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J D Klinger, B Tandler, C M Liedtke, T F Boat

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Research Article

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Proteinases of *Pseudomonas aeruginosa* Evoke Mucin Release by Tracheal Epithelium

Jeffrey D. Klinger, Bernard Tandler, Carole M. Liedtke, and Thomas F. Boat
Cystic Fibrosis Center, Departments of Pediatrics, Developmental Genetics and Anatomy, School of Medicine, Rainbow Babies and Childrens Hospital, and Department of Oral Biology, School of Dentistry, Case Western Reserve University, Cleveland, Ohio 44106

Abstract. We have determined the potential of exoproducts from pathogenic bacteria to stimulate the release of high molecular weight mucins from goblet cells of airway epithelium in a rabbit tracheal explant system. Culture supernatants from proteolytic strains of *Pseudomonas aeruginosa* and *Serratia marcescens,* but not supernatants from a number of non-proteolytic strains, released mucins from goblet cells. Highly purified elastase and alkaline proteinase from *P. aeruginosa* stimulated goblet cell mucin release in a dose-dependent fashion. Lipopolysaccharide, exotoxin A, and alginate of *P. aeruginosa* did not possess mucin release properties. Proteolytic activity was required for mucin release by *P. aeruginosa* elastase, but such release in goblet cells was not mediated by cyclic AMP. Morphologic studies suggested rapid release of mucins from goblet cells in response to elastase by a process resembling apocrine secretion. Several nonbacterial proteinases mimicked the effect of *Pseudomonas* proteases. These studies provide support for the hypothesis that bacterial and other proteinases play a role in the pathogenesis of mucus hypersecretion in acute and chronic lung infections.

Introduction

Lung disease in cystic fibrosis (CF)[1] is characterized by hypersecretion of mucus, mucus plugging of peripheral airways, and chronic airways infection (1). It has been difficult to establish whether excess mucus results from factors closely related to the gene defect or is secondary to airways infection. Evidence has been reported which supports both explanations. Specific pathophysiologic mechanisms linking infection and mucus hypersecretion in this disease have not been identified.

Recent evidence from in vitro studies suggests that bacterial exoproducts alter secretory functions of respiratory mucosa. Adler et al. (2, 3) demonstrated increased secretion of labeled macromolecules by guinea pig tracheal explants in response to cholera toxin or crude *Pseudomonas aeruginosa* filtrates. Stutts et al. (4) showed that supernatants from *P. aeruginosa* cultures significantly alter the bioelectric properties and presumably salt and water transport of canine bronchial epithelium. We therefore determined the potential of bacteria commonly associated with CF (1) or other patients with infected airways to stimulate mucin release and examined mechanisms of this effect in a well-characterized rabbit tracheal explant system. Results obtained with culture filtrates of *P. aeruginosa, Pseudomonas cepacia, Hemophilus influenzae, Klebsiella pneumoniae,* and *Serratia marcescens,* as well as purified bacterial products and mammalian proteinases, form the basis of this report.

Methods

Bacterial strains and products. Smooth and mucoid isogenic segregants of *Pseudomonas aeruginosa* were isolated from the sputum of an 11-yr-old CF patient. Both colonial morphologies (DGM, DGS) had the same serotype (International type 6/10) by slide agglutination using commercial antisera (Difco Laboratories, Detroit, MI). *P. aeruginosa* strain PA-103 (serotype 11) was isolated from sputum of an adult non-CF patient and has been used in numerous studies of exotoxin A (5); it was supplied by Dr. P. V. Liu, University of Louisville Health Sciences Center, Louisville, KY. *P. aeruginosa* strain WR-5, a blood isolate (serotype 12) (6), was supplied by Dr. Barbara Iglewski, University of Oregon Health Sciences Center, Portland, OR. PAKS-18 is the protease-negative ethyl methane sulphonate mutant of the wild-type *P. aeruginosa* urine isolate PAKS-1 (7). Both of the PAKS strains are serotype 9, and were supplied by Dr. Bengt Wretlind, Karolinska Hospital, Stockholm, Sweden. *Pseudomonas cepacia* strain 715j was isolated from the sputum of a 22-yr-old CF patient. Additional test organisms kindly provided by the Cleveland Clinic Foundation, Cleve-

Address correspondence to Dr. Klinger, Department of Pediatrics, Case Western Reserve University. Dr. Boat's present address is Department of Pediatrics, University of North Carolina, Chapel Hill, NC 27514.

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1. *Abbreviations used in this paper:* CF, cystic fibrosis; P_1, initial equilibration period; LPS, lipopolysaccharide; P_2, 15-min reincubation; TSB, tryptic soy broth.

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land, Ohio, included *Klebsiella pneumoniae* (derived from American Type Culture Collection Strain 27799), nonpigmented *Serratia marcescens* (CCF 33), and a serotype B *Hemophilus influenzae* (CCF 80).

All strains were isolated on tryptic soy agar (TSA), or, in the case of *H. influenzae,* on chocolate agar (Difco Laboratories). Five or six colonies from each isolate were subcultured into 250-ml flasks containing 50 ml tryptic soy broth (TSB; Difco Laboratories) and incubated for 20 h at 37°C in a reciprocating water bath at 140 excursions/min. Broth cultures of *H. influenzae* were grown in TSB that contained 1% lysed sheep erythrocytes and 1% Isovitalex supplement (Becton-Dickinson & Co., Cockeysville, MD). After incubation, bacterial cells were removed by centrifugation at 17,000 *g* for 30 min at 4°C. Supernatants were sterilized by filtration through 0.22-μm nitrocellulose membranes (Nalge Co., Rochester, NY) and immediately frozen at −20°C. Supernatant protein concentrations were determined by the Bradford procedure (8) using bovine serum albumin (CFG-7 powder, Reheis Chemical Co., Phoenix, AZ) as standard.

P. aeruginosa exotoxin A was purified from highly aerated TBS dialysate cultures of strain PA-103, as described by Taylor and Pollack (9). Exotoxin A preparations were shown to have ADP-ribosyltransferase activity (10), to form a single precipitin line against reference antitoxin in gel immunodiffusion assays, and to possess in vitro toxicity for mouse L-929 cells (11). Exotoxin A was stored at −70°C in 0.01 M Tris buffer, pH 8.

Lipopolysaccharide (LPS) was prepared by trichloroacetic acid extraction of reference International type 6 *P. aeruginosa* (strain 1369, supplied by P. V. Liu) using the method of Staub (12). LPS activity was assayed using Limulus amebocyte lysate (Associates of Cape Cod, Inc., Woods Hole, MA). Lyophilized LPS or 1-mg/ml solutions in pyrogen-free water were stored at 4°C.

P. aeruginosa elastase and alkaline proteinase were obtained commercially (Nagase Biochemicals Ltd., Tokyo, Japan). The purity of each was reconfirmed by demonstration of single bands when 25 μg of protein was analyzed by SDS-polyacrylamide gels according to the procedure of Laemmli (13). Lyophilized alkaline proteinase was dissolved in 0.01 M Tris-HCl, pH 8, aliquoted in 0.1–0.3-ml amounts, and stored at −20°C. *P. aeruginosa* elastase was suspended in 3.0 M $(NH_4)_2SO_4$, 10 mM sodium acetate, 2.0 mM $CaCl_2$, and 0.05 mM $ZnCl_2$, at pH 7–8. As needed, aliquots were dialyzed extensively against 0.01 M Tris-HCl, pH 8, and stored at −20°C. Elastase toxoid was prepared by treatment with 4% formaldehyde in 0.2 M borate buffer, pH 9 (14). Excess formaldehyde was removed by extensive dialysis against Tris buffer, pH 8. This toxoid was devoid of elastolytic activity, but formed a single precipitin line with rabbit anti-elastase serum in a double diffusion agarose system and retained its electrophoretic properties on SDS polyacrylamide gels.

Proteinase assays, inhibitors, and nonbacterial proteinases. General proteinase activity was assayed using a hide powder azure dye-release method (15). One-tenth-milliliter volumes of culture supernatants and 4.9 ml 0.01 M Tris buffer, pH 8, were incubated with 20 mg hide powder azure (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C in a shaking waterbath. Absorbance of dye released by proteinase was measured at 595 nm. Elastase activity was assayed as above except that 0.15 ml of each culture supernatant was incubated with 20 mg orcein-elastin in 2.5 ml Tris buffer, with shaking for 10 h. Released orcein was measured in the filtrate at 590 nm (16).

Purified phosphoramidon (17) was obtained from H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. Type XI bovine pancreatic trypsin (diphenyl cartamyl chloride-treated to inactivate chymotrypsin), crystallized porcine pancreatic elastase, and 1,10 phenanthroline (*o*-phenanthroline) were obtained from Sigma Chemical Co.

Tracheal explant cultures and mucin release assay. Tracheas were removed from 3 kg male New Zealand white rabbits after intravenous overdose of sodium pentobarbital. The epithelium and underlying connective tissue were dissected from the cartilaginous layer and cut into explants ~2.5 × 4.0 mm. Typically 20–30 explants were obtained from each trachea. Four explants, randomly selected with respect to donor animal and tracheal location, were cultured in each of 16–24, 35-mm tissue culture dishes containing 1.5 ml medium 199 (Gibco, Grand Island, NY) with 250 μg/ml ticarcillin (Beecham Laboratories, Bristol, TN) and 0.25 μg/ml amphotericin B. The mucous glycoprotein precursor $Na_2{}^{35}SO_4$ (20–40 μCi/ml final concentration; 500 mCi/mMol, New England Nuclear, Boston, MA) was also added to the culture medium. Plates were incubated in a water-saturated atmosphere of 40% O_2, and 5% CO_2 (balance air) at 35°C (18).

After an initial equilibration period (P_1) of 20–24 h for labeling mucins, media were collected and explants washed with two additional 1.5-ml aliquots of unlabeled medium. Incubation and wash media from each dish were pooled. Potential stimulators of mucin release were then added in fresh unlabeled medium, and dishes were reincubated for 15 min (P_2), except in time-course experiments. Control dishes received no additives. Labeled mucins secreted in both periods were isolated from other labeled macromolecules using an automatic BioGel A-5m gel filtration system (19) and counted using appropriate quench correction. DPM of $Na_2{}^{35}SO_4$ in 0.5 ml of the void volume fraction of P_1 and P_2 culture media were 16,440±410 (SEM)/24 h and 465±53/15 min, respectively, in a typical experiment.

A secretory index expressing the relative increase in rate of release of labeled mucins under experimental conditions was calculated for each culture dish as: [dpm per hour during P_2 (test)/dpm per hour during P_1 (test)]/[dpm per hour during P_2 (control)/dpm per hour during P_1 (control)]. This approach has been used in previous studies (18, 20, 21). The index theoretically is 1.0 if mucin release is not stimulated or inhibited. Differences between mean secretory indices and 1.0, i.e., no additives, were analyzed using a one sample *t* test with an expected numerator value of 1.0 (22). Significance of differences between mean secretory indices of response to different stimulators was calculated using a paired *t* test.

Ultrastructural studies. Tracheal explants were fixed in culture dishes by addition of an equal volume of phosphate-buffered, full-strength Karnovsky's (23) fixative to culture medium. The specimens were immediately transferred to fresh half-strength Karnovsky's fixative for 2 h at room temperature. After rinsing in buffered sucrose, they were postfixed for 2 h in phosphate-buffered 2% osmium tetroxide (24). Tissues were rinsed in distilled water and soaked overnight in aqueous 0.25% uranyl acetate. After another rinse in water, the explants were dehydrated in ethanol and embedded in Epon-Maraglas (25). Thin sections were sequentially stained with methanolic uranyl acetate (26) and lead tartrate (27) and examined in a Siemens Elmiskop 1a electron microscope. For light microscopy, sections 1-μm thick were stained with toluidine blue (28) and examined and photographed with a Zeiss Ultraphot II microscope.

Cyclic nucleotides and cAMP assay. Cyclic AMP was measured as previously described (29). Briefly, rabbit tracheal explants were cultured for 24 h in unlabeled Medium 199 as described for mucin release assays. They were then incubated at 35°C for 0.5–10 min in unlabeled medium containing 0.75–250 mg/ml of native or heat inactivated (30 min, 100°C) *P. aeruginosa* elastase. To stop the reaction, tubes containing the explants and medium were immersed

in a boiling water bath for 5 min, then cooled and freeze-thawed. Cyclic AMP levels were measured by the isotope dilution method of Brown et al. (30). Medium harvested from enzyme treated or control explants did not contain measurable amounts of cAMP for incubation times up to 20 min. Hence, this procedure assessed tissue levels of cAMP. Cyclic AMP levels were calculated as picomole per milligram protein and expressed as mean±SE. The explants were solubilized in 1 N NaOH for determination of protein levels by the method of Lowry et al. (31). ^{3}H-cAMP (34 Ci/mmol) was purchased from New England Nuclear. Dibutryl cAMP and 8-Br-cAMP were purchased from Sigma Chemical Co.

Results

Bacterial products and mucin release. Supernatants from TSB cultures of 10 strains representing five species of gram negative organisms were tested for their ability to release mucins from rabbit tracheal explants (Table I). The final cell densities were similar (mean colony forming units per milliliter was 1.81×10^{10}; range 0.55×10^{10} (PAKS 1) to 3.9×10^{10} [*S. marcescens*]). Only supernatants from *P. aeruginosa* and *S. marcescens* effected substantial mucin release at both concentrations tested (1:4, 1:16).

The supernatant of strain DGS (the smooth segregant of strain DGM) was the most active of any supernatant tested in the mucin release assay. The most stimulatory *P. aeruginosa* strains (DGM, DGS, WR-5, PAKS 1) were highly proteolytic and elastolytic. Elastolytic activity was not strictly related to total supernatant protein concentration. *P. aeruginosa* strains producing small amounts of elastase (PA-103, PAKS 18) showed little if any mucin release. TSB alone did not effect mucin release. Supernatants from other species having unde-

Table I. Stimulation of Mucin Release by Bacterial Culture Supernatants

Species	Strain	Supernatant protein concentration	Elastase A_{590}*‡	Protease A_{595}§	Supernatant concentration‖	Secretory index¶	Strain characteristics**
		μg/ml					
P. aeruginosa	DGM	32	0.30	0.86	1:4	2.7±0.2	Mucoid CF isolate
					1:16	1.7±0.1	
P. aeruginosa	DGS	36	0.13	0.83	1:4	4.4±0.5	Nonmucoid isogenic segregant
					1:16	2.3±0.2	of DGM
P. aeruginosa	PA-103	5.3	0.005	0	1:4	1.0±0.2	High exotoxin A/low
					1:16	1.3±0.3	proteinase producer
P. aeruginosa	WR-5	110	0.185	0.73	1:4	2.7±0.8	High proteinase/low exotoxin
					1:16	2.2±0.8	A producer
P. aeruginosa	PAKS 1	178	0.22	0.70	1:4	2.6±0.5	Non-CF clinical isolate
					1:16	1.9±0.3	
P. aeruginosa	PAKS 18	29	0.003	0.07	1:4	1.3±0.2	Low elastase mutant of
					1:16	0.67±0.5	PAKS 1
P. cepacia	715j	7.5	0.002	0.07	1:4	1.1±0.1	CF clinical isolate
					1:16	1.2±0.4	
H. influenzae	CCF 80	164	0	0.10	1:4	1.2±0.1	Type B clinical isolate
					1:16	1.3±0.2	
K. pneumoniae	ATCC 27709	6.0	0	0.01	1:4	1.4±0.1	Adult clinical isolate
					1:16	1.3±0.1	
S. marcescens	CCF 33	20	0.002	0.40	1:4	2.0±0.4	Nonpigmented clinical isolate
					1:16	1.7±0.2	
TSB	—	ND‡‡	0	0	1:4	1.0±0.1	(Culture medium)
					1:16	1.0±0.1	

* Assays of undiluted supernatants. ‡ A_{590} orcein-elastin assay; 0.15 ml cell-free supernatants. § A_{595} hide powder azure dye release; 0.1 ml undiluted cell-free supernatant. ‖ Final volume/volume concentration of supernatant to total media volumes. ¶ Mean±SEM, minimum of 3–4 repetitions. ** See Methods for detailed description of strains. ‡‡ ND, not done.

tectable or low levels of proteinases including elastase, i.e., *K. pneumoniae, H. influenzae, P. cepacia,* also had low stimulatory capacity. The *S. marcescens* supernatant which was moderately proteolytic (virtually no elastase activity) released some mucin, but less than elastolytic *P. aeruginosa* supernatants during the 15-min exposure period. No loss of ciliary motion was seen using a steroscopic microscope (25.2 times) and reflected surface lighting after 15 min incubation with any culture supernatants.

Four purified *P. aeruginosa* products varied widely in their ability to stimulate mucin release (Table II). Exotoxin A did not release labeled mucins at concentrations approaching probable toxic levels (10, 11), even when P_2 was extended to 0.5, 1.0, or 2.0 h. Lipopolysaccharide extracted from an International serotype 6 organism also failed to release mucins during a 15-min P_2 interval. At concentrations of 10 μg/ml both *Pseudomonas* proteinases increased mucin release; the mean secretory index for elastase was significantly greater than that of any of the other three products ($P < 0.005$). Panel *A* of Fig. 1 shows data for mucin release by tracheal explants in response to 0.1–100 μg/ml of *P. aeruginosa* elastase. The mean secretory index was 1.3±0.2 at 0.1 μg/ml, and increased to 3.4±0.3 at 100 μg/ml. Alkaline proteinase likewise gave a dose dependent, but more modest stimulation of mucin release (panel *B*, Fig. 1).

To further substantiate a role for enzymatically active elastase as a stimulator of mucin release we tested four preparations of inactivated or inhibited elastase (Table III). Chelation of magnesium (phenanthroline plus an excess of calcium) reduced the secretory index in response to 40 μg/ml *Pseudomonas* elastase to 58% ($P = 0.05$) of that with elastase alone. Likewise, the proteinase inhibitor phosphoramidon at 100-fold ($P < 0.04$) and 500-fold ($P < 0.01$) molar excess decreased the secretory index obtained with 10 μg/ml elastase to 32 and 41%, respectively. For these experiments, phosphoramidon and elastase were mixed and preincubated for 15–30 min at room temperature before addition to explants. In the dye release proteinase assay (15), phosphoramidon (100 and 500 M excess) reduced the activity of *P. aeruginosa* elastase (10 μg/ml) 29 and 86%, respectively. Lower concentrations of phosphoramidon did not block mucin release in response to

*Table II. Mucin Release in Response to P. aeruginosa Products**

Product	Concentration	Secretory index‡
	μg/ml	
Elastase	10	2.77±0.2
Alkaline protease	10	1.88±0.4
LPS	100	1.3±0.2
Exotoxin A	0.1	1.0±0.1

* See Methods for preparation and purity criteria.
‡ Mean±SEM, 4–14 repetitions.

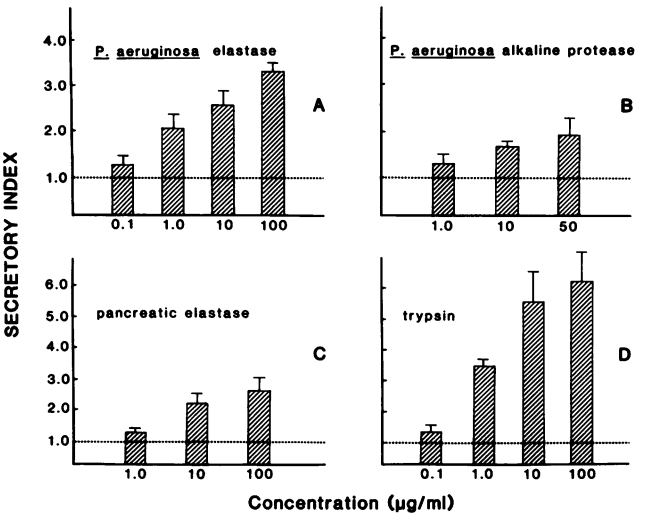

Figure 1. Mucin release by rabbit tracheal explants: dose responses to proteinases. (*A*) *P. aeruginosa* elastase. (*B*) *P. aeruginosa* protease. (*C*) Porcine pancreatic elastase. (*D*) Bovine pancreatic trypsin. Error bars show mean secretory index, ±SEM for 3–15 repetitions.

elastase. A 500-fold M excess (72 μg/ml) of phosphoramidon alone released little if any mucin.

The mean secretory index in response to elastase toxoid

Table III. Inhibition of P. aeruginosa Elastase Effect on Mucin Release

Additive	Elastase concentration	*n**	Secretory index‡
	μg/ml		
Elastase	40	4	3.1±0.6
1 mM, phenanthroline, 10 mM calcium	0	4	1.8±0.1
Elastase; 1 mM phenanthroline, 10 mM calcium	40	4	1.8±0.2
Elastase	10	3	3.7±0.3
72 μg/ml phosphoramidon§	0	3	1.4±0.5
Elastase; 14 μg/ml phosphoramidon	10	3	2.5±0.4
Elastase; 72 μg/ml phosphoramidon	10	3	2.2±0.2
Elastase	10	4	1.8±0.3
10 μg/ml elastase toxoid‖	0	4	1.0±0.2
Elastase	10	4	2.7±0.2
Elastase, 100°C, 10 min	10	4	1.3±0.1

* *n*, number of repetitions.
‡ Mean±SEM.
§ 72 μg/ml, 500-fold molar excess (versus elastase concentration).
‖ Formaldehyde-treated elastase; see Methods.

was lower than that of native elastase ($P < 0.05$). The preparation of elastase used as control in the toxoid experiment had been frozen and thawed several times and gave lower than expected results (secretory index: 1.8±0.33 vs. 2.8±0.2 (n = 14); see Table I). After five freeze-thaw steps, *P. aeruginosa* elastase retained 70.5% of the original activity against hide powder azure; after seven freeze-thaw steps only 57.3% of the activity remained. This lability provided additional evidence that native enzymatic activity is required for mucin release.

Boiling *Pseudomonas* elastase (10 μg/ml) for 10 min abolished stimulatory activity ($P < 0.005$). The secretory index obtained with heated elastase was not significantly different than that in control cultures.

Morphologic studies. After incubation for 20–24 h, control specimens of trachea were identical to those fixed immediately after extirpation from anesthetized rabbits (Fig. 2). The structure of the lining epithelium of the rabbit trachea has been described by Konradova (32) and will not be described in detail here. The tracheal epithelium consisted principally of ciliated, goblet cells, and basal cells. These cells were closely coherent. Goblet cells were abundant and had prominent thecas distended by numerous secretory granules. Only a few goblet cells (<15%) showed evidence of mucous discharge; such cells were characterized by bulging of their apical surface into the lumen, which created a "dome." Empty or nascent goblet cells were sparse in controls. These cells had at most only a few mucous droplets, and therefore a slender, columnar silhouette.

After exposure to *P. aeruginosa* elastase, specimens at all time points evidenced a similar spectrum of responses related to mucus discharge (Figs. 3 and 4). Between 55 and 100% of the goblet cells that abutted the lumen were domed. Moreover, many of the domes were elongated and contorted and contained most of the mucous droplets remaining in the cell (Figs. 5 and 6). Many of the stimulated specimens displayed detached pieces of goblet cells or free cytoplasmic tags. Few full goblet cells remained; most were completely exhausted of their secretory content, extremely attenuated, and had high cytosolic density. Regardless of the secretory status of the goblet cells, their junctions with adjacent ciliated cells were unaltered, and all epithelial lining cells were closely adherent (Fig. 7).

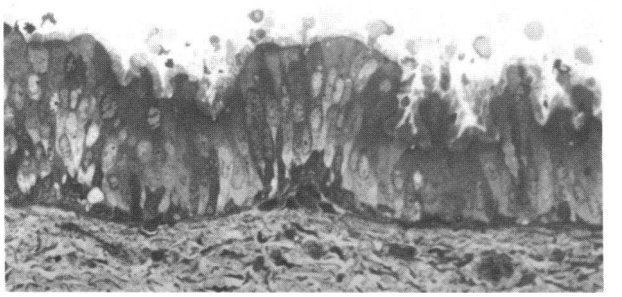

Figure 3. Rabbit trachea five min after exposure to 10 μg *P. aeruginosa* elastase. Masses of detached cytoplasm are present along the epithelial surface, as are cytoplasmic protrusions of goblet cells. Toluidine blue. × 230.

Cyclic AMP. Fig. 8 depicts changes with time in cAMP levels in response to 28 μg/ml elastase. Basal cAMP levels of 10 pmol/mg protein increased to 15 pmol/mg protein during the first 3 min of incubation. The tissue cAMP content decreased to basal levels after 5 min and remained there for incubation times up to 90 min. Heat-inactivated elastase did not alter cAMP levels in explants. As seen in Table IV, *P. aeruginosa* elastase effected a small dose-dependent increase of cAMP levels. Maximal response was attained with 75 μg elastase/ml. This pattern is unlike that for mucin release in response to enzyme, which did not plateau at concentrations of *Pseudomonas* elastase up to 100 μg/ml, and suggests that increased cAMP is not directly related to accelerated release of mucous glycoproteins.

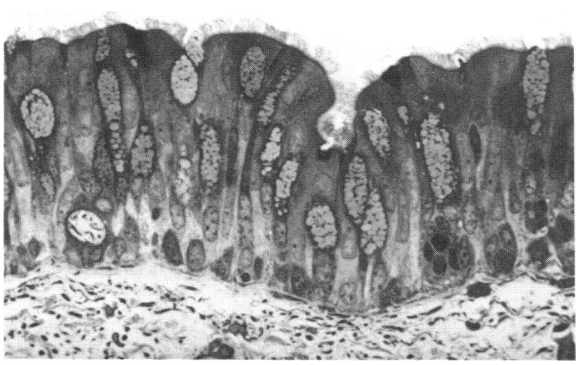

Figure 2. Photomicrograph of untreated rabbit trachea maintained in organ culture for 20 h. The pseudostratified epithelium is intact. Goblet cells, characterized by large numbers of mucus droplets, are abundant. Toluidine blue. × 360.

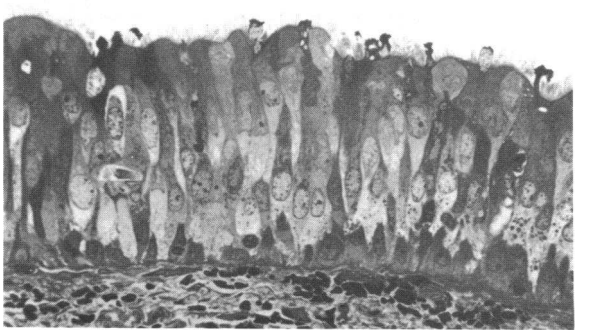

Figure 4. Rabbit trachea 30 min after exposure to 10 μg *P. aeruginosa* elastase. No full goblet cells remain. Many cells still show apical protrusions. Toluidine blue. × 360.

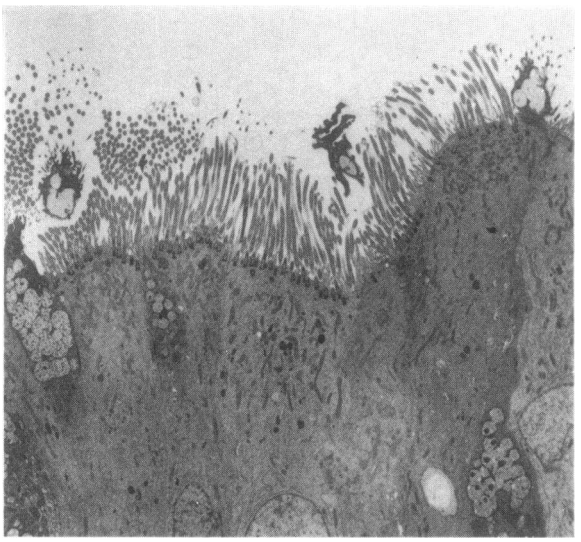

Figure 5. Electron micrograph of trachea 30 s after exposure to 10 μg *P. aeruginosa* elastase. Several detached pieces of cytoplasm containing mucous droplets are free in the lumen. The epithelium is otherwise intact. × 1,900.

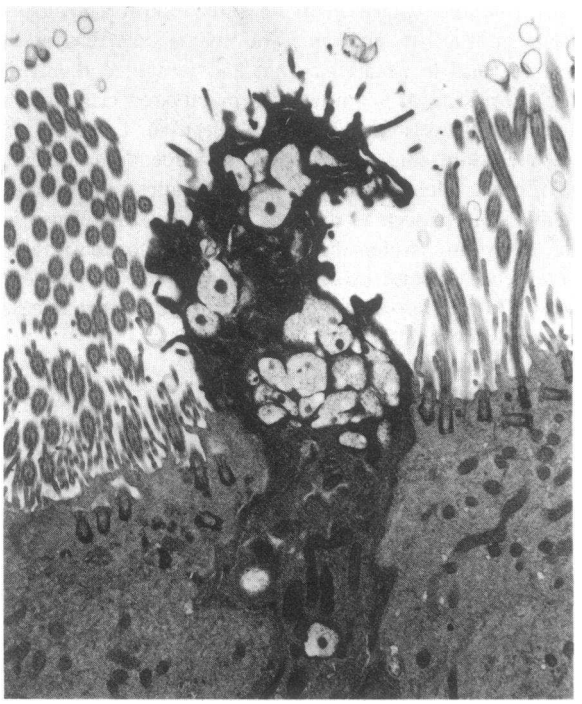

Figure 6. Electron micrograph of the apex of a goblet cell 30 s after exposure to 10 μg elastase. The cell protrudes into the lumen and is quite distorted. Some mucous droplets are still present, but the cytosol is unusually dense. Despite its obvious morphological alteration, this cell maintains its junctional connections and relationships with the flanking ciliated cells. × 6,500.

Figure 7. High magnification micrograph of the junctional region of a discharging goblet cell (*left*) and a ciliated cell (*right*). The tight junction has maintained its morphological integrity. 10 μg elastase, 30 s. × 56,000.

Additionally, exogenous cAMP failed to increase mucin release. Dibutryl-cAMP at concentrations of 10^{-5}–10^{-3} M caused no increase in secretory index after 15 min of incubation (secretory index = 1.02±0.05, n = 3 repetitions). Similar absence of effect was obtained using 10^{-5}–10^{-3} M 8-Br-cAMP.

Mammalian proteinases. Both porcine pancreatic elastase and chymotrypsin-free bovine pancreatic trypsin stimulated

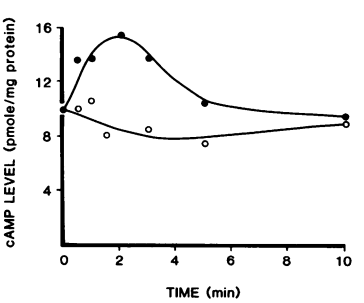

Figure 8. Effect of *P. aeruginosa* elastase on cAMP levels in rabbit tracheal explants. Explants were incubated for the indicated times with elastase (●) or heat-inactivated elastase (○) at a final concentration of 28 μg/ml. Each data point represents the mean of 4–6 determinations. Standard errors of each data point are <20% of the means.

Table IV. Effect of P. aeruginosa Elastase on Adenylate Cyclase Activity

Addition	Elastase concentration	cAMP level (pmol/mg protein)	
Basal (none)	0	10.0±2.0 (4)*	
P. aeruginosa elastase	0.75 μg/ml	8.4±0.6 (3)	$P < 0.05$
	7.5 μg/ml	10.6±1.0 (3)	NS
	25 μg/ml	11.9±0.7 (3)	$P < 0.05$
	75 μg/ml	12.8±1.8 (3)	NS‡
	250 μg/ml	11.9±0.5 (3)	$P < 0.0005$

* Mean±SE; number in parentheses is number of repetitions.
NS, not significant. Levels of significance determined by *t* test for paired samples (test vs. basal).
‡ Large SE.

mucin release in a dose-dependent fashion over the concentration range tested (panels *C* and *D*, Fig. 1). Of all the proteinases assayed in this study, trypsin was the most potent stimulator of mucin release.

Discussion

We have demonstrated that extracellular proteinases of *P. aeruginosa* stimulate mucin release by goblet cells in rabbit tracheal epithelium. Thus, proteinases can be added to a growing list of substances which trigger the release of mucins onto the airways surface, including autonomic agonists (21, 33, 34), polypeptides (35, 36), metabolites of arachadonic acid (37), cholera toxin (2), and serum components (18).

The impetus for our studies came from the observation of Adler et al. (3) that sterile culture filtrates of some *P. aeruginosa* strains effect 1.3–1.8-fold increases of labeled macromolecule secretion by guinea pig tracheal explants. Our studies confirm their basic observation, ascertain that the secretory effect specifically involves release of high molecular weight mucins, and identify exoproducts which mediate this effect. Our data support the suggestion that the secretory effect is not evoked by extracellular polyuronic acid (alginate) of mucoid *P. aeruginosa* (3). In addition, we have demonstrated that exotoxin A and lipopolysaccharide do not promote mucin release. Organisms of the same serotype may or may not generate mucin release activity, precluding a strict relationship between these properties.

Four proteolytic strains of *P. aeruginosa* elaborated mucin release activity, while two strains which produced minimal amounts of proteinases did not. This observation, coupled with the demonstration that highly purified elastase and to a lesser extent purified alkaline proteinase from *P. aeruginosa* stimulate mucin release, strongly suggest that proteinases are important, if not the sole products of this organism with mucin release properties. Adler et al. (3) reported that the effect of *P. aeruginosa* filtrates on macromolecule release is stable at 90°C for 30 min, a condition which would be expected to inactivate elastase and other proteinases. In fact, in our studies, enzymatic and mucin release activity of *P. aeruginosa* elastase were completely lost after treatment at 100°C for 10 min. Differing results of the two heat inactivation studies could relate to species differences in modulation of secretory responses. In addition, Adler's assay system measured all labeled secretory macromolecules, while our assay specifically quantitated the release of high molecular weight mucins (19) and may have been more sensitive to proteinase effects. Finally, we have not exhaustively eliminated the possibility that a bacterial exoproduct other than proteinases might participate in the mucin release response.

Mucins secreted in our in vitro system by rabbit tracheal epithelium are labeled with ^{3}H-glucosamine to a constant specific activity within 12 h (data not shown). Therefore, we have assessed release of all high molecular weight mucins rather than a select pool of these glycoproteins. Addition of radiolabeled precursor to medium only during the initial culture period (P_1) precludes the contribution of newly synthesized mucin to the secreted product during P_2. Consequently, we have examined only the release of preformed mucins in the studies. Rabbit trachea contains no, or only rudimentary, glands (18). This ensures that the mucin secretory response is a function of surface epithelium and presumably of its goblet cells. Additional studies are required to assess whether proteinases promote mucin biosynthesis or secretory activity of gland cells.

We have partially characterized the mechanism of action of proteinases on mucin-secreting cells. Several pieces of evidence firmly support a conclusion that proteolytic activity is required for the mucin release effect. Heat denaturation, or formaldehyde inactivation of enzyme activity with preservation of immunogenicity, completely abolish mucin release activity of *P. aeruginosa* elastase. In addition, removal of Zn^{+2} from the assay system with phenanthroline under conditions of calcium sufficiency significantly diminished the activity of this metalloproteinase (38). This approach was required because the simple addition of EDTA or other chelators of divalent cations alters the secretory response of tracheal epithelium (39). Finally, phosphoramidon, a competitive inhibitor of *P. aeruginosa* elastase and other metalloproteinases (17), significantly reduced mucin-release activity.

Results of our studies argue against generalized cytotoxic effects as a mechanism for release of labeled mucins. Specifically, we observed no damage to tracheal cells and no disruption of the integrity of surface epithelium. Ciliary motion was maintained during 15-min exposure periods at all concentrations of enzymes used.

Electron microscopic observations suggest goblet cell mucins are released rapidly in response to proteinases by a process resembling apocrine secretion. Masses of cytoplasm are expelled rapidly from goblet cells, and are accompanied by blebbing and distortion of their apical regions. The manner in which goblet cells release their secretory product has been a matter

of controversy. Originally, it was thought that goblet cells extrude a single mass of mucus through a break in the apical plasma membrane (40–45). Subsequent studies of intestinal goblet cells by Specian and Neutra (46) have demonstrated compound exocytosis which can be evoked by a variety of secretagogues (47). A similar sequence has been observed in goblet cells of the cat trachea (Tandler, B., unpublished observations). Trier and Rubin (48) have also noted that merocrine secretion is the norm in intestinal goblet cells, but that apocrine secretion occurs after a potent secretory stimulus. We suggest that proteinases similarly may be potent stimuli for goblet cells in large airways. This stimulus could be triggered by proteolytic modification of cell membrane proteins, although currently there is no evidence for such a mechanism.

A number of serine proteinases, including trypsin, chymotrypsin, pronase, subtilisin, protease from *Streptomyces griseus*, and a 30,000-mol wt proteinase from *Pseudomonas maltophilia* (49, 50), stimulate adenylate cyclase. We found that *P. aeruginosa* elastase also increases cyclic AMP levels in rabbit tracheal epithelium, although the response is a small one. On the other hand, mucin secretion by rabbit trachea is refractory to cyclic AMP analogues which enter these cells and which promote mucin release in cat tracheal explants (20). Therefore, it is highly unlikely that cAMP is an intracellular messenger of the proteinase effect on goblet cells. In this respect the response to proteinases is similar to the secretory response to cholera toxin by guinea pig tracheal explants (2).

Our work suggests that a broad range of proteinases has mucin release effects. *P. aeruginosa* elastase is a metalloproteinase with substrate specificity for peptides that contain a P_1' leucyl or phenylalanyl residue, similar to thermolysin (51). On the other hand, the serine proteinases, pancreatic elastase, and trypsin act on primary specificities of Ala(Ser)-, and Arg(Lys)-, on the carboxyl side of hydrolyzed bonds, and are less potent and considerably more potent, respectively, than *P. aeruginosa* elastase in the mucin release assay. More work is required to establish the relationship between substrate specificity and the response of goblet cells to proteolytic enzymes.

P. aeruginosa is a pathogen associated with both acute and chronic lung disease (52). For example, the airways of most patients with cystic fibrosis are permanently colonized with this organism (53). Several potential mechanisms for pathogenicity related to production of proteinases by *P. aeruginosa* in these conditions are recognized. *P. aeruginosa* elastase degrades a number of complement components (54) and IgG (55), which potentially reduces immunologic responses at the epithelial surface. *P. aeruginosa* elastase and other proteinases also may degrade structural elements in the airway walls and lead to bronchiectasis, bronchiolectasis, and air space enlargement (56). Finally, these studies strongly suggest that *P. aeruginosa* proteinases contribute to mucus hypersecretion and obstruction to airflow, both acutely and chronically. In addition, failure to clear excessive mucus may promote ongoing airways infection and set up a troublesome cycle of events. Under most circumstances the airways are protected from proteolytic effects by antiproteinases, such as the bronchial proteinase inhibitor, which is secreted locally (57, 58), and α_1-antiproteinase, which is acquired from serum by transudation (59). However, *P. aeruginosa* elastase complexes with and inactivates both α_1-antiproteinase (60) and the bronchial proteinase inhibitor. In fact *P. aeruginosa* elastase can release neutrophil elastase from complexes with the bronchial inhibitor (61). Therefore, one pathogenetic role of *P. aeruginosa* proteinases may be to render normal protective mechanisms inoperative, thus promoting the damaging effects of proteinases released by inflammatory cells. *P. aeruginosa* proteinases probably do not play a role in the ciliary inhibition (62) or in alterations of epithelial ion transport (4) which have been produced by sterile filtrates of *P. aeruginosa* culture media. Both of these activities are heat stable and possess other characteristics which distinguish them from a proteinase effect.

Organisms other than *P. aeruginosa* produce proteinases and could be responsible for acute and chronic mucus hypersecretion. We found that a *S. marcescens* filtrate also was proteolytic and triggered the release of goblet cell mucins. This is consistent with the demonstration of metalloproteinase production by this organism (63). As prepared for these experiments, supernatants from strains of *P. cepacia, H. influenzae,* and *K. pneumoniae* did not contain proteinases or mucin release activity in vitro. However, some strains of these organisms may be proteolytic under different conditions. Indeed, we have preliminary data showing that after long periods in culture, e.g., >26 h, *P. cepacia* strain 715j produces extracellular proteinase.

Additionally, proteinase production could be induced in vivo in strains which are not proteolytic in vitro; if so, the effect which we have described may have more pathophysiologic significance than our data currently suggest.

Instillation of proteinases into airways of laboratory animals produces emphysema, and is accompanied by striking goblet cell hyperplasia and metaplasia of conducting airways (64). We propose that intense or prolonged stimulation of mucin secretion by uninhibited proteinases may promote goblet cell proliferation and differentiation of small airways epithelium to a mucus-secreting type. Such changes may occur not only in laboratory animals treated with proteinases, but also in the chronic bronchitis and/or bronchiolitis produced by persisting airways infection. Specifically, in chronic *P. aeruginosa* infection in cystic fibrosis, release of bacterial enzymes may provide this stimulus, most likely in concert with inflammatory cell proteinases (65). Thus, the impressive mucus hypersecretion in airways of cystic fibrosis patients may relate to increased numbers of mucus-secreting units as well as an ongoing source of stimulation for mucus release, both attributable, at least in part, to persistent endobronchial infection.

We wish to emphasize that the implications of our data for alterations of airway structure and function in human lung infections are not yet established. In preliminary experiments, culture supernatant from *P. aeruginosa* (strain-DGS) increased

mucin secretion from explants of human nasal polyp epithelium as much as 2.4-fold. Additional studies are required to characterize this effect on human airways epithelium.

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