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Cystic Fibrosis. II. The Urinary Mucociliary Inhibitor

M. CAROL McNEELY, YOGESH C. AWASTHI, DON R. BARNETT, TATSUO IWASUMI, LARRY SCHNEIDER, SATISH K. SRIVASTAVA, AND BARBARA H. BOWMAN[37]

Department of Human Biological Chemistry and Genetics [M.C.M., Y.C.A., D.R.B., L.S., S.K.S., B.H.B.], Department of Physiology and Biophysics [T.I.], The University of Texas Medical Branch at Galveston, Galveston, Texas, USA

Summary

In the current study, the cystic fibrosis cationic mucociliary inhibitor has been purified from urine by ion exchange chromatography, gel filtration, lectin affinity chromatography, isoelectric focusing, and high performance liquid chromatography. The molecular size of the cationic mucociliary inhibitor was estimated to be in the range of 4,000 to 13,500 MW, by its elution on Sephadex G-50, and between 7,500 and 12,750 MW, by urea-sodium dodecyl sulfate polyacrylamide gel electrophoresis. In addition to the cationic mucociliary inhibitor, an anionic mucociliary inhibitor was also detected in the urinary fraction isoelectrically focused between pH 4.5 and 4.9. The identity of the mucociliary inhibitor as a glycoprotein was established in the current study by affinity chromatography on *Phaseolus lunatus* lectin, by radiolabeling the carbohydrate with galactose oxidase and tritiated sodium borohydride, and by determining the presence of a large concentration of glucosamine and small amounts of galactosamine by amino acid analysis. The amino acio analysis of the purified major component of the cationic mucociliary inhibitor reveals that the glucosamine concentration represents a high percentage of the composition of the glycoprotein.

Speculation

The purification of a cationic mucociliary inhibitor from cystic fibrosis urine will facilitate the construction of antibody reagents which can be utilized for feasibility studies of prenatal diagnosis and heterozygote detection.

Cystic fibrosis (CF), an autosomal, recessive inborn error of metabolism, is manifested by pulmonary disease, pancreatic insufficiency, intestinal malabsorption, and altered electrolyte concentrations in body fluids. The basic defect is not yet understood. Unique components of body fluids have been identified in CF by observing their biologic activity in a variety of assays. These components have been found in CF plasma (8, 18, 28, 33), in CF saliva and sweat (8, 24), and in tissue culture medium from cells derived from cystic fibrosis homozygotes and heterozygotes (7, 10, 19). Whether these factors are related to one another, or to the genetic basis of the disease, which is inherited as an autosomal recessive trait, has not been established. Comparison of the proteins detected by the various assays of CF factors has been hampered by the difficulty of purification: the factors are present in low concentrations in blood, and in addition, specimens of tissue and blood are difficult to obtain from individual cystic fibrosis patients, who are often seriously ill.

In preceding studies (16), the mucociliary inhibitor observed in cystic fibrosis sera was found to be associated with serum protein fractions of molecular weights in the range of 6,000 to 12,000 and was shown to have a charge heterogeneity, with isoelectric points corresponding to an anodal (pI 4.2–6.2) and a cathodal (pI ~9.0) fraction. Furthermore, at least some of the molecules in the inhibiting fraction contained carbohydrate.

The CF serum mucociliary inhibitor was initially found to be associated with the euglobulin fraction by Spock *et al.* (28) and was found to be noncovalently bound to IgG by our laboratory (4, 9, 13). This finding was later confirmed by Conod *et al.* (17) and Wilson *et al.* (33). Carson *et al.* (15) demonstrated that after the CF mucociliary inhibitor from plasma had been dissociated from IgG, it could reassociate with native IgG. The nature of this binding is not known.

In the present study, urine from CF subjects was evaluated as starting material for purification of the mucociliary inhibitor. Unlike plasma, urine can be collected conveniently from CF donors every day, and its collection need not be interrupted by illness. The presence of the mucociliary inhibitor was demonstrated in urine, and in terms of inhibitory activity, its concentration in urine of homozygotes appeared to be twice that in the urine of heterozygotes. The mucociliary inhibitor appears to be heterogeneous and appears to be composed of a family of related glycoproteins. The major cationic glycoprotein that demonstrates biologic activity has been purified and its amino acid composition has been determined.

EXPERIMENTAL

Urine specimens were collected from 11 CF homozygotes, seven heterozygotes, and six normal subjects. The patient donors in this study were relatively healthy young people between the ages of 9 and 25 and with Schwachman scores from 75 to 85. Normal controls were age matched with CF patients and heterozygotes. None of the patients demonstrated any detectable side effect of antibiotic therapy. Samples were collected with 0.005% NaN_3 as a preservative and were maintained at 4°C or were frozen. Pooled urine from each individual was filtered through sharkskin paper and desalted, using a KS 370 sectional column (30 cm × 15 cm, Pharmacia, Piscataway, NJ), packed with Sephadex G-15 in 0.005 M Tris HCl buffer, pH 8.6, at a flow rate of 5 liter/hr.

DEAE-CHROMATOGRAPHY

The desalted urine was subjected to DEAE (diethylaminoethyl) cellulose chromatography in a 5 cm × 40 cm column, equilibrated with the same buffer used in desalting. After elution of the initial unadsorbed protein fraction, a 1-liter linear ionic-strength gradient (0 to 0.4 M NaCl in equilibrating buffer) was applied to elute the proteins adsorbed to the column matrix. The fractions from DEAE-chromatography were concentrated to 10-ml volumes by using a membrane with molecular weight retention of approximately 1000 (UM 2, Amicon, Lexington, MA). After concentration, the fractions were dialyzed against distilled water by using Spectraphor tubing (Spectrum Medical Industries, Inc., Los An-

geles, CA) with a molecular weight retention of 3500, and then were lyophilized. The fractions were assayed for mucociliary inhibition.

GEL FILTRATION

Gel filtration of unadsorbed fractions from DEAE-chromatography, ranging from 5 to 25 mg protein/ml, was performed on a 5.0 cm × 150 cm column of Sephadex G-50 equilibrated in 0.05 M Tris HCL, 0.2 M NaCl, pH 8.6. The column was calibrated with proteins of known molecular weights: human serum albumin (68,000 MW), chymotrypsinogen (25,000 MW), myoglobin (17,000 MW), cytochrome c (11,700 MW), insulin (5,700 MW), and bacitracin (1,500 MW). Fractions eluted from the Sephadex G-50 column were dialyzed against distilled water and lyophilized. The void volume and total bed volume in each experiment were determined by the use of the markers, blue dextran and bacitracin (26). Aliquots of all fractions were tested for mucociliary inhibition.

LECTIN AFFINITY CHROMATOGRAPHY

Pooled fractions from gel filtration on Sephadex G-50 were subjected to lectin affinity chromatography. Lectin was isolated from lima beans (*Phaseolus lunatus*), according to the procedure described by Gould and Scheinberg (21). Lectin was tested for N-acetylgalactosamine specificity by agglutination of type A red blood cells. The purified lectin was conjugated to CNBr-activated Sepharose 4B beads (3). The Sepharose 4B conjugated with lectin was packed in a 6.5-ml disposable column (BioRad) and equilibrated for 24 hr at a rate of 6 ml/hr in a 0.2 M glycylglycine buffer, pH 7.0, containing 0.02 M $CaCl_2$, $MnCl_2$, and $MgCl_2$. The lyophilized Sephadex G-50 fraction was dissolved in 3 ml of the equilibration buffer and applied to the affinity column. The unadsorbed proteins were thoroughly washed off the column with the equilibration buffer containing 0.5 M NaCl. The adsorbed proteins were eluted with 0.1 M N-acetylgalactosamine in 0.5 M NaCl. Eluates were dialyzed against distilled water and lyophilized, and aliquots from the unadsorbed and the adsorbed fractions were tested for mucociliary inhibition.

PREPARATIVE ISOELECTRIC FOCUSING

Fractions from lectin chromatography and gel filtration on Sephadex G-50 were further purified by isoelectric focusing in a sucrose density gradient (LKB 8100-1 column), using ampholines in the range of pH 3.5 to 10. Narrow range focusing was conducted utilizing ampholines in the range of pH 4.0 to 6.5 (32). The column was focused at 300 V for 1 hr and at 500 V for the remaining time. After 42–48 hr, focused proteins were collected in 1.5-ml fractions. The pH and absorbance at 280 nm were recorded for each tube. The focused peaks were pooled and aliquots were tested for mucociliary inhibition after dialysis. Protein concentrations were determined by the procedure of Lowry *et al.* (23).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The cationic mucociliary inhibitor which was prepared by isoelectric focusing was further purified by high performance liquid chromatography on a Beckman model 332 system, utilizing an anionic Ultrasil AX column (4.6 mm × 25 mm) in 0.01 M phosphate buffer, pH 6.86. Elution rate was 0.5 ml/min, and 0.5-ml fractions were collected. Chart speed was 5 mm/min.

RADIOLABELING OF PROTEINS

Tritiation of protein fractions was performed by modifying the procedure described by Suzuki and Suzuki (29). Protein fractions (0.2 to 0.4 mg/ml of protein) in 0.02 M potassium phosphate buffer, pH 7.0, were treated with 500 units of galactose oxidase (Sigma) bound to Sepharose 4B. After enzymatic oxidation of galactose and N-acetylgalactosamine residues, the protein was reduced with tritiated $NaBH_4$. The protein solution was dialysed extensively against distilled water until radioactivity was not detectable in the dialysate.

ANALYTICAL THIN-LAYER ISOELECTRIC FOCUSING

Samples were subjected to analytical electrofocusing, using LKB Ampholine polyacrylamide gel plates in the pH range of 3.5 to 9.5 (35). The pI of fractions focused on plates was determined *in situ* by utilization of a surface-reading electrode (LKB, Rockville, MD).

POLYACRYLAMIDE GEL ELECTROPHORESIS

Estimations of the molecular weight of polypeptides were carried out according to the method of Swank and Munkres (30) by means of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The molecular weights of urinary polypeptides were estimated by comparing their mobilities to those of protein standards of known molecular weights ranging from 3,460 to 14,700. The method of Panyim and Chalkley (27) was used to analyze urinary fractions by urea polyacrylamide gel electrophoresis (15% polyacrylamide). The gels were stained following the procedure given by Carson and Bowman (16).

AMINO ACID ANALYSIS

Amino acid compositions were determined on Beckman automatic amino acid analyzers 121 M and 119 as described (22).

MUCOCILIARY INHIBITION ASSAY

The presence of a mucociliary inhibitor in experimental fractions was determined by assays that used preparations of ciliated epithelium from gills of *Crassostrea virginica* (12). Cessation of ciliary activity within 40 min after exposure to a sample denoted mucociliary inhibition. Authentic CF, normal (non-CF) sera, and seawater were used as controls in each assay. Each experimental fraction from every purification step was examined at least three times in independent assays where the identity of the experimental samples was unknown to the examiner. All fractions from simultaneous fractionations of urine from normal (non-CF) donors were examined on mucociliary assays. In dilution experiments, each serial dilution was examined three times in independent assays.

LASER LIGHT-SCATTERING SPECTROSCOPY

The mucociliary assay was monitored by following the ciliary response with a modification of laser light-scattering spectroscopy developed by Verdugo *et al.* (31) to measure ciliary frequency in rabbit tracheal explants. The procedure has been modified to be used with gill cilia of *C. virginica* (6). The system uses spectral analysis of the intensity fluctuations of soft laser light reflected from moving cilia. The light source was a helium-neon laser (Laser Spectra Physics 124B). The spectrum is dependent on the frequency and coherency of the movement of cilia and provides a quantitative measurement of ciliary activity that is recorded as hertz (Hz) by a Digital Spectrum Analyzer (EMR, model 1510). The assay conditions were the same as have been described earlier (12). The scattered light was monitored through a custom-built inverted microscope at a magnification of 625 and was detected on the Fourier plane of the objective on the 45° axis of the detector. In previous experiments (6), two peaks of spectral density were observed in oyster gill cilia preparations in the presence of seawater or normal serum fractions after 45 min. In contrast, the ciliary activity of sections of gill treated with CF serum fractions demonstrated a substantially different frequency spectrum with much slower frequencies recorded after 12 min. After 25 min in the presence of CF serum fraction, spectral analysis under laser light revealed no coherent movement.

QUANTITATION OF THE MUCOCILIARY INHIBITOR IN URINE OF CF HOMOZYGOTES AND HETEROZYGOTES

Lyophilized protein preparations of urinary fractions (DEAE and Sephadex G-50) from normals, CF homozygotes, and heterozygotes were dissolved in filtered seawater and examined for mucociliary inhibition (12). The total protein concentration was determined by the method of Lowry *et al.* (23), and serial dilutions were made by the addition of filtered seawater. Ten serial dilutions from 500 to 50 μg/ml were tested for mucociliary inhibition. The minimum protein concentration required for inhibition of mucociliary activity was determined for each fraction. Dilutions of fractions were examined from eight homozygotes and seven heterozygotes, so that a comparison of biologic activity in fractions of a group of homozygotes and heterozygotes would rule out variations among individuals or among sample pools. Standard deviations were calculated for each group of samples.

The minimum protein concentration necessary to cause mucociliary inhibition was determined for DEAE fractions from urine of seven heterozygotes, and the average minimum concentration of these seven fractions was designated as 1 inhibitory unit. The minimum protein concentration necessary to cause mucociliary inhibition was determined for DEAE fractions from urine of eight homozygotes, and again, the average minimum concentration was designated as 1 inhibitory unit. In a similar manner, inhibitory units were calculated for G-50 fractions from the homozygotes and three heterozygotes.

RESULTS: PURIFICATION OF MUCOCILIARY INHIBITOR

DESALTING AND ION EXCHANGE CHROMATOGRAPHY

Filtered urine (500 ml), desalted in Sephadex G-15 and applied to an ion exchange chromatography column of DEAE cellulose at pH 8.6, eluted four protein fractions (Fig. 1). An unadsorbed fraction (A) was eluted before application of the linear ionic-strength gradient; three fractions (B, C, and D) were collected after application of the gradient. The average protein concentration of each fraction obtained in each step is presented in Table 1. Urinary fractions from six normal subjects, 11 cystic fibrosis homozygotes, and seven heterozygotes exhibited similar elution profiles on ion exchange chromatography. Lyophilized aliquots of each of the four chromatographic fractions from urinary preparations of six normals, 11 homozygotes, and seven heterozygotes were dissolved in filtered seawater and tested for mucociliary inhibition. Only the unadsorbed DEAE fractions (fraction A, Fig. 1) of preparations from 10 homozygotes and seven heterozygotes inhibited mucociliary activity. The results from mucociliary assays of all fractions studies are summarized in Tables 2 and 3. The urinary fraction from one of the 11 CF subjects tested (J.W.) did not inhibit mucociliary activity, nor did fractions from additional urine collections from this subject. Why her urinary fractions failed to inhibit mucociliary activity is not known, although it is interesting that, on the other hand, her serum fractions, and the serum and urinary fractions from her parents (J.W. and R.W.), did repeatedly cause mucociliary inhibition.

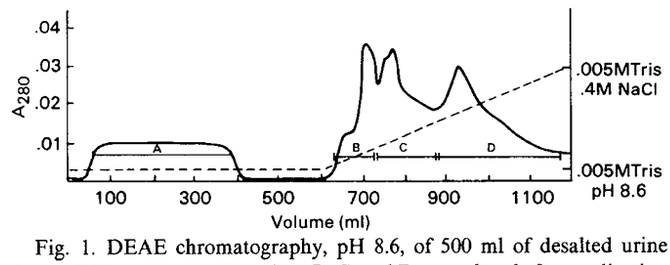

Fig. 1. DEAE chromatography, pH 8.6, of 500 ml of desalted urine from a CF homozygote. Fractions B, C, and D were eluted after application of the gradient. Only the unadsorbed urinary fraction A inhibited mucociliary activity.

Table 1. *Recovery and partial purification of the CF mucociliary inhibitor from urine of CF homozygotes*

Urine fraction	Concentration of protein (mg/liter of urine processed)
Desalted urine	140
DEAE unadsorbed	7.85
G-50 pool II (4,100–13,500 MW)	2.43
Lectin extracts	0.29
Isoelectrically focused pools	
Fraction C–D pI 4.2–6.2	0.011
Fraction H pI ~9.0	0.008
High Performance Liquid Chromatography	
Peak 2	0.002

Table 2. *Mucociliary inhibition studies of urinary preparations from DEAE, Sephadex G-50, and lectin affinity chromatograph*

Urinary fraction	No. homozygotes whose preparations inhibited	No. heterozygotes whose preparations inhibited	No. normals whose preparations inhibited
DEAE			
Fraction A	10/11	7/7	0/6
Fraction B	0/11	0/7	0/6
Fraction C	0/11	0/7	0/6
Fraction D	0/11	0/7	0/6
Sephadex G-50[2]			
>30,000 MW	0/5	0/3	0/5
1,100–13,500MW	5/5	3/3	0/5
1,500–4,500 MW	0/5	0/3	0/5
Phaseolus lunatus lectin[3]			
Unadsorbed (A, B)	0/3	Not tested	0/1
0.5 M NaCl wash (C)	0/3		0/1
N-acetylgalactosamine extract (D)	3/3		0/1

[1] Protein concentrations of the fractions examined were in the range of 0.3–0.99 mg/ml for CF genotypes and 0.3–2.0 mg/ml for normals.

[2] Protein concentrations of the fractions examined were in the range of 0.1–1.0 mg/ml.

[3] Protein concentrations of the fractions examined were in the range of 0.05–0.5 mg/ml.

None of the urinary DEAE fractions from six normals (non-CF), including two asthmatic children, inhibited mucociliary activity. When the protein concentrations of DEAE fractions from two normals were increased to 2.0 mg/ml (6.7-fold) the fractions still failed to inhibit. The DEAE fractions (B, C, and D in Fig. 1) from CF urinary preparations that were eluted from the DEAE column after application of the ionic gradient, failed to inhibit mucociliary activity, which indicated that the activity was confined to the unadsorbed fractions from urine of homozygotes and heterozygotes.

SEPHADEX G-50 GEL FILTRATION

Gel filtration on Sephadex G-50, pH 8.6, resolved the unadsorbed fractions from DEAE chromatography into fractions corresponding to 30,000 MW and above, 4,000 to 13,500 MW, and below 4,000 MW. After gel filtration of urinary preparations from five cystic fibrosis homozygotes and three heterozygotes, only those fractions corresponding to the 4,000 and 13,500 MW range inhibited mucociliary activity. In Figure 2, a plot of a representative elution profile on Sephadex G-50 is shown, with the *shaded*

area representing the molecular weight range of the fraction that inhibited mucociliary activity. Analogous fractions from gel filtration of urine from five normals never inhibited mucociliary activity (Table 2).

LECTIN AFFINITY CHROMATOGRAPHY

Biologically active fractions obtained from Sephadex G-50 gel filtration of urinary preparations from three CF homozygotes and the analogous fraction from one normal individual were subjected to lectin affinity chromatography. Lectin isolated from lima beans (*P. lunatus*), which exhibited a specificity for N-acetylgalactosamine, was used for this step. This lectin was chosen because the composition of the heterogeneous fraction (4,000–13,500 MW) obtained from Sephadex G-50 included galactosamine. The elution profile of the lectin affinity column is presented in Figure 3; fractions A and B correspond to unadsorbed fractions, whereas fraction C was eluted with the equilibration buffer containing 0.5 M NaCl, and fraction D was eluted with a solution of 0.1 M N-acetylgalactosamine. Only fraction D inhibited mucociliary activity (Table 2). These results have been consistent in preparations from three homozygotes. The analogous fractions from urine of a normal subject did not inhibit mucociliary activity. Polyacrylamide gel electrophoresis in acid urea revealed approximately eight electrophoretic bands in the urinary fraction extracted with N-acetylgalactosamine, and analytical isoelectric focusing of the same fraction between pH 3.5 and pH 9.5 also separated eight protein bands. The spectrophotometric tracing of the stained acid-urea polyacrylamide gel and a photograph of the stained isoelectric focusing gel of the same fraction are shown in Figure 4. The fraction eluted with N-acetylgalactosamine from lectin affinity chromatography had a protein concentration of approximately 0.29 mg/liter of urine processed (Table 1).

Table 3. *Mucociliary inhibition studies of urinary preparations from isoelectric focusing*

Urinary fraction	No. homozygotes whose preparations inhibited	No. heterozygotes whose preparations inhibited	No. normals whose preparations inhibited
Isoelectric focusing[1]			
pI 3.5–4.2	0/5	0/2	0/5
4.3–5.0	5/5	2/2	0/5
5.1–6.2	5/5	1/2	0/5
6.3–7.2	0/5	0/2	0/5
7.3–8.0	0/5	0/2	0/5
8.1–9.0	2/5	1/2	0/5
9.1–>9.5	5/5	2/2	0/5
Narrow range focusing[2]			
pI 3.3–3.8	0/2	0/1	0/3
3.9–4.0	0/2	0/1	0/3
4.1–4.2	0/2	0/1	0/3
4.3–5.0			0/3
5.1–5.2			0/3
5.3–5.35			0/3
5.4–5.5			0/3
5.6–6.1			0/3

[1] The protein concentrations of the fractions examined were in the range of 0.06–0.25 mg/ml.

[2] The protein concentrations of the fractions examined were in the range of 0.06–0.25 mg/ml.

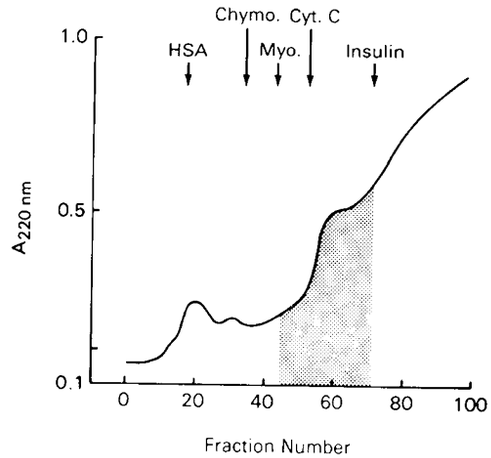

Fig. 2. Gel filtration (Sephadex G-50) of the unadsorbed fraction from DEAE chromatography of urine from cystic fibrosis homozygotes (4) and heterozygotes (3). The *shaded area* designates the molecular weight range of the fractions that inhibited mucociliary activity. The molecular weight standards were human serum albumin (HSA), chymotrypsinogen (Chymo), myoglobin (Myo), cytochrome c (Cyt. c), and insulin.

PREPARATIVE ISOELECTRIC FOCUSING

Biologically active fractions from Sephadex G-50 chromatography or Sephadex G-50 followed by lectin affinity chromatography, when subjected to isoelectric focusing, separated into eight protein fractions (A through H). Since similar results were obtained from urinary preparations of five homozygous individuals, a representative pattern is shown in Figure 5. Pools A through H were dialyzed extensively against deionized water and lyophilized. Aliquots were examined for mucociliary inhibition. In preparations from all five homozygotes studied, only those fractions which focused between pH 4.2 and 6.2 (fractions C and D) and those focusing around pH 9.0 (fraction H) inhibited mucociliary activity (Table 3). The protein concentration of each of the three biologically active fractions corresponded to approximately 0.01 mg/liter of urine processed (Table 1). Analytical isoelectric focusing of the three fractions is shown in Figure 6. The anionic fraction containing the mucociliary inhibitor consisted of one major band, which has been identified as a fragment of basement membrane collagen (14) and two minor bands. The electrophoretic pattern does not differ significantly from that of the analogous isoelectric focusing pattern of normal control urinary preparations. The cationic mucociliary inhibitor consists of a family of proteins focusing between pI 8.4 and pI 9.1. No mucociliary inhibition was associated with CF pools, A, B, E, F, and G (Fig. 5). Although there was no significant difference between the isoelectric focusing profiles of urinary fractions from CF patients and normal subjects, the analogous focused fractions from five normal subjects never inhibited mucociliary activity (Table 3).

The fraction focusing between pH 4.2 and 6.2 and associated with mucociliary inhibition was refocused in a narrow pH range between pH 4.0 and 6.5. Ten isoelectrically focused fractions were collected and tested for mucociliary inhibition. In urinary preparations of two CF homozygotes, the fraction with an isoelectric point between pH 4.55 and 4.9 inhibited mucociliary activity. Figure 7 is a representative pattern of the narrow-range isoelectric focusing of pooled fractions C and D. In Figure 7, the focused area designated as fraction 6 contained the mucociliary inhibitor. The basement membrane component was contained in fraction 4 in Figure 7. Analogous fractions from urinary preparations from three normal (non-CF) subjects gave similar elution patterns, although none of the focused fractions inhibited mucociliary activity.

MOLECULAR WEIGHT ESTIMATION ON SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

The molecular weight of the cationic urinary fraction containing the inhibitor (Fig. 6*B*) was estimated after polyacrylamide gel electrophoresis in the presence of SDS-urea. The fraction separated into three proteins of estimated molecular weight, 7,500, 8,800, and 12,750. Figure 8 is a photograph of the polyacrylamide

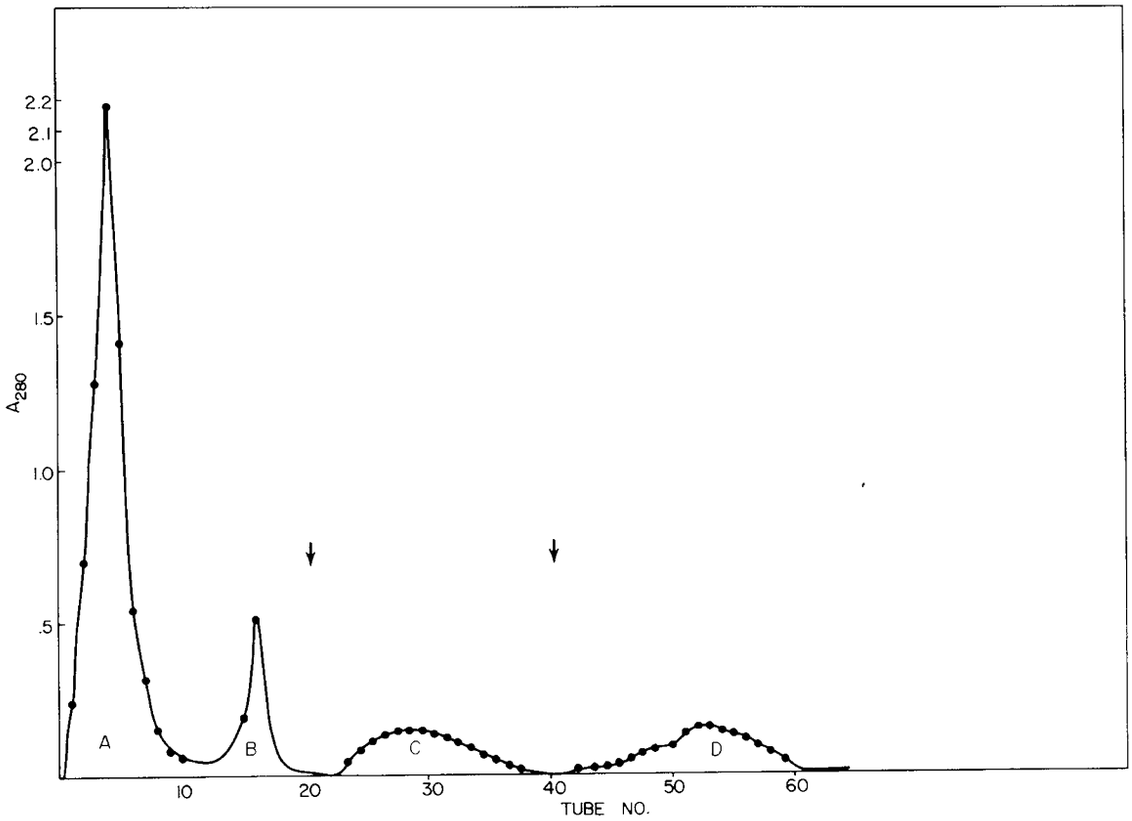

Fig. 3. Affinity chromatography of the Sephadex G-50 fraction (4,000-13,500 MW) on lectin (*P. lunatus*) conjugated to Sepharose 4B. The unadsorbed proteins were eluted in fraction A and B with a wash of equilibration buffer (0.2 M glycylglycine, pH 7.0, containing 0.02 M $CaCl_2$, $MnCl_2$, and $MgCl_2$). Fraction C was eluted after application of the equilibration buffer containing 0.5 M NaCl (shown by first *arrow*). Fraction D was eluted after application 0.1 M N-acetylgalactosamine in 0.5 M NaCl. Each tube contained 1.5 ml.

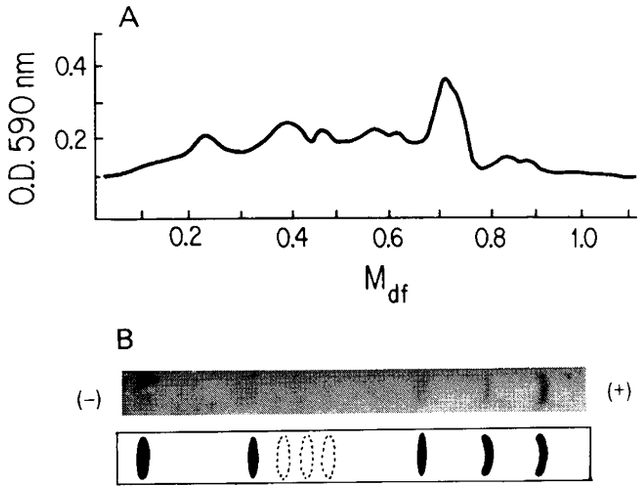

Fig. 4. Analysis of the urinary lectin extract that inhibited mucociliary activity. *A*, scan of acid-urea polyacrylamide gel electrophoresis, after staining; approximately eight electrophoretic bands can be seen. *B*, photograph of analytical isoelectric focusing gel between pH 3.5 and 9.5 that revealed approximately eight protein bands after staining. Mobility dye front is the mobility relative to the dye front.

gels. The migration of the anionic inhibitor (not shown) corresponded to the same molecular weight range.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The cationic mucociliary inhibitor which focused at pH ~9.0 on preparative isoelectric focusing was further purified by high performance liquid chromatography, which separated the fraction into one major and two or more minor peaks (Fig. 9). The major peak (designated as *2* in Fig. 9) represented about 33% of total protein and eluted as a single peak upon rechromatography on high performance liquid chromatography (Fig. 9, *lower pattern*). Examination of aliquots of fractions separated by high performance liquid chromatography on ciliated epithelium of *C. virginica* revealed that fractions 1, 2, 3, 4, and 5 in Figure 9 inhibited mucociliary activity. When urine of a normal subject was purified in an identical procedure, the pattern after high performance liquid chromatography could not be distinguished from the CF pattern shown in Figure 9. None of these fractions, however, inhibited mucociliary activity.

MONITORING MUCOCILIARY INHIBITION OF URINARY FRACTIONS WITH LASER BEAM LIGHT SCATTERING

An independent measurement of mucociliary inhibition was obtained by measuring cilia beat frequency by laser beam light scattering. Frequency measurements were obtained by following the ciliary activity when ciliated epithelium of *C. virginica* was exposed to purified cationic inhibitor from CF urine. Representative graphs of ciliary beat frequences are given in Figure 10 after exposure of cilia to the cationic cystic fibrosis mucociliary inhibitor, purified by high performance liquid chromatography and an analogous fraction from a normal control. Two ciliary frequencies of 10 Hz and ~20 Hz could be observed in both preparations after 12–15 min. After 23 min, cilia beat was not detected on the preparation exposed to purified cationic inhibitor (high performance liquid chromatography *pool 2* in Fig. 9). On the ciliated preparation exposed to the analogous fraction from normal urine, there was no significant change in ciliary frequency populations which remained in the 12 and 22 Hz ranges for longer than 40 min.

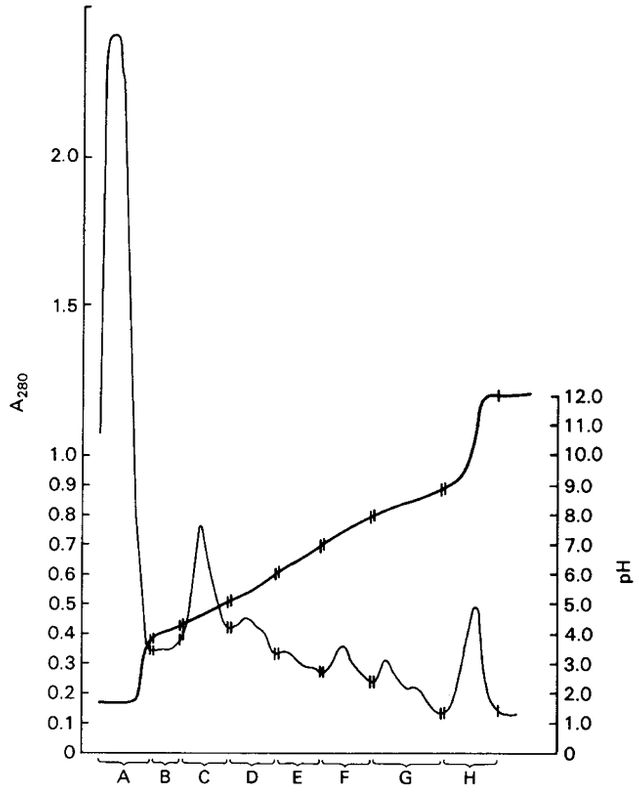

Fig. 5. Preparative isoelectric focusing pH 3.5–10.0 of the urinary lectin extract that inhibited mucociliary activity. Eight isoelectrically focused fractions were separated, A through H. Only fractions C, D, and H inhibited mucociliary activity.

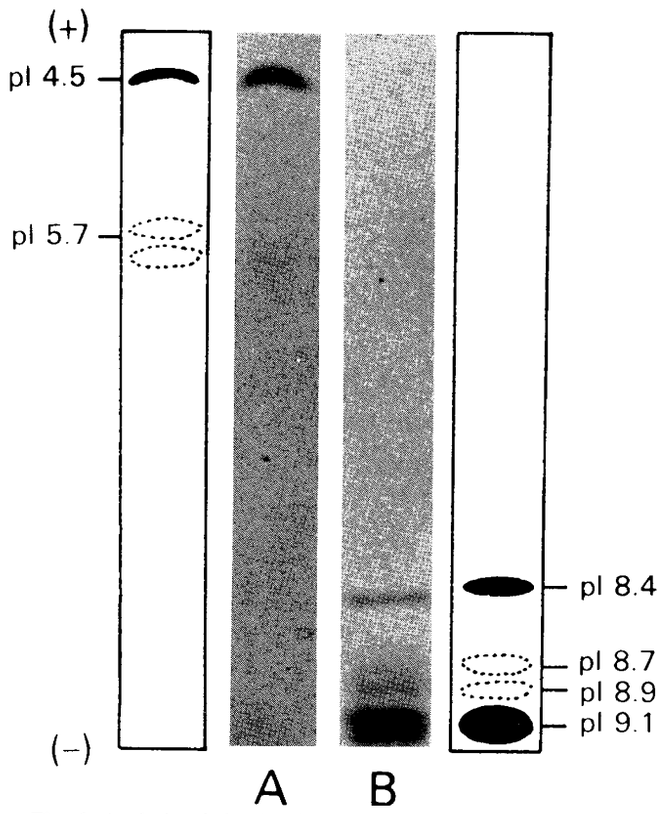

Fig. 6. Analysis of the fractions that inhibited mucociliary activity purified by isoelectric focusing between pH 3.5 and 10. The anionic fraction is composed of one major band (which is a fragment of basement membrane collagen) and two minor bands. *B*, the cationic mucociliary inhibitor is composed of a family of proteins that have isoelectric points from pI 8.4 to 9.1.

RADIOLABELING OF ISOELECTRIC FRACTIONS

Since adsorption and elution of the urinary factor on lectin affinity chromatography suggested the presence of N-acetylgalactosamine, radiolabeling was attempted with galactose oxidase and tritiated sodium borohydride. The two isoelectric fractions from one CF urinary preparation which inhibited mucociliary activity and which focused at a pH range between 4.2 and 6.2 and around 9.0 (Fig. 5) were subjected to radiolabeling with tritium by the modified procedure of the galactose oxidase-[^{3}H]-sodium borohydride technique (29). Both fractions were radiolabeled by this procedure when [^{3}H]-sodium borohydride with a specific activity of 250 mCi/mg was used. After radiolabeling, the anionic fraction (pI 4.2 and 6.2) contained approximately 840,000 cpm/mg protein, whereas the cationic fraction (pI ~9.0) contained approximately 142,500 cpm/mg protein, which indicated the presence of galactose or N-acetylgalactosamine moieties (or both) in both fractions. The relative contribution of galactose from the basement membrane contaminant in the anionic fraction is not yet known.

AMINO ACID COMPOSITION

The amino acid composition of the cationic major component of the mucociliary inhibitor, purified by high performance liquid chromatography (Fig. 9, *lower pattern*), is given in Table 4. The glycoprotein contained a highly significant concentration of glucosamine and smaller amounts of galactosamine. No half-cystine could be detected and only a small amount of methionine was present. The high isoelectric point of the glycoprotein suggests that many of the aspartic and glutamic acid residues must be present as their amides, glutamine, and asparagine. Amino acid analysis of aliquots of fractions 3, 4, and 5 (Fig. 9), separated by high performance liquid chromatography, revealed that the amino acid composition was impressively similar among all the fractions. The hexosamine content, however, differed in concentration in

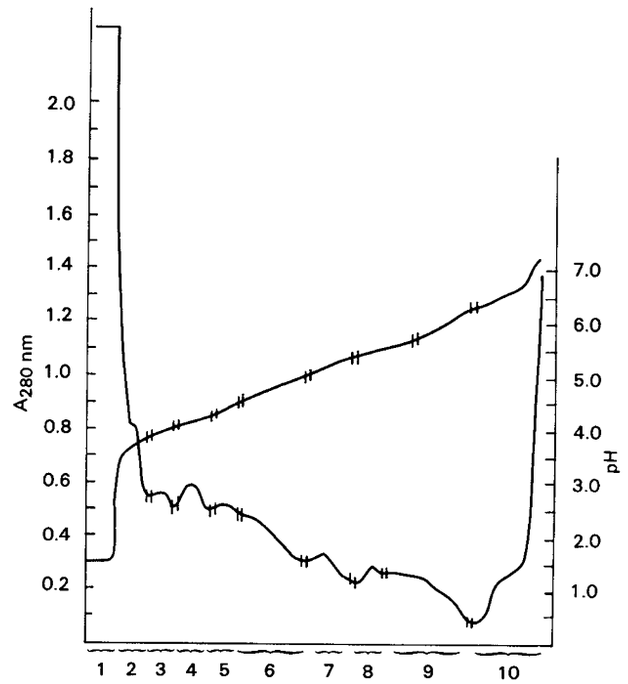

Fig. 7. Preparative narrow-range isoelectric focusing of the anionic urinary fraction containing the mucociliary inhibitor between pH 4.0 and 6.5. Only fraction 6, which focused between pH 4.5 and 4.9, inhibited mucociliary activity.

each fraction, which indicated that the heterogeneity observed in these proteins may have resulted from small differences in carbohydrate content.

RELATIVE CONCENTRATIONS OF THE MUCOCILIARY INHIBITOR IN URINARY PREPARATIONS FROM HOMOZYGOTES AND HETEROZYGOTES

An estimate of the relative concentrations of the CF mucociliary inhibitor in urinary fractions of homozygotes and heterozygotes

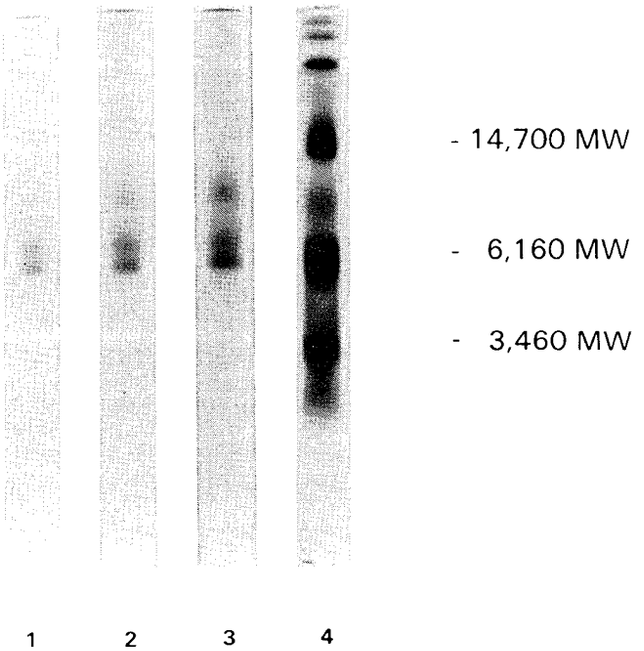

Fig. 8. Photograph of the cationic fraction containing the mucociliary inhibitor on SDS-urea polyacrylamide gel electrophoresis. Gels 1 through 3 contain cationic fractions in concentrations of 60, 150, and 300 μg, respectively. Gel 4 contains molecular weight standards.

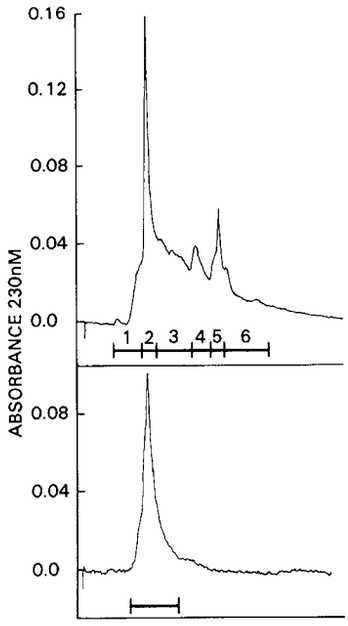

Fig. 9. High performance liquid chromatography of the cationic urinary fraction containing the CF mucociliary inhibitor, using an anionic column with 0.01 M phosphate buffer, pH 6.8. One major peak, 2, and at least two minor peaks were eluted. The major peak, 2, was rechromatographed and is seen in the *lower panel.*

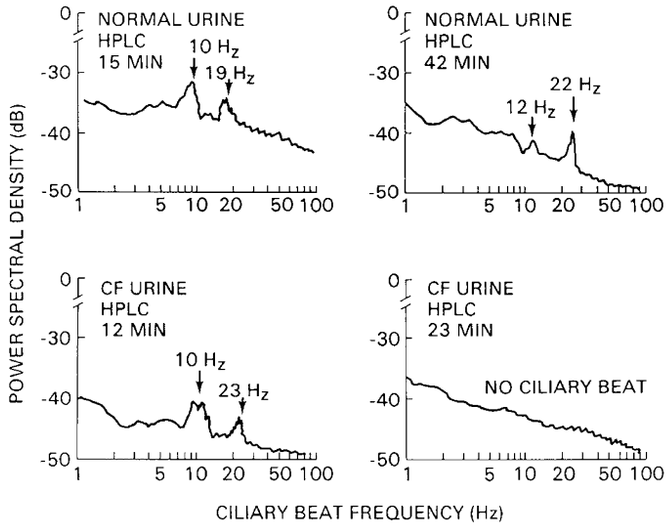

Fig. 10. Laser light scattering spectroscopy to follow ciliary frequency in gill preparations from *C. virginica*. After ciliated gills of *C. virginica* were treated with CF high performance liquid chromatography pool 2 (in Fig. 9) for 12 min, the ciliary frequencies (Hz) were 10 and 23 (*lower left*). After 23 min of exposure, no ciliary beat was recorded (*lower right*). The frequency spectrum of cilia in the presence of the analogous normal counterpart of the cationic mucociliary inhibitor consisted of two peaks of spectral density, 10 and 19 Hz after 15 min. After 42 min of exposure to the normal fraction the ciliary beats were recorded at 12 and 22 Hz (*upper panels*).

Table 4. *Composition of a cationic mucociliary inhibitor from CF urine*

Amino acid	Residues per 1000 amino acid residues
Aspartic acid	113.6
Threonine	91.6
Serine	126.8
Glutamic acid	94.2
Proline	30.2
Glycine	153.1
Alanine	70.1
Half cystine	ND[1]
Valine	61.0
Methionine	11.0
Isoleucine	29.2
Leucine	39.3
Tyrosine	20.4
Phenylalanine	18.3
Histidine	21.6
Lysine	67.7
Arginine	49.1
Glucosamine	696.7
Galactosamine	40.8

[1] Not detected.

was obtained by determining the minimum protein concentrations at which each fraction would inhibit mucociliary activity. Ten serial dilutions, from 500 to 50 μg/ml, were prepared from DEAE cellulose fractions of eight homozygotes and seven heterozygotes, and from Sephadex G-50 fractions of three homozygotes and three heterozygotes. The greatest dilution of each fraction that retained its capacity to inhibit mucociliary activity was specified as the minimum inhibitory protein concentration. The average minimum inhibitory protein concentration of the DEAE fractions from the seven heterozygotes was 346 ± 89 μg/ml, which was assigned a value of 1 inhibitory unit. The inhibitory units of the

Table 5. *Urinary fractions containing the CF mucociliary inhibitor: minimum concentrations[1] necessary for inhibition of ciliary activity and calculated inhibitory units*

	Homozygotes		Heterozygotes		
Urinary fractions	Minimum concentration	Inhibitory units	Minimum concentration	Inhibitory units	Inhibitory units, ratio heterozygous:homozygous
DEAE fraction	175 ± 43 μg/ml[2]	1.98	346 ± 89 μg/ml[3]	1	0.51
Sephadex G-50 fraction	96 ± 6 μg/ml[4]	3.6	223 ± 32 μg/ml[4]	1.5	0.42

[1] Protein concentrations were determined by the Lowry technique. By examinaing serial dilutions on oyster gill cilia, the protein concentration of the greatest dilution to retain the capacity to inhibit mucociliary activity was stipulated as the minimum concentration. Inhibitory units were calculated on the basis of the minimum concentration of the heterozygote DEAE fraction being 1 unit.

[2] Averages of minimum protein concentrations of fractions from eight individuals of this genotype (± S.D.).

[3] Averages of minimum protein concentrations of fractions from seven individuals of this genotype (± S.D.).

[4] Averages of minimum protein concentrations of fractions from three individuals of this genotype (± S.D.).

other preparations were calculated based on 346 μg/ml being 1 unit. These results are summarized in Table 5. Fractions from DEAE cellulose chromatography of the preparations from homozygotes required approximately 175 μg/ml, or one-half the concentration of fractions from heterozygotes' urine. A similar ratio, 0.42, was observed when the inhibitory units of Sephadex G-50 fractions from heterozygotes were compared with those from homozygotes.

DISCUSSION

CF is the most prevalent chronic inborn error of metabolism affecting Caucasians. Detection of the heterozygote, and antenatal diagnosis of the condition in the fetus, will dramatically improve genetic counseling, which is now limited to providing calculations of risk (11). A large variety of biologic systems has been used to search for an unambiguous laboratory means of detecting an enzyme deficiency or an abnormal function caused by a circulating factor in CF, but a screening technique is as yet unavailable. Whether any of the factors that are responsible for biologic reactions in the assays studied will be useful as diagnostic clinical tests for CF remains unknown. Two observations actuated the purification and characterization of the urinary mucociliary inhibitor: the 2:1 concentration of the mucociliary inhibitor in plasma, urine, and tissue culture medium of cells derived from cystic fibrosis homozygotes and obligate heterozygotes, and the absence of biologic activity in analogous fractions from normal controls (8, 12).

In the current study, the CF cationic mucociliary inhibitor has been purified from urine by ion exchange chromatography, gel filtration, lectin affinity chromatography, isoelectric focusing, and high performance liquid chromatography. Unlike enzyme purification, mucociliary inhibition could not be quantitated after every purification step because of the limited amount of material and the variable nature of the biologic assay (12). Each fraction from all purification steps was examined, however, for biologic activity, and the same fractions from preparations of different subjects of the same genotype gave consistent results.

The molecular size of the cationic mucociliary inhibitor was estimated to be in the range of 4,000 to 13,500 MW, by its elution on Sephadex G-50, and between 7,500 and 12,750 MW, by urea-SDS polyacrylamide gel electrophoresis. The molecular size of the urinary inhibitor is in agreement with that reported in previous studies, which estimated the CF mucociliary inhibitor in pooled plasma from CF heterozygotes and homozygotes to be in the range of 6,000 to 11,000 MW (13, 16), and 1,000 to 10,000 MW (18). These values are also in the range of molecular weight estimation for the CF mucociliary inhibitor reported in fibroblast and lymphocyte medium by Barnett *et al.* (5), Danes *et al.* (20), Conover *et al.* (18), and Beratis *et al.* (7).

The cationic property of the glycoprotein purified from urine in CF genotypes has been observed in a unique protein fraction identified in plasma of cystic fibrosis homozygotes by Wilson and Fudenberg (33) and by Altland *et al.* (2). In examining urinary fractions, however, we have been unable to detect an isoelectrically focused protein pattern unique to cystic fibrosis.

In the current study, in addition to the cationic mucociliary inhibitor, an anionic mucociliary inhibitor was also detected in the urinary fraction isoelectrically focused between pH 4.55 and 4.9, which confirmed results from fractionating pooled plasma of heterozygotes (16). The occurrence of two inhibitory fractions may be due to a posttranslational alteration of a single polypeptide chain. Immunologic analyses of serum and urine fractions containing mucociliary inhibitors have recently indicated shared immunologic determinants among these proteins (6a).

It is of interest that Wilson and Fudenberg (34) found an anionic plasma fraction from subjects with bronchial asthma that inhibited cilia. They suggested that this was a distinct molecular species and was not related to the CF protein. Two of the controls used in the current study, however, were from young non-CF subjects with bronchial asthma. None of the isoelectrically focused fractions in urinary preparations from these controls inhibited mucociliary activity.

The identity of the mucociliary inhibitor as a glycoprotein was established in the current study by affinity chromatography on *P. lunatus* lectin, by radiolabeling the carbohydrate with galactose oxidase and tritiated sodium borohydride, and by determining the presence of a large concentration of glucosamine and small amounts of galactosamine by amino acid analysis. Amino acid analysis of the heterogenous glycoproteins constituting the cationic mucociliary inhibitor indicates that the multiple bands observed after isoelectric focusing, SDS electrophoresis, and high performance liquid chromatography may reflect variations in carbohydrate composition occurring in one polypeptide chain.

The amino acid analysis of the purified major component of the cationic mucociliary inhibitor reveals that the glucosamine concentration represents a high percentage of the composition of the glycoprotein. The aspartic acid and glutamic acid must be present mainly in their amide forms. The high concentration of glucosamine indicates that asparagine must be highly glycosylated. The presence of small amounts of galactosamine may suggest O-linkages through serine or threonine residues of the polypeptide.

Alhadeff (1) reviewed the implications of glycoprotein metabolism in CF and suggested that the unique factors associated with cystic fibrosis may be products of abnormal glycoprotein metabolism, which results from a defect in specific glycosyltransferases. The mucociliary inhibitor, for example, could represent a normal protein that had been processed in an altered posttranslational event, thus gaining its peculiar biologic activity. It will be important in this regard to investigate the carbohydrate moieties of the cystic fibrosis mucociliary inhibitor and any analogous counterpart found in normal control urine and plasma.

The significance of the current study will relate to the availability of purified preparations of the cationic mucociliary inhibitor to serve the purpose of constructing antibody reagents. If antigenic determinants unique to the CF genotype can be identified, there will be some promise for a means of heterozygote detection and prenatal diagnosis. A similar antibody preparation obtained after injecting animals with the heterogenous cationic serum fraction from cystic fibrosis subjects has been described (25).

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37. Requests for reprints should be addressed to: Dr. Barbara H. Bowman, Professor and Chair, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550 (USA).

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