Effect of Human Tears on Acanthamoeba-Induced Cytopathic Effect

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Objective: To determine whether tears of healthy individuals provide protection against Acanthamoeba-induced cytopathic effect (CPE) in vitro.

Methods: Acanthamoebae were added to confluent cultures of corneal epithelium in 24-well plates, and co-cultures were incubated overnight in a serum-free medium containing varying amounts of tears or immunoglobulin A (IgA)–depleted tears. At the end of the incubation period, the cells were stained with Giemsa, and the extent of target cell damage (ie, CPE) was quantified.

Results: Acanthamoebae produced extensive CPE. The presence of even a low concentration of tears (10 µL of undiluted tears per milliliter of media) almost completely inhibited Acanthamoeba-induced CPE. The CPE was inhibited by pretreatment of the parasites with tears. In contrast, the pretreatment of host cells with tears was not protective. This finding suggests that the target of the inhibitory factor is the parasite. IgA-depleted tears also inhibited Acanthamoeba-induced CPE, albeit with a lower potency than total tears.

Conclusion: In addition to known IgA-dependent protective factors, human tears contain factors that inhibit Acanthamoeba-induced CPE independently of IgA.

Clinical Relevance: Identification and characterization of factors that protect against Acanthamoeba-induced CPE should help in the development of novel, rationally designed strategies to manage and protect against keratitis.

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proven by the internal review boards of Tufts University School of Medicine and the L.V. Prasad Eye Institute. Two different methods of tear collection were used: (1) nonreflex tears (approximately 10 µL from each person) were collected using microcapillaries and were stored in sterile microfuge tubes at −80°C until use, and (2) eye flush tears were collected from patients undergoing preparation for laser in situ keratomileusis or cataract surgery. Several drops of sterile isotonic sodium chloride solution were instilled into the eyes, the tear samples were collected using ophthalmic surgical sponge sponges, and the tears were retrieved by squeezing the sponge into a test tube containing 2 mL of phosphate-buffered saline. For each experiment, the eye flush tears from 3 or more donors were pooled and then concentrated using centrifugal tubes (Centricon YM-3; Millipore, Bedford, Massachusetts). Protein concentration was measured using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, California).

PARASITES AND HOST CELLS

For this study, an Acanthamoeba strain (MEEI 0184, Acanthamoeba castellani) derived from an infected human cornea was used. The ameba were axenically cultured in a proteose peptone/yeast extract/glucose medium. The host cells were immortalized rabbit corneal epithelial cells.9

EFFECT OF TEARS ON ACANTHAMOEBA-INDUCED CYTOPATHIC EFFECT

Cytopathic effect (CPE) assays were performed as described in a previously published study.9 Parasites (>95% trophozoites) were added to confluent cultures of corneal epithelium in 24-well plates (2 × 10³ parasites per milliliter of serum-free medium containing 0.4% bovine serum albumin [SFB medium]; 300 µL/well), and the co-cultures were incubated at 37°C and periodically examined using a phase-contrast microscope to assess the extent of CPE, as detected by the presence of cell-free plaques in the monolayer. At the end of the incubation period, the cells were stained with Giemsa, and the cell density in each well was estimated by using ImageQuant software (Molecular Dynamics, Sunnyvale, California). An unpaired 2-tailed t test was used for statistical analyses. Next, the impact of tears on ameba-induced CPE was examined. The protein concentration of the pooled tears was 5.0 µg/µL. To determine the effect of tears on ameba-induced CPE, the CPE assays were performed in the SFB medium containing varying concentrations of tear fluid (tear protein concentration per well: 1.5, 4.5, 7.5, 15, 45, and 75 µg; 300 µL of media per well).

Because early studies have shown that electrophoretic patterns of human tear samples collected using capillary and eye flush methods are comparable,10,11 it was of interest to determine the effect of eye flush tears on ameba-induced CPE. For this, the protein concentration of eye flush tears was adjusted to 5 µg/µL, and the CPE assays were performed as described in the previous paragraph. To determine whether the CPE inhibitory factor acts on host cells or the parasite, CPE assays were performed using parasites or host cells pretreated with tears. For this, before CPE assays, parasites (2 × 10³ ameba, >95% trophozoites) or epithelial cells were incubated with 300 µL of SFB medium containing tears (15 µg of protein per well) for 30 minutes in 24-well plates and were then washed twice with the SFB medium to remove traces of tear components. The CPE assays were then performed as described previously in this subsection in the absence of tears.

To remove IgA from tears, 400-µL aliquots of tears containing up to 1 mg of protein were incubated with 100 to 150 µL of anti-human IgA (α-chain specific)–conjugated agrose beads (Sigma-Aldrich Corp, St Louis, Missouri) (1 hour at 4°C) with gentle shaking. The beads were then separated by means of centrifugation, and the supernatant was analyzed for (1) protein concentration using the Bio-Rad Protein Assay reagent and (2) the presence of IgA by means of Western blot analysis using rabbit anti-human heavy-chain IgA (Jackson Immunoresearch Laboratories Inc, West Grove, Pennsylvania) as the primary antibody, horseradish peroxidase–linked goat anti–rabbit IgG as the secondary antibody (Vector Laboratories, Burlingame, California), and a chemiluminescence detection system (PerkinElmer Life Sciences, Wellesley, Massachusetts).

RESULTS

EFFECT OF TEAR COMPONENTS ON ACANTHAMOEBA-INDUCED CPE

Acanthamoebae parasites did extensive damage, that is, CPE, on corneal epithelial cells. After 4 to 6 hours of incubation, small cell-free plaques were observed in the monolayer (Figure 1A). Continuing the incubation, the cell-free areas increased in size, and ultimately the monolayer surrounding the large plaques lifted up from the plates, resulting in almost complete loss of the cell layer (Figure 1A). Epithelial cells incubated with parasites in the absence of tears were completely destroyed within 12 to 15 hours (Figure 1B). In contrast, epithelial cells incubated with parasites in the presence of tears were protected (Figure 1B). Nearly complete inhibition of CPE was consistently achieved at a tear concentration of 15 µg of protein per well (300 µL of media per well, ie, 50 µg/mL of tear protein) or higher. On average, at concentrations lower than 15 µg of protein per well, either moderate or no protection was detected (data not shown). Next, we tested the effect of eye flush tears on Acanthamoeba-induced CPE. As observed with unstimulated tears collected using the capillary method, eye flush tears almost completely inhibited the ameba-induced CPE inhibitory activity at a tear protein concentration 15 µg per well or higher (data not shown). Because similar results were obtained regardless of the procedure used for the collection of tears and it is more convenient to collect eye flush tears than undiluted tears, eye flush tears were used for the remainder of the study.

TARGET OF THE CPE INHIBITORY FACTOR OF TEARS

To determine whether the CPE inhibitory factor targets the parasite or the host cells, the CPE assays were performed using ameba or epithelial cells pretreated with tears instead of in the presence of tear components. Pretreatment of the parasites with tears markedly inhibited Acanthamoeba-induced CPE (Figure 2). In contrast, pretreatment of the epithelial cells with tears was not protective (Figure 2).
EFFECT OF IgA-DEPLETED TEARS ON ACANTHAMOEBA-INDUCED CPE

It is thought that ameba-specific IgA in tears provides protection against infection, presumably by blocking the adhesion of parasites to the host cells. Therefore, it was of interest to determine whether the IgA-depleted tears lack or possess CPE inhibitory activity. To remove IgA, aliquots of concentrated tears were incubated with anti-human IgA-conjugated agarose beads; the unbound material was separated by means of centrifugation and was analyzed for the presence of IgA and CPE inhibitory activity. Proteins bound to the beads were eluted in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (at 100°C for 3 minutes) and were also analyzed for the presence of IgA (bound fraction). Western blot analysis revealed that unfractionated tears contained a 56-kDa anti-IgA reactive component (Figure 3A). In contrast, the unbound fraction did not contain detectable levels of IgA (Figure 3A). This finding indicates that incubation with anti-IgA–conjugated agarose beads effectively depleted IgA from tears. As expected, the bound fraction contained copious amounts of IgA (Figure 3A). Next, the CPE assays were conducted in the presence and absence of unbound fraction. These experiments revealed that the unbound fraction lacking IgA also contained CPE inhibitory activity. In control experiments, unfracti0nated tears inhibited ameba-induced CPE at all 3 concentrations tested (15, 30, and 45 µg of tear protein per well) (Figure 3B and 3C). In contrast, the unbound fraction derived from tears.
aliquots containing at least 45 µg of protein was required to inhibit CPE (Figure 3B and 3C).

**COMMENT**

We demonstrated that human tears contain factors that provide protection against *Acanthamoeba*-induced CPE in vitro. Currently, the secretory IgA antibody is the only recognized component that is thought to account for the protection afforded by tears. The present study suggests that normal human tears contain IgA-dependent and IgA-independent protective factors. Regarding the mechanism of the IgA-mediated protective effect of tears, we recently determined that the normal human mucosal secretions, including tear fluid, milk, and saliva, contain amoeba-specific antibodies that inhibit the adhesion of parasites to host cells (N.P. et al, unpublished data, 2005). We know little about the nature of the putative IgA-independent inhibitory factor or the mechanism by which it renders the parasite nonpathogenic. To date, we have observed that major components of tears, including lipocalin and lactoferrin, do not inhibit amoeba-induced CPE (data not shown). Studies aimed at characterization of the mechanism by which *Acanthamoeba* produces CPE have shown that subsequent to the adhesion of parasites to the host cells, contact-dependent and contact-independent proteinases are produced and that these proteinases are critical in the ability of the parasite to induce CPE that leads to killing of the host cells, degradation of epithelial basement membrane and underlying stromal matrix, and penetration into the deeper layers of the cornea. In a recent study, we observed that human milk also contains IgA-mediated and IgA-independent *Acanthamoeba* CPE protective factors and that the IgA-independent protective factors of milk inhibit ameba-induced CPE by inhibiting proteinases produced by the parasite (Z.C. and N.P., unpublished data, 2007). It remains to be determined whether tear fluid also provides protection against ameba-induced CPE, at least to some degree, by alleviating the activity of 1 or more amebic proteinases. Whether human milk or tears contain specific inhibitors against amebic proteinases remains to be determined.

The presence of CPE inhibitory activity in tears of healthy individuals explains, at least in part, the low incidence of *Acanthamoeba* keratitis despite the ubiquitous distribution of the parasite. Likewise, the presence of CPE inhibitory factors in nonocular secretions helps explain the reason almost all nonocular tissues are resistant to *Acanthamoeba* infections in healthy individuals. Studies aimed at characterization of the CPE inhibitory factors of tears and other mucosal secretions should lead to a better understanding of the mechanism by which the cornea resists the infection and should

**Figure 3.** Immunoglobulin A (IgA)–depleted tears inhibit *Acanthamoeba*-induced cytopathic effect. A, Tear samples were incubated with anti-human IgA-conjugated agarose beads, the unbound material was separated by centrifugation, and the bound and unbound fractions were analyzed for the presence of IgA by Western blot analysis. Lanes 1 and 4 show tears containing 4.2 and 8.4 µg of protein, respectively; lanes 2 and 5, unbound fractions derived from tears equivalent to 4.2 and 8.4 µg of protein, respectively; lane 3, bound fraction eluted by boiling the beads in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer. Note that an intensely stained 56-kDa anti-IgA reactive component is present (arrow) in the unfractionated tears (lanes 1 and 4) and in the bound fraction (lane 3) but not in the unbound fraction (lanes 2 and 5). Arrowhead indicates dye front. B and C, Confluent cultures of corneal epithelium were incubated overnight with parasites in media alone (A), media containing tears (A + T), or unbound fraction derived from tears containing the indicated micrograms of protein (A + UB) (n=4 per group). *P < .05 compared with all groups in A + T panel and the 45-µg/well group in the A + UB panel. C, Representative photographs of the plates. Error bars represent SE. Cont indicates corneal epithelial cells incubated with media alone (negative controls).
help decipher circumstances that predispose to Acanthamoeba keratitis.

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REFERENCES


Ophthalmological Ephemera

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