Mucoglycoprotein hypersecretion in allergic rhinitis and cystic fibrosis

ATSUSHI YUTA, MUSHTAQ ALI, MARYBETH SABOL, ETHAN GAUMOND, AND JAMES N. BARANIUK
Division of Rheumatology, Immunology, and Allergy, Georgetown University Medical Center, Washington, District of Columbia 20007-2197

Yuta, Atsushi, Mushtaq Ali, Marybeth Sabol, Ethan Gaumond, and James N. Baraniuk. Mucoglycoprotein hypersecretion in allergic rhinitis and cystic fibrosis. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1203–L1207, 1997.—There is little information about specific changes in submucosal gland exocytosis in diseases such as allergic rhinitis (AR), nonallergic rhinitis (NAR), and cystic fibrosis (CF). Nasal lavage fluids were collected from normal, AR, NAR, and CF subjects. Concentrations of lysozyme, Alcian blue-staining mucoglycoconjugate material (AB+m), and human high-molecular-weight mucoglycoconjugates recognized by the 7F10 murine monoclonal antibody (7F10-immunoreactive mucoglycoconjugates [7F10-irm]) were measured. AB+m and 7F10-irm were characterized by Sepharose-2B column chromatography and glycosidase digestion. 7F10-irm was increased in AR (2.4-fold; P = 0.001) and AR (12.7-fold; P = 0.00007) subjects. AB+m was increased in CF (1.8-fold; P = 0.049) and AR (1.2-fold; P = 0.07) subjects. There were no changes in NAR subjects. On Sepharose-2B columns, AB+m peaks were at 1.3–3.0 × 10^6 and 0.36–0.65 × 10^6 Da. 7F10-irm showed four distinct peaks at 1.5, 1.2, 0.85, and 0.53 × 10^6 Da that were nearly identical in both normal and CF samples. Sialic acid was present in both 7F10-irm and AB+m. 7F10-irm and AB+m are mutually exclusive sialylated mucoglycoproteins that are significantly induced in AR and CF but not in NAR.

mucous hypersecretion; mucin; nasal mucosa; Alcian blue; 7F10-mucin

NORMAL NASAL SECRETIONS are derived from vascular leak (about one-third of protein content) and submucosal gland exocytosis (about two-thirds). Submucosal gland seromucous cells release lysozyme (15% total protein), secretory immunoglobulin A (15%), lactoferrin, hyaluronan, endothelin, neutral endopeptidase, and a host of other products (3–5, 18–20). Mucous cells stain with Alcian blue (AB) G8X, a copper-containing cationic isothiouronium dye (1,298.9 g/mol) that forms insoluble complexes with acidic carbohydrate polymers such as mucins and proteoglycans (25). The nature of the AB-staining mucoglycoconjugate material (AB+m) in human nasal mucus has not been characterized but may consist of sulfated mucins (11, 24). One specific population of mucoglycoconjugates is recognized by 7F10, a murine monoclonal antibody that was raised against human high-molecular-weight mucoglycoproteins synthesized by human bronchial explants in vitro (12). 7F10-immunoreactive mucoglycoconjugates (7F10-irm) are present in nasal and bronchial epithelial and submucosal gland cells (1, 2, 12).

Although the concept of mucus hypersecretion is well established, there are few quantitative data defining the changes in these specific glandular products in diseases such as allergic rhinitis (AR), nonallergic rhinitis (NAR), and cystic fibrosis (CF). Nasal secretions were collected by nasal lavage from subjects with these disorders, and 1) the concentrations of 7F10-irm, AB+m, and lysozyme were measured; 2) the size ranges of 7F10-irm and AB+m were determined by Sepharose-2B column chromatography; and 3) the nature of the sugars contributing to the physical properties of 7F10-irm and AB+m were partially characterized by glycosidase and chemical digestion.

METHODS

Subjects

Subjects with CF with chronic sinusitis (n = 7), active AR (n = 23), NAR with fibromyalgia and/or chronic fatigue syndrome (n = 52), and no nasal complaints (normal; n = 44) gave written informed consent for participation in this trial. None had an upper respiratory tract infection in the 6 wk before the study. The NAR group was selected from a population of fibromyalgia and/or chronic fatigue syndrome subjects with negative allergy skin tests. Rhinitis complaints are present in 76% of fibromyalgia and/or chronic fatigue syndrome subjects.

Nasal Provocation and Lavage

The subjects were seated, and preexisting nasal secretions were removed from each nostril with 24 sprays of sterile normal saline (100 µl each) with a Beconase AQ pump aspirator spray device (Glaxo, Research Triangle Park, NC). The subjects gently exhaled through their noses with their mouths closed to expel the lavage fluid into a paper cup. Ten minutes later, 12 sprays of saline were administered to each nostril, and saline-induced lavage fluids were collected. The lavage fluids were stored at −70°C.

Assays

Total protein. The total protein concentration in each sample was measured by a modified Lowry’s method as previously described (4, 13, 20). Human albumin standard or samples (10 µl) were placed in polystyrene microtiter plates (Dynatech Laboratories, Chantilly, VA) in duplicate, and assay reagents were added. The optical densities (650 nm) were measured with a microtiter-plate reader (Dynatech, Alexandria, VA). The protein concentrations in the samples were determined from the regression analysis of the standard curve.

Lysozyme. Lysozyme activity was measured by a turbidimetric assay based on the enzymatic hydrolysis of Micrococcus lysodeikticus (Sigma Chemical, St. Louis, MO) cell walls as previously described (19).

7F10-irm. 7F10-irm was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (12, 15). A “human nasal standard mucus” that had been collected from
one acute sinusitis patient and that was defined to contain 10^6 µU/ml of 7F10-irm was used on every ELISA plate. This material gave a log-log standard curve that was linear between 10^0 and 10^6 µU/ml. 7F10-irm was not present in human plasma taken from a series of volunteers (data not shown).

AB + m. Samples (25 µl) and standard type I-S bovine submaxillary gland mucins (12% sialic acid; 0.12–250 µg/ml; Sigma Chemical) were applied to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN) in a 96-well dot-blot apparatus (Schleicher & Schuell, Kene, NH), and a low vacuum was applied (17). The wells were washed two times with 250 µl of 50 mM sodium bicarbonate. The membranes were washed two times for 5 min each in deionized water, blocked in 5% bovine serum albumin for 5 min, and then washed two times in deionized water for 5 min each. The blot was stained with 3% AB 8GX, pH 2.5 (Poly Scientific, Bay Shore, NY), for 5 min and then washed three times in deionized water for 5 min each. The blot was dried in the dark at room temperature, covered with mineral oil (Sigma Chemical), and placed on a 96-well microtiter plate, and the optical densities (690 nm) were measured. Concentrations of AB + m were interpolated from the standard curve.

**Sepharose-2B Column Chromatography**

Human standard mucus and samples from four normal and six CF patients were reduced with 100 mM dithiothreitol at 42°C for 2 h, alkylated with 150 mM iodoacetamide at 22°C for 1 h in the dark, dialyzed against water, lyophilized, and then reconstituted to the original volume with 5 mM sodium phosphate, pH 6.8, and 6 M urea (urea-phosphate buffer). Sepharose-2B was washed in urea-phosphate buffer and packed at 4°C into a 0.7-cm-ID × 30-cm glass column. The column was allowed to settle by 17% in length for 2 wk to slow the rate of mucoglycoprotein elution. For each fraction run, the column was washed with two column volumes of urea-phosphate buffer, followed by a linear salt gradient of 0–1 M NaCl in the urea-phosphate buffer. Reduced and alkylated samples or standard were loaded, and the column was eluted with urea-phosphate buffer at a flow rate of 2 ml/h. Fractions (100 µl) were collected for 7F10-irm and AB + m measurements. The molecular weight of each fraction was estimated with blue dextran (mol wt 2,000,000) and thyroglobulin (mol wt 667,000) as standards. Blue dextran, thyroglobulin, and 6 M urea did not interfere with the 7F10-irm or AB + m assays (data not shown).

The means ± SE for 7F10-irm and AB + m for each fraction from the normal and six CF samples were calculated.

**Characterization of the Epitope Recognized by the 7F10 Monoclonal Antibody**

Human standard mucus in phosphate-buffered saline containing 1 ml/µl of 7F10-irm was treated by chemical, pepsinogen, and glycosidase digestion. 7F10-irm content was measured by ELISA, and the percentage of degradation was determined for each treatment.

Pepsinogen. Human standard mucus was incubated with 1 mg/ml of pepsinogen (Sigma Chemical) in 0.01 N HCl at 37°C for 18 h. After incubation, the acid was neutralized with 15 mM Na$_2$CO$_3$, pH 9.6, and 35 mM NaHCO$_3$, and then ELISA was performed.

HCl. HCl cleaves terminal fucose and sialic acid groups (11). Human standard mucus was incubated with 0, 0.01, 0.1, and 1 N HCl at 37°C for 24 h and then neutralized in carbonate buffer, pH 9.5, before 7F10-irm ELISA measurements.

Periodic acid. Periodic acid degrades carbohydrates and methionine (6, 9, 26). Human standard mucus was incubated with 0, 5, and 20 mM periodic acid (Poly Scientific) at room temperature for 1 h in the dark before ELISA measurements.

Sodium borohydride. Sodium borohydride (NaBH$_4$) reduces aldehyde groups of carbohydrates. Human standard mucus was incubated with 0 and 50 mM NaBH$_4$ (Sigma Chemical) at room temperature for 30 min to reduce aldehyde groups and prevent protein cross-linking, and then 7F10-irm was measured.

Glycosidases. To determine the terminal sugars contributing to the 7F10-epitope, human standard mucus was incubated with α-fucosidase (0.1 U/ml; Oxford Glyco System, Abingdon, UK) or endo-β-galactosidase (0.05 U/ml; Oxford Glyco System) at 37°C for 24 h, and then 7F10-irm was measured. Human standard mucus was also incubated with 0 (control), 0.01, 0.1, and 1 U/ml of sialidase (neuraminidase; Sigma Chemical) at 37°C for 18 h. After incubation, the concentrations of 7F10-irm were measured, and the percent degradation (mean ± SE) of 7F10-irm was calculated.

**Effects of Sialidase on AB Staining**

Human standard mucus and bovine type I (5% sialic acid) and type I-S (12% sialic acid) mucins (Sigma Chemical) were incubated with 0, 0.01, 0.1, and 1 U/ml of sialidase at 37°C for 18 h, and then AB staining was assessed as in AB + m.

**Statistics**

The means ± SE were calculated for the mucus components. Differences in concentrations between the rhinitis groups and between degradation treatments were tested by analysis of variance for multiple comparisons followed by unpaired two-tailed Student's t-test. Significance was assigned for $P < 0.05$.

**RESULTS**

**Concentrations of Mucus Constituents in Nasal Lavage Fluids**

Total protein was significantly elevated in AR (1.9-fold; $P = 0.023$; Table 1) compared with normal subjects. Lysozyme was not significantly elevated in any group. AB + m was significantly elevated in CF subjects (1.9-fold; $P = 0.049$), with a trend for an increase in AR subjects (1.2-fold; $P = 0.07$) compared with the normal subjects. 7F10-irm was significantly elevated in the AR (12.7-fold; $P = 0.00007$) and CF (2.4-fold; $P = 0.001$) subjects compared with the normal subjects. The ratios of 7F10-irm to AB + m concentration were similar in normal (0.14) and NAR (0.13) subjects and slightly increased in CF subjects (0.17). However, the ratio was greatly elevated in AR subjects (1.40), suggesting the potential modulation of mucoglycoconjugate expression in active atopy.

There were no significant differences between normal and NAR nasal lavage fluids.

**Sepharose-2B Column Chromatography of Mucoglycoconjugates**

In the nasal mucus standard, there were two broad peaks of AB + m at 1.3–3 (or larger) × 10^5 and 0.36–0.65 × 10^6 Da (Fig. 1). Between these two AB + m peaks, there was a broad peak of 7F10-irm. The peaks of...
7F10-irm and AB + m were present in mutually exclusive fractions.

Reduced and alkylated samples from normal and CF subjects were run in "settled columns" to improve peak resolution. However, because the elution rates were slow and the elution was prolonged, concentrations of AB + m were reduced to below the limits of detection by our assay. A distinct pattern of 7F10-irm elution was demonstrated in normal (n = 4) and CF (n = 6) samples, with 7F10-irm peaks at 1.5, 1.2, 0.85, and 0.53 × 10^6 Da in both groups (Fig. 2). There were no differences between normal and CF samples.

Characterization of the Epitope for 7F10 Monoclonal Antibody Binding

Pepsinogen degraded 87% of the 7F10-irm as determined by ELISA compared with the control sample, suggesting a protein component to 7F10-irm. The carbohydrate character of the 7F10-epitope was indicated by its 100% degradation in 5 and 20 mM periodic acid. 7F10-irm was degraded by 1 N HCl (100%) but not by 0.01 and 0.1 N HCl. NaBH₄ had no significant effect (19% degradation). Sialidase at 0.1 and 1 mM were reduced to below the limits of detection by our assay. A distinct pattern of 7F10-irm elution was demonstrated in normal (n = 4) and CF (n = 6) samples, with 7F10-irm peaks at 1.5, 1.2, 0.85, and 0.53 × 10^6 Da in both groups (Fig. 2). There were no differences between normal and CF samples.

### Table 1. Concentrations of total protein, lysozyme, AB + m, and 7F10-irm in normal, CF, AR, and NAR subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>CF</th>
<th>AR</th>
<th>NAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein, µg/ml</td>
<td>340 ± 75 (21)</td>
<td>509 ± 74 (7)</td>
<td>645 ± 103 (23)</td>
<td>331 ± 47 (52)</td>
</tr>
<tr>
<td>Lysozyme, µg/ml</td>
<td>30.4 ± 3.3 (15)</td>
<td>30.8 ± 2.9 (7)</td>
<td>40.8 ± 5.9 (10)</td>
<td>27.7 ± 1.8 (43)</td>
</tr>
<tr>
<td>AB + m, µg/ml bovine I-S mucin</td>
<td>364 ± 60 (44)</td>
<td>684 ± 119 (7)</td>
<td>444 ± 118 (9)</td>
<td>385 ± 62 (44)</td>
</tr>
<tr>
<td>7F10-irm, mU/ml</td>
<td>49.4 ± 8.0 (29)</td>
<td>119.0 ± 20.0 (7)</td>
<td>620.1 ± 332.4 (9)</td>
<td>50.2 ± 7.9 (39)</td>
</tr>
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Values are means ± SE; nos. in parentheses, no. of subjects. AB + m, Alcian blue-staining mucoglycoconjugates; 7F10-irm, 7F10-immunoreactive mucoglycoconjugates; CF, cystic fibrosis; AR, allergic rhinitis; NAR, nonallergic rhinitis. P values are compared with normal subjects (by Mann-Whitney U-test).

AB-Staining Properties of Human Nasal Mucus

Sialidase reduced AB + m in nasal mucus in a dose-dependent manner (Fig. 4). The degradation curve for the human mucus standard was intermediate between the purified bovine I type mucin (5% sialic acid) and bovine type I-S mucin (12% sialic acid), suggesting that the AB-staining component of human mucus contained ~8–9% sialic acid.

**DISCUSSION**

Submucosal gland seromucous cells, mucous cells, and vascular permeability each contribute about one-third of the protein contained in nasal mucus (18). Submucosal gland mucous cells are thought to secrete sulfated, sialylated, Alcian blue-staining "acid" mucus, whereas the serous cells secrete secretory immunoglobulin A, lysozyme, lactoferrin, hyaluronan, and some "neutral" mucus (3, 20). Changes in nasal mucus have been identified in nasal polyps (5), chronic sinusitis (8), and AR (7, 23), but the mucoglycoconjugate fractions have not been assessed. Although the consistency and constituents of tracheobronchial secretions in CF are greatly altered from normal, changes in precisely characterized mucus components in nasal and sinus secretions have not been assessed.

Human airway mucoglycoproteins (MGPs) have a broad range of molecular weights from 0.4 to 20 × 10^6 (16, 22). These MGPs consist of a mucin backbone polypeptide rich in serine, threonine, and proline residues. Several mucin backbone genes, including MUC4,
MUCSA/C, and MUC5B, are expressed in human nasal mucosa and the tracheobronchial mucin of Verma and Davidson (24) and Yuta (27). Each mucin polypeptide has several hundred oligosaccharide side chains attached to a threonine or serine residue by an O-glycosidic linkage. The average carbohydrate content of mucins is 80% by weight. The O-glycans may contain from 1 to 20 sugars (11). Our data indicate that sialic acid contributes 80% of the AB-staining characteristic of human nasal mucus. The residual AB staining may be due to sulfated galactose residues (11). The parallel degradation curves of human standard mucus and purified bovine mucins suggest that sialic acid represents ~8% of the carbohydrates in human nasal mucus.

Sialic acid contributes to the epitope recognized by the 7F10 monoclonal antibody because 5 mM periodic acid, which cleaves the exocyclic 7–8–9 hydroxyl positions of unsubstituted terminal sialic acid residues, and 20 mM periodic acid, which cleaves the terminal nonreducing sugars and some hexoses within the side chains (9), both reduced 7F10 immunoreactivity. The 7F10-epitope was not altered by α-fucosidase or endo-β-galactosidase. Degradation by pepsinogen indicates that 7F10-irm is an MGP. It is possible that proteolytically degraded peptide fragments may not stick to microtiter plates or permit a successful ELISA to be performed.

7F10-irm and the acidic AB+m were present in mutually exclusive fractions (Fig. 1) on Sepharose-2B columns, suggesting that 7F10-mucin is not an "acidic mucin." Additional subtle differences in MGPs may be discriminated that correspond to the various types of cells that can be identified by periodic acid Schiff base (PAS)-AB, pH 2.6, staining: AB−/PAS+ ("neutral mucin"); AB+/PAS−, where the AB staining disappears after sialidase treatment ("sialomucin"); AB+/PAS+ staining that is resistant to sialidase treatment; and those that stain in AB, pH 1.0 ("sulfomucins") (10, 14, 21). These mucoglycoconjugates have not been chemically delineated but may be of pathophysiological importance in AR and chronic sinusitis (14).

7F10-irm is clinically relevant because it is significantly increased in nasal secretions from both CF and AR subjects. AB+m was significantly increased in CF, suggesting an increase in the exocytosis and hyperplasia of goblet and submucosal gland mucous cells. In contrast, there were no changes in the serous cell product lysozyme. This would suggest the absence of seromucous cell hyperplasia in these subjects. This finding would be dissimilar to chronic sinusitis subjects who have increased seromucous cell products relative to normal subjects (8). Relative hyperplasia of epithelial goblet cells and submucosal gland seromucous and mucous cells has not been clearly demonstrated in human nasal mucosa in AR (14).

Although the 7F10-irm concentration was higher in CF than in normal secretions, the patterns of elution for 7F10-irm-containing fractions from Sepharose-2B columns were essentially identical. This suggested that 7F10-irm fractions were not qualitatively changed in CF but that the total amount of exocytosed 7F10-irm was increased in CF. The four 7F10-irm peaks differed in size by ~0.3 × 10^6 Da, suggesting that 1) four different mucin genes produce four different polypeptides to which the 7F10-reactive oligosaccharides were attached, 2) mRNA for a single mucin backbone protein was spliced into four specific variants, and 3) 7F10 monoclonal antibody recognized four products degraded from one mucin. Degradation or induction of novel mucin genes appears unlikely because the patterns were similar in all 10 samples. These data suggest increased production and secretion of this normal 7F10-irm in CF subjects. Mechanisms to explain the increase in 7F10-irm in AR require additional investigation.

In summary, 7F10-irm was significantly increased in CF compared with normal subjects. 7F10-irm was a series of four MGPs ranging in size from 0.4 to 1.5 × 10^6 Da. The size distribution in CF subjects was similar to that in normal subjects. 7F10-irm and AB+m hypersecretion in CF may reflect the underlying pathology within secretory cells induced by the mutated CF transmembrane conductance regulator or by inflammatory changes. There was no measurable glandular hypersecretion in this population of NAR subjects with fibromyalgia and/or chronic fatigue syndrome.
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Address for reprint requests: J. N. Baraniuk, Division of Rheumatology, Immunology, and Allergy, GL-008, Gorman Bldg., Georgetown University, Washington, DC 20007-2197.

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