Are Mucin and Mucin mRNA Reliable Markers for Hypersecretion in Humans with Bronchitis?

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY

AIR RESOURCES BOARD
Research Division

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Final Report
Contract No. A933-095

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October, 1992

ABSTRACT

Mucus production is a prominent feature of the airway's response to injury. Thus, increased mucus production can be interpreted as an indicator of injury and subsequent decreased production as an indicator of return to normal function. The goal of this work was to develop and validate methods for evaluating airway injury through monitoring mucus production. The first method measures mucin in lung fluids and the second measures mucin mRNA, the mucin precursor, in lung cells. Both the fluids and cells are obtained during bronchoscopy. In addition to providing detailed protocols for performing these assays, the report also shows that the first method, as predicted, detected significantly more mucin in the lung fluid of smokers than non-smokers. With respect to mucin mRNA, the assay detected a trend in the same direction.

Acknowledgements

Disclaimer

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their sources or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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Summary and Conclusions

The project completed under this contract permits the following conclusions:

(1) Mucin-like antigens detected in airway lavage fluid by ELISA assay are present in higher concentration in smokers than non-smokers (p<0.01).

(2) Mucin:ZO-1 mRNA ratios in airway epithelial cells, determined by quantitative PCR, show a trend to be higher in smokers than in non-smokers.

Significance of findings

The ELISA and PCR-based tests permit quantitative detection of mucin overproduction *in vivo* in human airways. That the smokers participating in our study showed relatively mild symptoms suggests that the tests are capable of detecting early bronchitic changes in individuals exposed to airborne irritants. The tests can also be used to evaluate the ability of candidate drugs to attenuate hypersecretion.

Recommendations

The ELISA and PCR tests can be applied to samples obtained from subjects exposed to irritants under controlled conditions or to subjects spontaneously exposed to differing concentrations of atmospheric pollutants. In future studies, PCR measurements should be made on nasal, tracheal and lung epithelial cells to determine the site most reflective of injury. Because of their proximity to inspired irritants and their accessibility, nasal cells may prove the most useful .

While the ELISA test is somewhat easier to perform than the PCR test, the latter may ultimately be preferred since, in principle, it could detect elevations in mucin mRNA within hours after an injury, whereas several days would probably be required for ELISA-detectable mucin elevations to occur.

BODY OF REPORT

INTRODUCTION

Persistent airway irritation can lead to the development of chronic bronchitis, a condition characterized by cough, mucus hypersecretion, and airway obstruction (1) (2), (3), (4), (5)   Complications associated with chronic bronchitis include bacterial infection (6) and an increased risk of bronchogenic cancer (7).

The morbidity and mortality associated with chronic bronchitis have stimulated interest in identifying specific pollutants capable of inducing, and therapeutic agents capable of attenuating, this disorder. The potential benefit of identifying such agents is suggested by studies demonstrating an improvement in lung function after smoking cessation (8),(9). The task of identifying both bronchitis-inducing irritants and bronchitis-attenuating drugs would be facilitated by the availability of assays by which to detect and quantify airway mucus hypersecretion. The purpose of these studies was to develop such assays and to assess their ability to discriminate between groups of individuals with healthy vs. bronchitic airways.

In designing these tests, we focused on the glycoprotein mucin, the molecule responsible for the viscoelasticity of mucus. Mucin synthesis occurs in goblet and mucous gland cells of human airways (10; 11) and, like the synthesis of other glycoproteins, requires RNA transcription, protein translation and glycosylation (12). Since mucin production increases in chronic bronchitics (13), we predicted that appropiate probes would detect increased amounts of mucin and mucin mRNA in the airways of individuals with chronic bronchitis. We chose smokers and non-smokers for our study population because cigarette smoke is known to induce chronic bronchitis, as evidenced by mucus hypersecretion and mucous gland hypertrophy (14; 15; 16).

In this report, we show that mucin can be detected in bronchial lavage fluid by enzyme-linked immunosorbent assay (ELISA) and mucin mRNA can be detected in

brushed airway cells by the reverse transcription polymerase chain reaction (RT-PCR) method followed by Southern blotting. Using these methods, we observed that mucin is elevated in smokers' airways as compared to controls. The data on mucin mRNA indicate a trend in the same direction. This work has appeared in preliminary form (17).

MATERIALS AND METHODS

Subjects

Twelve subjects were studied: six smokers and six non-smokers. The subjects had no history of pulmonary or cardiac abnormalities contraindicating elective bronchoscopy. The subjects had no history of atopy and no history of a respiratory infection in the six weeks prior to bronchoscopy. Subjects were screened by a medical history, physical examination, spirometry and flow-volume curves. The spirometry was performed according to American Thoracic Society criteria, and interpreted with respect to the predicted normals of Knudson and co-workers (18). Coagulation status was determined to exclude subjects with bleeding disorders. All subjects gave informed consent as approved by the University of California, San Francisco Committee on Human Research.

Bronchoscopy, bronchial washings and bronchial brush specimens

Bronchoscopy was performed after application of topical anesthesia to the mouth, pharynx and larynx. Bronchial washes were obtained by instilling 20 milliliter aliquots of sterile normal saline solution into each of six segments of a single lung. Each instillate was immediately aspirated to minimize dwell time. The bronchial wash returns were pooled, and the total volume recorded. Bronchial samples obtained in this manner are known to be enriched for airway (as opposed to alveolar) cells and proteins (19); (20); (21). The specimens were stored at -20 °C until ELISA and total protein analysis.

Measurement of total protein in bronchial wash samples

To determine whether solutes were mixed uniformly in lavage fluid independent of the amount of fluid returned after each instillation, and whether total protein concentration differed between smokers and non-smokers, we measured the

concentration of total protein in each sample using the Pierce BCA reagent (Pierce, Rockford IL). Samples were assayed in duplicate.

Characterization of mucin-like antigen recognized by monoclonal antibody A10G5

In an earlier study (22), gel filtration analysis (Sepharose 4B) of cystic fibrosis sputum followed by immunoassay of each fraction showed that antibody A10G5 recognized an epitope carried on high molecular weight components eluting in the column exclusion volume, Vo. Based on the properties of Sepharose 4B, these components correspond to molecules exceeding 10^6 daltons in size. In airway secretions, such molecules include mucins (23; 24), proteoglycans (13) and hyaluronic acid (13). To investigate the potential affinity of antibody A10G5 for proteoglycans and hyaluronic acid, we obtained Vo material by gel filtration analysis of human tracheal organ culture medium radiolabelled with ^{3}H-glucosamine. The organ culture medium was DME H21/ Hams F12 (50/50) with 100 µg/ml gentamicin, 2.5 µg/ml fungizone, 100 U /ml penicillin, 100 µg/ml streptomycin and ^{3}H-glucosamine. After 16 h incubation, the medium was dialyzed and lyophilized and the macromolecules reconstituted in 0.1 M sodium acetate, 0.15 M sodium chloride, 2 mM PMSF and 1 mM calcium chloride. This sample was chromatographed on Sepharose Cl-4B and Vo fractions pooled. An aliquot of the Vo was treated with hyaluronidase (10 U/ml), in 0.4 M sodium chloride, 0.1 M sodium acetate, and 0.1 mg/ml BSA, and incubated for 24 hours at 37°C, while another aliquot was incubated under the same conditions with no hyaluronidase. Both samples were then rechromatographed on the same column to detect a potential shift of radioactivity to the included volume. An ELISA was performed on Vo fractions from both samples using antibody A10G5 to detect potential loss of antigenicity due to hyaluronidase treatment.

Amino acid compositions of the Vo before and after hyaluronidase digestion were determined in a Beckman 6300 high performance amino acid analyzer (Beckman Instruments, Palo Alto, CA) after acid hydrolysis (6N HCl, 100 $^{\circ}$C, for 24 h under vacuum). Carbohydrate composition was determined by high performance liquid chromatography with pulsed amperometric detection (25).

ELISA procedure on bronchial washings.

A sandwich type ELISA was performed on each bronchial wash specimen using a method described previously (26). Monoclonal antibody A10G5, an IgG$_1$, was purified prior to use in the ELISA using a Protein G column. Aliquots of purified A10G5 were biotinylated prior to use (27).

Each 96 well microtiter plate (Immulon 2; Dynatech Laboratories, Alexandria, VA) was coated with 100 µl/well of a 5 µg/ml solution of A10G5 purified (unbiotinylated) antibody. The plates were incubated at 4°C overnight. The plates were washed with PBS containing 0.1% Tween 20 ®(PT). The plates were then blocked with 1% normal goat serum (Sigma Chemical Co., St. Louis, MO) / 0.1% Tween 20 in PBS (GTP) for two hours. During this period, each bronchial wash specimen was incubated at 37°C for 15 minutes in the presence of 1 mM EDTA, 0.02% sodium azide, 0.5 mM phenylmethylsulphonylfluoride and 0.1% dithiothreitol (DTT). After 15 minutes, an equal volume of PBS was added. The presence of a residuum of DTT (0.05%) had no effect on antigen-antibody interaction as demonstrated by superimposable absorbance values obtained with DTT-treated and untreated serial dilutions of the reference mucin.

Experimental samples were serially diluted in PT and plated in duplicate on microtiter plates. Wells containing no bronchial wash specimen (no antigen) served as negative controls and wells containing human mucin-like material (Sepharose Cl 4B Vo, hyaluronidase-treated) served as positive controls on each plate. The plates

were incubated at room temperature for one hour. After washing in PT, biotinylated antibody (10 mg/ml in GTP) was applied to each well and incubated for one hour. The plates were then washed with PT and incubated with ß-galactosidase steptavadin conjugate (GIBCO BRL), (1:1000 in PT). After washing with PT, a ß-galactosidase substrate (p-nitrophenyl -b-galactopyranoside: 1 mg/ml) was applied to each plate. After 15 min at 37°C, absorbance values were read at 405 nm, using a Biorad microplate reader.

Mucin-like antigen: standard curve

A standard curve was constructed for the A10G5 antibody using the hyaluronidase-treated Sepharose Cl-4B Vo fraction of human airway secretion as "reference mucin". A Hill function was fitted to absorbance values for the smokers and non-smokers from the undiluted lavage fluid by the Gauss-Newton-Marquardt-Levenberg method for non-linear estimation (28). From the standard curve, we estimated mucin concentrations in undiluted lavage fluid for each subject and derived the median mucin concentration for each group.

RNA extraction and cDNA synthesis

To obtain airway cell RNA, we took three bronchial brush specimens from a proximal site (second generation bronchus) and three from a more distal site (fourth generation bronchus). Brushing was performed with a "protected " brush to avoid contaminating airway cells with those potentially brought down from the oropharynx by the bronchoscope. RNA was extracted separately from cells obtained at each site, according to Chomczynski (29). Briefly, the bronchial specimens from proximal and distal sites were each agitated in guanidinium thiocyanate, phenol, sodium acetate and chloroform-isoamyl alcohol (29). The extracted RNA was precipitated twice in isopropanol, and washed twice in ethanol / DEPC water. RNA was then

converted to cDNA using 100 pmol of random hexamers per sample, 1 mM each dNTP, 20 units RN'ase inhibitor (Promega), 5X reverse transcriptase buffer, and 200 Units of Moloney Murine Leukemia Virus reverse transcriptase (BRL) in a total volume of 20 μl. The reaction was incubated for 1 hour at 37°C, and stopped by chilling on ice.

Polymerase chain reaction (PCR)

Mucin cDNA was selectively amplified using the nested primer modification (30; 31) of the polymerase chain reaction (32). Because of the risk of harvesting non-epithelial cells (eg. neutrophils, macrophages) as contaminants in the brush specimens, we expressed mucin cDNA values with respect to the amount of cDNA encoding the epithelial cell tight junction protein ZO-1 (33) (present in all columnar cells) in each sample. The ratio "mucin cDNA:ZO1 cDNA" reflects the concentration of mucin mRNA in a sample of total columnar epithelial cell mRNA. This parameter would be expected to increase in mucin-overproducing airways.

Sample cDNA derived from brushed cell RNA served as a template for two MUC-2 mucin gene-specific (MU-2, 5' and MU-2, 3', see Table 4), and two ZO-1 gene-specific (ZO-1, 5' and ZO-1, 3', see Table 4), intron-flanking primers selected from non-repetitive regions of the human intestinal mucin cDNA, SMUC 41 (34) and a ZO-1 cDNA (35). We have previously shown that the human MUC 2 gene is expressed in the human airway (36). The PCR reaction mixture included 10 pmol 5' and 3' mucin and ZO-1 primers, 200 umol each dNTP, 3.75 mM magnesium chloride, 10X PCR buffer, and 2.5 Units of *Thermus acquaticus* polymerase (Perkin-Elmer Cetus), in a total of 100 ml. Each cycle included denaturation at 94°C for 1 minute, annealing of primer to cDNA template at 51°C for 1 minute, and primer extension at 72°C for 1 minute. Amplification was performed in a Perkin-Elmer Cetus DNA Thermocycler.

After 25 cycles of amplification using two mucin and two ZO-1 primers, two additional mucin'primers (MU-2(N), 5' and MU-2(N), 3', see Table 4) internal to the first (also from the non-repetitive region) were used for a second round of co-amplification (20 cycles) with the same ZO-1 primers used in the first round of amplification (ZO-1, 5' and ZO-1, 3', see Table 4) using 5 µl of a 1:10 dilution of the PCR product from the first round of cycles.

Southern Blot Analysis of PCR Products

The PCR products from the second round of amplification were analyzed by agarose gel electrophoresis. The gel was stained with ethidium bromide prior to Southern blot analysis (37). The blot was hybridized first with a mucin-specific $\gamma^{32}P$ end-labelled oligonucleotide probe derived from MUC 2, which was internal to the nested mucin primers ("Mucin (Probe)," Table 4). After stripping, the blot was re-hybridized with a ZO-1-specific $\gamma^{32}P$ end-labelled oligonucleotide probe ("ZO-1 (Probe)," Table 4). After hybridization for 18 hours, each blot was washed at high stringency and exposed to Kodak X-Omat film at -70° C for 24 hours. After development, densitometry analysis was performed on the mucin and ZO-1 hybridization signals, and the values expressed as a mucin: ZO-1 ratio.

Statistical analysis

For the ELISA, the amount of mucin-like antigen (µg/ml) was calculated for each lavage sample by reference to the standard curve (see above) and medians were obtained. For the PCR data, the mucin:ZO-1 ratios were calculated for each brushing site and means were obtained. Statistical comparisons for both the ELISA and PCR-based assays, as well as the total protein assay, were performed using the Mann-Whitney U (non-parametric) test because the

distribution of data elements within the two study groups was not Gaussian. Statistical significance was accepted for $p < 0.05$.

RESULTS

Patient Characteristics

The mean age of the smokers was 43.1±15.35 (± S.E.M.) and their mean pack-year smoking history was 46.6 years. The mean age of the non-smokers was 27.6 ±2.65 (± s.e.m.). Although the FEV1/FVC (%) was lower in the smokers (67.3 ±19.4, mean ± S.E.M.) than the non-smokers (85.33±6.1, mean ± S.E.M.) the difference was not statistically significant primarily due to high variability among the smokers. Indeed, three of the smokers (S1,2,3 Table 1) had no evidence of airway obstruction despite a history of smoking and sputum production.

Fluid returns for smokers vs. non-smokers were not significantly different (smokers' mean return = 38.1 ±7.3 ml (S.E.M.) ; non-smokers' mean return = 45.3 ± 2.10 ml (S.E.M.); p > 0.5 .

Total protein concentrations in bronchial lavage fluid samples

The size of the return fluid volume did not affect protein concentration as evidenced by the fact that mean protein concentrations for the six lavage samples with lowest, and the six with highest, fluid volume returns, were not significantly different (low volume returns: 198.1±36.3 mg/ml; high volume returns: 177.1±43.4 mg/ml, mean ± S.E.M., p>0.5). Total protein concentration also did not differ significantly between smokers and non-smokers (smokers: 238.0 ±45.7mg/ml; non-smokers: 136.0±10.3 mg/ml, mean ± S.E.M., p>0.05).

Characterization of antibody

Monoclonal antibody A10G5 was isolated from the spleen of a mouse immunized with dialyzed, unfractionated human tracheal secretions and was selected based on its recognition (by immunofluorescence) of secretory granule

constituents of human airway gland and goblet cells (22). Gel filtration chromatography (RIA of column fractions) and SDS/PAGE (Western blot) revealed the A10G5 antigen was a high molecular weight molecule, possibly representing a mucin, proteoglycan or hyaluronic acid. Hyaluronidase digestion of the high molecular weight molecules was performed to better define the epitope.

The hyaluronidase used in these experiments degraded hyaluronic acid (HA), chondroitin 4 and 6 sulfates (C4S and C6S) and dermatan sulfate (DS) standards to completion under the conditions used. It also shifted approximately 30% of the radiolabelled Vo fraction (Figure 1A) to the column inclusion volume (Figure 1B), whereas it did not inhibit the ability of A10G5 to recognize its antigen (Figure 2). We conclude that A10G5 does not recognize epitopes carried on HA, C4S, C6S or DS, which are among the most common contaminants of airway mucin. The amino acid and sugar composition of the hyaluronidase-insensitive material showed mucin-like features including a combined serine-threonine composition of 38%, large amounts of fucose, glucosamine and galactose, little mannose and no uronic acid (Tables 2 and 3). Taken together, these findings indicate that the antibody recognizes mucin-like molecules. A standard curve was constructed using antibody A10G5 and hyaluronidase-insensitive Vo material (Figure 3).

Quantification of mucin-like antigen in lavage fluid from smokers and control subjects

Absorbance values obtained using antibody A10G5 were dependent on the lavage fluid dilution factor (Figure 4). Using the Hill function with reference to the standard curve, we estimated mucin concentrations in undiluted lavage fluid for each subject. The median mucin concentration for the smokers was 52.2 µg/ml (range: 16.3-4,860.0) whereas that for the non-smokers was 12.7 (range: 4.5-22.9). The difference between smokers and non-smokers was statistically significant (p $\leq$ 0.01).

Polymerase chain reaction and Southern blot

Amplification of the tight junction protein ZO-1 nucleotide sequence from either colon or bronchus RNA (using primers ZO-1, 5' and ZO-1, 3'; see Table 4) produces two bands (433 bp and 193 bp) on ethidium bromide-stained agarose gels. These two bands are expected due to alternative splicing of mRNA transcripts (35). Amplification of the mucin nucleotide sequence using primers MU-2, 5' and MU-2, 3' (see Table 4) produces a single band at 457 bp. After twenty five cycles of amplificaton using both sets of primers (Round 1), ethidium bromide-stained agarose gels of the PCR products showed bands corresponding to ZO-1, but not mucin. A 1:100 dilution of the reaction product from the first round was used as a template for a second round of 20 cycles of PCR, using "nested" mucin primers (MU-2(N), 5' and MU-2(N), 3') and the original ZO-1 primers (Round 2). An ethidium bromide-stained agarose gel of the second round products showed ZO-1 bands but weak or no mucin bands. To gain sensitivity, we prepared Southern blots of products from the second round. Figure 5 shows results from proximal and distal airway samples for each subject, with the exception of several samples that yielded too few cells for processing. Adequate samples could not be obtained from the distal airway sites of two non-smoker subjects (NS 2 and NS 4), from the proximal site of smoker S 6, and both proximal and distal sites from smoker S 5.

By Southern blot, ZO-1 bands were detectable for all samples providing adeqate cells for processing. Mucin bands were present for all processed samples from the smokers, and from eight out of the ten processed samples from the non-smokers. Scanning densitometry demonstrated that ZO-1 band intensity did not differ between groups (smokers: 13,948±3597; non smokers: 20,172±2963 densitometry units, mean ± S.E.M., p>0.05). This indicated that ZO-1 mRNA represented a fixed denominator with respect to which mucin mRNA values could be

evaluated. Results were therefore expressed as a ratio between mucin and ZO-1 hybridization band intensity. The overall mean ratio ± S.E.M. (including both proximal and distal brush sites) was 3.53 ±1.71 for the smokers and 0.36 ± 0.14 for the non-smokers. The ratio for the proximal brush site was 2.41 ± 2.34 in the smokers, and 0.24 ± 0.18 in the non-smokers; for the distal site, the ratio was 4.43 ±2.60 in the smokers, and 0.47 ±0.22 (mean ± S.E.M.) in the non-smokers. Despite large differences between means for the two groups, these differences did not reach significance.

DISCUSSION

The results of this study demonstrate the feasibility of monitoring mucin and mucin mRNA levels *in vivo* in human airways. The ability to make such measurements should significantly aid efforts to identify air pollutants that increase (38), and candidate drugs that decrease, mucus secretion. The demonstrated cross-reactivity of the A10G5 antibody with rat airway mucin (39) will also permit analysis of the effects of irritants and candidate drugs in rats.

The immunoassay used to detect mucin in airway lavage fluid relies on a monoclonal antibody (A10G5) originally isolated for its ability to recognize human airway secretory cell granules (22). This antibody recognizes granules in serous and mucous gland cells in addition to goblet cells. Preliminary characterization revealed that A10G5 recognized a high molecular weight antigen eluting in the column exclusion volume (Vo) during Sepharose 4B chromatography (22). This was consistent with the epitope being present on mucin, proteoglycan or hyaluronic acid, each of which has been detected in high molecular weight fractions of human airway secretions (13). The present data, showing that hyaluronidase digestion did not remove antigenicity from the Vo, strongly suggests that the epitope is carried on a mucin because hyaluronidase digestion would have shifted proteoglycan and hyaluronic acid epitopes to the column inclusion volume. This view was supported by an amino acid and sugar analysis of the hyaluronidase-insensitive material (Tables 2 and 3). With 38% hydroxyamino acids, little mannose and no uronic acid, the antigenic material is clearly "mucin-like."

Antibody A10G5 detected higher concentrations of the mucin-like antigen in airway lavage fluid from smokers than non-smokers, indicating its utility in discriminating between normal subjects and those with bronchitic airways. The

relatively high variability in these data, especially those of the smokers' group, is consistent with that seen in previous measurements of lavage fluid solutes including albumin, immunoglobulins and total protein (40) (41). While the sources of the solute variability are not known, they do not appear to include variability in fluid volume returns, because we (this report) and others (40) find that low and high volumes have equal protein concentrations. That the mucin ELISA was able to detect a significant difference between two groups consisting of only 6 subjects each is attributable to the large difference between group medians.

The parameter used to assess mucin mRNA levels was a ratio between the intensities of mucin and ZO-1 hybridization signals in each PCR-amplified sample. ZO-1 is a tight junction protein present in epithelial cells that functions to block permeability across the epithelium by sealing the apical portions of adjacent cells (33) (35). In the airway epithelium (42), as in other epithelia (43), only those cells whose apical surfaces reach the lumen contain tight junctions. In the airway, tight junction-containing cells correspond to columnar cells. Thus, the mucin:ZO-1 ratio reflects the concentration of mucin mRNA in a sample consisting of total columnar cell mRNA. The use of this parameter excludes the effects of unknown numbers of non-epithelial (i.e., inflammatory) cells in the brushing samples. That we could detect no differences between the concentration of ZO-1 mRNA in samples from smokers and non-smokers led us to regard ZO-1 mRNA as a stable denominator with respect to which mucin mRNA values could be expressed and compared between groups.

Southern blot analysis of products from two rounds of PCR permitted detection of ZO-1 and mucin cDNA in most brush samples. Although there was a 10-fold increase in the mucin: ZO-1 ratios for smokers vs. non-smokers, variability prevented this difference from achieving statistical significance. As mentioned above, variability is a common finding in studies of this type (40) (41). In this

particular study, despite efforts to select subjects with homogeneous smoking histories, the smoking group contained subjects with widely varying disease severity (see Table 1). Notably, the two smokers (S4 and S6) showing the most intense mucin signals and highest mucin :ZO-1 ratios by Southern blot (Figure 5) had the lowest FEV1/FVC (%) values (indicating airway obstruction) of those for whom adequate RNA samples were collected. Among the non-smokers, variability may have derived from varying degrees of passive exposure to smoke and other irritants.

Despite our inability to detect statistically significant elevations in mucin: ZO-1 ratios in the smokers, mucin mRNA was likely to be elevated since the ELISA showed that mucin, itself, was being overproduced. We have previously shown elevations in mucin mRNA steady state in rats suffering from experimentally induced chronic bronchitis (44). Although it is possible that the A10G5 antibody recognizes an epitope carried on a mucin encoded by a gene other than the one from which our primers were constructed (MUC 2; (34)), the large elevation in mean mucin:ZO-1 ratios observed in the distal airways suggests that the MUC 2 gene is indeed upregulated in bronchitics. With a larger sample size and/or more uniform subject groups, the difference between groups would likely attain statistical significance.

In summary we have developed two techniques, an ELISA and a PCR-based assay, that can quantitatively detect mucin and mucin mRNA *in vivo* in human airways. These tests should prove useful in detecting airway injury in response to diverse air pollutants (45; 46) as well as in evaluating the ability of candidate drugs to attenuate hypersecretion.

Glossary

Annealing - binding

ELISA - Enzyme-linked immunosorbent assay. A method for the detection and quantification of a specific molecule using antibodies

FEV1 - Forced expiratory volume attained in one second; the amount of air exhaled during the first second of an FVC maneuver.

FVC - Forced vital capacity. The amount of air that can be exhaled after a maximal inspiration.

Nascent - immature, developing

PCR - Polymerase chain reaction. A method by which to amplify specific gene sequences using primers homologous to the gene of interest. The final amount of amplified product is proportional to the amount of starting material.

periodate sensitivity - susceptibility to destruction by periodate oxidation (selectively shown by sugars)

Pmol - picomol

Primer - synthetic DNA consisting of 10-20 nucleotides corresponding to known sequences in specific genes; used in the amplification of these sequences from small amounts of cellular material such as that harvested by a bronchoscopy brush

Southern blot - procedure by which DNA that has been electrophoresed in a gel is transferred to a nitrocellulose filter. The DNA on the filter is then incubated with a radiolabeled cDNA corresponding to a specific gene. The location of bound radiolabeled cDNA (indicated by autoradiogrphy) denotes the position of migration of the DNA of interest.

Tween - a detergent used to block non-specific hydrophobic binding of antibody to non-antigen molecules.

Vo - Void volume fraction containing the largest molecules in the chromatographed sample.

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FIGURE LEGENDS

Figure 1A. Elution profile of [^{3}H]-glucosamine-labeled molecules released from human trachea in organ culture (24 h). Organ culture medium was dialyzed against distilled water, lyophilized and applied to a Sepharose Cl-4B column as described in Methods. V_0 and V_t refer to elution positions of blue dextran and phenol red.

Figure 1B. Elution profiles obtained by re-chromatography of the V_0 fraction peak from Figure 1A with and without hyaluronidase digestion (10µ/ml, 24 h. 37°C). Approximately 30% of the V_0 counts were shifted to the included column volume after digestion.

Figure 2. ELISA results showing that the affinity of antibody A10G5 for V_0 material was unchanged by hyaluronidase digestion.

Figure 3. Standard curve constructed using antibody A10G5 and hyaluronidase-insensitive V_0 material.

Figure 4. ELISA data showing recognition of mucin-like antigen in bronchial lavage fluid from smokers and non-smokers. Antibody A10G5; mean ± S.E.M.

Figure 5. Southern blot of PCR products hybridized with mucin and ZO-1 oligomer probes, demonstrating a mucin product of 304 base pairs, and a ZO-1 product of 433 base pairs. A mucin product is detectable in all nine samples from smokers (S) and in eight out of ten samples from the non-smokers (p = proximal airway; d = distal airway)
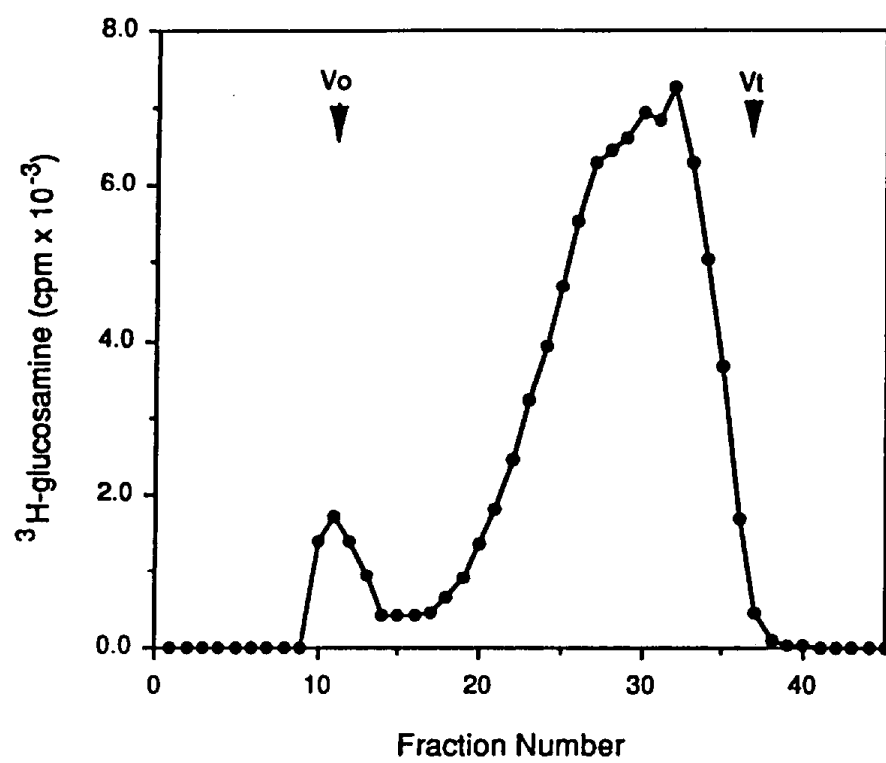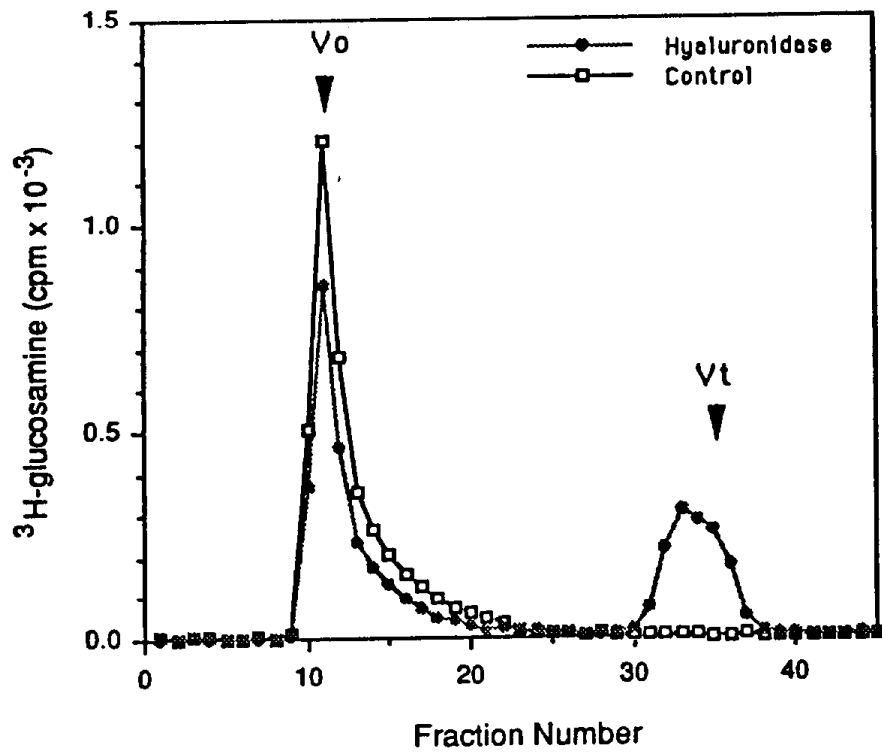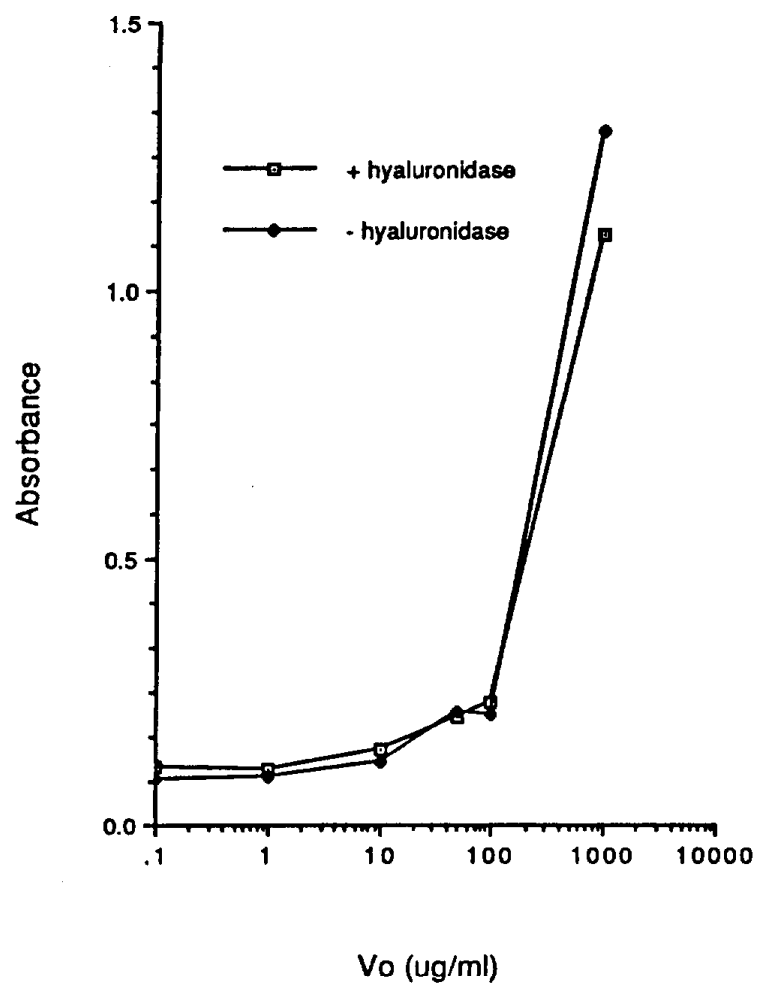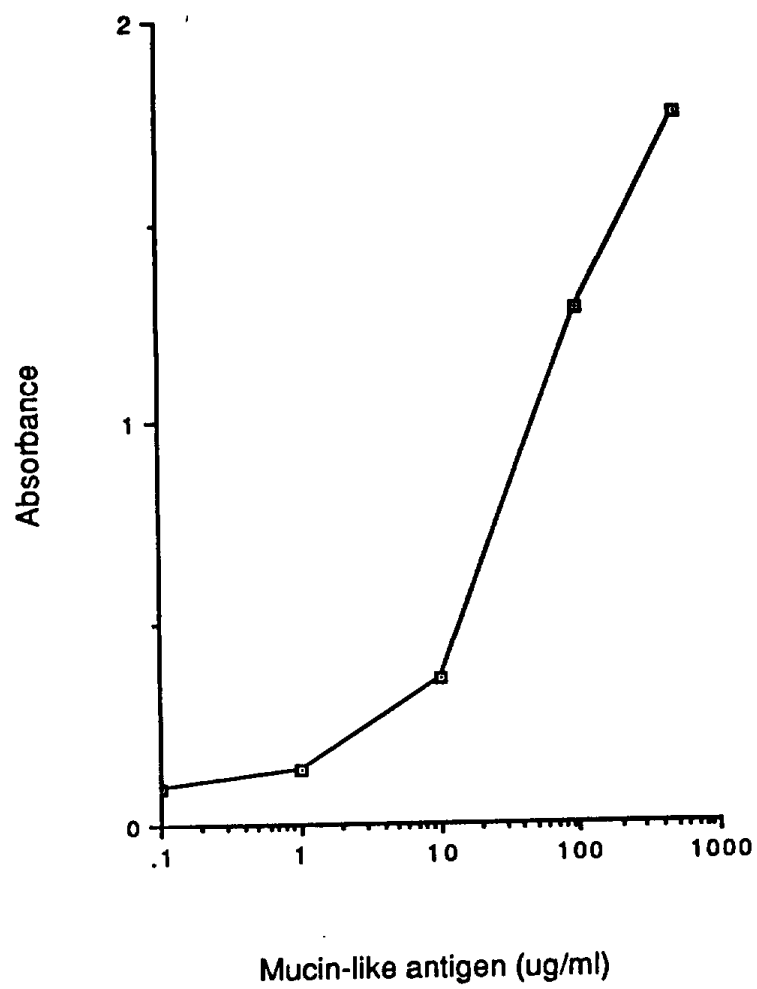
Fig 1a

Fig 1b

Fig 2

Fig 3

Fig 4

Concentration of Lavage of Lavage Fluid Solutes
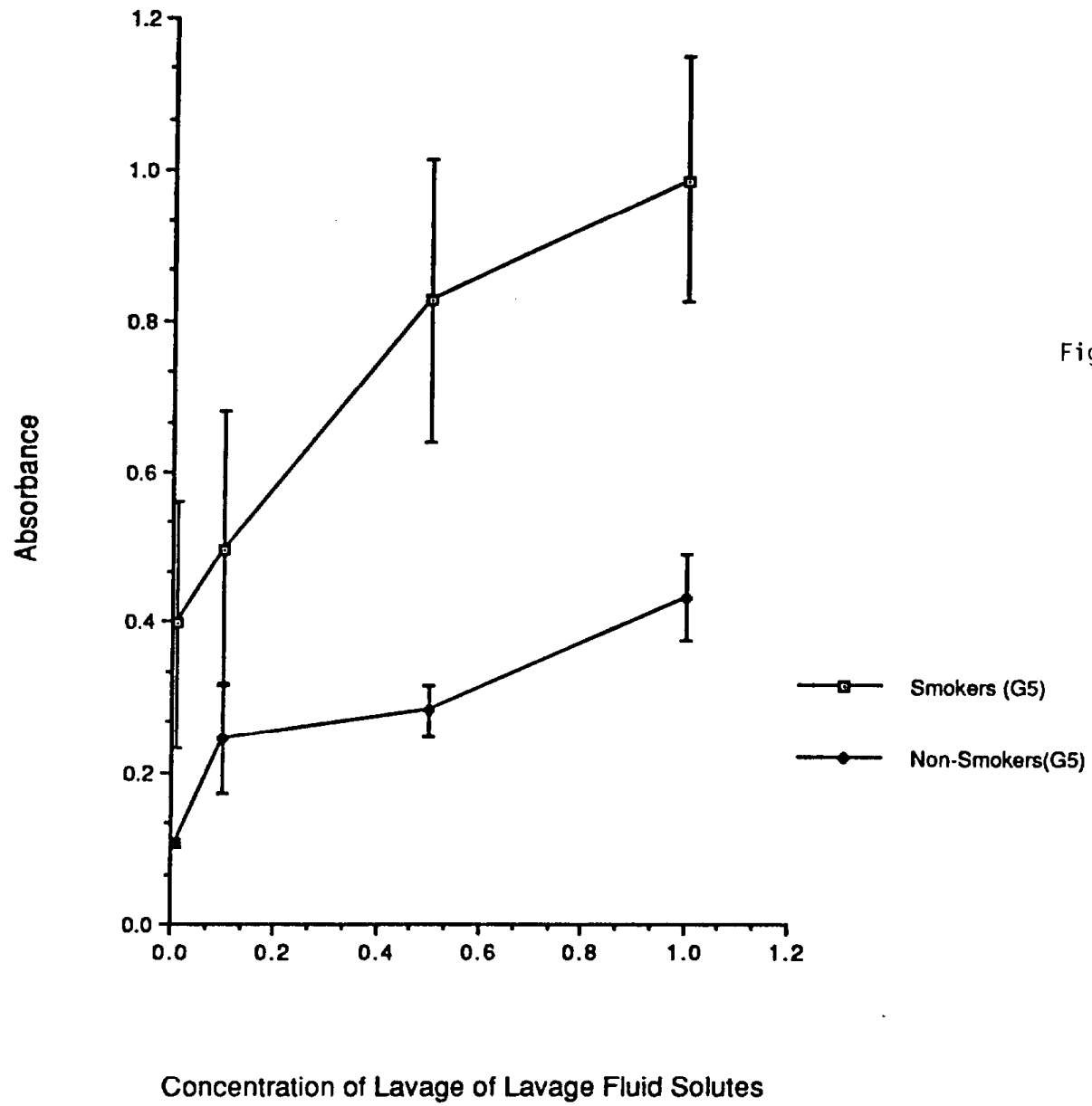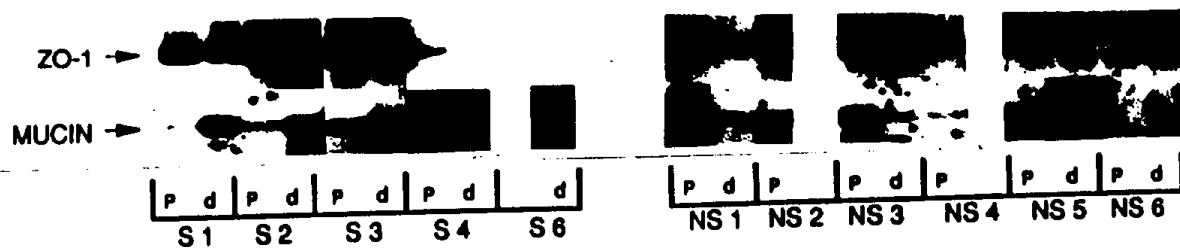
Fig 5

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SUBJECT	SEX/AGE	PACK YEARS	FEV$_1$/FVC	Vol. (ml) Bronchial Wash IN/OUT	Brush Site	B.W. Site
S 1	M/28	15	4.6/5.8 81%	120 / 37.5	LLL/LUL	RML/RLL
S 2	M/29	15	4.9/6.0 81%	120 / 32.5	LLL/Lingula	RML/RLL
S 3	M/36	15	4.8/5.6 85%	120 / 67	LLL/LUL	RML/RLL
S 4	M/58	60	1.9/4.3 44%	120/12	RLL/RUL	LLL/Lingula
S 5	M/65	100	1.6/3.8 42%	120/45	RUL/RML	LLL/Lingula
S 6	M/43	75	3.5/4.9 71%	120/35	LLL/LUL	RML/RLL
NS 1	M/32	0	5.7/7.3 78%	120 / 40	RLL/RML	LLL/Lingula
NS 2	M/27	0	4.6/5.3 86%	120 / 50	LLL/LUL	RML/RLL
NS 3	F/29	0	3.7/4.2 88%	120 / 40	LLL/LUL	RML/RLL
NS 4	M/25	0	4.6/5.1 90%	120 / 50	LLL/LUL	RML/RLL
NS 5	F/28	0	4.6/5.0 92%	120 /42	LLL/LUL	RML/RLL
NS 6	M/25	0	5/6.4 78%	120 / 50	LLL/LUL	RML/RLL

TABLE 1. Patient characteristics and bronchoscopy data. (S) = Smoker, (NS) = Non-smoker. LLL = left lower lobe, LUL = left upper lobe, RLL = right lower lobe, RML = right middle lobe. B.W. = bronchial wash.

Amino Acid	Concentration (mol %)
ASP	7.1
THR	20.4
SER	17.7
GLU	7.3
GLY	14.4
ALA	11.5
VAL	3.7
LEU	6.6
HIS	2.2
LYS	2.8

TABLE 2. Amino acid analysis of hyaluronidase treated Vo from human airway secretions.

Carbohydrate	Concentration of Sample (mol %)
Fucose	17.5
Galactosamine	11.3
Glucosamine	48.5
Galactose	19.2
Glucose	2.8
Mannose	0.03

Table 3. Carbohydrate analysis of hyaluronidase treated Vo from human airway secretions.

PRIMER	SEQUENCE (5' -3')	cDNA AND LOCATION (bp)	
MU-2 5'	CAAGCACAGCACCGATTGC	SMUC 41	(380-398)
MU-2 3'	GTGAGGAGGTGTACAACGGC	SMUC 41	(818-837)
MU-2 (N) 5'	CTACCACCTGCCATTGAGATG	SMUC 41	(499-519)
MU-2 (N) 3'	TTATCTGCTGTGTCCTGAACG	SMUC 41	(779-799)
ZO-1 5'	CATAGAATAGACTCCCCTGG	ZO-1	(10-30)
ZO-1 3'	CTGCTGGCTTGTTTCTCTAC	ZO-1	(423-443)
ZO-1 (Probe)	TTCAGGCGAAAGGTAAGGGA	ZO-1	(74-94)
Mucin (Probe)	ACGAGCGGAGGCCACACACTGTC	SMUC 41	(562-584)

Table 4. Oligonucleotide sequences of the primers used for PCR and probes used for Southern blot hybridization. 5' denotes that the primer was homologous to the 5' end of the specific target gene; 3' denotes that the primer was homologous to the three prime end of the specific targhet gene. N = nested primer.