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Mucin gene (*MUC 2* and *MUC 5AC*) upregulation by Gram-positive and Gram-negative bacteria

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Abstract

Bacterial infection of the lung is associated with mucin overproduction. In partial explanation of this phenomenon, we recently reported that supernatant from the Gram-negative organism *Pseudomonas* (*P.*) *aeruginosa* contained an activity that upregulated transcription of the *MUC 2* mucin gene [J.-D. Li, A. Dohrman, M. Gallup, S. Miyata, J. Gum, Y. Kim, J. Nadel, A. Prince, C. Basbaum, Transcriptional activation of mucin by *P. aeruginosa* lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease, Proc. Natl. Acad. Sci. U.S.A., 94 (1997) 967–972]. The purpose of the present study was to determine whether mucin genes other than *MUC 2* are so regulated and whether Gram-positive organisms also contain mucin stimulatory activity. Results from in situ hybridization and RNase protection assays showed that *P. aeruginosa* upregulates *MUC 5AC* as well as *MUC 2* in both bronchial explants and cultured airway epithelial cells. The upregulation of both genes by *P. aeruginosa* can be mimicked by lipopolysaccharide (LPS) and can be blocked by the tyrosine kinase inhibitor genistein. In addition, both genes are upregulated by a variety of Gram-positive as well as Gram-negative organisms showing the same rank order of potency. These data indicate the existence of a general mechanism by which epithelial cells respond to the presence of bacteria by increasing mucin synthesis.

Keywords: Mucin gene; Mucin synthesis; Bacterial infection; Lipopolysaccharide; Mucin overproduction

1. Introduction

Mucin overproduction exacerbates bacterial pneumonia and cystic fibrosis (CF) lung disease. Recently we observed by in situ hybridization that *MUC 2*, a gene cloned from the intestine [1] and generally expressed weakly in the airways [2], is expressed strongly in CF airways [3]. We determined that a factor contributing to this upregulation was the common CF pathogen *P. aeruginosa* [3]. We wondered whether the phenomenon was specific to *P. aerugi-*

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nosa and the *MUC 2* gene or might extend to other bacteria and mucin genes as well. If the phenomenon is a general one, are the regulatory mechanisms the same or different for each mucin gene (*MUC 1, 3–8* [1,4–10](12)) and bacterium? Answers to these questions could lead to therapeutic approaches for blocking bacterially-induced mucin overproduction.

To this end, we examined the bacterial inducibility of the mucin gene *MUC 5AC*, which shows relatively high levels of expression even in healthy human airways [2] and has been found to show an altered tissue distribution in CF airways [11]. We found that it too is upregulated by *P. aeruginosa* and that the regulatory mechanisms share similarities with those previously described for *MUC 2*. In addition, we found that both *MUC 2* and *MUC 5AC* are inducible by supernatants from a wide variety of Gram-negative and Gram-positive bacteria. The generality of the phenomena observed so far offers promise that a single class of inhibitors aimed at an epithelial cell signaling molecule may eventually control mucus hypersecretion induced by both Gram-negative and Gram-positive organisms.

2. Methods

2.1. Tissue procurement and preservation for in situ hybridization

To determine whether *MUC 5AC*, like *MUC 2* is upregulated in CF airways, we examined human nasal polyps as well as bronchial samples from donors and recipients at the time of lung transplantation. All tissue types were removed and processed according to UCSF policy and were directly fixed or placed in vitro and exposed to *P. aeruginosa* as described below. Tissue samples were fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 4 h and cryoprotected in 30% sucrose/0.1 M phosphate buffer overnight at 4°C. The next day, samples were embedded in OCT compound (Miles, Elkhart, IN) and quickly frozen in liquid nitrogen-cooled Freon-22. The frozen tissue was sectioned (6 μm), placed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and air dried quickly. The sections were stored at −80°C until use.

2.2. Human bronchial explant cultures

Segmental to subsegmental bronchi from lung donors and recipients were sectioned to yield bronchial rings 0.5 mm in length. These were rinsed in sterile PBS before incubation in a serum-free, 1:1 mixture of Dulbecco's modified Eagle's (DME) and Ham's F-12 (F12) supplemented with penicillin, streptomycin, gentamycin and amphotericin B.

2.3. Human airway epithelial cell cultures

NCI H292 cells were grown in RPMI 1640 + 10% FCS. Medium was changed every 3 days. Incubation with *P. aeruginosa* supernatant was at 3 days post confluence.

2.4. Bacterial culture and preparation of cell-free supernatants

P. aeruginosa strain PAO1 was grown in M9 buffer [12] for 72 h at 37°C (to late log phase). All other bacterial strains were grown to late log phase in LB buffer. Cell-free supernatant was obtained by centrifugation at 10,000 rpm for 60 min at 4°C and by filtration through a 0.22 μm filter (Corning). Supernatant was aliquoted and stored at −80°C until used.

2.5. Exposure of tissues and cells to bacterial cell-free filtrates

Incubation was as described in Ref. [13]. Briefly, cells or organ culture specimens were washed twice with PBS at 37°C. Samples were then incubated with bacterial supernatant or buffer (M9 or LB) diluted 1:4 with RPMI 1640 for 6 h. Total RNA was obtained from pelleted cells scraped from the culture dish [14]. Lactic dehydrogenase release was measured (LDH 320, Sigma) to detect any cell lysis.

2.6. RNA probes

A single *MUC 5AC* cRNA probe was used for both in situ hybridization and RNase protection assay (RPA). This probe was constructed using PCR with primers corresponding to nucleotides 580–604 and

851–875 of the cDNA sequence described in Ref. [9]. The resulting 295 bp cDNA was TA-cloned into pCRII vector (Invitrogen, San Diego, CA). The *MUC 2* probe for RPA was constructed using PCR with primers corresponding to nucleotides 423 to 639 of the cDNA sequence described in Ref. [15]. The resulting 216 bp cDNA was TA-cloned into pCRII vector (Invitrogen). The *MUC 2* probe for in situ hybridization was generated from the 90 bp HAM 1 insert [16] in pBluescript plasmid (Stratagene, La Jolla, Ca). [^{32}P] or [^{35}S] UTP-labeled RNA probes were synthesized from the cDNA in linearized pBluescript or pCRII plasmid using T7, T3, or SP6 polymerases. Antisense and sense probes were used at concentrations of $2–5 \times 10^5$ cpm/μl. The linearized template for the housekeeping gene cyclophilin was purchased from Ambion. The pTRI-cyclophilin–human anti-sense control contains a 103 bp cDNA insert of a highly conserved region of the human cyclophilin gene spanning exons 1 and 2 (nucleotides 38–140 of Accession# X52856). The probe was prepared as above using [^{32}P] UTP and T7 polymerase according to manufacturer's instructions.

2.7. In situ hybridization

Frozen sections of human nasal polyp or bronchus were air dried quickly, heated at 55°C for 10 min, fixed with 4% paraformaldehyde in PBS for 10 min, washed with 2 × SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0), immersed in 0.1 M triethanolamine HCl (pH 7.5) containing 0.25% acetic anhydride for 10 min, rinsed with 2 × SSC, dehydrated with ethanol and air dried. RNA probes described above were applied in a hybridization mixture containing deionized formamide (50%), dextran sulfate (10%), tRNA (0.5 mg/ml), Ficoll 400 (0.02% (w/v)), salmon sperm DNA (1 mg/ml), polyvinylpyrrolidone (0.02% (w/v)), 10 mM DTT, 0.3 M NaCl, 0.5 mM EDTA, 10 mM Tris–HCL, and 10 mM $NaPO_4$ (pH 6.8). The mixture was heated to 70°C for 15 min and chilled on ice. Fresh DTT was added to achieve a concentration of 20 mM. Then 100 μl of the mixture was applied to each section and parafilm coverslips were applied. Hybridization was carried out in humid chambers overnight at 55°C. Coverslips were removed in 5 × SSC, 10 mM DTT, at 55°C. Sections were washed three times in wash buffer (2 × SSC, 1 mM EDTA, 10 mM β-mercaptoethanol (βME)) for 5 min at room temperature. Subsequently, they were treated with 20 μg/ml of RNase A in 500 mM NaCl, 10 mM Tris (pH 8.0) for 30 min at room temperature. This was followed by 2 × 5 min changes of wash buffer and a high stringency wash in 4 l of a wash solution containing 0.1 × SSC, 1 mM EDTA and 10 mM βME (2 h at 55°C). Slides were then washed 5 min at room temperature in 0.5 × SSC without βME and EDTA. Finally, sections were dehydrated with ethanol and air-dried. The slides were exposed to Ilford K5D emulsion, and stored in the dark at 4°C until developed after 3–10 days exposure.

2.8. Imaging and quantification

We used NIH Image software. Because polyp epithelium is irregular in shape, we evaluated in situ hybridization labeling density by obtaining a series of brightness measurements for areas of epithelium at fixed intervals and averaging them. We analyzed four slides of nasal polyps from nine CF and 11 non-CF individuals in this way. In bronchial explants, the epithelium consisted of rectangular areas in each cross section. We established brightness thresholds and collected binary image data consisting of total pixels per unit length of epithelium in four slides each for three CF and three non-CF subjects. For the *P. aeruginosa* explant incubation study, we analyzed four slides of airway epithelium from treated and four from untreated explants in two separate studies based on two individual lung donor subjects.

2.9. RNase protection assay

MUC 2 and *MUC 5AC* cDNA inserts were generated by PCR as described above and subcloned into TA vector. The vector was linearized with *Bam*HI in a 100 μl vol at 37°C overnight. The DNA was digested with Proteinase K before extracting with equal volumes of phenol/chloroform/isoamyl alcohol. The extract was precipitated with ammonium acetate and ethanol for 15 min at −80°C and redissolved in DEPC water. Riboprobe was synthesized in the presence of ^{32}P-labeled UTP using linearized and purified DNA template. Then it was subjected to RNase-free DNase I at 37°C, phenol-extracted and ethanol-precipitated. The pellet was resuspended in

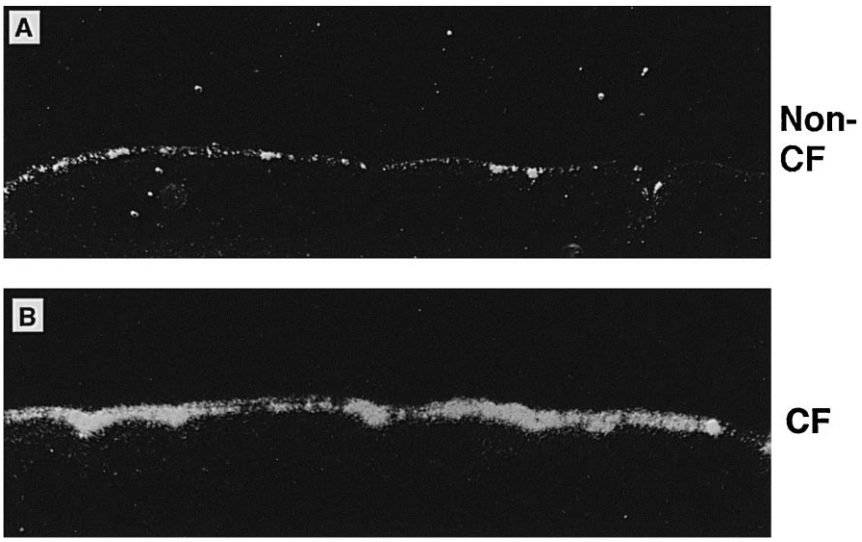

Fig. 1. In situ hybridization analysis of nasal polyp epithelium from non-CF (A) and CF (B) subjects showing considerably more *MUC 5AC* mRNA in the CF than the non-CF sample. Both were probed with an ^{35}S labeled *MUC 5AC* probe containing non-repetitive sequence.

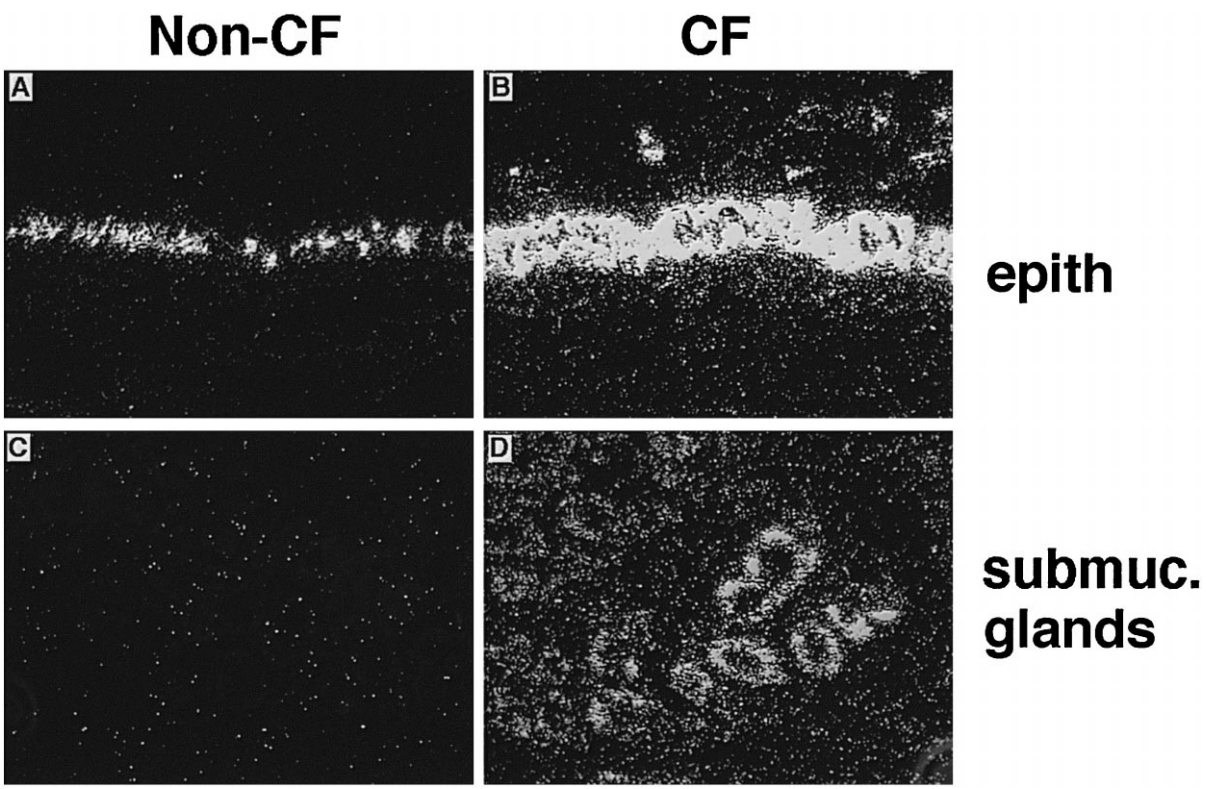

Fig. 2. In situ hybridization analysis of bronchial epithelium from non-CF (A and C) and CF (B and D) subjects. Epithelium (epith.) and submucosal glands (submuc. glands) are different fields from the same tissue sections. Samples were probed as above.

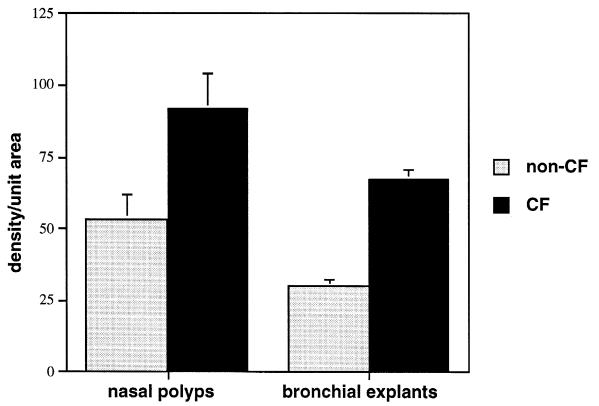

Fig. 3. Morphometric analysis of *MUC 5AC* mRNA autoradiographic grain density in nasal polyps and bronchial explants of CF and non-CF subjects. Nasal polyps were taken from 11 non-CF and nine CF subjects; bronchial explants were taken from three non-CF and three CF subjects.

gel loading buffer. The probe was purified on a polyacrylamide/urea gel kept at 300 V for 1 h. The target band on the gel was visualized by exposing the gel to film for 15 s. The band was excised and incubated in elution buffer (RPA II kit, Ambion) at 37°C overnight.

Experimental RNA samples were precipitated and dissolved in hybridization buffer. A total of 10 μl of probe was added, the samples were heated and allowed to hybridize overnight. After hybridization, RNase A and T (RPAII kit, Ambion) were added to each sample and incubated at 37°C for 30 min. The samples were precipitated, redissolved in gel loading buffer, denatured at 95°C, and run on a standard 30×38 cm, 6% polyacrylamide/8 M urea sequencing gel at 250 V for 1–2 h. The gel was dried and placed on film at -80°C for 2–7 days.

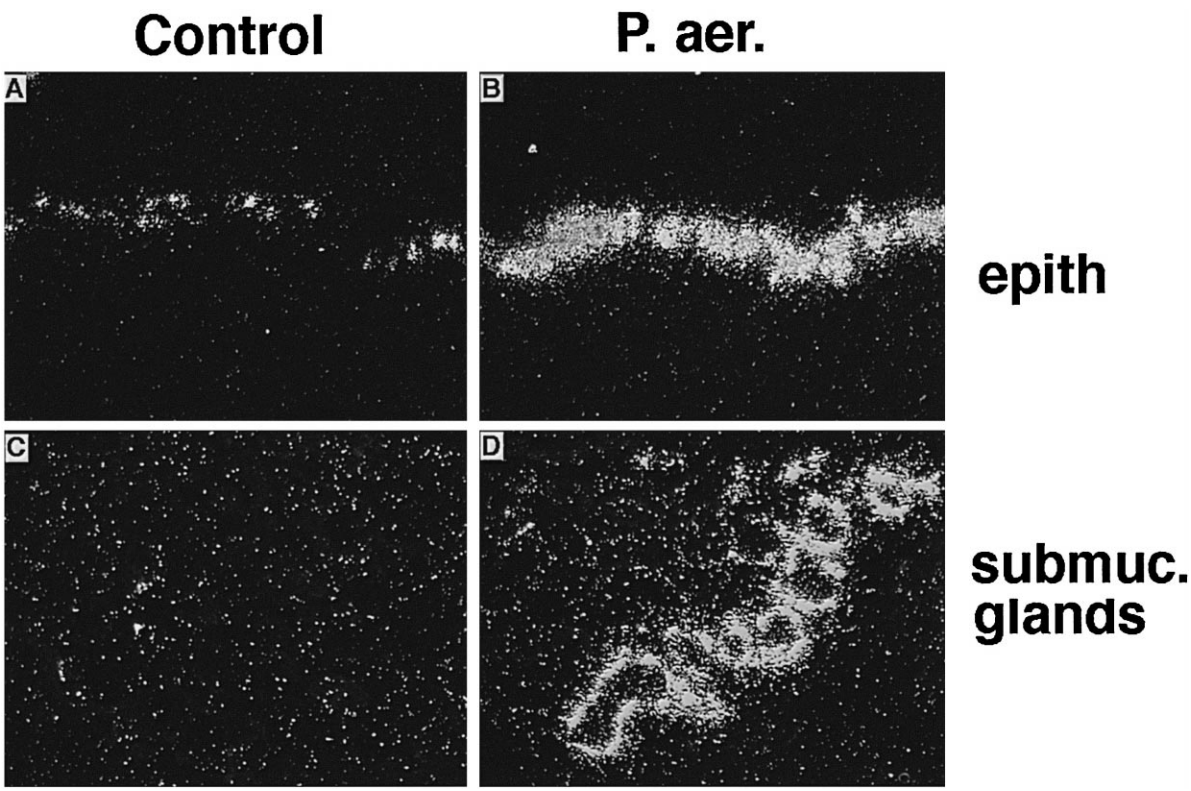

Fig. 4. In situ hybridization analysis of *MUC 5AC* mRNA in bronchial explants from a lung donor. Explants were exposed to *P. aeruginosa* supernatant (P. aer.) or M9 buffer (control) for 6 h as described in Section 2. epith = surface epithelium; submuc. glands = submucosal glands.

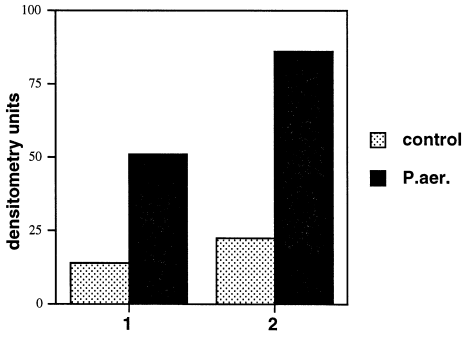

Fig. 5. Morphometric analysis of *MUC 5AC* mRNA autoradiographic grain density in bronchial explants that had been treated with *P. aeruginosa* (P. aer.) or M9 buffer (control) as described in legend for Fig. 4. Data from experiments 1 and 2 are shown.

2.10. ELISA to detect mucin glycoprotein released by epithelial cells in presence and absence of P. aeruginosa exposure

ELISA was performed as previously described [17]. Briefly, antigen diluted in 0.05 M sodium bicarbonate was dried down overnight at 37°C onto 96 well plates (Immulon 2, Dynatech). After blocking with phosphate-buffered saline containing 0.3% triton and 1% normal goat serum, plates were incubated with anti-human mucin monoclonal antibody 10G5 [17] diluted 1:10 for 1 h at room temperature. Antigen–antibody binding was detected using the Vectastain ABC-AP kit (Vector Labs) with *p*-nitrophenyl phosphate as substrate.

3. Results

In situ hybridization revealed that *MUC 5AC* mRNA levels are elevated in CF nasal polyps (Fig. 1) and bronchi (Fig. 2) with respect to analagous samples from non-CF subjects. As previously reported for *MUC 2* [3], both surface epithelium and submucosal glands showed *MUC 5AC* upregulation. *MUC 5AC* mRNA occupied more of the linear surface of CF than non-CF mucosal epithelium and was more abundant on a per cell basis. Quantification of autoradiographic grain density confirmed this (Fig. 3).

P. aeruginosa is a common pathogen in CF airways. To examine the possibility that it participates in upregulation of *MUC 5AC*, we exposed bronchial explants from two individual lung donors to *P. aeruginosa* supernatants for 6 h in vitro and then examined them by in situ hybridization. We observed increased *MUC 5AC* expression in both surface epithelium and submucosal glands (Fig. 4). Morphometric analysis for two separate experiments is shown in Fig. 5.

Due to the cellular complexity of human bronchial explants, it was unclear from these experiments whether *P. aeruginosa* supernatant was acting directly on epithelial cells or through resident inflammatory cells to upregulate *MUC 5AC*. To investigate this, we tested the ability of *P. aeruginosa* to upregulate *MUC 5AC* in human bronchial epithelial cell (NCI H292) cultures lacking inflammatory cells.

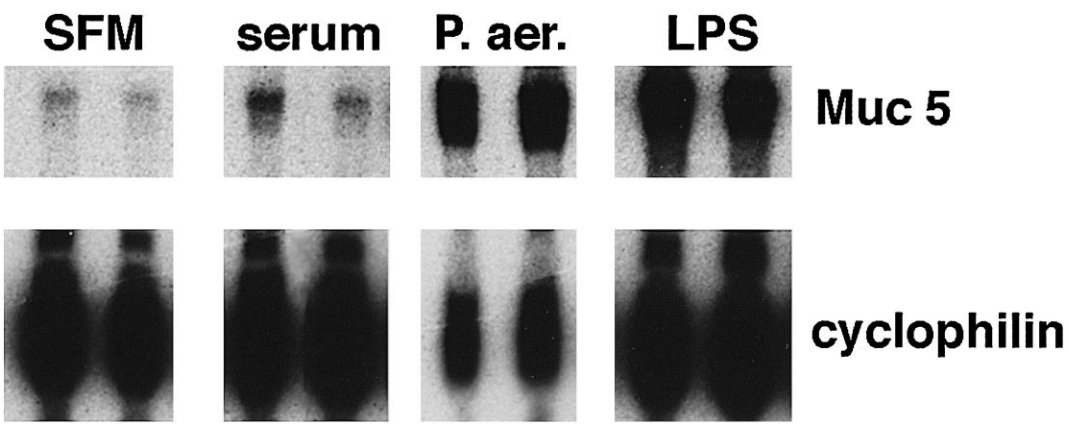

Fig. 6. RNase protection assay showing that both *P. aeruginosa* supernatant (P. aer.) and LPS (10 μg/ml) strongly and directly upregulate steady state levels of *MUC 5AC* mRNA in cultured airway epithelial cells (NCI H292). Serum has a weak stimulatory effect. Compare with *MUC 5AC* signals from cells incubated in serum-free medium (SFM). Cyclophilin mRNA levels are monitored to assess the amount RNA used in each hybridization reaction.

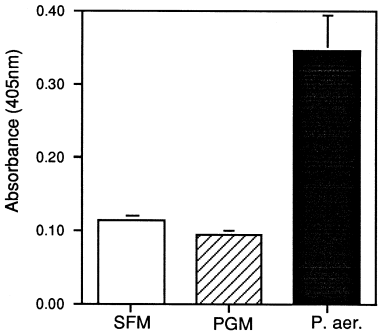

Fig. 7. ELISA data showing a greater abundance of mucin immunoreactive material in supernatants from NCI H292 epithelial cells exposed to *P. aeruginosa* (P. aer.) than in supernatants from cells incubated in serum-free medium (SFM) or *P. aeruginosa* growth medium (PGM); mean ± s.e.m., $N = 4$.

Fig. 6 shows RNase protection assay (RPA) data indicating that these epithelial cells responded to *P. aeruginosa* and to *P. aeruginosa* LPS by greatly increasing their pools of steady state *MUC 5AC* mRNA. It can be seen from these data that serum, added as a source of LPS binding protein, has a relatively weak stimulatory effect on *MUC 5AC* when used alone. The *P. aeruginosa*-induced increase in *MUC 5AC* mRNA is accompanied by an increase in mucin glycoprotein as shown by ELISA analysis (Fig. 7). Sensitivity to inhibition by genistein (Fig. 8) indicates that the mucin upregulation response is dependent on tyrosine phosphorylation.

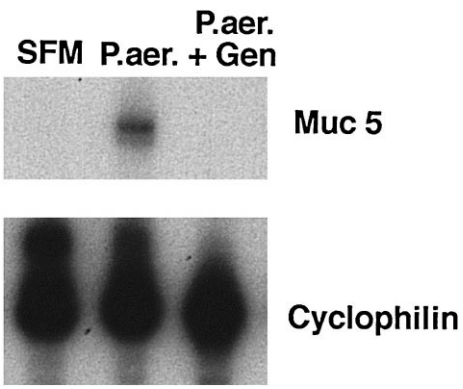

Fig. 8. RNase protection assay showing that the tyrosine kinase inhibitor genistein (Gen) inhibits the *P. aeruginosa* induction of *MUC 5AC* in cultured airway epithelial cells (NCI H292). SFM = serum-free medium, P. aer. = *P. aeruginosa* supernatant.

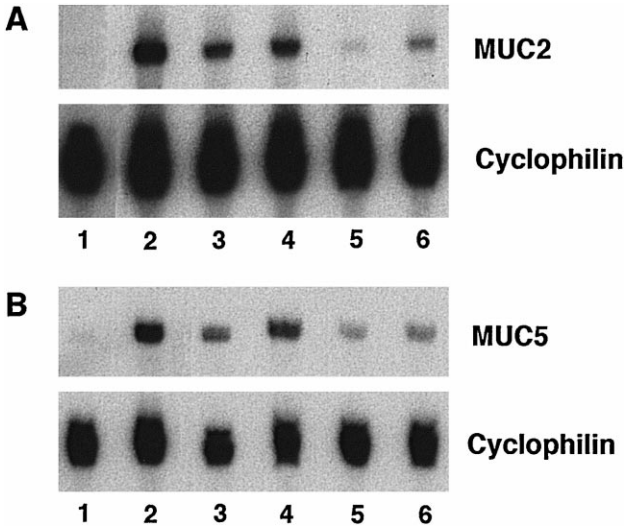

Fig. 9. RNase protection assay showing the effects of various bacterial supernatants on *MUC 2* and *MUC 5AC* steady state mRNA levels in airway epithelial cells (NCI H292). Lane 1: serum-free medium; lane 2: *P. aeruginosa*; lane 3: *E. coli*; lane 4: *S. aureus*; lane 5: *S. epidermidis*; lane 6: *Str. pyogenes*.

To determine whether Gram-positive as well as Gram-negative bacteria are capable of mucin upregulation, we exposed epithelial cells to the Gram-negative bacteria *P. aeruginosa* and *E. coli* as well as to the Gram-positive bacteria *Staphylococcus aureus*, *S. epidermidis* and *Streptococcus pyogenes*. As shown in Fig. 9, all bacteria tested were capable of upregulating both *MUC 2* and *MUC 5AC*, but to varying degrees. That the rank order potency for *MUC 2* is similar to that for *MUC 5AC* suggests that common mechanisms operate with respect to both genes.

4. Discussion

Bacterial infection of the airways is often accompanied by mucin overproduction, a phenomenon that can lead to airflow obstruction, respiratory insufficiency, and secondary infection. The link between infection and mucin overproduction is poorly understood. Recently we found that *P. aeruginosa* culture supernatant stimulates transcription of the *MUC 2* mucin gene [3]. As *MUC 2* is only one of nine known human mucin genes (*MUC 1*, *3–8* [1,4–10](12)) and *P. aeruginosa* is only one of many bacterial pathogens, we wondered whether the effect

of *P. aeruginosa* on *MUC 2* might be a specific example of a general phenomenon whereby epithelial cells respond to bacteria by coordinately activating the expression of several mucin genes.

Our data provide evidence favoring this possibility in showing that *P. aeruginosa* acts on airway epithelial cells to activate not only *MUC 2* but also *MUC 5AC* [5,9,18]. Furthermore, both genes are upregulated by a variety of Gram-positive and Gram-negative bacteria.

Our initial observations were made on nasal polyps excised therapeutically from CF and non-CF subjects. Polyps are intrinsically complex, reactive structures, containing inflammatory cells and mediators. The presence of ongoing inflammatory reactions in both CF and non-CF polyps may explain the relatively high levels of *MUC 2* and *MUC 5AC* RNA seen there, as the inflammatory mediator TNF α is known to activate mucin transcription [19]. Despite an overall high level of *MUC 5AC* expression in the polyps, we detected more *MUC 5AC* mRNA in the CF than in the non-CF polyps and subsequently reproduced this finding in CF vs. non-CF bronchi.

Our detection of elevated mucin mRNA levels in CF airways by in situ hybridization supports earlier histopathological and pulse-labeling studies suggesting that mucin synthesis is upregulated in CF airways [20] [21]. Our data extend the earlier observations by suggesting that an important stimulus for mucin synthesis in CF airways is the common CF pathogen *P. aeruginosa*. Not only does this pathogen upregulate mucin mRNA, but it also upregulates mucin protein as shown in Fig. 7. That the fold increase in mucin protein is smaller than the fold increase in *MUC 2* and *MUC 5AC* mRNA may be due to the fact that the antibody-based mucin assay collectively monitors the contributions of nine or more mucin genes, not all of which may require *P. aeruginosa* for high level expression, contributing to a relatively high baseline. Components of *P. aeruginosa* that could mediate *MUC 2* and *MUC 5AC* upregulation include a variety of proteases including elastase and alkaline proteinase [22] [23], exotoxin A (20), exoenzyme S [24], rhamnolipids [25] hydrocyanic acid [24], phenazine pigments (23), leukocidin (24), *Pseudomonas* autoinducer, a <1 kD component that induces IL-8 [13] and endotoxin (LPS) [26]. Based on our in vivo studies showing mucin induction in rat airways by LPS [27], we administered LPS to isolated cultures of epithelial cells and obtained evidence supporting the existence of a direct effect of LPS on epithelial cells ([3] and the present data).

That the effect of *P. aeruginosa* on *MUC 2* was at least partly at the level of transcription was clear from transient transfection assays with a *MUC 2* luciferase reporter gene [3]. Our recent cloning of the *MUC 5AC* promoter permitted parallel studies in which we observed that *MUC 5AC* transcription is also responsive to *P. aeruginosa* [28]. It will be informative to compare response elements and transcription factors involved in each gene's response to various bacteria. How, for example, do responses to Gram-positive organisms, which lack LPS, differ from responses to Gram-negative organisms? Do multiple mucin genes respond to each bacterium similarly? The increasing availability of mucin gene promoters will facilitate studies of this kind.

Acknowledgements

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