Degradation of Uniquely Glycosylated Secretory Immunoglobulin A in Tears from Patients with Pseudomonas aeruginosa Keratitis

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PURPOSE. To investigate the integrity of secretory (S)-IgA in tear fluid during bacterial keratitis and to evaluate the significance of specific Pseudomonas aeruginosa extracellular proteases in the observed degradation of S-IgA.

METHODS. The integrity of component chains of S-IgA in tear fluid from patients with keratitis caused by P. aeruginosa, Streptococcus group G, Moraxella catarrhalis, Staphylococcus aureus, coagulase-negative staphylococci, and the IgA1 protease-producing Streptococcus pneumoniae were compared with S-IgA in tear fluid, colostrum, and saliva. S-IgA degradation was observed only in tears from P. aeruginosa-infected patients, in a pattern similar to that observed in incubation of tear samples with P. aeruginosa strains and to an extent closely correlated with their expression and activity of individual proteases. Experiments using isogenic mutants of P. aeruginosa PAO1 lacking either elastase or alkaline protease indicated that several proteases were working in concert.

RESULTS. Secretory component (SC) of S-IgA secreted by the lacrimal glands was more abundantly glycosylated than SC in colostrum and saliva. S-IgA degradation was observed only in tears from P. aeruginosa-infected patients, in a pattern similar to that observed in incubation of tear samples with P. aeruginosa strains and to an extent closely correlated with their expression and activity of individual proteases. Experiments using isogenic mutants of P. aeruginosa PAO1 lacking either elastase or alkaline protease indicated that several proteases were working in concert.

CONCLUSIONS. Surprisingly, SC of tear S-IgA is more abundantly glycosylated than SC of S-IgA in other secretions, a difference of potentially great functional significance. Primarily, SC and α-chains are partially degraded in vivo during Pseudomonas keratitis by the concerted action of several proteases, including elastase and alkaline protease. (Invest Ophthalmol Vis Sci. 2008;49:4939–4944) DOI:10.1167/iovs.07-1198

Contact lens-associated keratitis causing sight-threatening corneal ulcers is often caused by Pseudomonas aeruginosa, which readily adheres to damaged corneal epithelium, forms a biofilm, and, by the production of destructive proteases, rapidly invades the stroma. The pathogenesis of P. aeruginosa-induced keratitis is thought to be multifactorial and is dependent on the extent of contamination of the contact lens (CL), the virulence of the infecting strain, the integrity of the corneal epithelium, and the local mucosal immune defense.1-3

The immune defense of the ocular surfaces is confronted by a unique challenge, in that not only must integrity be maintained against microbial, inflammatory, and physical assault, but it must be done while minimizing the risk of loss of corneal transparency by inflammatory reactions.4 The primary specific mediator of this defense is secretory (S)-IgA, which prevents viral adhesion and internalization, bacterial, and parasitic attachment, colonization, and activity, and reduces antigen-related damage in mucosal sites.5,6 The secretory component (SC) of S-IgA is added to the dimeric IgA complex by the secretory epithelium during the active secretion process, and contributes to the structural stability of the antibody molecule in the enzymatically hostile environment at mucosal sites.6 Recent evidence shows that SC, in addition, actively contributes to the protective functions of S-IgA by binding selected bacteria and bacterial toxins and by anchoring S-IgA antibodies and immune complexes to the mucus lining on surface epithelia. These nonadaptive defense mechanisms are mediated by the abundant N-linked carbohydrate moiety of SC combined with O-linked carbohydrate side chains associated with the immunoglobulin heavy chains.7,8 In agreement with these concepts, some published observations indicate that S-IgA antibodies may protect the eye against P. aeruginosa keratitis. Thus, purified S-IgA from human milk containing specific anti-pseudomonas antibodies significantly inhibits P. aeruginosa binding to murine scarified corneal epithelium in an in vitro organ culture model in a concentration-dependent manner. Furthermore, topical ocular immunization with heat-killed P. aeruginosa elicits anti-pseudomonas S-IgA antibodies, which reduce the severity of keratitis in an in vivo mouse model.9 Studies of another important cause of keratitis, Acanthamoeba, also demonstrated that induction of mucosal immunity as well passive application of monoclonal IgA antibodies to Acanthamoeba trophozoites protect against experimental ocular infection in various animal models.9,10

One factor in the pathogenesis of corneal bacterial infections may be proteolytic S-IgA degradation induced by the infecting microorganism. Thus, some of the most frequently isolated eye pathogens Streptococcus pneumoniae, Haemophilus influenzae, and the closely related Haemophilus aegyptius produce highly specific IgA1 proteases that cleave human S-IgA1 and serum IgA1 in the hinge region.11 IgA1 protease activity separates the antigen-binding part of S-IgA1 from its Fc-SC mediated secondary effector functions such as inhibition of bacterial adhesion, agglutination, and mucus-binding. In vivo evidence indicates that the ensuing coating of bacteria with monomeric antigen-specific Fab fragments facilitates adhesion and subverts other parts of the immune system.11,12

P. aeruginosa produces a considerable number of potent proteases, including elastase, alkaline protease, and the more recently identified protease IV, which has been associated with corneal virulence.13,14 Previous reports based on in vitro experiments suggest that P. aeruginosa elastase is capable of...
cleaving S-IgA and myeloma proteins of both IgA subclasses and that alkaline protease degrades myeloma IgA. However, the activity of potential S-IgA-degrading proteases of *P. aeruginosa* and other eye pathogens during the pathogenesis of corneal infection in humans has not been determined. In the present study, tear fluid from patients with keratitis infected with *P. aeruginosa* and other bacterial species was examined for in vivo degradation of S-IgA. Degradation patterns were compared to those observed in vitro by incubating S-IgA with clinical isolates and experimental mutants of *P. aeruginosa* with different proteolytic activity. The study surprisingly demonstrates that SC of tear S-IgA is more abundantly glycosylated than S-IgA of other secretions and that SC is proteolytically degraded in vivo during pseudomonas keratitis by the concerted action of several proteases.

**Methods**

**Tear Samples**

Tear samples (5–100 μL) were obtained from 16 patients with keratitis before local antibiotic treatment and from five healthy volunteers. The samples were collected with a 50-μL glass capillary tube inserted into the lower fornix and were transferred to a 1.5-mL tube (Eppendorf, Fullerton, CA) and immediately frozen at −70°C. Corneal scrapings from the 16 patients yielded growth of *P. aeruginosa* (n = 5), *S. pneumoniae* (n = 6), *Moraxella catarrhalis* (n = 2), *Streptococcus* group G (n = 1), *Staphylococcus aureus* (n = 1), and coagulase-negative staphylococci (n = 1). The patients infected with *P. aeruginosa* were all contact lens wearers without other eye or systemic diseases, except for one patient who subsequently was found to have diabetes mellitus. Informed consent was obtained from the patients and volunteers. The study complied with the tenets of the Declaration of Helsinki and was approved by the local ethics committees in Jutland, Denmark.

**SDS-PAGE and Western Blot Analysis**

SDS-PAGE and Western blot analysis were performed as previously described. Samples of tear fluid, saliva, or S-IgA purified from human colostrum as described and dissolved in PBS at a concentration of 0.155 mg/mL were subjected to electrophoresis under reducing conditions using 4% to 20% gradient SDS polyacrylamide gels. After electrophoresis for 50 minutes at 200 V, the proteins, including molecular weight markers, were transferred to a PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA). The membrane was then blocked with 2.5% gelatin (wt/vol) in a buffer containing 0.05 M Tris (pH 7.4), 0.85% NaCl, 0.05% NaN₃, and 0.05% Tween 20, followed by incubation for 2 hours with affinity-purified rabbit antibodies against human S-IgA component chains: SC, and immunoglobulin α, κ, and λ-chains, respectively (Dako, Glostrup, Denmark). After the membranes were washed three times, they were incubated with alkaline-phosphatase-conjugated swine anti-rabbit immunoglobulin (Dako) for 2 hours and again washed three times, and the reactions were visualized by addition of 2.5 mg nitroblue tetrazolium in 25 mL 0.1 M Tris buffer (pH 9), containing 100 μL MgCl₂ and 375 μL indolyl phosphate.

**In Vitro Degradation of S-IgA**

Samples of pooled tear fluid collected from five healthy individuals were incubated for 2, 8, and 24 hours at 32°C with an equal volume of protease preparations from *P. aeruginosa* strains including elastase (LasB) and alkaline protease (AprA) insertion deletion mutants of the reference strain PAO1 (PAO1ΔlasB and PAO1ΔaprA), which has been genome sequenced. The isogenic mutant strains were kindly provided by Michael A. Curtis and Anastasia Papakonstantinopoulou (MRC Molecular Pathogenesis Group, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, London, UK). In vitro reactions were stopped by addition of an equal volume of reducing sample buffer followed by boiling of the samples for 5 minutes, where after the samples were frozen at −70°C. The protease and S-IgA preparations used as the control were subjected to identical conditions.

**Preparation of *P. aeruginosa* Extracellular Proteases**

Seven *P. aeruginosa* strains showing different gelatinolytic activity profiles previously determined by gelatin SDS-polyacrylamide electrophoresis (SDS-PAGE) (Table 1) and the PAO1 wild-type and deletion mutants were grown in tryptic soy broth (TSB) at 37°C during continuous shaking at 200 rpm for 24 hours. The supernatants were harvested after centrifugation at 20,000 rpm for 30 minutes and were immediately frozen at −80°C. This procedure leaves the activities of the *Pseudomonas* proteases intact.

**Deglycosylation of Secretory Component of S-IgA**

Complete deglycosylation of S-IgA in samples of human colostrum, saliva, and tear fluid was achieved with the enzyme PNGase F (peptide-N-acetyl-β-glucosaminyl)-asparagine amidase N-Glycosidase F) from *E. coli* (Genetex, Inc., Seattle, WA) and with PNGase F from *E. coli* (Peptide-N-Glycosidase F) provided by the manufacturer.

Partial partial deglycosylation of all component chains of S-IgA in tear samples was obtained by overnight incubation with *Streptococcus mitis* biovar-2 strain SK96, which is capable of depleting S-IgA of all of its N-acetyl neuraminic acid and N-acetyl galactosamine residues, and the majority of N-acetylglucosamine, galactose, mannose, and fucose residues while keeping the polypeptide chain intact.

**Results**

**Comparison of S-IgA in Tears and Other Secretions from Healthy Individuals**

S-IgA in tear samples from each of five healthy volunteers was compared by SDS-polyacrylamide gel electrophoresis combined with Western blot analysis to S-IgA in colostrum and saliva collected from three healthy individuals. Immunostaining of the blots with antibodies specific for α heavy chains, κ- and λ-light chains, and SC revealed identical migratory patterns of heavy and light chains (not shown). Surprisingly, SC of S-IgA in tear fluid consistently migrated in the gel corresponding to an apparent molecular size exceeding that of SC from all colostrum and saliva samples by approximately 10 kDa. Complete deglycosylation of the samples of colostrum, saliva, and

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* The activities of elastase and alkaline protease were graded by gelatin SDS-polyacrylamide electrophoresis, in which the individual protease activities were identified according to their characteristic electrophoretic mobilities reflected by clear zones in the protein-stained gel.
Tear S-IgA Degradation in Keratitis

In Vivo Degradation of S-IgA in Tears from Patients with Keratitis and Healthy Controls

Examination by Western blot analysis of each of the component chains of S-IgA in tear samples from patients with keratitis revealed detectable degradation only in samples from *P. aeruginosa*-infected patients. No proteolytic fragments of S-IgA were detectable in the eleven samples from keratitis cases involving *S. pneumoniae*, *M. catarrhalis*, *S. aureus*, and coagulase-negative staphylococci. Representative results are demonstrated in Figure 2.

The results obtained from the five cases of *P. aeruginosa* keratitis are shown in Figure 3. The major part of the chains appeared intact. However, in addition to the intact α chain (MW = 60 kDa) most samples showed a fragment at 50 kDa reactive with anti-α chain antibodies (Fig. 3A1, lanes P1–P5), which was absent in tears collected from healthy control subjects (representative control sample in Fig. 3A1, lane C). Likewise, in the blot stained with antibodies against SC two fragments of approximate molecular masses of 60 and 48 kDa were evident in tears from all patients (Fig. 3C1, lanes P1–5) and absent in the control sample (Fig. 3C1, lane C). In addition, the major SC band was reduced in molecular mass from an apparent 85 to 75 kDa (representative control sample from healthy individuals in Fig. 3C1, lane C). No degradation of light chains was detected (Fig. 3B1, lanes P1–P5).

In Vitro Degradation of S-IgA by *P. aeruginosa* Exoproteases

In vitro incubation of a pool of tear samples from healthy controls with supernatants of *P. aeruginosa* strains revealed maximum cleavage of S-IgA after 8 hours with no further degradation occurring on extended incubation. The extent of degradation of the component chains of S-IgA induced by the clinical isolates of *P. aeruginosa* varied between isolates and correlated closely with their proteolytic activity, as determined in a gelatin degradation assay (Table 1). The strains F10 and AAB15, which produced high amounts of elastase and alkaline protease, caused detectable degradation of heavy and light chains as well as SC, the latter chain being the most extensively degraded (Figs. 3A2, 3B2, 3C2; lanes 1 and 3). Strain ME19, which showed less activity of alkaline protease and elastase in the gelatin assay, showed a similar pattern of S-IgA degradation, but less extensive. Among the numerous cleavage fragments detected, in particular as a result of degradation of SC, some resembled fragments detected in tear fluid of patients with *P. aeruginosa* keratitis. The most noticeable similarities were the 50-kDa fragment of α-chains and the 48-kDa fragment of SC (Fig. 3, arrowheads). The more limited effect of the remaining strains is compatible with their lower activities in the gelatin assay. For example, strain 6206, which did not induce detectable S-IgA degradation, did not produce elastase and only small amounts of alkaline protease (Table 1).

As the carbohydrate moiety of glycoproteins normally adds to their resistance to proteolytic attack, we examined the ability of the *P. aeruginosa* protease preparations to degrade the component chains of S-IgA after preincubation with *Streptococcus mitis* biovar 2 strain SK96, which is capable of depleting S-IgA of all its N-acetyl neuraminic acid and N-acetyl galactosamine residues, and the majority of N-acetyl-glucosamine, galactose, mannose, and fucose residues while keeping the polypeptide chain intact.19 The representative results shown in Figure 3 (lanes with d after the sample number) indicate that deglycosylation of the substrate did not significantly increase the degradation induced by *P. aeruginosa* strains with limited proteolytic activity, such as ME 19 (Figs. 3A2, 3C2, lane 2d), MiB 8 (Fig. 3C2, lane 4d), and 6206 (Fig. 3C2, lane 5d). However, an increased ability to degrade deglycosylated SC was seen with strain PAO1 (Fig. 4, lane 1d compared with lane 1), which had high alkaline protease and elastase protease activity.

The fact that strain PAO1 showed significantly less ability to degrade SC than did strain F10 (Fig. 4, lane 4) and AAB15 (Fig. 3, lane 3) suggests that other proteases than alkaline protease and elastase contribute to the ability to degrade S-IgA. To examine the relative contributions of alkaline protease and elastase, we incubated tear fluid with protease preparations derived from strain PAO1 (wild-type) and the two deletion mutants PAO1ΔaprA and PAO1ΔlasB. Comparison of the results verified the observations made in vitro.

**FIGURE 1.** Immunoblot of representative samples of colostrum (lane 1), saliva (lane 2), and tear fluid (lane 3) after separation of component proteins according to molecular mass by SDS-polyacrylamide gel electrophoresis. The blot was stained with anti-serum specific for human SC and shows that the size of SC of S-IgA in tears exceeded that of SC in the other secretions by approximately 10 kDa. The size difference was completely eliminated by deglycosylation of SC in tears (lane 3d), saliva (lane 2d), and colostrum (lane 1d). MW, molecular weight markers. Magnification, ×1000.

**FIGURE 2.** Immunoblots showing no in vivo degradation of S-IgA in tear samples collected from four patients with keratitis caused by *S. pneumoniae* (lanes 3 and 4), *Streptococcus* group G (lane 5), and *S. aureus* (lane 6). Shown are bands reactive with antibodies against α chains (A) and SC (B). For comparison, lane 1 shows intact colostrum S-IgA, and lane 2 shows colostrum S-IgA incubated overnight with *S. pneumoniae*. In vitro incubation of S-IgA with *S. pneumoniae* caused deglycosylation of both α chains (A, lane 2) and SC (B, lane 2), resulting in marked reductions in size of the otherwise intact chains and partial cleavage of α chains into Fc and Fd fragments (A, lane 2). Immunoblots developed with antibodies against light chains showed no sign of degradation (not shown).
action mixtures in a blot stained for SC (Fig. 4) showed that all three protease preparations reduced the approximate molecular mass of SC from 85 to 75 kDa. Furthermore, the comparison indicated that the wild-type strain with both alkaline protease and elastase activities caused more degradation of the 75-kDa band than any of the two deletion mutants and that the mutant lacking alkaline phosphatase, in contrast to the mutant lacking elastase, retained the ability to release fragments of approximate molecular masses of 32 and 27 kDa. The same patterns were seen when deglycosylated S-IgA was used as the substrate (Fig. 3). These results indicate that, while elastase seems to be the most potent of the two proteases with regard to degradation, these results indicated that, while elastase seems to be the most potent of the two proteases with regard to degradation of SC, both seem to contribute to the phenotype of PAO1 in this context. No detectable degradation of heavy and light chains was observed with PAO1 or any of the two mutants.

Control experiments confirmed that none of the protease preparations yielded bands reactive with the primary or secondary antibodies used for developing the blots.

**DISCUSSION**

The host–parasite relationship between *P. aeruginosa* and the human eye is one of considerable complexity. Like several other pathogens, the species *P. aeruginosa* shows considerable genetic diversity reflected in size variations of the total genome and variable presence of genes considered important for virulence. The infected host produces both innate and adaptive immune factors potentially capable of inhibiting bacterial adherence and colonization and the activity of their secreted proteins. The present study focused on an additional layer of this complex relationship (i.e., the ability of clinical isolates of *P. aeruginosa* and other bacteria that cause eye infections, to challenge the antibody response of the host by degrading secretory immunoglobulins).

An unexpected observation resulting from our comparison of S-IgA from various human secretions was the significantly larger molecular size of SC of S-IgA from tear fluids than from samples of colostrum and saliva. The finding that this difference in size could be eliminated by complete enzymatic depletion of the N-linked carbohydrate moiety of SC shows that SC of S-IgA in tear fluid is more abundantly glycosylated. Glycosylation polymorphisms among S-IgA molecules purified from colostrum have been noted previously. Thus, Royle et al. showed that most carbohydrate side chains on human colostrum S-IgA are fully galactosylated, nonbisected, biantennary structures, but that some triantennary and a small proportion of tetra-antennary structures are also present. Although most structures were sialylated, variations were noted in the content of fucose including outer-arm fucose residues. To our knowledge, our observation is the first demonstration of a systematically aberrant glycosylation pattern of SC secreted from lacrimal glands, which conceivably is of biological significance. Apart from the recognized protection against proteolytic attack, which was confirmed in some of our experiments with *P. aeruginosa* strain PAO1, important functions of the carbohydrate moiety of SC have been recently reported. Thus, the carbohydrate side chains of SC provide S-IgA with further binding sites for selected bacteria and bacterial toxins in addition to the four traditional Fab-binding sites, thus enabling S-IgA to participate in both innate and adaptive immunity at mucosal surfaces. The biological consequences of these interactions are further enhanced by the anchoring of S-IgA, via the carbohydrate moiety of SC, to mucus lining the epithelial surface, thus preventing bacterial penetration and enhancing subsequent clearance of the microbial challenge. On this background it is conceivable that the observed aberrant glycosylation of SC of tear S-IgA is of functional significance to the unique challenges of the protection of the eye. Obvious possibilities are that the more abundant glycosylation affects the binding specificity and capacity of SC.

The observed pattern of proteolytic degradation of S-IgA in tears from the keratitis patients infected with *P. aeruginosa*
were in accordance with the in vitro observations, although in vivo degradation was less pronounced. This is not unexpected considering the difference in effective contact between protease and substrate. Conceivably, a functional impact of S-IgA cleavage during an infection is dependent on cleavage of S-IgA molecules with binding affinity for the infectious microorganism rather than on complete degradation of all S-IgA present in the secretion. Elastase released by neutrophil polymorphonuclear leukocytes accumulating in the inflamed eye theoretically contributes to degradation of S-IgA. However, the lack of detectable S-IgA fragmentation in tears of patients with keratitis caused by other pyogenic microorganisms than *P. aeruginosa* does not support this hypothesis.

The immunoglobulin heavy chains and SC were the principal targets of the proteolytic attack by *P. aeruginosa*. The extent of cleavage was closely related to the observed activity pattern of the two proteases elastase and alkaline protease, which appeared to work in concert and were supported by additional proteases released by the bacteria. Our findings are in partial agreement with those reported by Heck et al. who showed in vitro degradation of both SC and heavy chains by a *P. aeruginosa* protease identified as elastase but less cleavage of S-IgA by alkaline protease. None of the studies confirm the exclusive cleavage in the IgA hinge region by *P. aeruginosa* elastase reported by Döring et al. Although the band at 31 kDa observed in our Western blot analysis may represent the Fc part of the IgA heavy chains, the observation that strains without elastase activity also exhibited bands at this location rules out that elastase alone is responsible.

Clinical *P. aeruginosa* strains express different molecular forms and varying in vitro activities of elastase that are associated with their site of isolation. The fact that isolates from keratitis, together with isolates from wound infections, the environment, and feces show high activity compared to CLARE (contact lens associated red eye) isolates and strains from urine and lungs, may indicate that elastase plays a role in the pathogenesis of keratitis in patients without predisposing conditions. However, neither alkaline protease nor elastase was essential for initiating or maintaining corneal infection in mouse and rabbit keratitis models. Significant differences in the primary structure of S-IgA from different species make it impossible to extrapolate to S-IgA cleavage during eye infections in humans.

The annotation of the genome sequence of *P. aeruginosa* strain PAO1 identified more than 30 genes encoding known or putative proteolytic enzymes (Comprehensive Microbial Resource, www.tigr.org/ J. Craig Venter Institute, Rockville, MD, and La Jolla, CA). As the expression of many of these proteases are under regulation and are likely to be differentially expressed under different environmental conditions we cannot exclude that other proteases may play a role in S-IgA degradation during active infection. For example, alkaline protease, but not elastase activity, was demonstrated during experimentally induced *P. aeruginosa* keratitis in mice infected with a *P. aerugi- nosa* strain producing both enzymes in vitro. The fact that partial or complete carbohydrate depletion induced by other bacteria such as streptococci may increase the susceptibility of S-IgA to proteolytic degradation renders the in vivo situation more complex.

The lack of detectable S-IgA degradation in tear fluids from the six patients infected with the IgA1 protease-producing species *S. pneumoniae* may be surprising. Although IgA1 cleavage fragments characteristic of IgA1 protease activity have been observed in human secretions under conditions that allow prolonged exposure of S-IgA to the responsible bacteria, recent observations in nasal and vaginal secretions of subjects colonized by IgA1 protease-producing bacteria suggest that cleavage may be restricted to the microenvironment of the bacteria. The effect of IgA1 proteases is probably a local functional IgA deficiency in the microenvironment immediately adjacent to the bacteria, which may be difficult to detect due to the constant flow of tears.

Collectively, this study demonstrates that SC of S-IgA secreted by human lacrimal glands is more abundantly glycosylated than S-IgA from colostrum and saliva, a difference of potentially great functional significance to the glycosylation-dependent protective functions of SC. Furthermore, this is the first report of in vivo degradation of human S-IgA in tears induced during active keratitis by proteolytic activity of *P. aeruginosa*. Local degradation of S-IgA is likely to jeopardize the local immune defense mechanisms until protease-neutralizing antibodies are induced. The results suggest that proteolytic cleavage of S-IgA by the concerted action of several proteases plays a role in the pathogenesis of *P. aeruginosa* keratitis, particularly in patients without otherwise compromised defense mechanisms.

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**References**


