trophozoite binding.

Having demonstrated that oral immunization produced functional anti-Acanthamoeba IgA, we sought to examine the effects of this immunization on established Acanthamoeba keratitis. We suspected that if oral immunization protects by eliciting the appearance of parasite-specific IgA in the tears that prevent the binding of the trophozoites to the corneal surface, then initiating oral immunization after corneal infection has been established would not affect the clinical course of the corneal disease. To test this, two groups of pigs were infected, and oral immunizations were initiated 1 week after the infections were established. Neither the severity nor the chronicity of the infection was diminished when oral administration of Ac-CT was initiated after corneal infection had been established (Fig. 5A). These results suggested that oral immunizations could not affect ongoing corneal infections with Acanthamoeba. However, if oral immunization acts to prevent the binding of the parasite to the corneal surface, we predicted

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933203/ on 11/26/2018)
that tear IgA would develop in the orally immunized animals that would protect against future corneal infections with Acanthamoeba. The results (Fig. 5B) confirm this prediction and show that only one of the five pigs immunized orally with Ac-CT showed evidence of keratitis when rechallenged with parasite-laden contact lenses. By contrast, all control pigs immunized orally with PBS after the first corneal infection and rechallenged with parasite-laden contact lenses showed severe Acanthamoeba keratitis.

**DISCUSSION**

The results reported here and previously indicate that oral immunization with aqueous extracts of Acanthamoeba induces strong immunity against the development of Acanthamoeba keratitis. By contrast, intramuscular immunization with the same antigenic preparation stimulates the production of serum IgG antibodies and delayed-type hypersensitivity responsiveness to Acanthamoeba antigens, yet fails to prevent Acanthamoeba keratitis. Thus, neither parasite-specific IgG nor delayed-type hypersensitivity is able to protect the cornea against Acanthamoeba infection. The most conspicuous difference between orally immunized hosts and hosts immunized intramuscularly is the appearance of parasite-specific IgA antibodies in mucosal secretions, including the tears.

The present results confirm that oral immunization prevents Acanthamoeba keratitis when initiated before corneal challenge with parasite-laden contact lenses. In a companion study performed in Chinese hamsters, we have shown that oral administration of either irrelevant antigens mixed with cholera toxin (i.e., lysozyme) or cholera toxin alone did not protect against Acanthamoeba keratitis. Thus, the immunity induced by oral immunization with Ac-CT is antigen specific.

The present results indicate that oral immunization initiated after infection has been established fails to affect the progression of corneal disease. This clearly shows that oral immunization is ineffectual once the parasite has invaded the cornea and suggests that the protective mechanism induced by oral immunization acts to prevent the early phases of the infectious process.

Acanthamoeba trophozoites initiate pathogenesis by binding to epithelial cells. Adhesion to the corneal epithelium is mediated by a mannos-specific lectin present on Acanthamoeba. The current results suggest that parasite-specific IgA antibodies in the tears of orally immunized hamsters may inhibit the adhesion of Acanthamoeba trophozoites to the corneal epithelium and prevent the crucial first step in the development of corneal infection. Immunization with Ac-CT produced IgA specific for Acanthamoeba that was detectable in tears and enteric washes. The minute volume of tears that could be collected from pigs precluded our testing the inhibitory effect of tear IgA in vitro. However, suitable quantities of enteric washes from the same hosts were collected and found to contain anti-Acanthamoeba IgA. Of importance, the immune enteric washes inhibited the binding of the parasites to corneal epithelial cells. Moreover, removal of IgA from the washes removed the inhibitory effect and confirmed that the active component was parasite-specific IgA.
Acanthamoeba cholera toxin (Ac-CT) inhibit adhesion of corneal epithelial cells. Acanthamoeba trophozoites were radiolabeled overnight, washed three times, and incubated for 15 minutes in enteric washes from pigs that were immunized orally with Ac-CT or phosphate-buffered saline (PBS). Trophozoites were added to 96-well plates containing confluent monolayers of Chinese hamster corneal epithelial cells and incubated 45 minutes. Wells were washed three times and the remaining counts transferred to scintillation vials. Percentage of adherence was calculated relative to the total number of counts added per well. PBS, donors immunized orally with phosphate-buffered saline; Ac-CT, donors immunized orally with Acanthamoeba antigen mixed with cholera toxin. Error bars, SEM; *P < 0.05, Student’s t-test.

We hypothesized that oral immunization produces a polyclonal IgA response against Acanthamoeba antigens. Tears containing high quantities of these antibodies could continually coat the cornea and be readily available before and during ocular exposure to Acanthamoeba trophozoites. This would suggest that antibody-coated trophozoites are removed with the tears through the nasolacrimal duct. This hypothesis is consistent with previous studies in which it was shown that antigen-specific tear IgA blocks bacterial and viral adhesion to the corneal surface.31,52

Because oral immunization did not affect ongoing keratitis, it is possible that once the trophozoites penetrate the corneal epithelium, they are inaccessible to IgA in the tear film. Immunoglobulin penetration of epithelial surfaces is facilitated by polyimmunoglobulin receptors. However, corneal epithelial cells appear to be devoid of such receptors.33,34 In the absence of polyimmunoglobulin receptors, it is unlikely that surface IgA could reach the stroma of the cornea through an intact epithelium. However, during periods of epithelial ulceration, trophozoites residing in the stroma may be exposed to tear IgA. Even if tear IgA were to reach intrastromal parasites, it is questionable whether the IgA would have a deleterious effect on the trophozoites, because anti-Acanthamoeba IgA in enteric washes from orally immunized animals fails to induce either encystment or lysis of trophozoites in vitro.53 This is consistent with the observations that IgA does not activate the classical complement cascade17 and that Acanthamoeba spp. are resistant to complement-mediated lysis.55 It is possible that parasite-specific IgA in the tears acts as an opsonin and promotes phagocytosis by macrophages. That oral immunization failed to affect ongoing Acanthamoeba keratitis argues against the latter scenario and supports the hypothesis that oral immunization protects by preventing the initial establishment of infection, presumably by preventing the adhesion of the trophozoites to the corneal epithelial surface.

The weight of evidence strongly suggests that oral immunization protects by activating the common mucosal immune system and thereby inducing the appearance of anti-Acanthamoeba IgA antibodies in the tears. The capacity of mucosal anti-Acanthamoeba IgA to prevent binding of trophozoites to corneal epithelial cells supports the notion that IgA acts in vivo to prevent the initial adhesion of trophozoites to the corneal surface.

**Figure 3.** Enteric washes from pigs orally immunized with Acanthamoeba–cholera toxin (Ac-CT) inhibit adhesion of Acanthamoeba trophozoites to corneal epithelial cells. Acanthamoeba trophozoites were radiolabeled overnight, washed three times, and incubated for 15 minutes in enteric washes from pigs orally immunized with Ac-CT or phosphate-buffered saline (PBS). Trophozoites were added to 96-well plates containing confluent monolayers of Chinese hamster corneal epithelial cells and incubated 45 minutes. Wells were washed three times and the remaining counts transferred to scintillation vials. Percentage of adherence was calculated relative to the total number of counts added per well. PBS, donors immunized orally with phosphate-buffered saline; Ac-CT, donors immunized orally with Acanthamoeba antigen mixed with cholera toxin. Error bars, SEM; *P < 0.05, Student’s t-test.

**Figure 4.** Anti-Acanthamoeba-specific IgA inhibits adhesion of Acanthamoeba trophozoites to corneal epithelial cells. Enteric washes from pigs immunized orally with Acanthamoeba–cholera toxin (Ac-CT) were treated with anti-pig IgA bound to protein A beads or with protein A beads bound to normal rabbit serum. Radiolabeled trophozoites were preincubated for 15 minutes with one of the above or with enteric washes from pigs orally immunized with Ac-CT or phosphate-buffered saline (PBS). Trophozoites were transferred to 96-well plates containing confluent monolayers of Chinese hamster corneal epithelial cells and incubated for 45 minutes at 37°C. Wells were washed three times and the remaining cells transferred to scintillation vials. Percentage of adherence was calculated relative to the total number of counts added per well. PBS, donors immunized orally with phosphate-buffered saline; Ac-CT, donors immunized orally with Acanthamoeba antigen mixed with cholera toxin. Error bars, SEM; *P = 0.003, Student’s t-test compared with the PBS group. †P < 0.05, Student’s t-test, compared with anti-IgA-treated Ac-CT.
Acanthamoeba infection after the oral immunization regimen was completed. PBS, pigs immunized orally with PBS pigs immunized orally with antigen mixed with cholera toxin = 5).

FIGURE 5. Oral immunization does not mitigate established Acanthamoeba keratitis. Pigs were infected with Acanthamoeba-laden contact lenses and immunized orally at weekly intervals. Oral immunizations consisted of 500 µg Acanthamoeba antigen (Ac-ag) mixed with 10 µg Acanthamoeba-cholera toxin (Ac-CT) or phosphate-buffered saline (PBS) once weekly for 4 weeks. (A) Severity was measured according to the amount of corneal infiltration, corneal neovascularization, and corneal ulceration (n = 5). Error bars, SD. The data were not significantly different at any time point. (B) Pigs in (A) were challenged to a second ocular Acanthamoeba infection after the oral immunization regimen was completed. PBS, pigs immunized orally with PBS (n = 5); Ac-CT, pigs immunized orally with Acanthamoeba antigen mixed with cholera toxin (n = 5).

References


