STIMULATION OF SECRETORY ACTIVITY BY CF FACTOR

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY IN MOLECULAR

BIOLOGY IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

COLLEGE OF NATURAL AND SOCIAL SCIENCE

DEPARTMENT OF BIOLOGY

BY

MARIE WRIGHT WOOTEN, B.S.

DENTON, TEXAS

AUGUST, 1983

The Graduate School

Texas Woman's University

Denton, Texas

July 8, 1983

We hereby recommend that the dissertation prepared under our supervision by Marie Wright Wooten

entitled Stimulation of Secretory Activity by CF Factor

be accepted as fulfilling this part of the requirements for the Degree of Doctor of Philosophy.

Committee:

Michael J. Rudick
Chairman

Lorna Glasscock

Victoria J. Rudick

Accepted:

Provost of the Graduate School

ACKNOWLEDGMENTS

There is no one individual that has supported this effort, rather it has been the contributions of several associates.

I owe a debt of gratitude to Dr. Michael J. Rudick, my mentor and committee chairman, who was always a source of valuable suggestions, support, inspiration and who provided me throughout my graduate studies with a 'drive' to accomplish my goals.

I wish to thank my committee members: Dr. Victoria Rudick, Dr. Lynda Uphouse, Dr. Gunter Gross, Dr. Alan Cockerline, and Dr. Elaine Jacobson, for providing criticism, advice, training, and stimulating discussion concerning this research.

I am indebted to Dr. Louise Higgins for her help in the EM studies and for the many hours she spent in the darkroom on my behalf. Also, I wish to express my appreciation to Ms. Laurie Hammett, my typist, for her excellent work and preparation of the format for the dissertation.

I am especially grateful to my husband and best friend, Michael, for providing encouragement and understanding of

my 'missionary work', and also my parents, Roy and Lucretia, for just being there and always believing in me.

Last but not least, I wish to acknowledge a very special friend and associate, Dr. Ken Davis, who "sparked" my interest in the field of biochemistry and research.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure | Page

INTRODUCTION

Cystic fibrosis (CF) is the most common genetic disorder among caucasian populations. It has a reported frequency of 1 in 1600 births (Bowman, 1976a and b; Bowman and Barnett, 1976; Mangos, 1976) or some studies have suggested a frequency as high as 1 in 500 births (DiSant'Agnese and Davis, 1976; Mangos, 1976). There is evidence to suggest that CF is inherited as an autosomal recessive (Bowman and Barnett, 1976c; Sing et al., 1982). Therefore, the Hardy-Weinberg Law would predict that 1 in 16 caucasians is a carrier, if it is the expression of a single aberrant allele (Sing et al., 1982). Although CF does occur in other races, it has a much lower frequency, approximately 4 per million births among orientals and blacks (DiSant'Agnese and Davis, 1976). Clinical manifestations of the disease include: dysfunction of exocrine glands, pulmonary disease, gastrointestinal involvement, and elevated levels of sweat electrolytes (Bowman and Barnett, 1976c; Mangos, 1976; Davis and DiSant'Agnese, 1980). Generally, CF individuals suffer from hypersecretion of mucus into airways and gastrointestinal tract by the epithelial cells which line these organs. Often this leads to nutritional problems due to malabsorption, pulmonary disease in advanced stages,

and chronic respiratory failure. However, there is a considerable amount of variability of expression among CF individuals, manifesting one or several symptoms. The most well documented aspect of the disease is the apparent alteration in glycoprotein metabolism from which other symptoms are derived, contributing to the majority of deaths (Aldaheff, 1978).

The obstruction of organ passages produces most of the clinical manifestations of CF. However, there is some evidence that exocrine glands are normal morphologically before onset of the secondary effects of the disease (Boat and Cheng, 1978; Davis and DiSant'Agnese, 1980). The mucus secreted by these individuals is abnormally viscous (Boat et al., 1977; Boat and Cheng, 1978; Clamp and Gough, 1979; Frates et al., 1983), highly sulfated (Alhadeff, 1978; Boat and Cheng, 1978; Frates et at., 1983), and contains calcium ion associated with the negatively charged sialic acid residues causing precipitation of the mucus (Boat and Cheng, 1978; Frates et at., 1983; Gugler et al., 1967). However, it has been difficult to imagine how these symptoms are related to the presence of abnormal sweat electrolytes. Some studies have postulated that the sweat gland itself being impermeable to Cl^- is abnormal thus leading to elevated sweat Cl^- (Katz, 1978; Moriarty et al., 1982; Quinton,

1983). In the normal individual sweat chloride is found in levels ranging from 20 to 40 meq/l, and in the CF patient this level rises to 70 meq/l (Katz, 1978). Although impermeable to certain ions, the gland morphology has been shown to be normal (Katz, 1978). Therefore, any presumptive model concerning CF would have to attempt to explain this consistent clinical finding.

Research regarding CF has been extensively reviewed (DiSant'Agnese and Davis, 1976; Bowman and Barnett, 1976c; Bowman, 1976a and b; Mangos, 1976). Most of it has focused on the search for a single aberrant protein to account for the disease symptoms, either an abnormal functional protein, or a defective macromolecule. However, the CF literature, although vast, contains many contradictory and inconclusive studies. Most of these have been conducted on relatively small populations of CF individuals with insufficient controls, and few or no heterozygotes. Moreover, control subjects which could be mistaken for heterozygotes were not considered. Therefore, it has been difficult to make any conclusions regarding clinical symptoms and biochemical data in relation to the genetics of CF. Possibly the most well documented and consistent findings have been the discovery of factor(s) which is(are) thought to be responsible for these widespread dysfunctions (Nagy _et al_., 1979).

Assay procedures have been employed using irregularity in ciliary rhythm as a means to assess the presence of the active factor (Conover et al., 1974; Carson et al., 1976; Danes et al., 1973; Bowman, 1976a and b). Investigators have utilized two bioassays to detect and purify these factors: an oyster gill (Crassostrea virginica; Carson and Bowman, 1982) or a tracheal explant from the rabbit (Gabridge et al., 1979). It is not known whether these two bioassays respond to the same material, since each has unique metabolic requirements. Utilizing these systems factors have been isolated from serum (Carson and Bowman, 1982), urine (McNeely et al., 1982), and saliva (Impero et al., 1981) of CF patients. The factor has been shown to be associated with IgG (Carson et al., 1976; Conover et al., 1974; Harper et al., 1976), mainly IgG_1 (Carson et al., 1976; Danes et al., 1973), in a non-covalent fashion. The reason for the association is unknown. However, some studies have shown that the factor-IgG complex is not analagous to an antigen-antibody association: work by Danes et al. (1973) demonstrated that the factor is able to bind to the Fc portion of the heavy chain and not to the antigen binding sites (Fab). After treatment of the factor-IgG complex with either guanidine-HCl (Carson and Bowman, 1982) or urea (Blitzer and Shapira, 1982a), a low

molecular weight active peptide can be obtained in relatively pure form (Blitzer and Shapira, 1982a). The factors have been shown to have some characteristics in common: they are heat and pH labile (Spock *et al*., 1967; Bowman *et al*, 1973; they are cationic (Barnett *et al*., 1973b); they contain carbohydrate residues (Blitzer and Shapira, 1982a), and thus are glycoprotein in nature. The oligosaccharide chain has been shown to have an altered carbohydrate composition when compared to that on the normal serum counterpart (Blitzer and Shapira, 1982a) and this may be a generalized defect in glycoprotein metabolism which affects the CF patient (Aldaheff, 1978). Also, isolated factors have reported molecular weights between 4,500-11,000d (Blitzer and Shapira, 1982a). This wide range in apparent molecular weight has been explained by the existence of dimers depending upon the pH and ionic strength of the buffer used in isolation (Blitzer and Shapira, 1982a).

In an attempt to find an *in vitro* system to study production and secretion of factor several workers have employed mononuclear leukocytes (Wilson *et al*., 1981), fibroblasts (Bowman *et al*., 1973; Barnett *et al*., 1973a) and amniotic fluid cells (Beratis *et al*., 1973) from CF patients. All cell types have been reported to secrete

proteins (factors) which induce ciliary dyskinesis in each of the bioassays (Wilson et al., 1981; Beratis et al., 1973; Bowman et al., 1973; Barnett et al., 1973a). However, particularly with the use of fibroblasts have come conflicting data as to whether these cells actually produce CF factor and, if they do, there is little or no clear information on the requirements for its biological activity.

There are several contradictory reports within the literature. For example in the purification of these factors from in vitro sources some have found that the addition of IgG to the incubation medium is a prerequisite for the ciliary-inhibitory activity (Conover et al., 1976; Beratis et al., 1973), while others do not find this to be the case (Conover et al., 1973; Tegner et al, 1981; Tegner and Toremaln, 1981). Thus, the relationship between IgG and factor is unclear. It has been shown that normal cells secrete a factor which does not alter ciliary rhythm (Carson et al., 1976; Bogart et al., 1977; Bogart et al., 1978; Bowman et al., 1973; Bowman and Barnett, 1976c; Carson and Bowman, 1982). Furthermore, it has been assumed that the fibroblast protein has many properties in common with the serum counterpart based upon the IgG requirement, but, in fact, there has not been sufficient documentation to either support or refute this assumption.

However, the CF patient retains normal ciliary function and, therefore, findings regarding factors based on ciliary-inhibitory activity bear little, if any, relationship to the etiology of CF.

Some reports have indicated the presence of yet another activity associated with factor, that is a mucus stimulatory activity (Seale et al., 1980; Nagy and Sturgess, 1976; Kulandsky et al., 1980; Boat et al., 1982; Fleming and Sturgess, 1981), and it is this activity which could be related to one of the major clinical symptoms of CF, hypersecretion of mucus. No factor from CF fibroblast medium has been shown to possess this mucus stimulatory activity (Conover et al., 1973; Conover et al., 1976); however, it has not been made clear, in part because of the bioassay employed, whether mucus stimulatory activity and ciliotoxic activity are attributes of one and the same factor. The generalization which can be drawn from the literature is that ciliary-inhibition activity is a serum complement induced phenomenon bearing no apparent relationship to CF (Kennedy et al., 1982; Sanderson and Sleigh, 1981), while the mucus stimulatory activity may be specific for the CF allele, since it does bear some relationship to the disease symptoms. Still any basic defect must be demonstrated to be related to the presence of an abnormal gene, and not merely a secondary consequence of the disease.

CF is probably best classified as an epithelial disease and, therefore, the putative factor presumably should have its effect upon epithelial tissue. Boat et al. (1982) have developed a system for quantitating release of glycoproteins labeled to equilibrium with $[^{35}SO_4]^{=}$ and $[^{3}H]$ glucosamine from rabbit tracheal explants and have demonstrated that 50% CF serum induces release of mucus over that of control serum. Furthermore, they were able to demonstrate with partially fractionated serum that IgG was not the serum component involved in the stimulation of mucus release.

Some investigators have also proposed that the factor may be responsible for increased calcium uptake by tracheal explants (Bogart et al., 1977; Bogart et al., 1978; Conover and Conod, 1978). This could explain the ciliary-inhibitory phenomenon attributed to CF factor, since calcium ion influx has been reported to arrest ciliary activity (Walter and Satir, 1978). Moreover, there is well documented evidence from numerous systems (e.g. amylase secretion (Eimerl et al., 1974) and histamine release (Kagayama and Douglas, 1974)) which demonstrates that calcium uptake mediates exocytosis leading to increased secretion (Cochrane and Douglas, 1974; Douglas, 1981; Eimerl et al., 1974; Foreman et al., 1973).

Calcium is a ubiquitous intracellular regulator of secretion (Selinger et al., 1974; Rubin, 1982; Foreman et al., 1973). Using fibroblasts and leukocytes derived from CF individuals it has been shown that these cells accumulate more calcium than normal cells and calcium accumulation by normal cells can be increased to CF levels by exposure to CF serum (Feigal and Shapiro, 1979). Ionophores are agents which alter the membrane permeability to certain ions (Foreman et al., 1973), and, since CF serum apparently increases intracellular levels of calcium, some investigators have suggested an ionophore-like effect produced by the CF factor (Feigal and Shapiro, 1979; Bogart et al., 1977; Bogart et al., 1978). In each case serum from heterozygotes caused an intermediate increase in the levels of calcium.

Adrenergic receptors have been studied due to the role they play in intracellular calcium relocation and control of secretion (Quissell and Barzen, 1980; Kawai and Arinze, 1983). CF leukocytes demonstrated a decreased response to cAMP after β-adrenergic stimulation. The response, however, was not a consequence of decreased receptors (Davis and Laundon, 1979), but, rather, a defect in the coupling of β-receptor and adenylate cyclase. Studies regarding β-adrenergic responses in CF fibroblasts were contradictory

due to within-line variability of the cells, and thus, no consistent findings could be made (DiSant'Agnese and Davis, 1976).

Although the factor literature has had a history of controversy, contradictions, irreproducible results, and subjective bioassays, the consistent observation is that in fact there is some component of serum and possibly other body secretions (urine and saliva) which does have a presumptive role in the etiology of CF. The question remains, what is the source of factor? Much work has been done on the protease-antiprotease interactions of CF serum (Harpel *et al*., 1973; Parsons and Romero, 1980). CF factors would be present as a result of deficiencies in proteolytic activity that would normally break them down and/or inactivate them. To further support this idea, CF patients have been reported to have a deficiency in arginine esterase activity because of an abnormal interaction of serum proteases with α_2-macroglobulin (α_2M) which normally converts them from proteases to peptidases resulting in an altered specificity (i.e., their specificity is altered from large substrates to small). This may account for the existence of 'CF-factors' which are reported as small peptides. Normally, they would be degraded by proteases converted to peptides by α_2M but in CF, due to abnormal protease-antiprotease interactions, this degradion would not occur.

To advance this hypothesis Shapira et al. (1976a and b; 1977a and b) and Wilson and Fudenberg (1976) have documented an abnormal α_2M in the CF individual, others have reported that in CF serum the IgG has a trypsin binding capacity (Romeo et al., 1978; Parsons and Romeo, 1980). In fact the IgG may bind trypsin much more avidly than α_2M and could compete for circulating trypsin in CF serum. Coupled with the observation that CF α_2M is abnormal the IgG could bind trypsin that is not capable of associating with α_2M. This has been the focus of much debate over factors which are found non-covalently associated with IgG; they do not represent an abnormal gene product but are a secondary phenomenon.

And yet another plausible explanation for the existence of the CF-factor is the organism Pseudomonas aeruginosa (PA) (Klinger et al., 1978). The CF patient is colonized with and harbors the organism either in a dormant form very early in his life, or in an active infectious form during chronic respiratory involvement. The environment of the respiratory tract coupled with the primary disease symptom, excess mucus, is such that colonization and infection are frequent. Due to the excessive mucus the less frequently found mucoid strain is associated with these infections (Klinger et al., 1978). The normal individual or the

heterozygous parent are never colonized with or harbor the mucoid strain (Dr. Robert Kramer, personal communication). These organisms produce exotoxins which could seep into the circulatory system via the close association of the alveolar walls of the respiratory system and the capillary beds of the circulatory system. Some reports have indicated that the CF individual 'builds up' antibodies to the secreted products of PA and antigen-antibody complexes form (Klinger et al., 1978). Thus, the proteins which are found associated with IgG could be a PA secreted product acting as an antigen. Although plausible, these results do not explain the existence of factor in heterozygotes, since to date no reports have shown the heterozygote individual to harbor PA of the mucoid strain, nor do they explain the presumptive association with IgG and some factor found in normal serum.

In any study dealing with the putative factor treatment of the serum with protease inhibitors specific for the type of proteins secreted by PA should rule out this possibility and also the use of heterozygotes would be essential in determining the presence of such a factor.

Whatever the source of factor, the present bioassay systems have been found to be unreliable, qualitative, and subject to great variability. Moreover, only a select

group of cells is affected in the CF individual, and so proof of the involvement of factor in CF etiology must entail a demonstration of its limited locus of action. The bioassays thus far employed have been limited by their complexity of different tissues, as well as the many cells which make up these tissues, making it very difficult to determine which cells respond to the factor. If the factor does exist, its mechanism of action would be difficult if not impossible to determine. Thus, there has been a need for a cell culture system for the detection and assay of putative factor eventually leading to elucidation of its mode of action. Also any such study should contain appropriate numbers of controls (orientals, blacks and caucasians with no family history of CF, and other diseases closely resembling CF), a large group of CF individuals (to account for the variability of the disease), and heterozygote parents so that statistical inferences can be made from the data gathered.

An epithelial system would be ideal in that CF is more generally described as a disease of epithelial origin (Boucher _et al_., 1983). Recently, a method for the isolation and culture of epithelial cells derived from the hamster trachea (HTE) has been reported (Goldman and Baseman, 1980a). These are pure cultures of epithelial cells

which proliferate *in vitro* and can be passaged retaining both their morphologic and biochemical differentiated characteristics. These cells have been further characterized by histochemical and immunocytochemical means (Rudick and Rudick, 1982). Also, they have been shown to be active in the synthesis of high molecular weight 'mucin-like' glycoproteins (Goldman and Baseman, 1980b; Rudick and Rudick, 1982; Rudick *et al*., 1983). With such a cell culture system a more detailed and quantitative study of CF factor(s) is(are) possible. Thus the HTE system would allow investigation into:

1. the stimulation of epithelial cell secretory activity by CF factor(s).
2. determination of the role the factor(s) may play in calcium permeability.
3. characterization of the mechanism of action of CF factor(s).

In this study the HTE cell system was used as a means to detect the presence of CF factor(s) by virtue of its presumed stimulatory effect on mucus secretion by these cells. This would be the first indication that a CF disease symptom can be duplicated *in vitro*.

The rationale behind the research reported herein is to study extensively a well documented feature of the disease, i.e. the presence of a circulating factor(s), in an attempt to:

1. study secretion induced by CF serum or CF fibroblast media employing ultrastructural examination of hamster tracheal explants.

2. demonstrate the presence of a unique protein from CF serum and fibroblast culture medium involved in the secretory process of the HTE cell culture system.

3. study the association or requirement for IgG in the presumptive stimulatory effect induced by CF serum.

4. study the involvement of CF factor(s) and calcium in the secretory process because of the well documented role of calcium in exocytosis.

5. show a relationship between CF factor(s) and clinical symptoms of the disease.

Lastly, pharmacological agents such as ionophore A23187 (specific for increasing the permeability of the membrane to calcium, Reed and Lardy, 1976), verapamil (specific for blocking calcium channels, Lee and Tsien, 1983), and adrenergic blockers will also be employed in an attempt to determine the mechanism of action of putative factor and its role in the regulation of HTE cell secretion.

MATERIALS AND METHODS

Materials

The following were obtained from Bio-Rad Laboratories, Richmond, CA: DEAE Affi-Gel Blue; Acrylamide; N,N,N'N'-tetraethylmethylenadiamine (TEMED); ammonium persulfate; Coomassie Brilliant Blue R-250 (CBB-250); Bio-Gel P10 (100-200 mesh); and Bio-Rad Silver Stain Kit. Ultrapure urea was from Schwartz-Mann, Inc., Spring Valley, N.Y. Sodium dodecyl sulfate (SDS) was obtained from Pierce Chemical Company, Rockford, Il. Bis-acrylamide and Kodak X-OMAT AR films were from Eastman Kodak Co., Rochester, N.Y. Aquasol, EN^{3}HANCE, $^{45}Ca^{2+}$ as calcium chloride (4-50 Ci/g) and $^{22}Na^+$ (as sodium chloride) were purchased from New England Nuclear, Boston, MA. Sheep anti-human IgG was from Cappel Laboratories, West Chester, VA. Chymostatin and L-Propanolol were from Sigma Chemical Co., St. Louis, MO. Beta Phase scintillation cocktail was from West Chem, San Diego, CA. EM-Grade glutaraldehyde (8%), OsO_4 (4%), and Mollenhauer epon araldite were obtained from EBTEC, Agawam, MA. Amicon Diaflo UM2 and UM10 membranes were obtained from Amicon, Danvers, MA. Hams F12 tissue culture medium was purchased from Gibco Laboratories, Grand Island, N.Y. Fetal calf and pooled human serum were obtained from

KC Biological, Inc., Lenexa, KA. Thermolysin was from Calbiochem-Behring Corp., La Jolla, CA. Spectropore Low Molecular Weight Cut-Off Dialysis Tubing (3,500 dalton cutoff) was purchased from Arthur H. Thomas Co., Philadelphia, PA. The following radiolabeled materials were purchased from ICN, Irvine, CA: [U-^{14}C]L-Threonine (180-220 mCi/mmole); $^{45}Ca^{2+}$ as calcium chloride (5-30 Ci/g); [4,5-^{3}H]L-Leucine (55 Ci/mmole); and [G-^{3}H]L-Threonine (22 Ci/mmole) was obtained from Amersham, Arlington Heights, Il. Human skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J. The following were supplied as generous gifts: verapamil and ionophore A23187 were from Dr. Eugene Quist, Department of Pharmacology, The Texas College of Osteopathic Medicine, Ft. Worth, TX., and retinoate was from Dr. Aaron Heifetz, Department of Biochemistry, University of Texas Health Science Center, Dallas, TX. All other chemicals were reagent grade or the highest purity available.

Methods

Fibroblast Cell Culture

Human skin fibroblasts (GM 3349 and GM38 normal human fibroblasts; GM 1348, 142 and 770 cystic fibrosis fibroblasts all having classical symptoms of CF) were routinely

grown in 100 mm culture dishes at 37°C in a humidified 95% air- 5% CO_2 atmosphere.

Cells were monitored for mycoplasma using the Hoechst reagent (Chen, 1977). To determine cell number the medium was removed from the dish, the cells were washed twice with 2-3 ml of phosphate buffered saline containing glucose (PBS-glc: 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 2.0g glucose per l, pH 7.4) and then detached from the surface of the dish with 2 ml of 0.02% EDTA in PBS-glc. After 5-10 min., the cells were diluted with either an equal amount of growth medium or with PBS to a maximum density of 8,000 cells/ml. The cell number was determined with a Coulter Particle Counter (Coulter Electronics Co.) equipped with a 100 μm orifice.

Secreted Proteins of Fibroblast in Culture

Prior to collection of secreted proteins from near confluent fibroblast cultures the growth medium was removed and replaced with growth medium lacking fetal calf serum. The cells remained in serum-free conditions for 48 hrs. after which the medium was collected. The Ham's F12 components were removed by Bio-Gel P10 column chromatography. The secreted proteins were radiolabeled by the addition of [^{3}H]leucine (2μCi/ml) during the serum-free period.

Bio-Gel P10 was employed to removed Ham's F12 components from serum-free medium containing fibroblast secreted proteins. The medium was applied to a Bio-Gel P10 column (2.5 cm X 35 cm) and eluted with PBS, pH 7.4. The eluted fractions were monitored for absorbance at 220 nm and for radioactivity by counting 1μl samples in Aquasol by liquid scintillation spectrometry. Those proteins which eluted at the void volume (molecular weight $\geq$10 kd) and those in the included volume (molecular weight $\leq$10 kd) were dialyzed against water using Spectropore dialysis tubing (low molecular weight cut-off (3,500 daltons), followed by lyophilization. The column was calibrated using bovine serum albumin (64 kd), soybean trypsin inhibitor (28 kd), lysozyme (14 kd) and bovine trypsin inhibitor (6 kd) at concentrations of 500 μg each prior to the elution of experimental material.

Tracheal Organ Cultures

Male Golden Syrian hamsters less than 4 months of age were used as tracheal donors. Animals were anesthetized with CO_2. Hamster tracheae were surgically removed under sterile conditions, as described by Collier (1976). After excision the tracheae were placed in PBS, and any excess connective tissue was gently removed with forceps. The cleaned tracheae were then placed into Ham's F12 medium,

cut into rings, and transferred to F12 plus 10% FCS and incubated at 37°C in a humidified 95% air-5% CO_2 atmosphere for a period of 24 hrs. prior to experimental treatment. Rings were examined microscopically for damage before use. For experimentation rings were placed into 35 mm culture dishes with Ham's F12 medium and test protein. Samples were taken with time and prepared for observation with either the scanning electron microscope or the transmission electron microscope.

Scanning Electron Microscopy

After being exposed to test protein in F12 medium, rings were washed in PBS and fixed in 3% glutaraldehyde-PBS, at 4°C overnight. The fixed rings were washed twice in PBS, and transferred to 1% OsO_4 in PBS for 1h at room temperature. The osmicated tissue was briefly rinsed in water before being dehydrated through an ethanol series (70-100%), and then placed in a critical point dryer, using CO_2, overnight. After drying the rings were mounted and coated with gold palladium. Samples were observed with the AMR 1200 scanning electron microscope (Amray, Inc.).

Transmission Electron Microscopy

After being exposed to test protein in F12 medium, rings were washed in McDowells buffer, pH 7.4, followed by post-fixation in 2% osmium made up in cacodylate buffer,

pH 7.4. The tissue was then dehydrated through an ascending ethanol series (50-100%) after which the tissue was placed first in 1:1 absolute ethanol/propylene oxide, second in propylene oxide, third in 1:1 epon/propylene oxide, and fourth in epon. Each step was carried out for 5-10 min. at room temperature. Polymerization was accomplished at 60°C overnight. Ultrathin sections were obtained using a Sorvall MT2-B microtome. The sections were mounted on copper grids, poststained with saturated aqueous uranyl acetate for 30 min. and with lead citrate for 10 min., and were then examined with a Siemens 101 electron microscope (Siemens, Corp.) at 80 kv.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) slab gels were prepared according to Laemmli (1970), using a discontinuous buffer system. The acrylamide to bis-acrylamide ratio was 37:1 and polymerization was initiated by the addition of TEMED and ammonium persulfate to final concentrations of 0.025% v/v and 0.03% w/v, respectively. The stacking gel was 3% acrylamide containing 0.125M Tris-HCl, pH 6.8, and 0.1% SDS. Electrode buffer was 0.02M Tris-0.192M glycine, pH 8.3, containing 0.1% SDS.

For electrophoresis of serum proteins, which contain species of various molecular weights, linear acrylamide

gels (7-20%) were prepared, while for analysis of high molecular weight species a 4-10% linear gradient was routinely used. The gradient gels were prepared by mixing equal volumes of complete gel mixtures to achieve the desired type of gradient. Acrylamide stock, gel buffer, and water were mixed to give the desired concentration of the upper portion of the gradient, i.e. 20%. This solution was placed in a small flask with a small magnetic stir bar; the flask was then packed in ice in a small plastic box and placed onto a stir plate. The same procedure was carried out for the lower portion of the gradient, while this flask was placed on top of the packed ice. The ice packing was used as a means of slowing down the polymerization reaction while casting the gradient. An LKB peristaltic pump (LKB2115 Multiperpex) equipped with dual channels was used to generate a linear gradient and insure equal mixing and constant flow during the casting period. One end of the tubing was placed into the high concentration of acrylamide mixture and the other end in between the plate to which was attached a microliter pipet tip; while the other tubing was placed into the low concentration of acrylamide mixture and its opposite end into the high concentration of acrylamide. At this time the stir plate was turned on to a very low speed while TEMED and ammonium persulfate were added to the

volumes of gel mixtures, after which the pump was turned on and the acrylamide mixture was allowed to flow between the plates until the gradient was completely formed. After polymerization a stacking gel was formed, as previously mentioned.

Samples were prepared for electrophoresis by using a 2:1 v/v ratio of dissociating solution consisting of 800 µl 0.125M Tris-HCl, pH 6.8, 400 µl 10% SDS and 50 µl beta-mercaptoethanol. The samples were boiled for 3 min. after which 10-25 µl of glycerol and 2-5 µl of a 0.1% bromophenol blue tracking dye was added to each sample followed by vortexing.

After sample application, electrophoresis was carried out at 15mA during stacking and then at 30mA until the bromophenol blue tracking dye reached the bottom of the gel.

Gels were fixed with 50% trichloroacetic acid (TCA) overnight, then stained with 0.1% Coomassie Brilliant Blue R-250 in 50% TCA for 1h at 37°C and destained by repeated washings in 7% acetic acid, as described by Laemmli (1970).

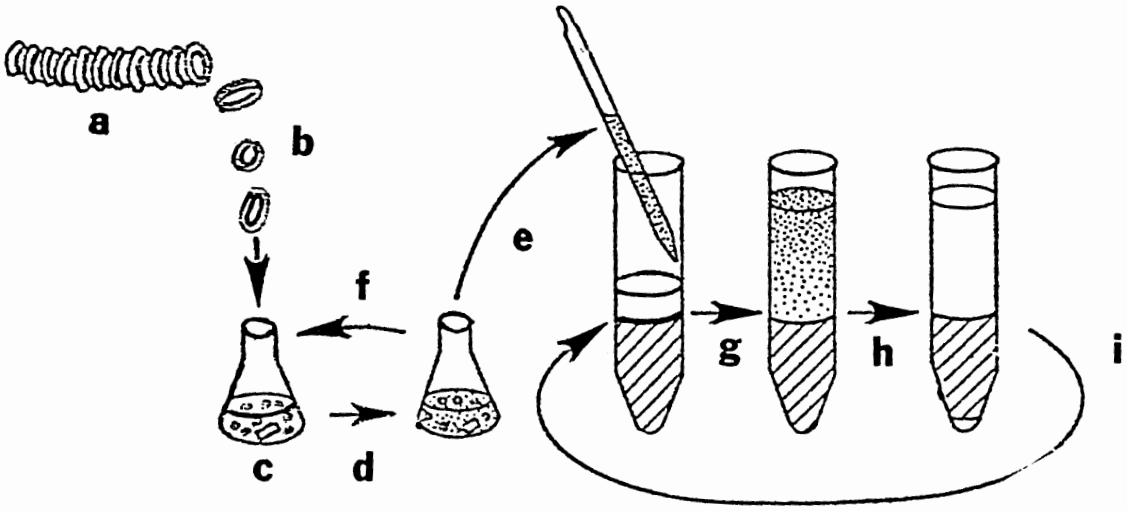
Alternatively, nanogram amounts of proteins in the gel were detected with the silver stain. Gels were fixed with 10% TCA-10% acetic acid-40% methanol with constant shaking at room temperature overnight. The fixing solution was removed and replaced with oxidizer for 5 min., followed by

numerous washes in ultrapure water for at least one and a half hours. The gel was then placed in silver reagent for 5 min., followed by a quick ultrapure water wash and immediately placed in developer. The developing reaction was stopped, when protein bands reached a desired intensity, by placing a 5% acetic acid wash onto the gel. All solutions were made in ultrapure water, as chloride ion interferes with the developing step, and carried out at 37°C in a shaking water bath, as described in the Bio-Rad Silver Stain Kit (Bio-Rad Laboratories).

Detection of Radiolabeled Proteins (Fluorography)

Radiolabeled proteins resolved by slab gel electrophoresis were detected by fluorography. The destained gel was impregnated with EN^{3}HANCE scintillant (New England Nuclear). The treated gel was placed in a solution of 5% glycerol, 5% polyethylene glycol, 5% methanol and 10% acetic acid, agitated for 15 min., and then removed and dried under vacuum with heat (60-70°C) on a slab gel dryer (Savant SGD-200 Slab gel dryer). The dried gel was overlaid with Kodak X-OMAT AR X-Ray film and kept at -80°C for 2 wks. to 1 month before developing. The film was developed using Kodak X-ray developer for 5 min., followed by a 30 sec. stop bath. The film was fixed using Kodak fixer for 10 min. and then was extensively washed with tap water for up to 15 min. The film was then allowed to dry.

Figure 1. Procedure for obtaining pure populations of hamster tracheal epithelial cells (HTE). 'Dissociated Cell Rescue' technique of Goldman and Baseman (1980a).


a
b
c
d
e
f
g
h
i

Hamster tracheal epithelial cell cultures (HTE)

Tracheae were asceptically excised from 1-5 male Golden Syrian Hamster, as described in the "organ culture" section, and were then cut transversely between rings (Figure 1a and b). The ring was sliced once against its membranous portion allowing it to open up slightly. This technique apparently improves cell yield. All solutions used in the isolation were prepared sterile. A stock solution of thermolysin was prepared (0.01% (v/v) in 0.1M Tris, pH 8.0, with 2mM $CaCl_2$) and frozen in 1 ml aliquots. For use the stock was diluted 1:16 with PBS. Final pH of the enzyme solution was 7.5 to 7.6. The rings were placed into a vessel containing 5 ml of the thermolysin solution with a small magnetic spin bar to agitate the rings for 15 min. (Figure 1c). Then the enzyme solution containing dissociated cells was removed from the vessel and carefully layered onto the top of a gradient (Figure 1d and e) prepared in a plastic conical centrifuge tube, as follows: PBS containing 3% bovine serum albumin w/v (BSA), 9 mg glucose, and 10^{-6}M ethylenediamine tetracetate (EDTA) on the bottom; with 3 ml of diluted thermolysin solution on the top. An additional 5 ml of diluted thermolysin was added back to the rings in the vessel and the stirring begun again (Figure 1f). The gradient (Figure 1g) was centrifuged at 400xg for 5 min.

pelleting the cells at the bottom of the tube well separated from the thermolysin in the upper layer (Figure 1h). The top 5 ml was removed and reused as enzyme solution. The entire process was repeated for a total of 5 centrifugations (Figure 1i) and the dissociated cells collected each time were combined. Finally the cells were twice carefully resuspended in 10 ml of 1% BSA in PBS and centrifuged. This is basically the dissociated cell rescue method of Goldman and Baseman (1980a) used in obtaining pure populations of epithelial cells. The isolated cells were resuspended in Ham's F12 medium containing 10% FCS and retinoate (1×10^{-7}M) and grown in a humidified 95% air-5% CO_2 atmosphere at 37°C. Cells were routinely grown in 100 mm cell culture dishes. For all experiments cultures of 5 passages or less were utilized and stock cultures were never allowed to reach confluency or remain for more than 4 days in culture before use. This ensured that the cells remained in an active metabolic state. Alternatively the cells could be cryogenically preserved. Cell number was determined, as previously described.

Collection of Sera

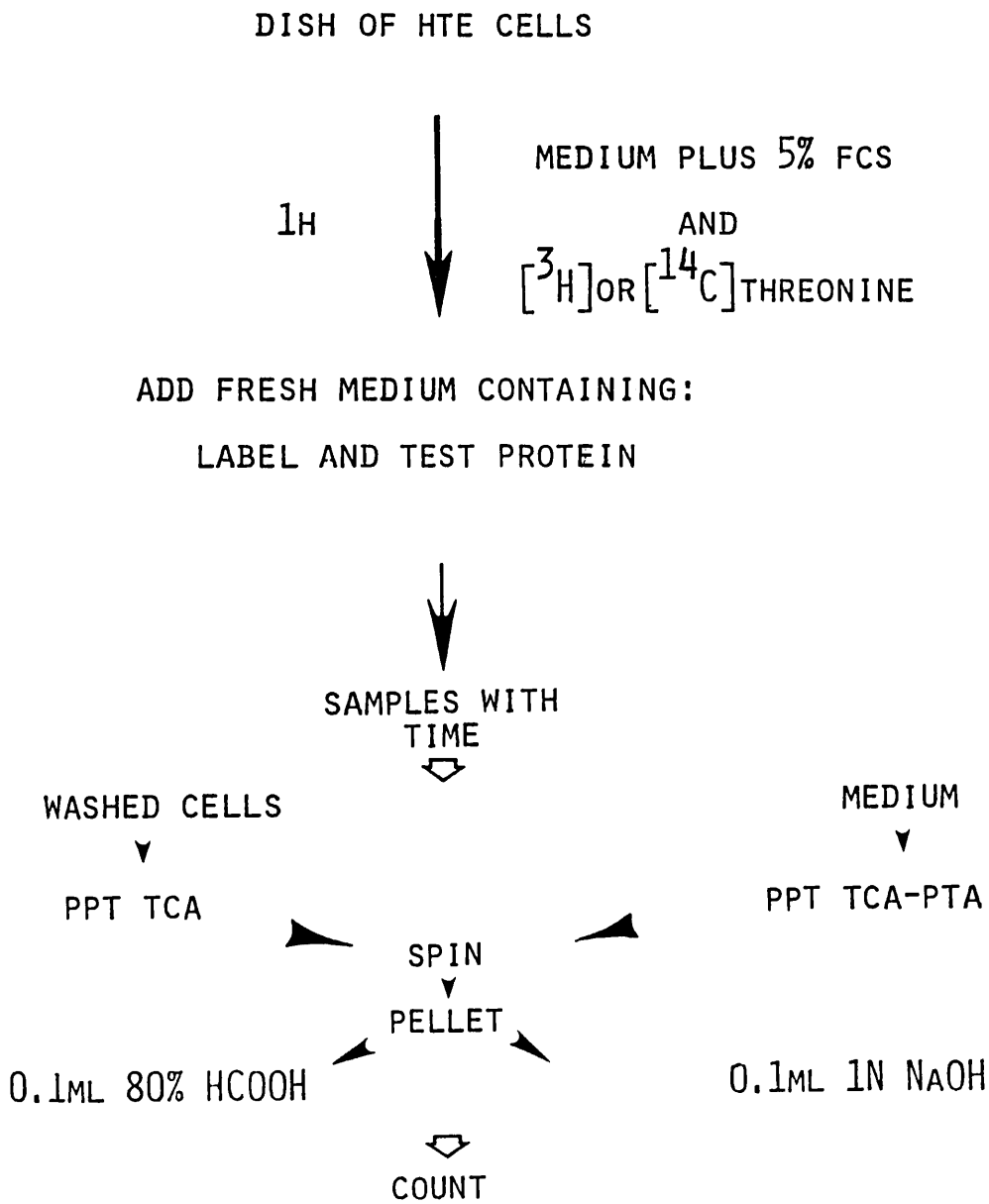
Blood samples from cystic fibrosis (CF) patients 1-30 yr. of age and having the typical respiratory and/or gastrointestinal symptoms, along with elevated sweat

electrolytes (chloride concentration greater than 70 meq/l) were collected by venipuncture under the auspices of the staff of the Cystic Fibrosis Foundation Care and Teaching Center in Dallas, TX, after obtaining informed consent from the donor or donor's parents. This study was approved by the Human Subjects Review Committee at U.T.H.S.C., Dallas, TX, and was conducted under the direction of Dr. Robert Kramer (Professor of Pediatrics, U.T.H.S.C., and Director of its CF Clinic). Blood samples from parents (obligate heterozygotes) and normal black or oriental individuals were also collected. Blacks or orientals were chosen, because the frequency of the mutant allele in these groups is low compared to the frequency in caucasians, and thus the chances of confusing heterozygotes for normals is significantly decreased. Also, sera were collected from normal healthy caucasians who were not taking any medication and had no family history of CF. Both sexes were equally represented in the study.

Whole blood samples were drawn into plastic syringes, immediately transferred into glass tubes, allowed to clot at room temperature for 15-20 min., centrifuged and the serum collected. The serum was stored at -20°C until use. Samples collected at the CF clinic were transported to TWU on dry ice.

Figure 2. Procedure of the hamster tracheal epithelial (HTE) stimulation secretion assay.

HTE SECRETION STIMULATION ASSAY



HTE Secretion Stimulation Assay

Cells of passage 5 or less were seeded into 35 mm cell culture dishes and grown to near confluency (less than 100,000 cells/dish) overnight. For all experiments cells were seeded from a single inoculum, which insured that the plate to plate variation in cell number was at a minimum. The complete medium was removed and replaced with the appropriate radiolabeled precursor (0.3 μCi/ml-0.5 μCi/ml of either [^{3}H]threonine or [^{14}C]threonine), in 0.5 ml F12 medium containing 5% FCS (Figure 2). After 1h, the medium was aspirated off the cells and replaced with fresh F12 minus FCS but containing the same radiolabeled compound and either whole serum or fractionated serum proteins from CF, heterozygous, or normal individuals at various concentrations. Routinely, a 20% serum concentration was used unless otherwise stated. Duplicate or triplicate plates were taken at various times, the medium was removed, and the cells were washed with 0.5 ml PBS. The combined medium and wash was treated with either an equal volume of cold 10% TCA-0.5% phosphotungstic acid (PTA) to precipitate all proteins, or with 1.3 times the volume of medium with alcian blue solution (0.1% in 0.05M acetate buffer, pH 5.8, containing 25mM $MgCl_2$). Hall et al., (1980) reported that alcian blue can be used to precipitate polyanionic glycoproteins. HTE

cells have been reported to be active in the synthesis of 'mucin-like' glycoproteins (Rudick and Rudick, 1982). Since mucin is highly charged and polyanionic due to the types of carbohydrate residues found within the oligosaccharide chain, alcian blue can be used to precipitate these types of glycoproteins and the radiolabeled precursor which is incorporated and precipiated by alcian blue will be referred to as 'mucin-like'.

Five percent TCA (1.0 ml) was applied to the washed cells which were then scraped from the dish and placed in an eppendorf tube with another 0.5 ml of TCA wash. Proteins were allowed to precipitate at 5°C overnight. After precipitation overnight at room temperature, alcian blue precipitates were washed twice with 40% ethanol in 0.05M acetate buffer, pH 5.8, containing 25mM $MgCl_2$, then dissolved in 100 µl of 80% formic acid. The medium precipitates were washed twice with cold 5% TCA-0.25% PTA and were dissolved in 100-150 µl 0.1N NaOH, while the cell precipitates were dissolved in 100 µl 80% formic acid. All dissolved pellets were placed in Aquasol and counted by liquid scintillation spectrometry (Beckman Model LS-9000 Scintillation Counter).

In some cases the alcian blue precipitates were dissolved in 200 µl of dissociation buffer (10% SDS, 0.125M Tris-HCl, B-mercaptoethanol) followed by SDS-PAGE and

fluorography (as described previously) to detect the types of proteins which were secreted by HTE cells.

In some experiments the following agents were included in secretion assays at various concentrations: EGTA, ionophore, calcium, verapamil or propanolol.

Uptake of $^{45}Ca^{2+}$ by HTE Cells

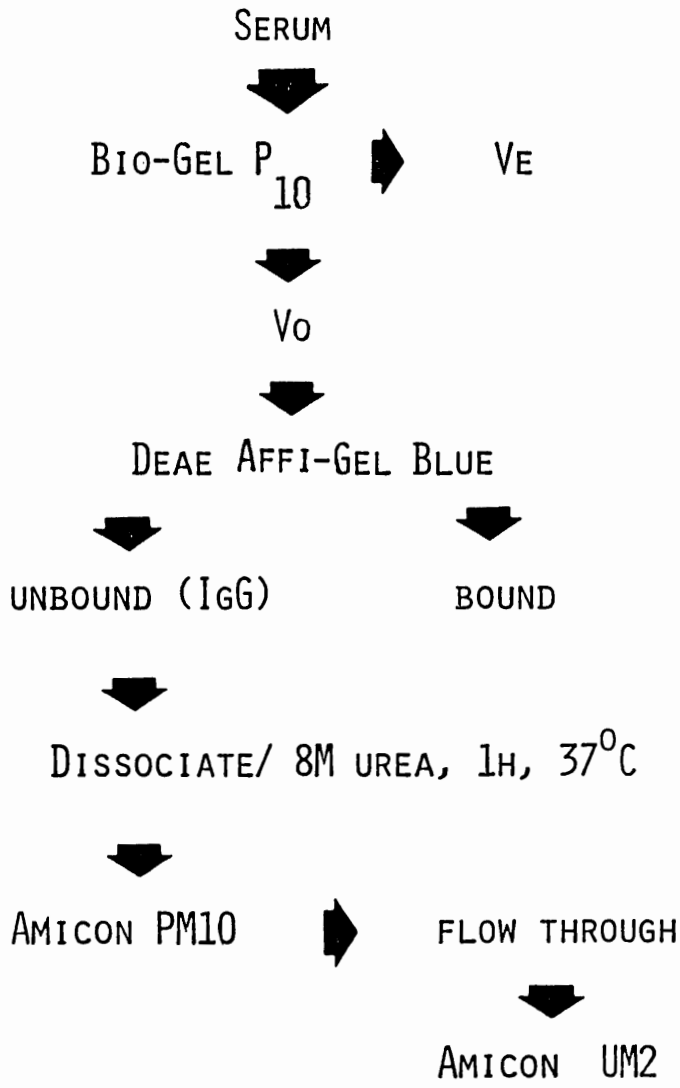
HTE cells were seeded exactly as described in the HTE secretion stimulation assay. The complete medium was removed and replaced with 0.5 ml F12 containing either whole serum or fractionated serum proteins from CF, heterozygote, or normal individuals and 5.0 μCi/ml $^{45}Ca^{2+}$. Duplicate or triplicate plates were taken at various times, the medium was removed, and the cells were thoroughly washed with an excess of PBS, at least three times. Then the following were sequentially added to each plate: 0.5 ml 1N NaOH, 0.5 ml 1N HCl, and 0.5 ml H_2O. Each solution was transferred from the plate to 10 ml of Beta-Phase scintillation fluid and counted by liquid scintillation spectrometry. In some experiments the following agents were added at various concentrations to the plates along with $^{45}Ca^{2+}$: EGTA, verapamil, ionophore, and calcium.

Sodium Transport by HTE Cells

The influx of sodium was monitored by the addition of $^{22}Na^{+}$(0.5 μCi/ml) during concomitant uptake of $^{45}Ca^{2+}$ (5 μCi/ml) as carried out for Ca^{2+} uptake experiments.

Figure 3. Isolation of IgG and dissociation of non-covalently bound proteins.

SCHEME OF PURIFICATION



Sodium efflux was observed by loading the HTE cells overnight with $^{22}Na^{+}$ (0.5 μCi/ml) in 0.5 ml of Ham's F12 medium plus 10% FCS under standard culture conditions. The medium was removed, the cells washed thoroughly with PBS and test protein was added in 0.5 ml of Ham's F12 medium. Duplicate or triplicate samples were taken at various times. The medium was collected and transferred to 10 ml of Beta-Phase scintillation fluid which was counted by liquid scintillation spectrometry.

Purification of Serum Factor

The factor associated with serum IgG was partially purified using a modified procedure of Blitzer and Shapira (1982a). The sera from CF individuals were pooled and pooled normal human serum was obtained from KC Biological, Inc. The serum from several affected CF individuals was pooled to account for possibility of heterogeneity of the disease.

The pooled sera from CF or normals (Figure 3) was applied to a Bio-Gel P10 column (5 cm X 90 cm) equilibrated with PBS. The void volume (as determined by blue dextran exclusion) containing those proteins with a molecular weight $\geq$ 10 kd were collected by monitoring the elution of the column at 280 nm while the included volume containing proteins with a molecular weight $\leq$ 10 kd was determined by the

220 nm absorbance. These proteins (P10Vo) were dialyzed extensively against an excess of 0.02M potassium phosphate buffer, pH 8.0, at 4°C. The dialyzed P10Vo was applied to a DEAE Affi-gel blue affinity column which was used to isolate serum IgG. The column was equilibrated and eluted with 0.02M potassium phosphate buffer, pH 8.0. Routinely an 8:1 ratio of gel matrix to serum was used to prepare the column. Fractions of approximately the sample size applied to the column were collected and column elution was monitored at 280 nm. The first peak of protein was collected to give eight times the volume of sample applied to the column. These unabsorbed fractions were tested against sheep anti-human IgG in double diffusion Ouchterlony plates (Ouchterlony, 1968), and were analyzed by SDS-PAGE. The proteins bound to the DEAF Affi-gel blue were recovered by elution with 1.4M NaCl in the elution buffer. The matrix was routinely reused by regenerating with 2 bed volumes of 6M guanidine hydrochloride in 0.02M potassium phosphate, pH 8.0. All column chromatography was performed at 4°C.

Dissociation of Proteins Bound to IgG

The IgG fraction from the DEAE Affi-gel blue column was dialyzed extensively against water at 4°C. The proteins were then lyophilized, and reconstituted in 0.05M ammonium

bicarbonate, pH 7.8. Solid urea (ultrapure) was added to a final concentration of 8M and stirred for 1h at 37°C.

Proteins with molecular weights less than 10 kd were collected by ultrafiltration through an Amicon PM10 Diaflo Membrane. This fraction was concentrated to 10 ml using an Amicon Diaflo UM2 Membrane (molecular weight cut-off < 1,000d). After concentration the volume of the retentate was increased to 200 ml with distilled water and this was reconcentrated to 10 ml. This procedure removed very low molecular weight components and also the remaining urea. All ultrafiltration steps were carried out at room temperature. The concentrated fraction was lyophilized and stored at -20°C until use.

The electrophoretic profile during each step of purification was assessed by SDS-PAGE (7-20% linear gradient) as described previously, and stained with Bio-Rad Silver Stain.

Protein Determination

Proteins were routinely determined by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Establishment of Hamster Tracheal Ring Bioassay

Rings which were processed for SEM after immediate removal from the hamster (Figure 4a) upon observation revealed that the luminal surface was predominately composed of ciliated epithelial cells. Ultrastructural observation by TEM (Figure 4b) revealed the luminal surface to be a pseudostratified epithelium composed of ciliated, basal, and goblet cells. The goblet cell is the mucus producing cell. Freshly dissected rings were not used in the bioassay, but were incubated overnight to allow assessment of damage. Only tracheal rings which appeared undamaged, as judged by phase contrast microscopy, and had active ciliary brush borders and lack of mucus release from goblet cells were used in the assays.

Tracheae were excised and rings prepared, as described in Methods. The rings were placed in 0.5 ml of F12 medium in 35 mm cell culture dishes and various sera were added at 25% concentration. After incubation for 1h, the rings were removed and processed for observation by electron microscopy. Gabridge *et al*. (1979) used this method to observe discharge of mucus by tracheal goblet cells. The resulting scanning and transmission electron micrographs demonstrated

Figure 4. A. Scanning electron micrograph of hamster tracheal ring taken directly after surgical removal and prior to overnight incubation in F12 medium (500x).

B. Transmission electron micrograph of hamster tracheal ring taken under conditions described in (A) (19,000x).

The luminal surface is a pseudostratified epithelium composed of mucus or goblet cells (m), ciliated cells (c), and basal cells (b).

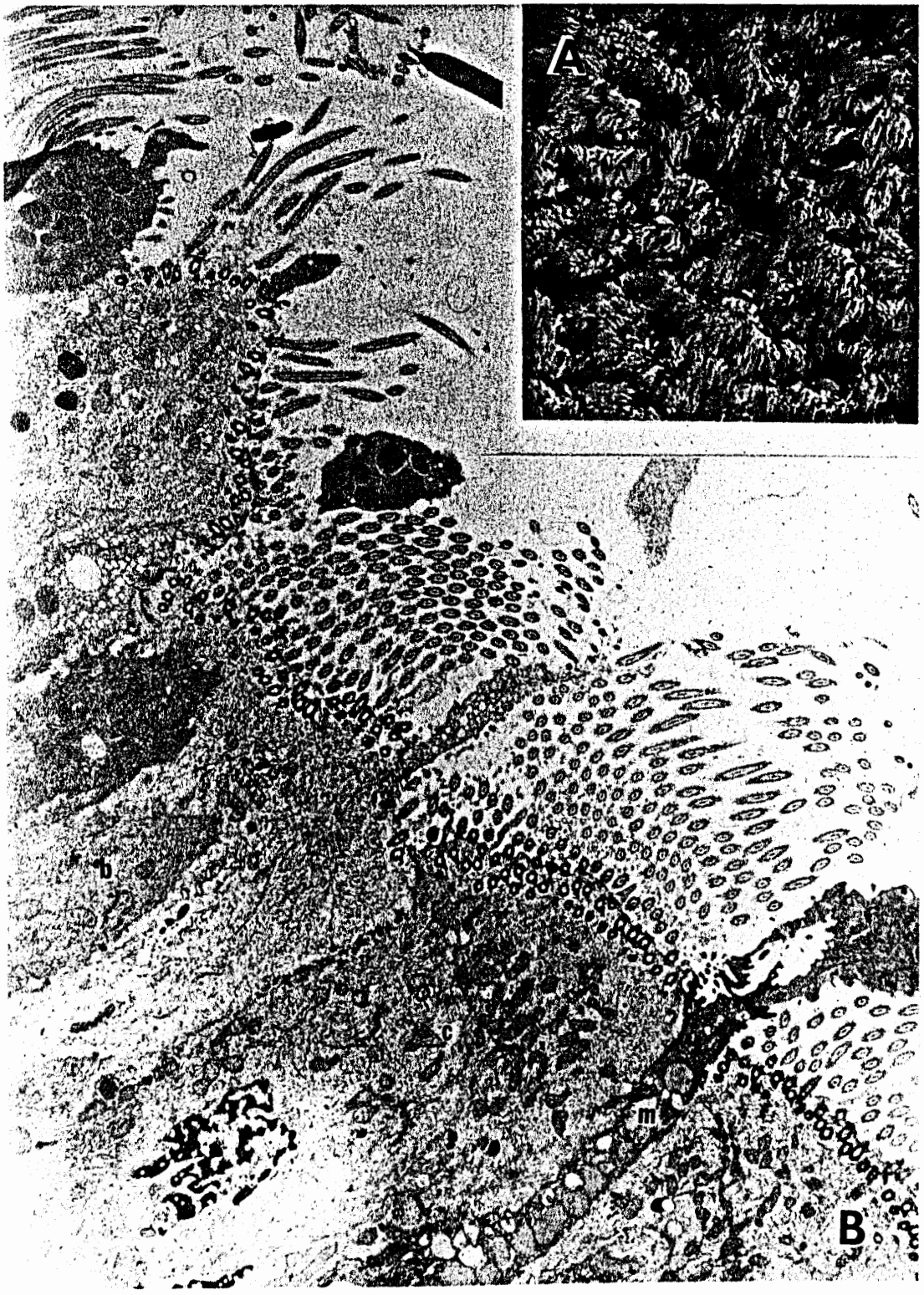

A
B
b
c
m

Figure 5. Electron micrographs of hamster tracheal rings which were exposed to 25% pooled normal human serum. Rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in 0.5 ml fresh medium plus 25% serum for 1h. The data shown are from five experiments in which duplicate rings were tested and prepared for observation.

A. SEM of ring treated with NHS (500x).

B. TEM of ring treated with NHS (19,000x).

C. SEM of ring treated with NHS (2,000x).

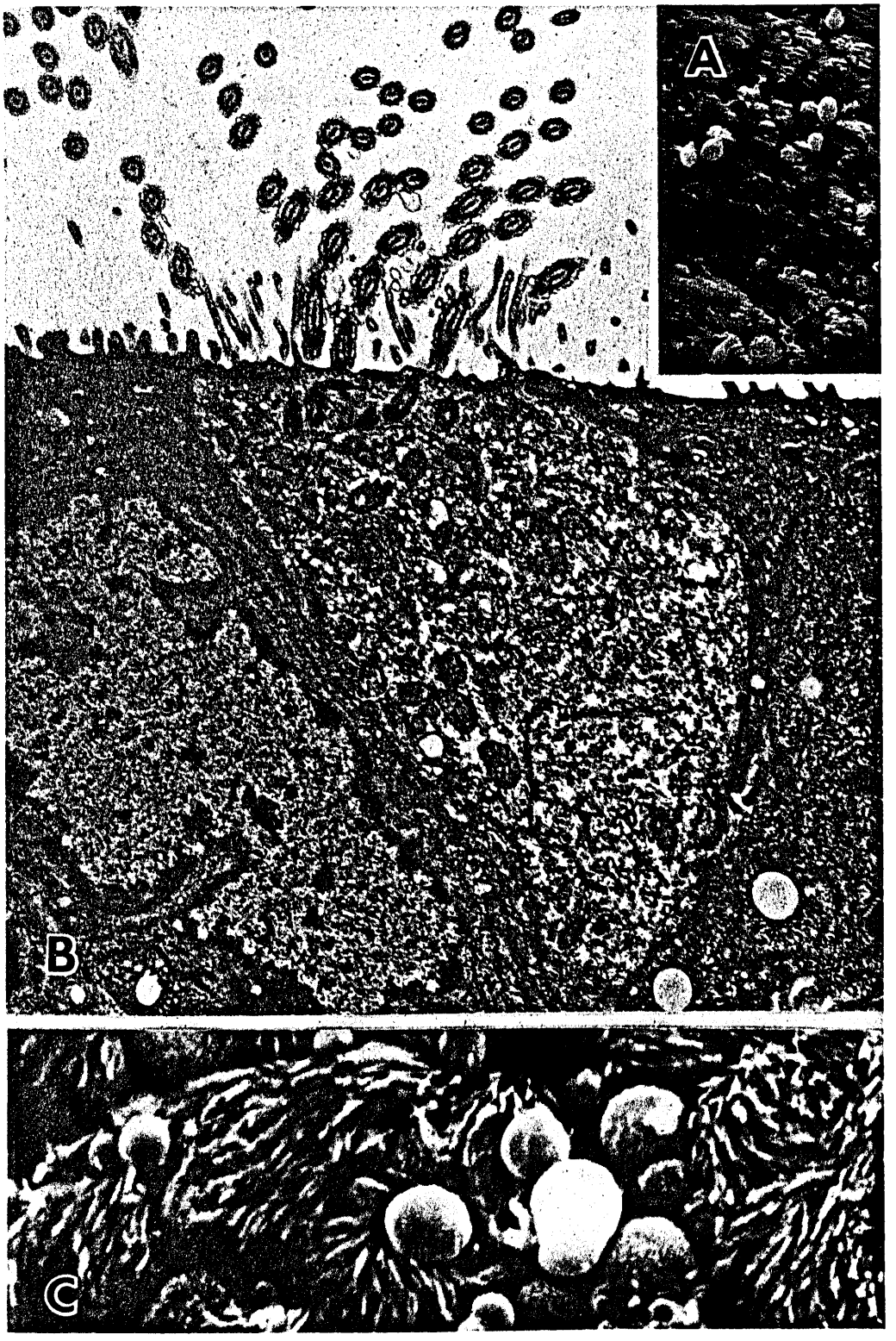

A
B
C

Figure 6. Scanning electron micrographs of hamster tracheal rings treated with pooled cystic fibrosis serum. Rings were prepared as described in Methods, incubated for 24h in Ham's F12 medium, then placed in fresh 0.5 ml F12 medium plus 25% serum for 1h.

A. Low magnification of the luminal surface of the hamster tracheal ring (200x).

B. High magnification of the same tracheal ring; arrow points to mucus droplets (1,200x).

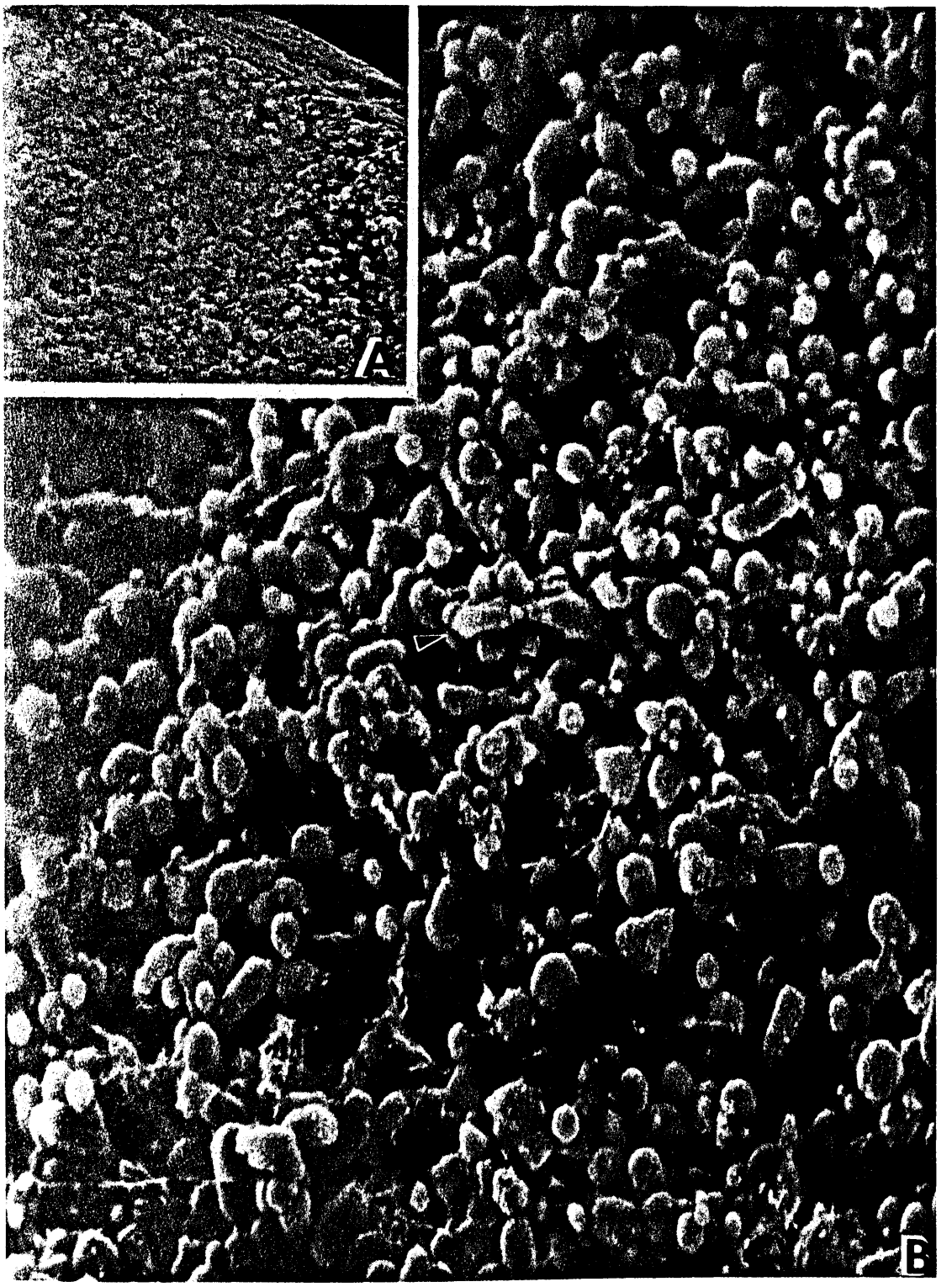

A
B

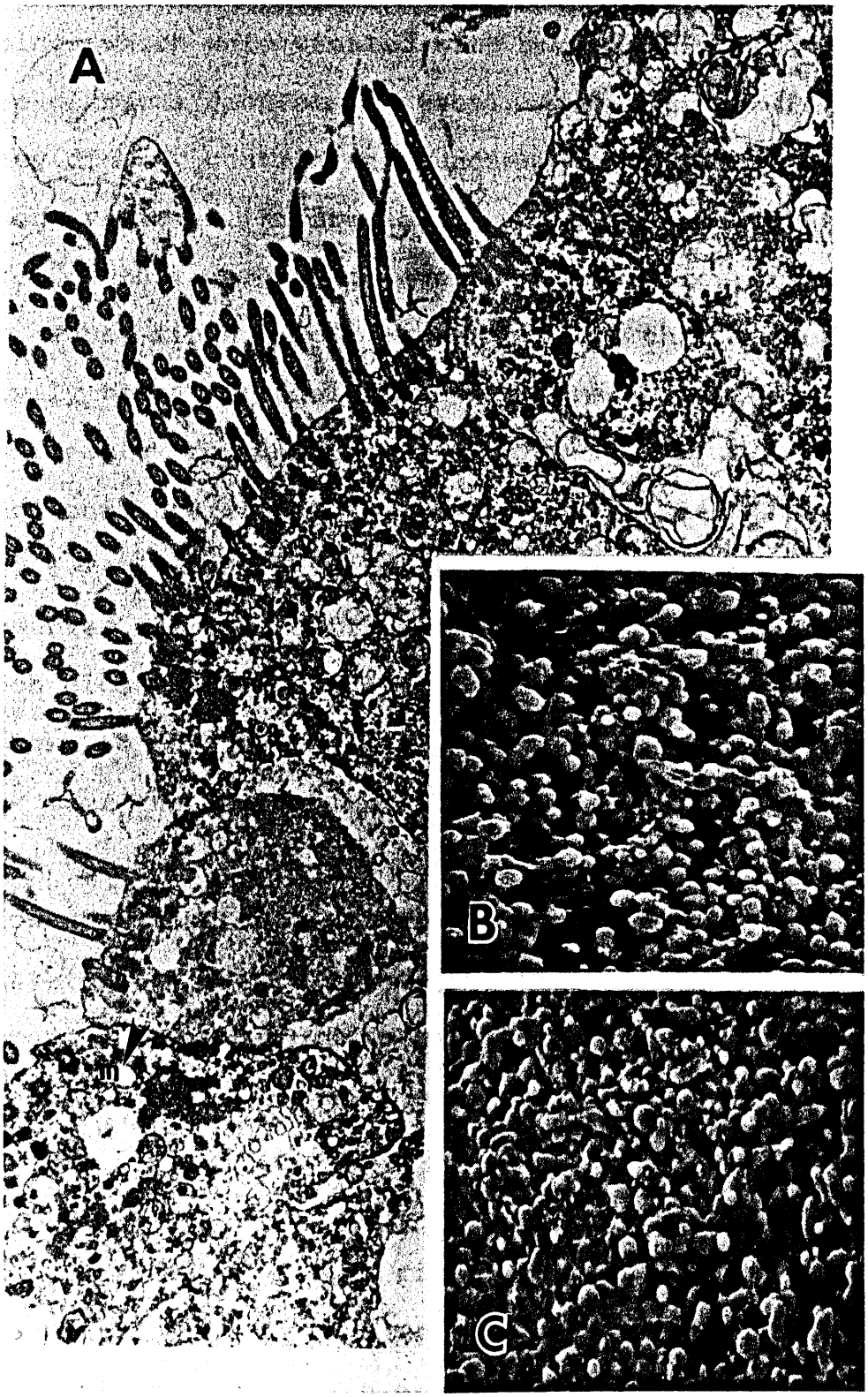
Figure 7. Electron micrographs of hamster tracheal rings treated with pooled cystic fibrosis serum. Hamster tracheal rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in 0.5 ml fresh F12 medium plus 25% cyctic fibrosis serum for 1h.

A. TEM of hamster tracheal ring exposed to CF serum (19,000x).

B. SEM of hamster tracheal ring exposed to CF serum from 6 CF patients (500x).

C. SEM of hamster tracheal ring exposed to CF serum from 4 CF patients (500x).

The arrow points to intracellular secretory material, presumedly mucus (m).


A
B
C

that rings which were treated with pooled NHS were found to be free of mucus droplets on the luminal surface (Figure 5a) and they did not contain an abundance of intracellular secretory vesicles (Figure 5b). Upon closer observation (Figure 5c) they were also devoid of any fibrous network of mucus. Thus, rings which were treated in this manner had a relatively normal morphology, as compared to those rings taken directly from the animal (Figure 4).

In contrast, rings which were treated with CFS (Figure 6) stimulated the release of mucus. This effect was observed over the entire luminal surface (Figure 6a) and upon closer observation (Figure 6b) mucus droplets were also shown to be associated with mucus strands forming a network over the luminal surface. Similarly, rings which had been exposed to CFS and processed for observation by TEM showed an apparent increase in intracellular secretory activity (Figure 7a). The goblet cells appeared swollen and numerous secretory vesicles were present at the apical region of the cells. The same rings were processed for SEM and the luminal surface contained numerous droplets (Figure 7b and c).

In comparative studies rings were treated with various sera and after 1h the rings were prepared for SEM (Figure 8). Treatment of the rings with either 25% fetal calf (FCS)

Figure 8. Scanning electron micrographs of hamster tracheal rings. Hamster tracheal rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in 0.5 ml fresh F12 medium plus various types of sera for 1h. The data shown are from one experiment in which duplicate samples were tested and prepared for observation. The results are representative of 6 independent experiments conducted with other lots of sera under the same conditions (660x).

A. Out of the animal, no treatment.

B. Fetal calf serum (25%).

C. Pooled normal human serum (25%).

D. Pooled CF serum (25%).

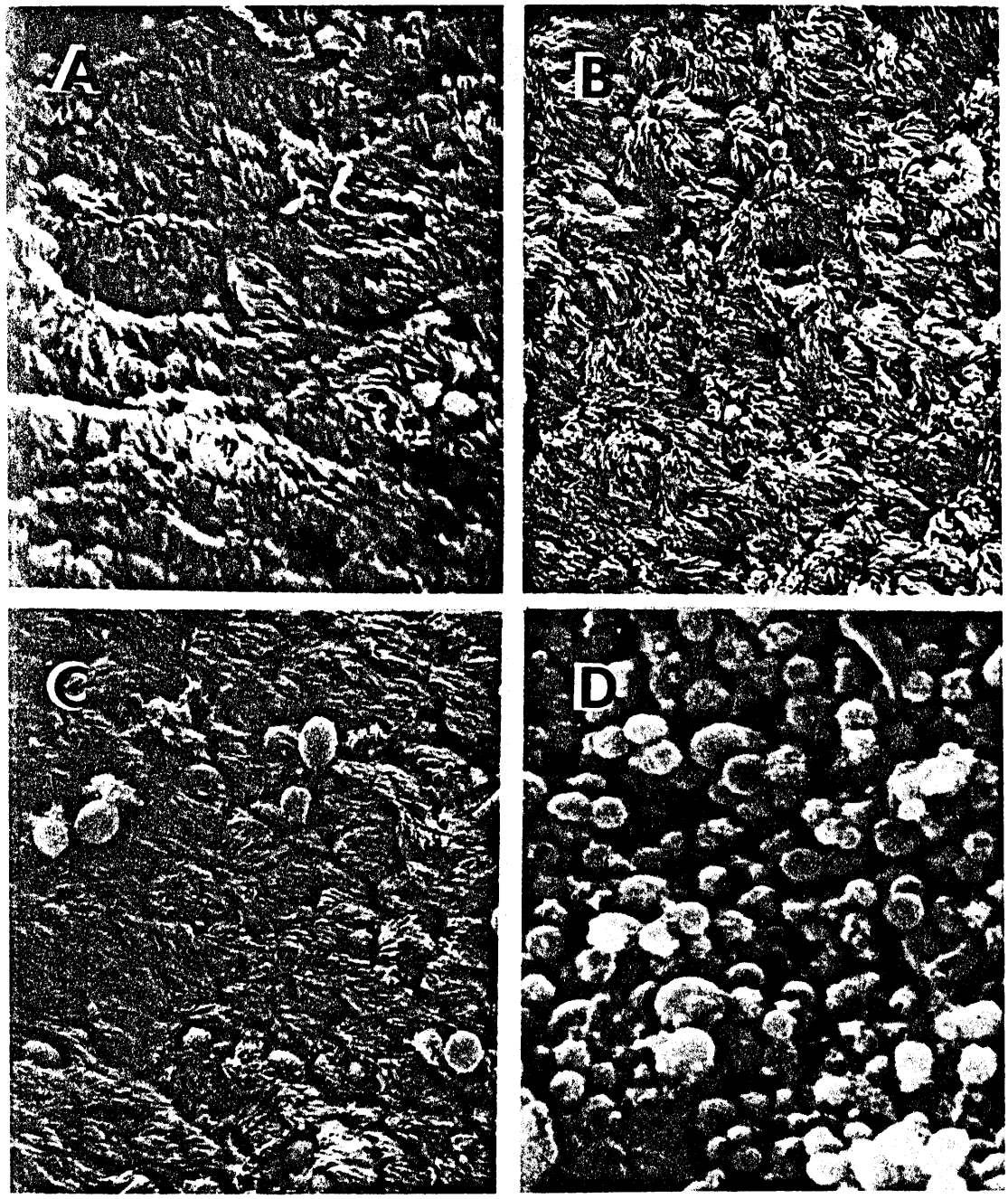

A
B
C
D

Figure 9. Scanning electron micrographs of hamster tracheal rings. Hamster tracheal rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in 0.5 ml fresh F12 medium plus various serum concentrations for 1h. These data are from one experiment in which duplicate samples were tested and prepared for observation. The results are representative of three independent experiments conducted in the same manner (660x).

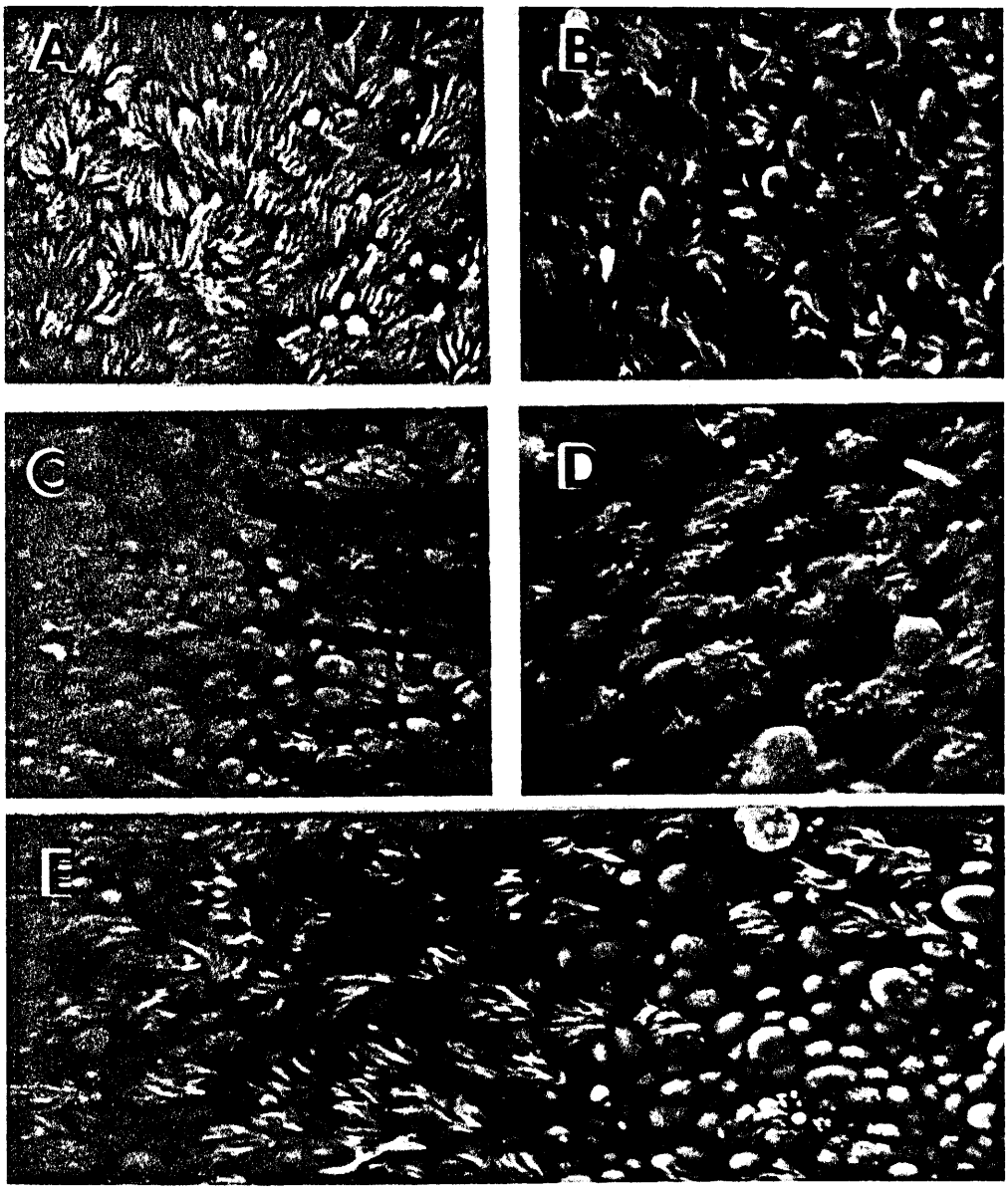
A. Normal human serum 10%.

B. Normal human serum 5%.

C. Normal human serum 2%.

D. Normal human serum 1%.

E. Normal human serum 0%.


A
B
C
D
E

(Figure 8b) or NHS (Figure 8c) resulted in no apparent changes, when compared with rings taken directly out of the animal (Figure 8a) or with rings incubated for 24h with 10% FCS. On the other hand, treatment with pooled CFS resulted in dramatic mucus accumulation at the luminal surface (Figure 8d). In blind comparison studies of the effects of normal and CF serum, concentrations as low as 10% were tested and in all cases positive identification of CFS effects could be made using the ring assay with SEM.

Effects of Serum Depletion

The effects of serum concentration in the assay were determined by varying the concentration of NHS applied to the rings. Figure 9 demonstrates a typical response, as observed by SEM, in which the serum concentration was varied from 0 to 10% for 1h of incubation. Explants which were treated with serum concentrations of less than 5% displayed extensive damage (Figures 9a-e). This qualitative assessment was made on the basis of the apparent loss of cilia from the luminal surface which was also accompanied by goblet cell exposure. When explants were incubated for longer than 1h mucus accumulation similar as to that observed with rings treated with CFS was a common occurrence.

Effects of Fibroblast Secreted Proteins on Hamster Tracheal Explants

The media obtained from CF or normal fibroblast cultures were tested using the tracheal ring bioassay and the rings were processed for SEM. These media were used without desalting or concentrating at a concentration of 100 µl/ml. Figure 10 shows that CF and normal media elicited identical responses from the tracheal explants. The luminal surface displayed marked morphological changes, the normal ciliated surface appearing to be extensively damaged, as exhibited by loss of cilia and some mucus release. In general, the rings appeared to be morphologically similar to those treated with low serum concentrations (Figure 9).

The effects induced by CFS and NHS may have been due to osmotic changes which would occur as a result of low protein concentration. To test this possibility the collected fibroblast media were desalted using Bio-Gel P10 column chromatography, as described in Methods, and the lyophilized proteins were concentrated 10 fold by reconstituting in PBS. These proteins were used in the tracheal ring bioassay at a concentration of 1 mg/ml. When concentrated proteins were tested alone, effects similar to those

Figure 10. Scanning electron micrographs of hamster tracheal rings. Hamster tracheal rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in medium (0.5 ml) which had been collected from either near confluent normal or CF fibroblast cultures and incubated with time. These data shown are from one experiment in which duplicate tracheae were treated and prepared for observation. The results are representative of experiments in which other CF (GM 770, 768, 142) or N (GM 38) fibroblast cell culture media were tested, under identical conditions (660x).

A. Normal secreted proteins (GM3349), 1h.

B. Normal secreted proteins (GM3349), 6h.

C. Normal secreted proteins (GM3349), 12h.

D. CF secreted proteins (GM 1348), 1h.

E. CF secreted proteins (GM1348), 6h.

F. CF secreted proteins (GM 1348), 12h.

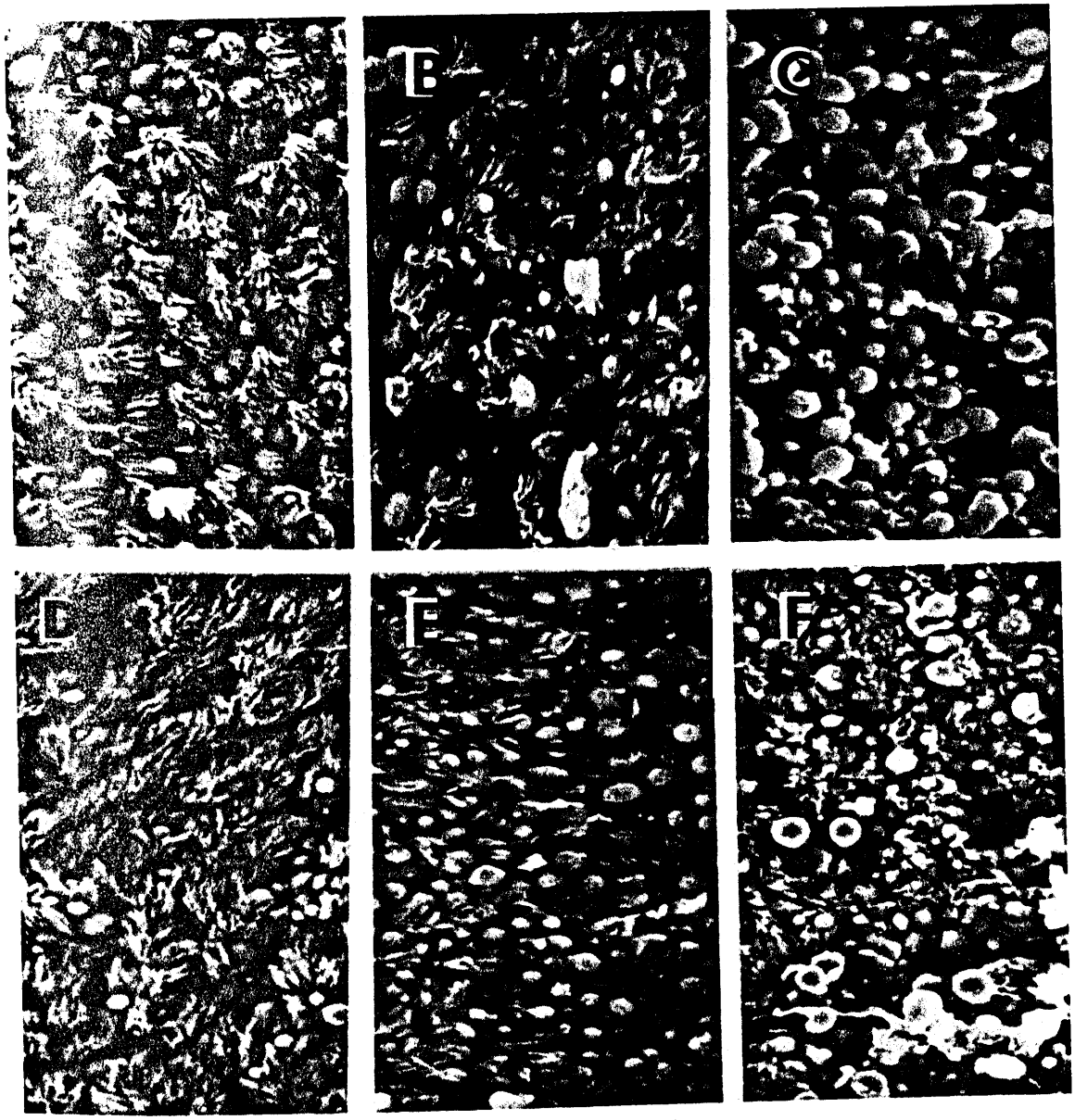

B
C
D
E
F

Table 1. The Effects of Secreted Proteins Obtained from Normal or Cystic Fibrosis Fibroblasts on Hamster Tracheal Rings.

Cell Line	Concentrated Protein[1]	Concentrated Protein+FCS[2]	Concentrated Protein+IgG[3]	Concentrated Protein+FCS/IgG[4]
GM 38 (normal) VoP10	+	-	+	-
GM 38 (normal) VeP10	+	-	+	-
GM1348(CF) VoP10	+	-	+	-
GM1348(CF) VeP10	+	-	+	-

Secreted proteins were obtained from near confluent fibroblasts in culture, as described in Methods. The collected media were fractionated into high molecular weight proteins (Vo $\gtrsim$ 10kd) and low molecular weight proteins (Ve $\lesssim$ 10kd) using BioGel P10 column chromatography. These proteins were then concentrated 10 fold to a final concentration of 1mg/ml. The tracheal rings were incubated with 200-300µl of sample to achieve equivalent protein concentrations. (+) mucus accumulation on luminal surface; (-) absence of mucus on luminal surface.

[1] 10 fold concentrated proteins.
[2] 10 fold concentrated proteins plus 10% FCS.
[3] 10 fold concentrated proteins plus 1% IgG.
[4] 10 fold concentrated proteins plus 10% FCS and 1% IgG.

observed with serum depletion (cilia loss and epithelial damage) occurred (Table I), but tracheal rings which had been incubated with concentrated protein and 10% fetal calf serum displayed a normal typical morphology. The addition of normal human IgG to the concentrated proteins at a physiological concentration of 1% did nothing to prevent damage to the luminal lining. However, the combination of concentrated proteins, FCS, and IgG had an effect similar to just FCS alone. The same results were obtained whether the source of the concentrated proteins was normal or CF fibroblasts.

Lastly, to rule out the possibility that a CF factor might not have been present among proteins of the Vo fraction of Bio-Gel P10, the included proteins (Ve) were also applied to the rings at proteins concentrations of 1 mg/ml. The results obtained with Ve proteins from normal and CF fibroblasts were the same as those obtained with the Vo protein fractions.

Labeling of Secreted Fibroblast Proteins

Fibroblasts from either CF (GM768 and 1348) or normal (GM38 and 3349) individuals were allowed to incorporate [^{3}H]leucine for 48 hrs., as described in Methods. The medium was collected and desalted using Bio-Gel P10 column

chromatography. Elution from the column was monitored by recording the absorbance at 220 nm and counting 1 μl aliquots of the fractions by scintillation spectrometry. The radiolabeled proteins eluted in the void volume were lyophilized, reconstituted in water, and subjected to SDS-PAGE, as described in Methods. The resulting fluorograms, although revealing discrete protein bands of various molecular weights, showed no differences between the secretions of CF or normal fibroblasts.

Effects of Serum on Tracheal Rings

While the hamster trachea did not respond to secreted fibroblast proteins, apparently the cells lining the luminal surface did respond to some component of CF serum resulting in stimulation of secretion. However, prior to attempting to study the effects of CF serum on individual cells it was important to establish that this response was consistent from patient to patient. Although assessment by scanning electron microscopy is a nonquantitative method, a qualitative estimation of the effects could be made.

More experiments were conducted in order to observe the effects of other sera in the tracheal ring bioassay. Fetal calf serum was tested (FCS) (Figure 11a) as well as serum from individuals who were affected by chronic

Figure 11. A comparison by scanning electron microscopy of hamster tracheal rings treated with various types of sera. Rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in fresh F12 medium (0.5 ml) plus 25% serum for 1h.

A. Fetal calf serum (660x).

B. Bronchitic serum (660x).

C. Normal serum (660x).

D(1-6). Each represents a ring tested with serum from a different CF patient (660x).

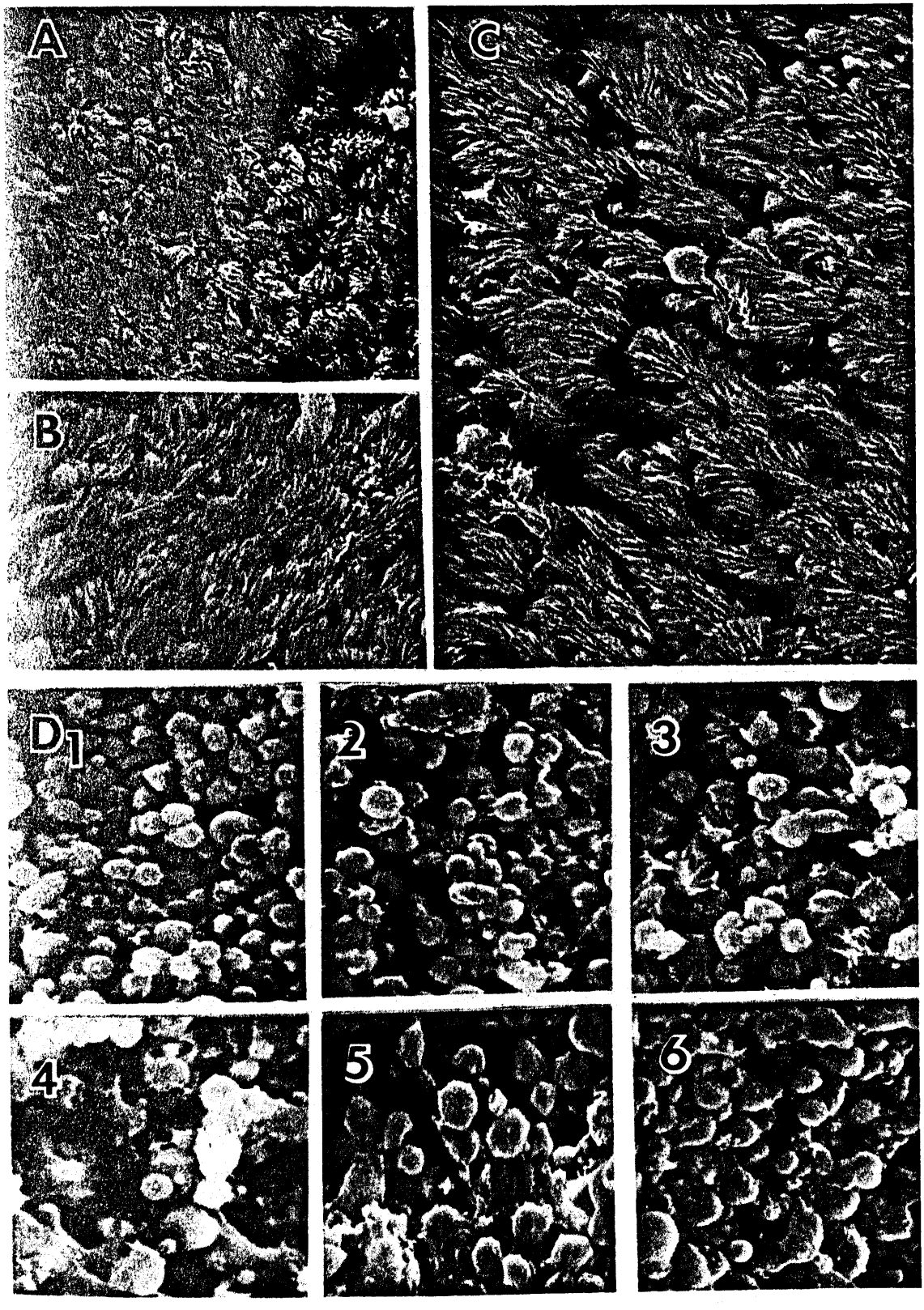
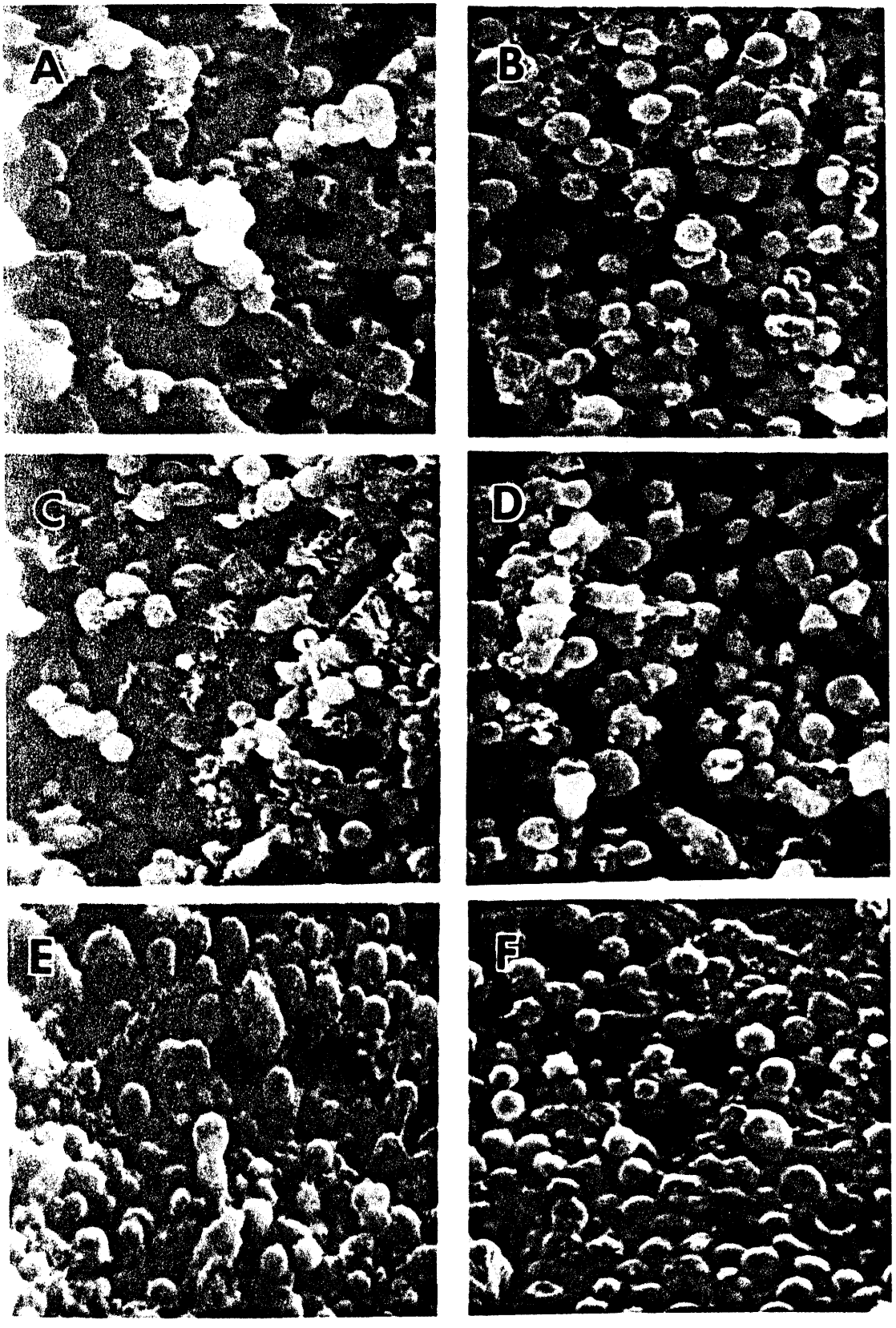

A
B
C
D1
2
3
4
5
6

Figure 12. A - F. Scanning electron micrographs of hamster tracheal rings exposed to serum from six individuals heterozygous for CF. The rings were prepared as described in Methods, incubated for 24h in F12 medium, then placed in 0.5 ml fresh F12 medium plus 25% heterozygous serum for 1h. Each heterozygote corresponds respectively to the homozygous child tested in the previous experiment (Figure 11, D1-6) (660x).


A
B
C
D
E
F

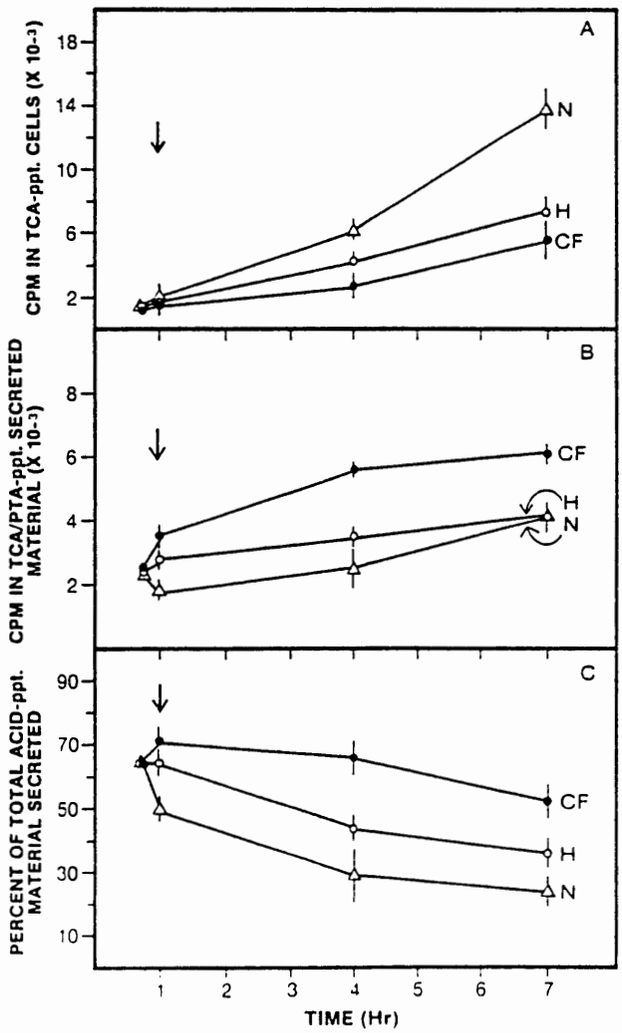
bronchitis (Figure 11b) and NHS (Figure 11c). Preliminary observations indicated that sera from bronchitic individuals affected the tracheal epithelium in a manner analogous to that of normal serum. In each case the sera obtained from CF individuals elicited obvious stimulatory responses (Figure 11d). Sera from several CF patients (n = 49) and from normal individuals (n = 15) were tested for their ability to induce expulsion of mucus droplets from tracheal explants. All of the CF sera (used in concentration ranges from 10-50% and with exposure times ranging from 30 min. to 24 hrs.) have augmented mucus release over and above that elicited by normal sera. In addition, the heterozygous parents were tested in the tracheal bioassay. All samples tested (n = 15) induced the rings to release mucus (Figure 12), but it was difficult to determine whether there was a difference in the number of droplets formed in the presence of heterozygote serum proteins as compared to those found in response to CF serum.

Influence of CF serum on HTE Cell Secretion

The effects observed with the tracheal explants established the rationale for the isolation of epithelial cells from hamster trachea. Hamster trachea epithelial cells (HTE) were isolated, as described in Methods. Near confluent cells were routinely prelabeled for 1h in 0.5 ml

Figure 13. The effects of serum from normal, heterozygous, and cystic fibrosis individuals on secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 containing 5% FCS and 0.3 μCi/ml [^{14}C]threonine. After 1h, the cells were washed and 0.5 ml F12 medium containing label and serum were added. The arrow represents the addition of the appropriate serum proteins at 20% concentration. The results are expressed as the mean ± S.E. of 5 CF, 5 heterozygotes, and 4 N individuals run in duplicate.

$$\text{Percent total acid precipitable secreted material} = \frac{\text{TCA-PTA secreted material (cpm)}}{(\text{TCA-PTA secreted material (cpm)}) + (\text{TCA ppt. material (cpm)})}$$


A
B
C
CPM IN TCA-ppt. CELLS (X 10-3)
CPM IN TCA/PTA-ppt. SECRETED MATERIAL (X 10-3)
PERCENT OF TOTAL ACID-ppt. MATERIAL SECRETED
TIME (Hr)
N
H
CF

F12 medium containing 5% FCS and 0.3 μCi/ml [^{14}C]threonine; after 1h the medium was removed and fresh F12 containing the radiolabeled compound plus either 20% cystic fibrosis (CFS), heterozygote (HHS) or normal human serum (NHS) was added. At various times, samples of the media and cells were collected and processed. The radioactivity incorporated into acid precipitable secreted material, acid precipitable cell associate material, and the percentage of the total acid precipitable material secreted was calculated at each time point. Figure 13 shows the results when sera from 5 CF, 5 heterozygotes, and 4 normals were tested individually. The standard error bars represent the variability between individual plates and indicate a minimal variation. In the presence of CFS the cell bound radioactivity decreased (Panel A), while there were higher levels of acid precipitable material in the medium (Panel B). This resulted in a net stimulation of secretion over that of cells treated with NHS. Heterozygote sera gave an intermediate response. Panel C shows that at every time point there was a 1.5 to 2.5 fold stimulation of secretion in the presence of CFS compared to that of NHS. Also, the values for the HHS effect fell between the other two. Thus, after 6h there was a 2.2 fold difference between NHS and CFS, a

Table II. The Effects of Serum Proteins from Normals, Heterozygotes, and Cystic Fibrosis Patients on Secretion of Radiolabelled Proteins.[1]

Individuals	Age (yr)	Sex	Total Acid-Precipitable Material Secreted in 3 h (%)[2]
Normals			
1	10	F	32
2	11	M	34
3	24	M	40
4	25	F	37
5	28	M	37
6	29	F	27
7	29	F	40
8	29	F	40
9	35	M	31
10	36	F	48
11	38	M	48
12	-	-	27
			Mean ± S.E. (37.08 ± 2.10)[3]
Heterozygotes			
1	-	F	44
2	-	F	59
3	-	F	44
4	-	F	43
5	-	F	33
6	-	F	73
7	-	F	64
8	-	F	36
9	-	M	44
10	-	F	38
11	-	F	55
12	-	F	47
13	-	F	41
14	-	F	39
15	-	F	32
			Mean ± S.E. (46.13 ± 3.03)[4]

Individuals	Age (yr)	Sex	Total Acid-Precipitable Material Secreted in 3 h (%)[2]
Cystic Fibrosis Patients			
1	1	F	55
2	7	F	50
3	7	M	78
4	8	M	59
5	10	F	60
6	10	F	67
7	10	M	41
8	12	F	68
9	13	F	83
10	17	M	51
11	17	F	41
12	19	F	78
13	19	M	52
14	21	F	35
15	21	M	62
16	24	M	52
17	25	M	63
18	29	M	33
19	30	M	40
			Mean ± S.E. (56.21 ± 3.35)

1. HTE cells prelabelled for 1 h with [^{14}C] threonine (0.5 μCi/ml) in F_{12} medium plus 5% FCS were placed in fresh F_{12} medium plus radioisotope with the appropriate 25% serum additions. Cells were allowed to secrete for 6 h before medium was precipitated with TCA/PTA and the cells with TCA and processed as described in Methods.

2. The data are calculated by summing the acid-precipitable counts secreted and cell-bound and then determining the percent of the radioactivity that was actually secreted by the cells after 3 h. Each individual was tested in duplicate.

3. $P < 0.01$ (normals vs heterozygotes); $P < 0.01$ (normals vs CF patients).

4. $P < 0.01$ (heterozygotes vs CF patients).

1.5 fold difference between NHS and HHS, and a 1.4 fold difference between HHS and CFS.

Table II shows the percent of total acid precipitable. material that was released in 3h, when individual serum samples were applied to HTE cells. The standard error of the mean is negligible. In a limited number of cases, blood has been drawn from the same individual on separate occasions and tested for percentage secretion after 3h. In each case the samples elicited the same results. When the effects of the three serum types are compared, there are statistically significant ($p \leq 0.01$) differences in the means of NHS versus HHS, NHS versus CFS, and HHS versus CFS. Although there is some overlap of values, in general the HHS produces results which fall midway between values obtained using CFS and NHS.

Calcium Uptake and Cellular Secretion

In an attempt to further characterize the hypersecretory effect on HTE cells induced by CF, serum studies involving the uptake of radiolabeled $^{45}Ca^{2+}$ were undertaken. It is well documented that calcium has a role in secretion (Williams and Lee, 1974; Douglas, 1981). Many studies have indicated that extracellular and/or intracellular calcium is required for secretion to occur. In order to begin to define and characterize the mechanism of

Figure 14. The effect of either pooled normal or cystic fibrosis serum on the uptake of $^{45}Ca^{2+}$ by HTE cells. The cells were allowed to take up radiolabeled calcium (5.0 μCi/ml) in 0.5 ml F12 medium containing 25% serum. Samples were taken with time by removing the medium and processing, as described in Methods, for liquid scintillation spectrometry. The data are representative of four independent experiments and are expressed as the Σ mean ± S.E. of duplicate samples. NHS:normal human serum; CFS:cystic fibrosis serum.

$$\text{Relative percent } ^{45}Ca^{2+} \text{ uptake} = \frac{\text{uptake } ^{45}Ca^{2+} \text{ for individual samples (cpm)}}{\text{maximum uptake } ^{45}Ca^{2+} \text{ (cpm)}}$$

maximum uptake = 100%

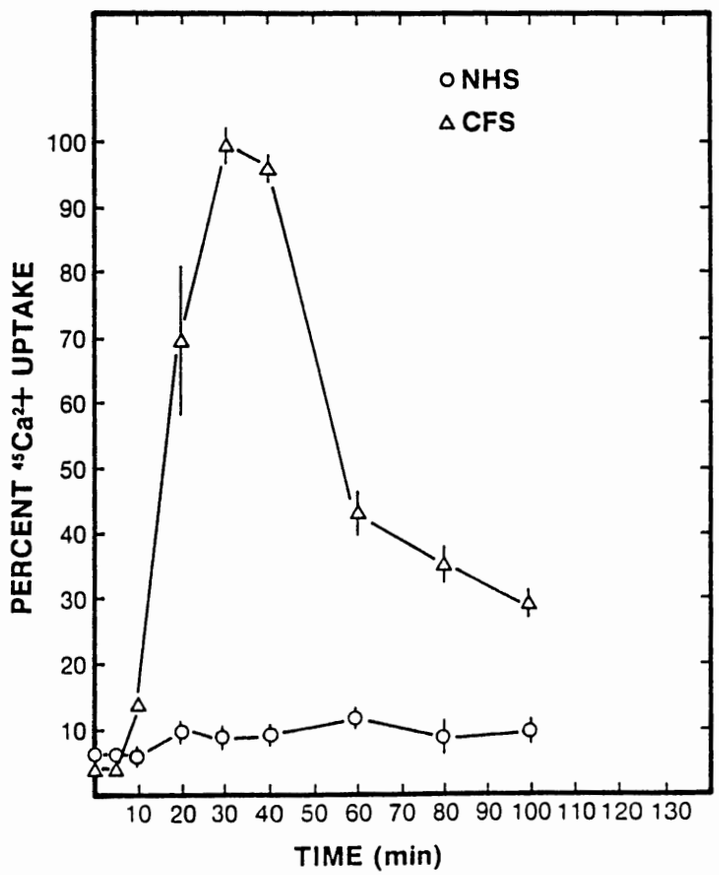

NHS
CFS
PERCENT $^{45}Ca^{2+}$ UPTAKE
100
90
80
70
60
50
40
30
20
10
10 20 30 40 50 60 70 80 90 100 110 120 130
TIME (min)

Figure 15. The effect of normal, CF, or FCS on the uptake of $^{45}Ca^{2+}$ by HTE cells. The cells were allowed to take up radiolabeled calcium ion (5.0 μCi/ml) in 0.5 ml F12 medium; serum was added at either 10 or 20% concentration. Samples were taken with time and processed, as described in Methods, for liquid scintillation spectrometry. The data are representative of two independent experiments and are expressed as the mean of duplicate samples. NHS: normal human serum; CFS: cystic fibrosis serum; FCS: fetal calf serum.

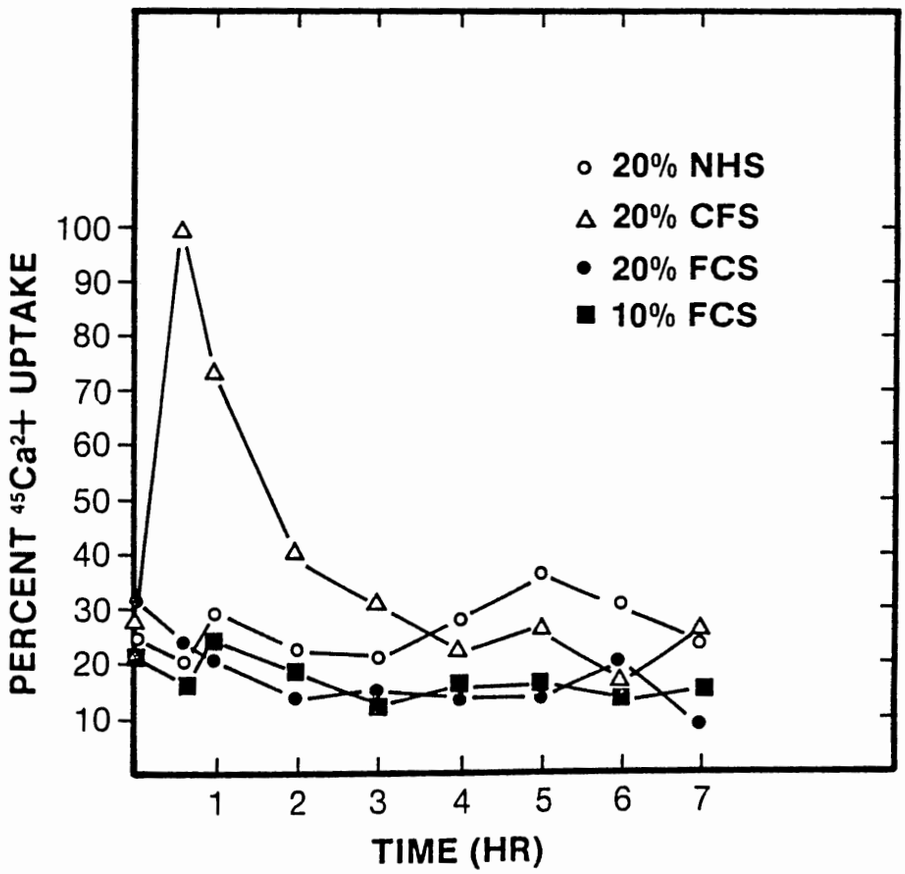
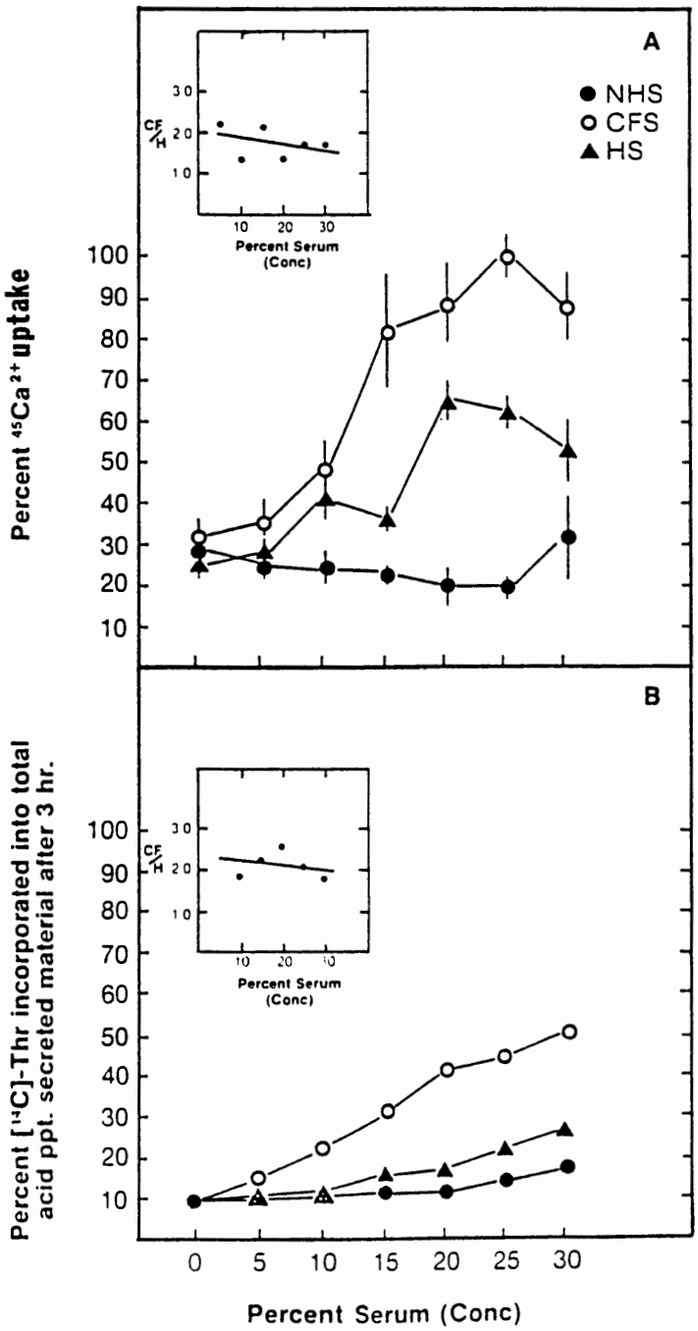

20% NHS
20% CFS
20% FCS
10% FCS
PERCENT $^{45}Ca^{2+}$ UPTAKE
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
TIME (HR)

Figure 16. A. Effects of normal, heterozygous, or CF serum on calcium ion uptake by HTE cells. Cells were incubated in 0.5 ml F12 medium, containing 5.0 μCi/ml $^{45}Ca^{2+}$ and serum concentrations ranging from 0-30%. The cells were allowed to take up $^{45}Ca^{2+}$ for 30 min., the medium was aspirated off and the cells processed for liquid scintillation spectrometry, as described in Methods. The data are the mean ± S.E. of four separate experiments in which duplicate plates were run. Inset: Least sums of the ratio of CF/HS percent uptake as a function of percent serum was calculated.

B. Effects of normal, heterozygous, or CF serum on secretion of [^{14}C]threonine labeled proteins by HTE cells. Serum was added at concentrations ranging from 0-30% in 0.5 ml of F12 medium plus 0.3 μCi/ml threonine. The cells were allowed to secrete for 3 hrs., medium was collected and precipitated with TCA-PTA, the cells were precipitated with TCA, and processed for liquid scintillation spectrometry, as described in Methods. The data are the means of duplicate plates for 2 independent experiments. Inset: Least sums of the ratio of CF/HS percent secreted proteins as a function of percent serum was calculated. NHS:normal human serum; CFS:cystic fibrosis serum; HS: heterozygous serum.


A
NHS
CFS
HS
Percent $^{45}Ca^{2+}$ uptake
CF/H
Percent Serum (Conc)
B
Percent [^{14}C]-Thr incorporated into total acid ppt. secreted material after 3 hr.
Percent Serum (Conc)

such a factor in promoting cellular secretion, experiments were conducted to establish the role calcium plays in the hypersecretory phenomenon.

The uptake of $^{45}Ca^{2+}$ was measured, as described in Methods. Influx in the presence of NHS was a minimum (Figure 14), while in the presence of CFS there was a rapid influx of calcium ten minutes after exposure to the serum which peaked at 20 to 30 min. The intracellular level then declines over the remaining period of the assay.

The effects of 20% NHS and CFS on $^{45}Ca^{2+}$ uptake were tested over the period of time used in the secretion assays (Figure 15). Only one spike of influx was seen in the presence of CFS. Also, 10% FCS, the concentration used in propagating the HTE cells, and 20% FCS were tested. Figure 15 shows that neither concentration of FCS nor NHS produced an effect. Therefore, the observed spike seen with CFS was not due just to the changes in concentration or type of serum placed onto the cells.

Serum Concentration Effects on Calcium Uptake and Secretion

Figure 16a demonstrates the effect of increasing concentrations of pooled NHS, HHS, or CFS on the uptake of calcium ion after 30 min. of incubation, the time at which the spike of Ca^{2+} influx occurred with CFS. The results

indicate that NHS had essentially no effect over the range of concentrations tested. Both CFS and HHS caused cellular calcium levels to increase starting with 5% serum and reaching a maximum at 20-25%. The least sums ratio of CFS/HHS for calcium ion uptake was approximately two throughout the range of serum concentrations tested. A similar pattern was observed, when the secretion stimulation assay was conducted (Figure 16b). NHS did not stimulate secretion appreciably, while increasing amounts of CFS caused a linear response in the rate of secretion over and above that elicited by NHS or HHS. HHS responses were intermediate between CFS and NHS. The CFS/HHS ratio for secretion also remained approximately at two. Although concentrations as high as 100% were tested in each case, these were omitted due to possible cellular damage induced at such high serum concentrations.

Individual Differences in Calcium Uptake

Several individual sera were tested for their ability to stimulate $^{45}Ca^{2+}$ uptake by HTE cells. Figure 17 shows the results of five CFS, five HHS, and four NHS. The peak uptake was found to occur on the average between 20 and 60 min., depending on the CF individual assayed. When the results were averaged, it appeared that $^{45}Ca^{2+}$ was taken up

Figure 17. Effects of normal, heterozygous, or CF serum on calcium ion uptake by HTE cells. Cells were incubated in 0.5 ml F12 medium containing 5.0 μCi/ml $^{45}Ca^{2+}$ and 20% serum concentration. Samples were taken with time by removing the medium and processing the cells for liquid scintillation spectrometry, as described in Methods. The data are represented as the mean ± S.E. of 5 normal, 5 heterozygotes, and 5 CF individuals which were tested in duplicate.

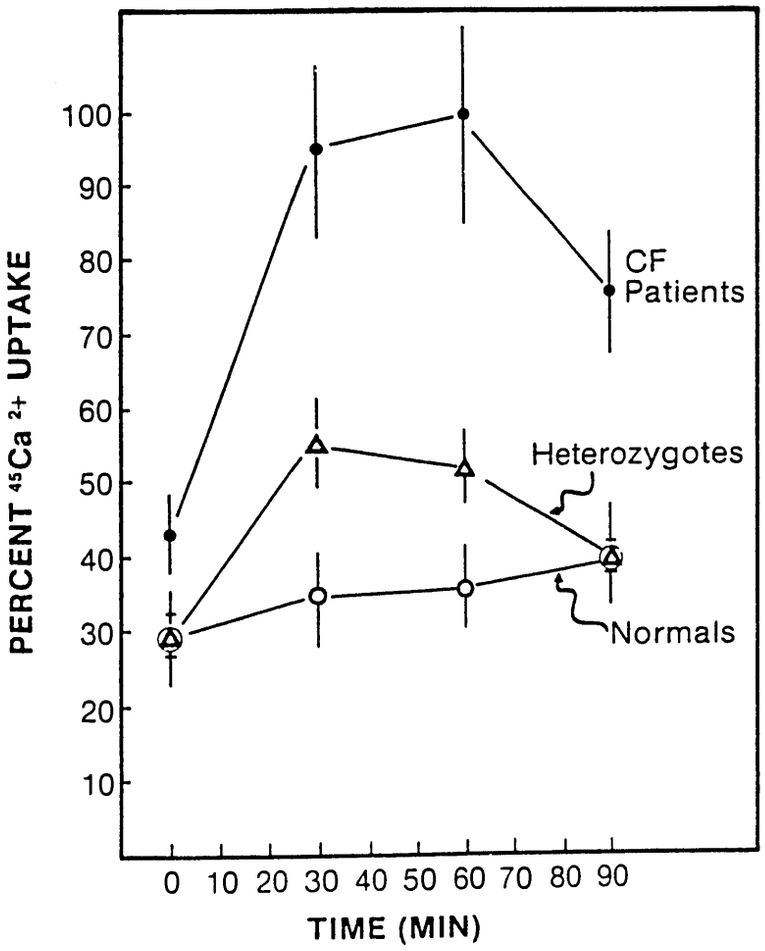

PERCENT $^{45}Ca^{2+}$ UPTAKE
100
90
80
70
60
50
40
30
20
10
0 10 20 30 40 50 60 70 80 90
TIME (MIN)
CF
Patients
Heterozygotes
Normals

Figure 18. Effect of EGTA on calcium ion uptake by HTE cells. The cells were incubated for 30 min. in 0.5 ml F12 medium containing 20% CF serum, 5.0 μCi/ml $^{45}Ca^{2+}$ and concentrations of EGTA ranging from 0.33 mM to 4.0 mM. After 30 min. incubation the cells were processed for liquid scintillation spectrometry, as described in Methods. The data are represented as the Σmean ± S.E. of 2 independent experiments in which duplicate plates were run.

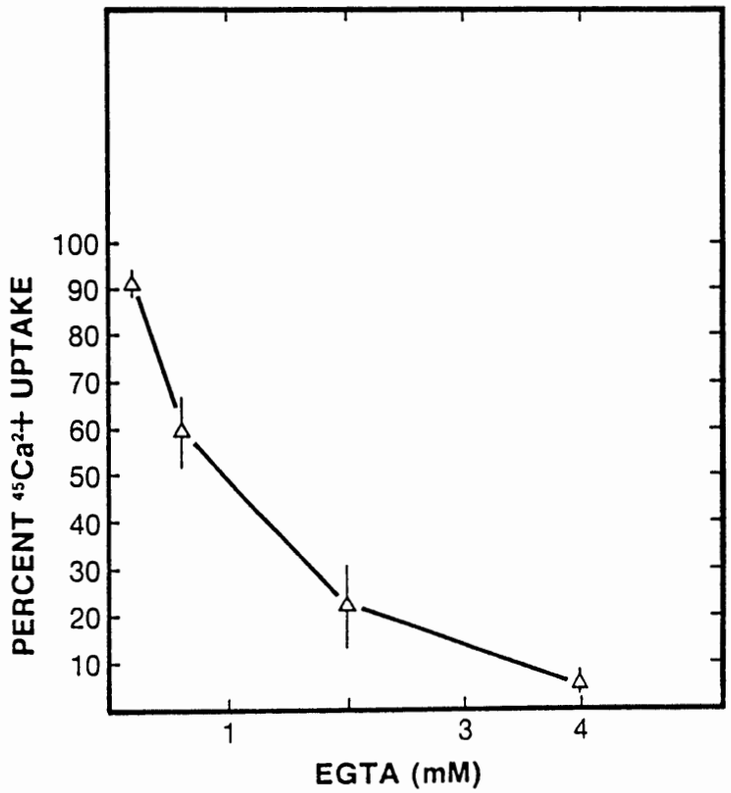

PERCENT $^{45}Ca^{2+}$ UPTAKE
100
90
80
70
60
50
40
30
20
10
1
3
4
EGTA (mM)

Figure 19. The effects of EGTA on secretion of [^{14}C] threonine labeled proteins by HTE cells. Normal or CF serum was added at 20% concentration in 0.5 ml F12 medium containing 0.3 μCi/ml threonine ± EGTA. The cells were allowed to secrete for 3h after which the medium was precipitated by the addition of TCA-PTA, while the cells were precipitated with TCA and processed for liquid scintillation spectrometry, as described in Methods. The results are expressed as the mean ± S.E. Five individual serum samples from either CF or normal individuals were tested in duplicate. N:normal human serum; C:cystic fibrosis serum.

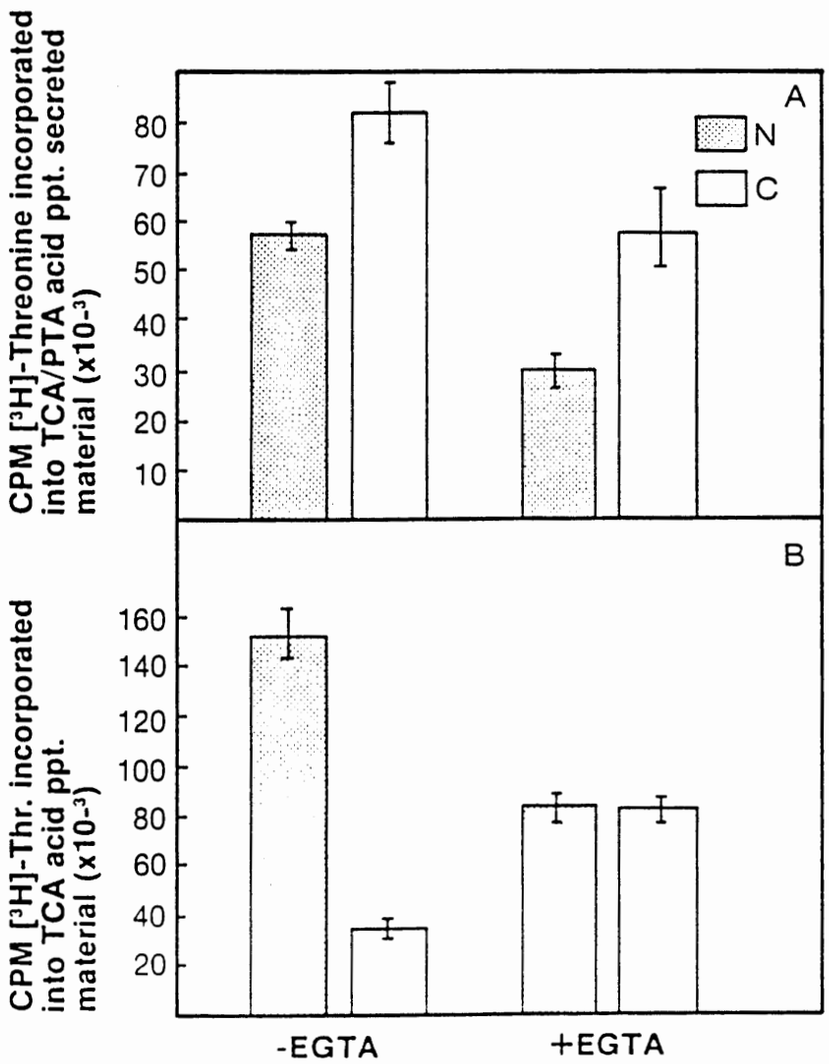

CPM [3H]-Threonine incorporated into TCA/PTA acid ppt. secreted material (x10-3)
80
70
60
50
40
30
20
10
A
N
C
CPM [3H]-Thr. incorporated into TCA acid ppt. material (x10-3)
160
140
120
100
80
60
40
20
B
-EGTA
+EGTA

Table III. The Effects of Serum Proteins from Normals, Heterozygotes, and Cystic Fibrosis Patients on $^{45}Ca^{++}$ Uptake by HTE Cells.[1]

Individuals	Age (yr)	Sex	Percent $^{45}Ca^{++}$ Uptake at 30 min[2]
Normals			
1	10	F	24
2	11	M	13
3	24	M	10
4	25	F	15
5	25	F	20
6	28	F	20
7	28	M	10
8	29	F	18
9	29	F	32
10	29	M	6
11	35	M	35
12	35	M	41
13	36	F	16
14	38	M	24
15	40	F	14
			Mean ± S.E. (19.27 ± 2.6)[3]
Heterozygotes			
1	-	F	43
2	-	F	53
3	-	F	25
4	-	F	29
5	-	F	50
6	-	F	70
7	-	F	50
8	-	F	31
9	-	M	36
10	-	F	26
11	-	F	19
12	-	F	15
13	-	F	22
14	-	F	50
			Mean ± S.E. (37.1 ± 1.6)[4]

Individuals	Age (yr)	Sex	Percent $^{45}Ca^{++}$ Uptake at 30 min[2]
Cystic Fibrosis Patients			
1	1	F	69
2	7	F	100
3	8	M	27
4	10	F	73
5	10	F	27
6	10	M	49
7	12	F	94
8	13	F	85
9	17	M	41
10	17	F	47
11	19	M	35
12	19	F	65
13	21	F	64
14	21	M	74
15	24	M	100
16	25	M	40
17	29	M	84
18	30	M	72
			Mean ± S.E. (63.67 ± 1.9)

[1]Near confluent HTE cells were exposed to $^{45}Ca^{++}$ (5 μCi/ml) in F12 medium containing the appropriate 20% serum additions and after 30 min were processed as described in Methods.

[2]The data represent averages from two experiments. During the experiment each individual was tested in duplicate.

[3]$P < 0.001$ (normals vs heterozygotes); $P < 0.001$ (normals vs CF patients).

[4]$P < 0.001$ (heterozygotes vs CF patients).

maximally by 30 min. and remained at this level for 60 min. Once again HHS was intermediate in its effect.

To further document the differences observed among CFS, HHS, and NHS in their ability to cause calcium ion uptake by HTE cells, several individual sera were tested with sex and age equally represented. Also, individuals donating NHS were blacks, orientals, or caucasians with no known history of CF. The CF patient sera were chosen at random without regard to disease severity to account for variability of the disease. CFS ($n = 18$), HHS ($n = 14$), and NHS ($n = 15$) were assayed at 30 min. of incubation. Table III shows the data for all individuals tested utilizing the student's t-test for significance of means and variability between individuals; the data clearly demonstrate that: 1) CFS stimulated uptake more than NHS or HHS ($p \leq 0.001$); 2) HHS stimulation was greater than that of NHS ($p \leq 0.001$).

Effects of EGTA

Increasing concentrations of EGTA (0.33-4mM) were added to media containing 20% pooled CFS in order to determine the concentration required to chelate available calcium. It was found the 4mM EGTA inhibited $^{45}Ca^{2+}$ uptake by 96% (Figure 18). The same concentration of EGTA prevented CFS stimulation of secretion (Figure 19). In

general treatment with EGTA resulted in: 1) decreased CFS stimulation of secretion by decreasing the TCA-PTA acid precipitable secreted material to the NHS level; 2) a concomitant increase in the TCA acid precipitable cell bound material in the presence of CFS plus EGTA. Apparently, chelation of available calcium ion resulted in reversion of the cells to the basal level of secretion observed in the presence of NHS.

Effects of Ionophore A23187

Ionophores are drugs which increase membrane permeability to ions (Rubin, 1982) and A23187 is fairly specific for Ca^{2+} (Reed and Lardy, 1972). To establish a relationship between calcium ion influx stimulated by CFS and subsequent secretion such a drug should be able to stimulate secretion by itself or in the presence of NHS to mimic the effects seen with CFS. It was important to establish the concentration of ionophore which would cause increased calcium ion influx comparable to that stimulated by CFS without damaging the cells. Table IV shows the results of two independent experiments in which various concentrations of ionophore were tested with time at fixed external calcium ion concentration (that of F12 medium, 0.33mM). Maximum uptake occurred at 10 μg/ml of ionophore. Several concentrations of calcium ion ranging from 0.33mM to

Table IV. Percent $^{45}Ca^{++}$ Uptake by HTE Cells Using Various Concentrations of the Ionophore A23187[1]

Ionophore Concentration (μg/ml)	Time (min) 0	30	90
0.5	15 ± 1.1[2]	25 ± 1.2	33 ± 5.5
1.0	16 ± 1.3	30 ± 1.9	30 ± 1.0
5.0	20 ± 1.0	51 ± 3.3	47 ± 12.2
10.0	26 ± 1.8	100 ± 3.0	66 ± 4.2
20.0	30 ± 1.1	66 ± 4.9	90 ± 7.3

[1]Near confluent cultures of HTE cells were exposed to varying concentrations of the ionophore A23187 in the presence of $^{45}Ca^{++}$ (5 μCi/ml) as described in Methods. The data represent two separate experiments with duplicate cultures run at each time point.

[2]± S.E.

Table V. The Effects of Varying the Concentration of Calcium on Secretion of Radiolabeled Proteins by HTE Cells in the Presence of the Ionophore A23187[1].

Concentration of $CaCl_2$ (mM)	CPM in TCA-ppt (Cells)	CPM in TCA/PTA ppt. (Secreted Material)	Total Acid-ppt. Material Secreted (%)
0.33[2]	1849 ± 430[3]	3056 ± 356	62
5.40	1887 ± 658	3262 ± 9	63
5.40 + EGTA	4318 ± 61	1099 ± 69	20

[1]Near confluent cultures of HTE cells were prelabeled with [^{14}C] thr (0.3 μCi/ml) in F12 medium containing 5% FCS. At one hour, the cells were washed and 0.5 ml of serum-free F12 medium containing label and the ionophore A23187 (10 μg) + 4 mM EGTA were added. After 3 hrs, the medium was precipitated with TCA/PTA and the cells with TCA and processed for scintillation spectrometry as described in Methods. Each value represents the average of duplicate samples.

[2]The normal concentration of calcium in F12 medium is 0.33 M.

[3]± S.E.

Figure 20. The effects of ionophore A23187 and extracellular calcium ion on the uptake of $^{45}Ca^{2+}$ by HTE cells. The cells were exposed to 5 and 10 μg/ml of ionophore A23187 in 0.5 ml F12 medium, containing 5.0 μCi/ml $^{45}Ca^{2+}$ supplemented with increasing concentrations of calcium ion as calcium chloride (2.75, 4.08, 5.40 mM) in the absence of serum. The medium was removed and the cells processed for liquid scintillation spectrometry, as described in Methods. The data are represented as the mean of duplicate plates for two independent experiments. The error was less than 5% of the mean.

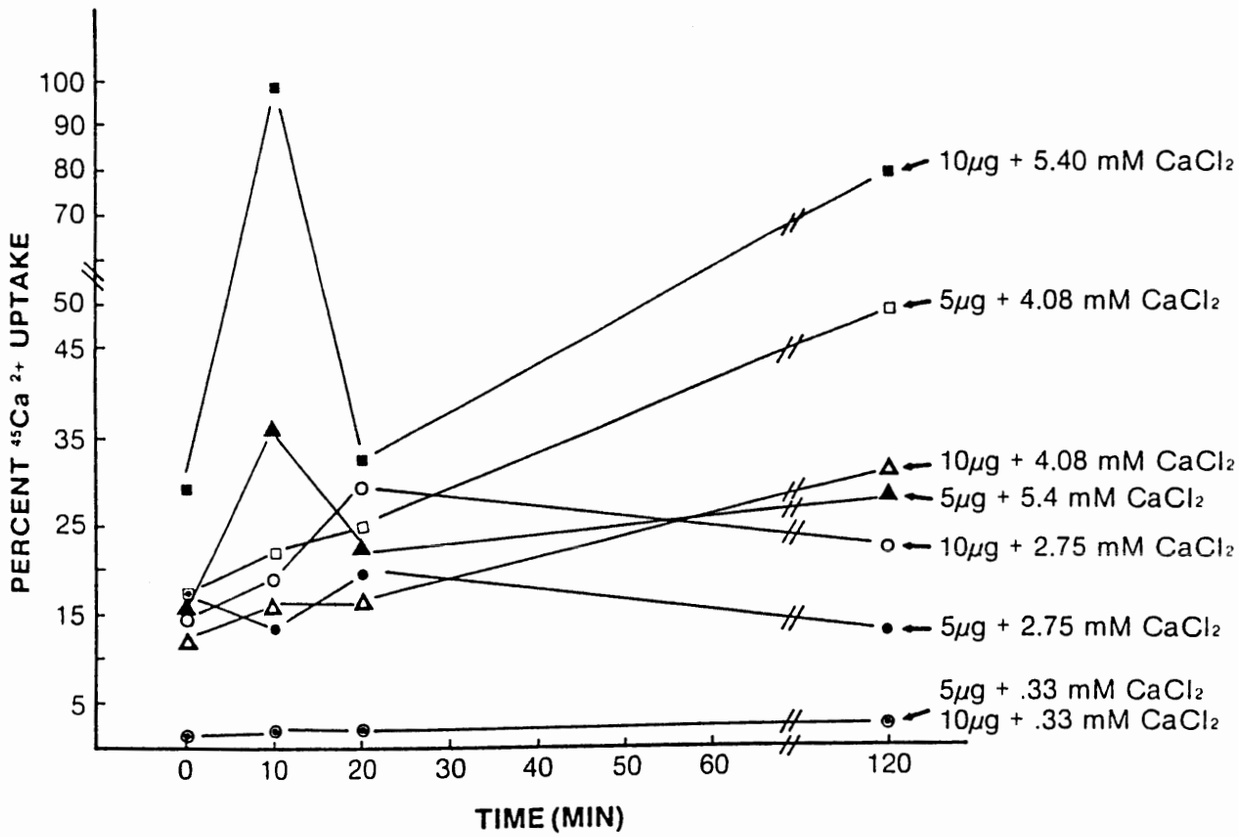

PERCENT $^{45}Ca^{2+}$ UPTAKE
100
90
80
70
50
45
35
25
15
5
0
10
20
30
40
50
60
120
TIME (MIN)
10μg + 5.40 mM CaCl2
5μg + 4.08 mM CaCl2
10μg + 4.08 mM CaCl2
5μg + 5.4 mM CaCl2
10μg + 2.75 mM CaCl2
5μg + 2.75 mM CaCl2
5μg + .33 mM CaCl2
10μg + .33 mM CaCl2

Table VI. The Effects of the Ionophore A23187 on the Secretion of Radiolabeled Proteins by HTE Cells in the Presence of Serum ± EGTA[1].

Human Serum (20%)	Ionophore (10 μg)	EGTA (4 mM)	Calcium Conc (mM)	CPM Secreted	Percent Secreted
CF	0	0	0.33[2]	16,529 ± 159[3]	100
Normal	0	0	0.33	9,342 ± 197	56
Normal	+	0	0.33	21,288 ± 2468	128
Normal	+	+	0.33	8,113 ± 1014	49
Normal	+	0	5.40	21,531 ± 2422	130
Normal	+	+	5.40	10,906 ± 1120	66

[1]Near confluent cultures of HTE cells were prelabeled with [^{14}C] thr (0.3 μCi/ml) in F12 medium containing 5% FCS. At one hour, the cells were washed and 0.5 ml F12 medium containing label plus either normal or CF sera were added. After 3 hrs, the medium was precipitated with TCA/PTA and processed for scintillation spectrometry as described in Methods. Each value represents the average of duplicate samples.

[2]The normal concentration of calcium in F12 medium is 0.33 M.

[3]± S.E.

5.40mM at 5 μg/ml and 10 μg/ml ionophore were tested on calcium uptake (Figure 20). The amount of $^{45}Ca^{2+}$ taken up by the cells increased over a 2h period. These same concentrations of calcium ion and ionophore were tested in the secretion stimulation assay (Table V). The cells were placed in various concentrations of calcium ion and 10 μg/ml ionophore in F12 medium lacking serum for a period of 3h after which they were processed as usual. Secretion was stimulated independently of the amount of calcium ion in the medium. The addition of EGTA caused a decrease in amount of protein secreted, while the cellular radioactivity increased.

Experiments were conducted to establish whether or not NHS in the presence of ionophore could mimic the CFS response. Table VI shows the results in which NHS in the presence of either low calcium (0.33mM) or high calcium (5.40mM) plus ionophore stimulated secretion to the level obtained with CFS and the addition of EGTA abolished the secretory response to basal level. Moreover, since NHS placed in the presence of high calcium ion (5.40mM) did not cause an increase in secretion (data not shown) when compared to results obtained with the low calcium ion concentration (0.33mM), the secretion stimulatory effect required the presence of ionophore.

Figure 21. The effects EGTA and verapamil on secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium, containing 5% FCS and 0.3 μCi/ml [^{14}C]threonine. After 1h the cells were washed and 0.5 ml F12 medium containing label, normal or CF serum, and either 4 mM EGTA and/or 10^{-8} M verapamil were added. The cells were allowed to secrete for 3h before being processed for liquid scintillation spectrometry. The results are expressed as the mean of 5 plates. NHS: normal human serum; CFS: cystic fibrosis serum.

Inset: The effect of verapamil on $^{45}Ca^{2+}$ uptakeby HTE cells in the presence of CF serum proteins. The cells were allowed to take up $^{45}Ca^{2+}$ (5 μCi/ml) in 0.5 ml F12 medium containing 20% CFS in the presence of increasing concentrations of verapamil. The cells were processed for liquid scintillation spectrometry, as described in Methods. The data are represented as the mean ± S.E. of triplicate plates.

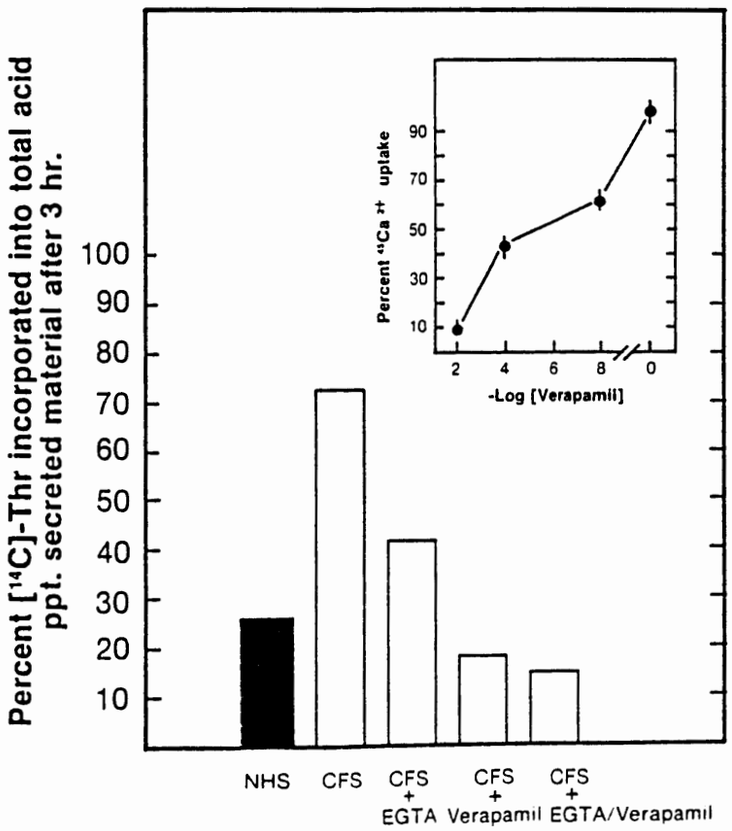
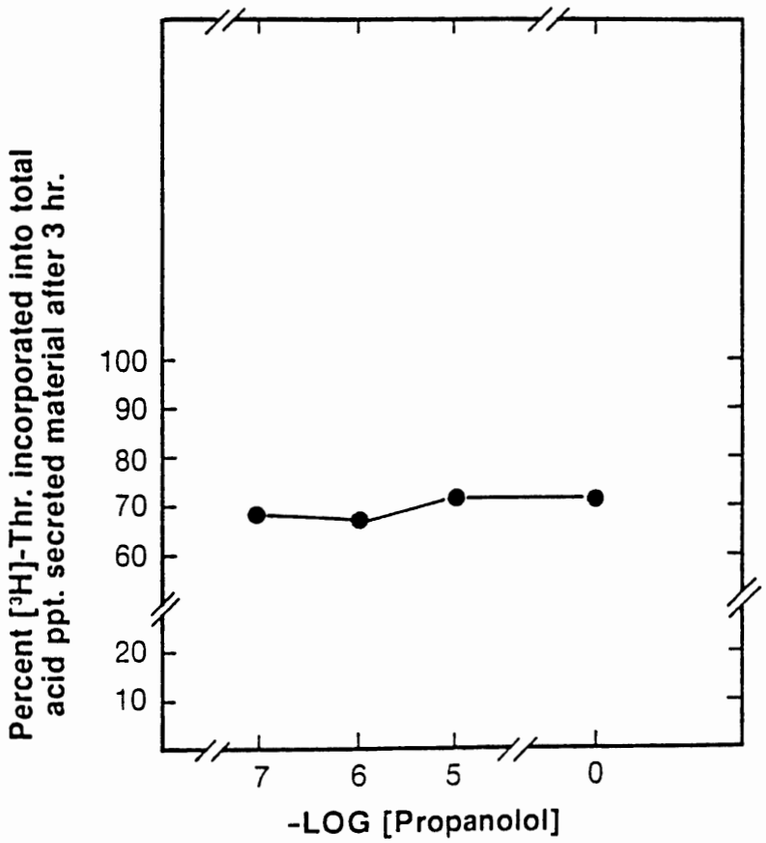

Percent [14C]-Thr incorporated into total acid ppt. secreted material after 3 hr.
100
90
80
70
60
50
40
30
20
10
NHS
CFS
CFS + EGTA
CFS + Verapamil
CFS + EGTA/Verapamil
Percent 45Ca ++ uptake
90
70
50
30
10
2
4
6
8
0
-Log [Verapamil]

Figure 22. The effects of propanolol on secretion of [^{3}H]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium containing 5% FCS, 1.5 μCi/ml [^{3}H]threonine, and propanolol ranging in concentration from 10^{-7} to 10^{-5}M. After 1h the cells were washed and 0.5 ml F12 medium containing label, 20% CFS and propanolol were added. The cells were allowed to secrete for 3h before being processed for liquid scintillation spectrometry, as described in Methods. The results are expressed as the mean of triplicate samples.


Percent [³H]-Thr. incorporated into total acid ppt. secreted material after 3 hr.
100
90
80
70
60
20
10
7
6
5
0
-LOG [Propanolol]

Effects of Verapamil on Ca^{2+} Uptake and Secretion

An attempt was made to determine what route calcium ion was taking to enter the HTE cells by using verapamil, a calcium channel blocker (Lee and Tsien, 1983). The drug was added to 20% CFS and $^{45}Ca^{2+}$ uptake was allowed for 30 min. Approximately 45% inhibition of Ca^{2+} uptake was observed at 10^{-8}M verapamil (Figure 21, inset). When this concentration of verapamil was tested in the secretion assay, it brought the secretory response to the basal level obtained with NHS.

Effects of Adrenergic Blocker on Secretion

Two types of adrenergic receptors are found on mammalian calls, alpha and beta. The alpha receptor acts via a calcium dependent pathway (Quissell and Barzen, 1980), while the beta receptor operates via cAMP (Quissell and Barzen, 1980; Rubin, 1982; Kawai and Arinze, 1983). In a preliminary attempt to determine if the stimulatory response was dependent upon cAMP activation the beta blocker propanolol was administered at various concentrations to HTE cells during prelabeling and readministered upon the addition of 20% CFS. Figure 22 shows the results obtained after 3h of secretion. The blocker had no observable effect upon the stimulatory response of CFS.

Sodium-Calcium Exchange

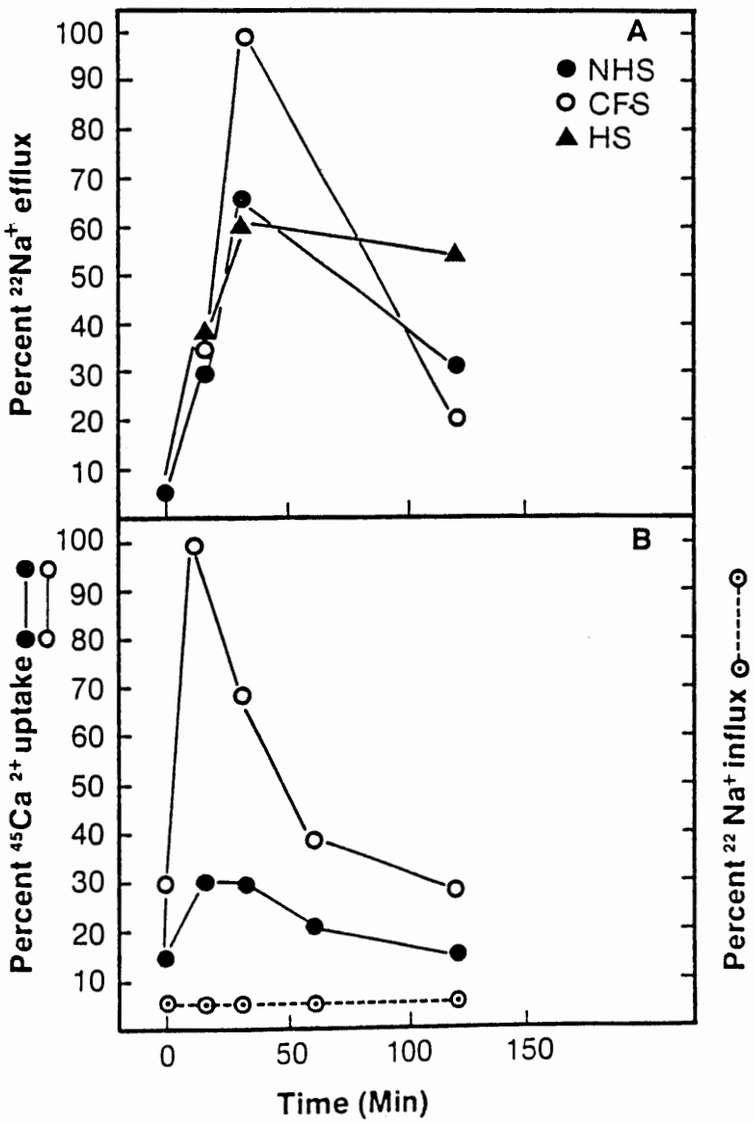
Although calcium ion has been shown to be a ubiquitous intracellular component and plays a defined role in secretion, other ions as well are coupled to the influx or efflux of calcium. For example, in extensively studied systems there is a well documented catio-anion exchange in which as calcium enters the cell sodium leaves the cell, if the calcium electrochemical gradient is large enough to motivate this efflux. Therefore, in an attempt to define the HTE system with regard to the role other electrolytes play in the movement of calcium, initial studies were undertaken using sodium ion.

Cells were incubated in medium containing $^{22}Na^{+}$ for a period of 24h to 'load up' intracellular pools, then they were washed and placed into medium containing 20% CFS, HHS, or NHS and processed, as described in Methods. Figure 23a shows that a rapid sodium efflux occurred upon the addition of CFS to the incubation medium with a peak occurring at approximately 30 min. which is the time of maximum calcium uptake. NHS and HHS produced only half the effect seen with CFS. The influx of Na^{+} and uptake of Ca^{2+} were simultaneously measured by the addition of both radiolabeled ions to the incubation medium. Figure 23b

Figure 23. A. The effect of normal, heterozygous, and CF serum on efflux of sodium from HTE cells. Cells were incubated overnight in 0.5 ml F12 medium containing 0.5 μCi/ml $^{22}Na^{+}$ and 10% FCS. The medium was removed and replaced with 0.5 ml F12 medium containing either NHS, HHS, or CFS at 20% concentration. Samples were taken with time by collecting the medium and processing for liquid scintillation spectrometry, as described in Methods. The data are represented as the mean of triplicate plates.

B. The effect of normal and CF serum on the influx of sodium and uptake of calcium by HTE cells. The medium was removed and replaced with 0.5 ml F12 containing 5μCi/ml $^{45}Ca^{2+}$ plus 0.5 μCi/ml $^{22}Na^{+}$ and either normal or CF serum at 20% concentration. Samples were taken with time by removing the medium and processing the cells for liquid scintillation spectrometry, as described in Methods. The data are represented as the mean of triplicate plates.

NHS: normal human serum; CFS: cystic fibrosis serum; HS: heterozygous serum.


A
NHS
CFS
HS
Percent $^{22}Na^{+}$ efflux
B
Percent $^{45}Ca^{2+}$ uptake
Percent $^{22}Na^{+}$ influx
Time (Min)
0
50
100
150
10
20
30
40
50
60
70
80
90
100

shows the usual calcium spike was seen with CFS, but these was no measurable Na^+ influx with either CFS or NHS.

Purification of Factor Associated with IgG

It has been reported that CF serum factor is associated with serum IgG (Blitzer and Shapira, 1982a and b; Danes *et al*., 1973; Carson *et al*., 1976; Barnett *et al*., 1973b; Bowman, 1976a; Carson and Bowman, 1982; Conover *et al*., 1973; Conover and Conod, 1978; Kurlandsky *et al*., 1980), and that the factor itself has a molecular weight between 4,500-11,000d (Blitzer and Shapira, 1982a and b; Carson and Bowman, 1982; Kurlandasky *et al*., 1980). Therefore, the sera obtained were desalted by Bio-Gel P10 column chromatography and the material eluting at the void volume (VoP10) was collected (Figure 24), dialyzed extensively against water, and lyophilized. Before use the lyophilized proteins were dissolved in PBS to the original serum volume and used in the secretion assay as with serum. In the tracheal ring assay CF VoP10 stimulated mucus extrusion more than normal human VoP10 (Figure 25a compared with b).

Next the VoP10 was tested in the secretion assay. The cells were prelabeled for 1h with 0.5 ml [^{3}H]threonine in F12 medium lacking FCS. Serum or VoP10 was added to a final concentration of 25% and samples of medium were collected with time for processing. The results in Figure 26

Figure 24. Bio-Gel P10 column chromatography of pooled sera. The total bed volume was 2000 ml. The column was equilibrated and eluted with PBS, pH 7.4. Ten milliter fractions were collected by monitoring the absorbance at 280 nm ●——●, and 220 nm o——o.

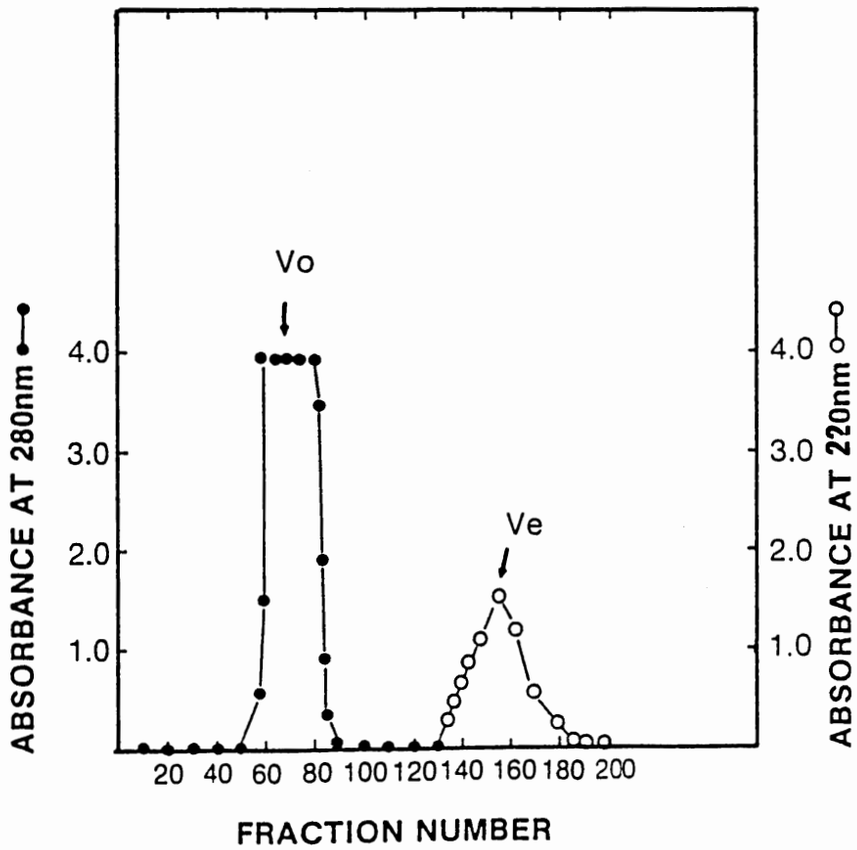
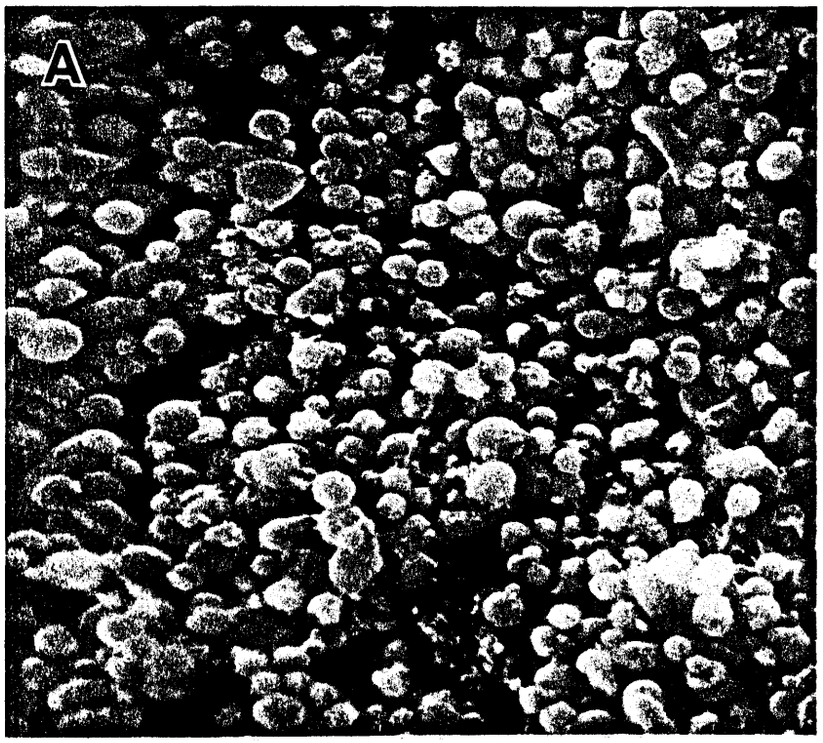

Vo
Ve
ABSORBANCE AT 280nm
ABSORBANCE AT 220nm
4.0
3.0
2.0
1.0
20 40 60 80 100 120 140 160 180 200
FRACTION NUMBER

Figure 25. Scanning electron micrographs of hamster tracheal rings which were treated with either normal or CF proteins which had been chromatographed through Bio-Gel P10 and emerged in the void volume (Vo). Hamster tracheal rings were incubated for 24h in F12 medium after being removed from the animals, then placed in fresh medium plus 25% VoP10 serum proteins for 1h.

A. Ring treated with VoP10 from CFS (660x).

B. Ring treated with VoP10 from NHS (660x).


A

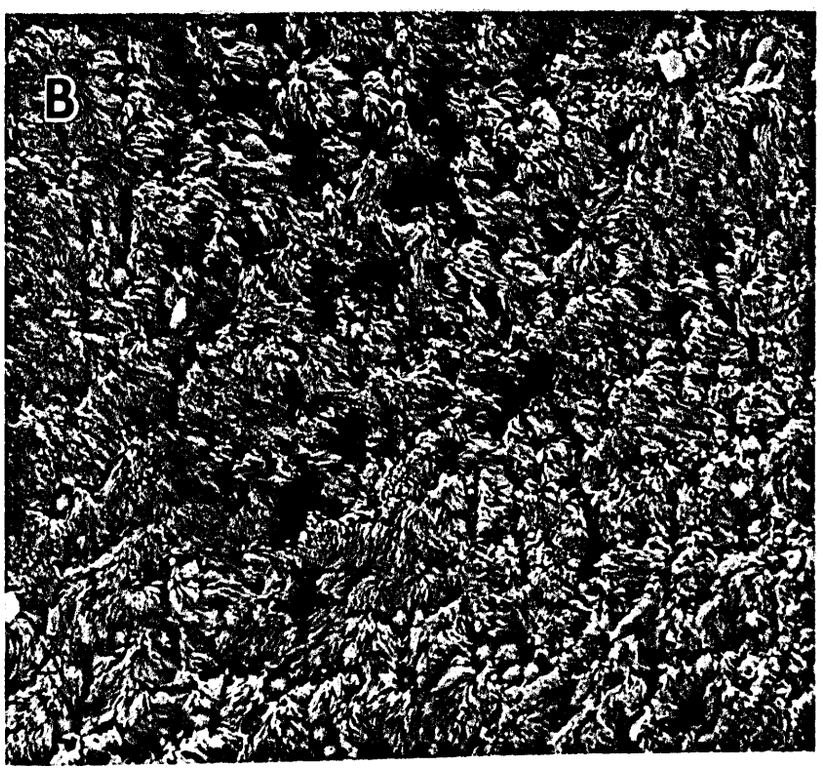

B

Figure 26. The effects of serum proteins and partially fractionated (VoP10) from either normal or CF serum on the secretion of [^{3}H]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium containing 0.5 μCi/ml [^{3}H]threonine. After 1h either fractionated or whole serum proteins were added at 25% concentration to 0.5 ml F12 medium containing label. The cells were allowed to secrete in the presence of test protein, the medium was collected and precipitated with TCA-PTA and processed for liquid scintillation spectrometry, as described in Methods. The arrow represents addition of appropriate serum proteins after 1h prelabel. The data are expressed as the mean of triplicate samples taken at each time for each treatment.

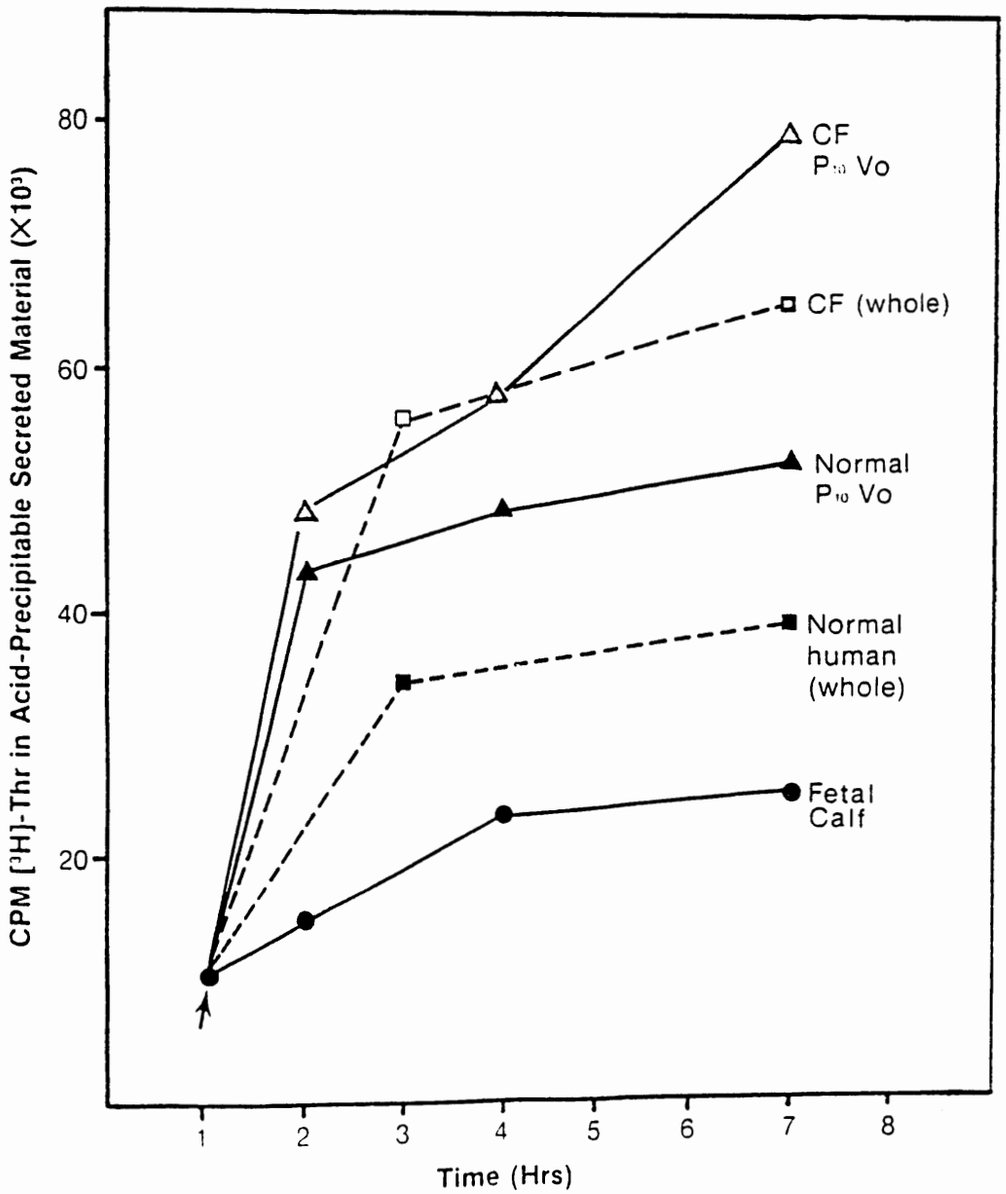

CPM [³H]-Thr in Acid-Precipitable Secreted Material (X10³)
80
60
40
20
CF
P₁₀ Vo
CF (whole)
Normal
P₁₀ Vo
Normal
human
(whole)
Fetal
Calf
1
2
3
4
5
6
7
8
Time (Hrs)

Table VII. The effects of serum proteins from normal and cystic fibrosis patients on secretion of [^{3}H]-threonine-labeled proteins by HTE cells[A].

Serum	Total precipitable activity per dish (cpm)	Percent of total acid-precipitable material secreted
Fetal Calf	164,289	15.1
Normal P_{10} Vo	228,534	23.1
CF P_{10} Vo	159,543	49.9
Normal Human (whole)	166,650	23.1
CF (whole)	95,898	67.3

A. HTE cells pre-labeled for 1 h with [^{3}H]-thr. in serum-free medium were placed in fresh medium with the appropriate 25% serum additions. Cells were allowed to secrete for 6 h before medium was precipitated with 10% TCA/0.5% PTA and the cells with 5% TCA. Values are the average of 3 samples.

show that the amount of secretion in the presence of CF VoP10 was at least 1.5 times greater than that with normal VoP10. Also, as reported above, on the average CFS stimulated secretion two-fold more than did NHS.

When the percent of total acid precipitable material secreted was calculated, the results were much more dramatic after 6h of secretion (Table VII). In the presence of CF VoP10 or CFS 49-67% of total acid precipitable material was secreted, compared to 23% for NHS or normal VoP10.

The VoP10 fraction was dialyzed against 0.02M potassium phosphate buffer, pH 8.0, and applied to a DEAE Affi-gel blue column (Figure 27). These proteins were collected, as described in Methods, dialyzed against water and lyophilized. Before use in the HTE assay the proteins were reconstituted to their original volume of serum and used in the assay at physiological concentration. The cells were prelabeled with 0.3 μCi/ml of [^{14}C]threonine plus 5% FCS in 0.5 ml F12 medium. After 1h the medium was removed, replaced with fresh medium containing radiolabel and the unbound fraction was added, and samples were taken with time. The unbound fraction was mainly IgG, as demonstrated by double immunodiffusion with sheep anti-human IgG and the SDS-PAGE electrophoretic profile, showing both heavy and

Figure 27. DEAE Affi-gel blue column chromatography of normal or CF Bio-Gel VoP10 serum proteins. An 8:1 ratio of matrix to serum was used to prepare the column. The column was equilibrated and eluted with 0.02 M potassium phosphate buffer, pH 8.0. Fractions were collected which equalled approximately the sample volume applied to the column. The unbound fraction, mainly IgG, was pooled by collecting the first protein peak to equal eight times the sample volume. The proteins which remained bound to the matrix were eluted by the addition of 1.4 M NaCl to the elution buffer.
●——● 280 nm absorbance.

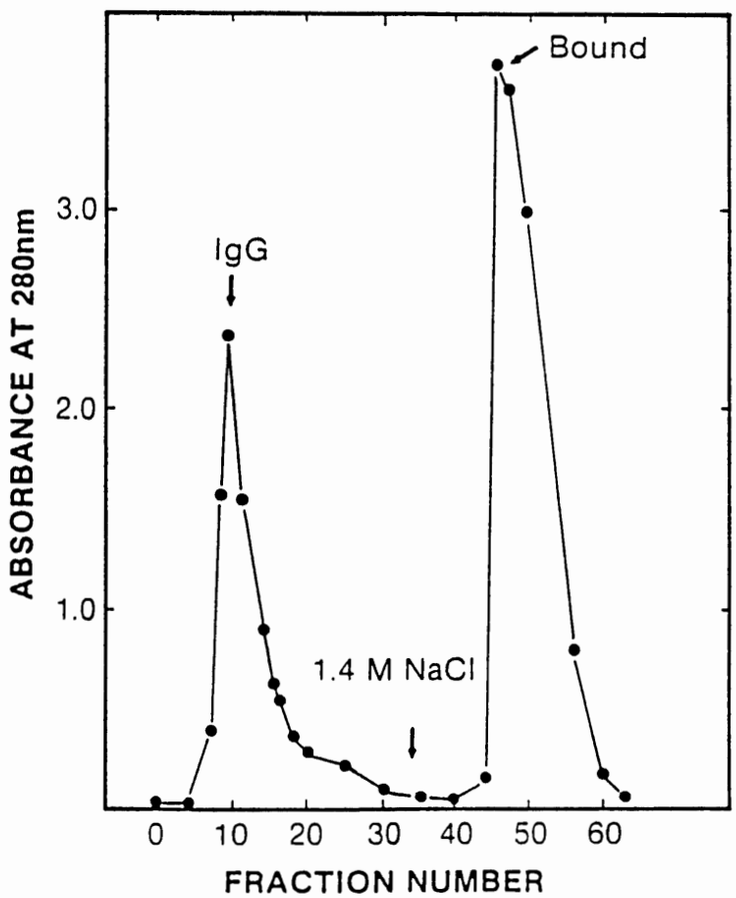
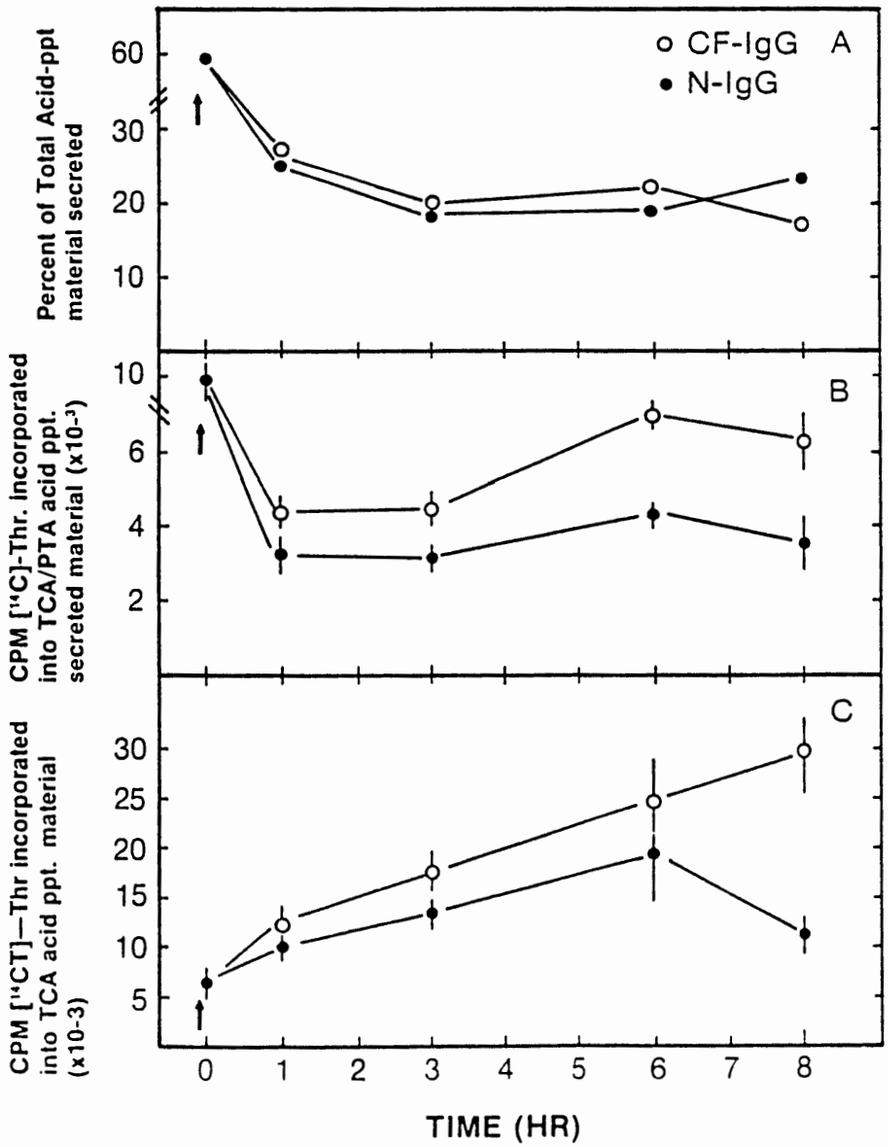

Bound
IgG
1.4 M NaCl
ABSORBANCE AT 280nm
3.0
2.0
1.0
0
10
20
30
40
50
60
FRACTION NUMBER

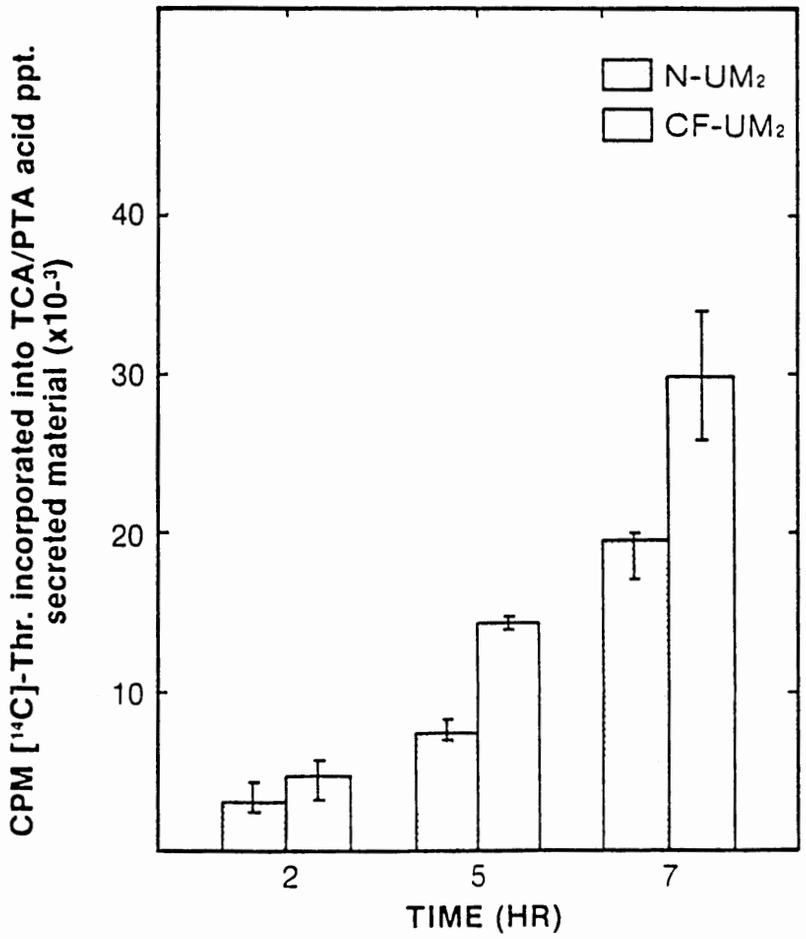
Figure 28. The effects of IgG from CF or normal serum on secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium containing 5% FCS and 0.3 μCi/ml [^{14}C]threonine. After 1h prelabel the cells were washed and 0.5 ml F12 containing label and IgG was added at 1% concentration. The arrow represents the addition of IgG from either normal or CF serum after 1h prelabel. The cells were allowed to secrete and samples were taken with time. The medium was collected and precipitated with TCA-PTA and the cells were precipitated with TCA. The samples were processed for liquid scintillation spectrometry, as described in Methods. The results are expressed as the mean ± S.E. The data are representative of 5 independent experiments in which different preparations of IgG were used. CF-IgG: cystic fibrosis serum IgG; N-IgG: normal serum IgG.


CF-IgG
N-IgG
A
B
C
Percent of Total Acid-ppt material secreted
CPM [14C]-Thr. incorporated into TCA/PTA acid ppt. secreted material (x10-3)
CPM [14CT]—Thr incorporated into TCA acid ppt. material (x10-3)
TIME (HR)

light chains of the IgG molecule. Figure 28, Panel A, shows the results of such an experiment. The results are expressed as the mean ± S.E. of one representative experiment. Several experiments of this type were conducted in which IgG was isolated from several different individuals. Little or no stimulation of secretion was seen in such experiments. The secreted material and the cell bound counts remained approximately constant (Figure 28, Panel B and C). Therefore, the percentage of the total acid precipitable radioactivity that was secreted also remained constant.

Since the IgG fraction apparently did not stimulate the release of any secreted material it was important to establish whether or not the proteins bound to IgG would have an effect. The noncovalently attached proteins were dissociated by treatment with 8M urea followed by ultrafiltration, as described in Methods. These proteins were reconstituted in PBS, and tested in the HTE assay. The cells were prelabeled for 1h in 0.5 ml medium containing 0.5 μCi/ml [^{14}C]threonine. After 1h prelabel, protein concentrations ranging from 1 μg/ml to 50 μg/ml were added to fresh medium containing label and sampled with time. These concentrations had previously been reported to stimulate the release of mucus droplets from rabbit tracheal

Figure 29. The effects of proteins dissociated from IgG on secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were incubated in the presence of 0.5 ml F12 medium and 0.5 μCi/ml [^{14}C]threonine. After 1h 50 μg/ml of protein from either normal or CF IgG was added to the incubation medium. The cells were allowed to secrete for various times after which the medium was collected and precipitated with TCA-PTA and processed for liquid scintillation spectrometry, as described in Methods. The results are expressed as the Σmean ± S.E. from two independent experiments in which duplicate samples were tested. CF-UM_2;N-UM_2 : cystic fibrosis or normal serum peptides obtained after dissociation of serum IgG with 8 M urea and subjected to PM10 and UM2 ultrafiltration, as described in Methods.


N-UM2
CF-UM2
CPM [14C]-Thr. incorporated into TCA/PTA acid ppt. secreted material (x10-3)
40
30
20
10
2
5
7
TIME (HR)

epithelium (Blitzer and Shapira, 1982a). Below 50 μg/ml there was no stimulation of secretion. At 50 μg/ml the release of secreted material was stimulated by approximately 1.2 times over that of protein from normal serum (Figure 29).

Although an apparent effect upon secretion was observed an actual hypersecretory phenomenon was not seen with these proteins in any of the experiments conducted. That is, a decrease in cell bound radioactivity did not accompany the release of secreted material in the presence of the proteins. Thus, the percentage of total acid precipitable secreted material secreted remained constant throughout the experiment.

In conclusion it was found that: 1) IgG alone did not stimulate secretion; 2) no noncovalently IgG-associated proteins had stimulatory activity.

Stimulation of Secretion by Fractionated CF Serum

After elution of the IgG fraction from DEAE Affi-gel blue, the absorbance was allowed to return to baseline after which 1.4 M NaCl was added to the elution buffer. This procedure was used to recover the absorbed serum proteins minus the majority of the IgG fraction. When these bound proteins were tested in the HTE secretion assay, an active stimulatory component was present

Figure 30. The effects of IgG and DEAE Affi-gel blue-bound fractions of CF and normal serum on the secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium containing 5% FCS and 0.3 μCi/ml of [^{14}C]threonine. After 1h the cells were washed and 0.5 ml F12 containing label and fractionated serum proteins were added at physiological concentrations, at the time indicated by the arrow. The cells were allowed to secrete for various times and the medium was collected and precipitated by the addition of TCA-PTA and processed for liquid scintillation spectrometry, as described in Methods. The results are expressed as the mean ± S.E. of one experiment in which triplicate plates were tested. The results are representative of four independent experiments. CF-B, N-B: CF and normal serum bound fraction from DEAF Affi-gel blue column; CF-IgG, N-IgG: serum IgG fraction from either CF or normal serum.

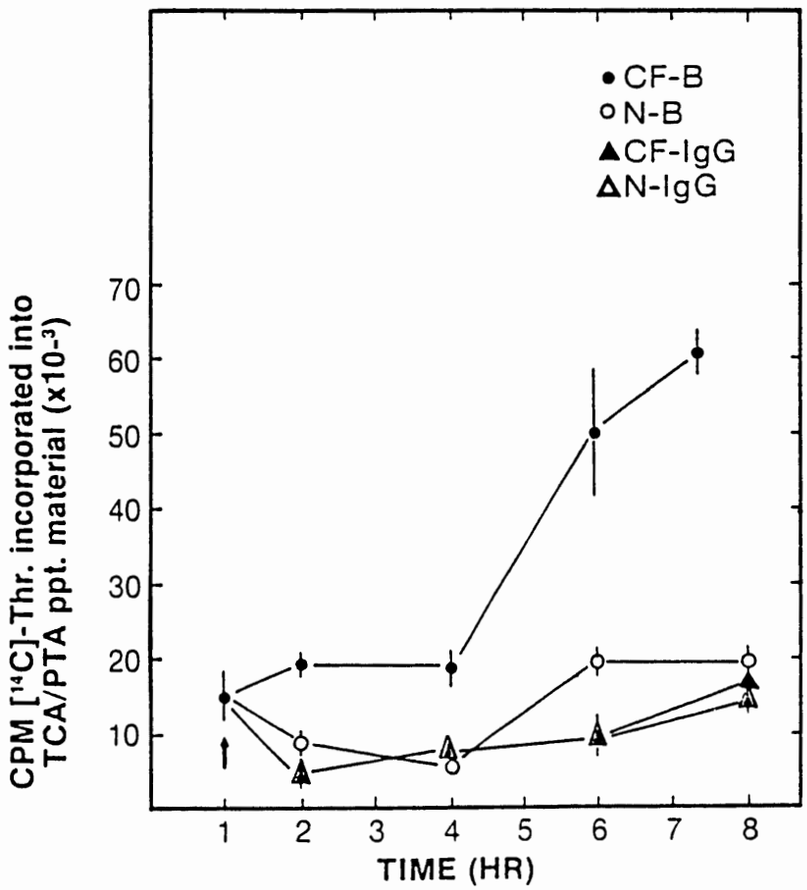
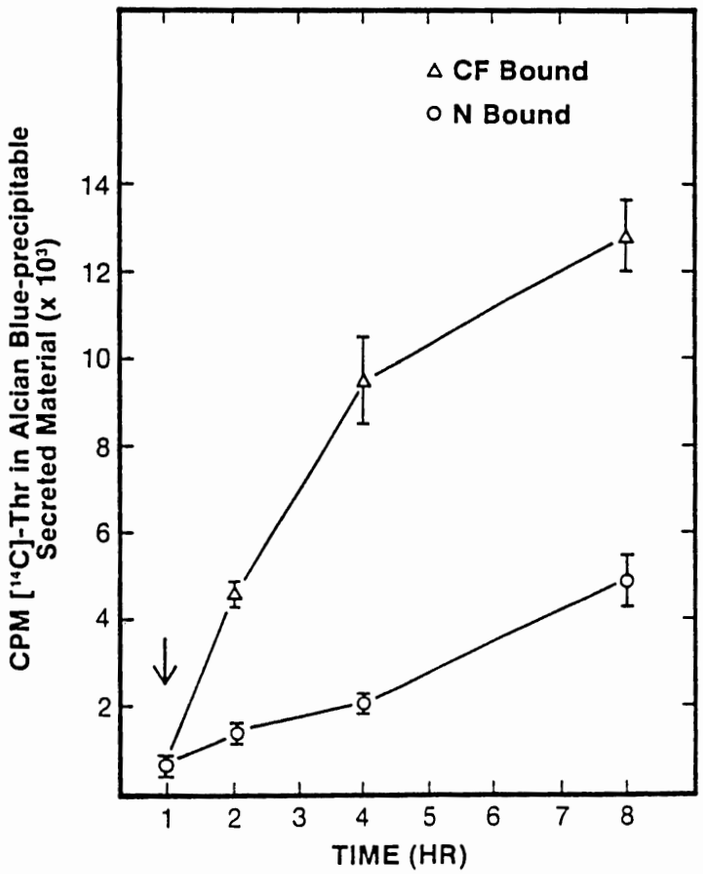

CF-B
N-B
CF-IgG
N-IgG
CPM [14C]-Thr. incorporated into TCA/PTA ppt. material (x10-3)
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
TIME (HR)

Figure 31. The effects of DEAE Affi-gel blue-bound proteins from either normal or CF serum on the secretion of [^{14}C]threonine labeled alcian blue precipitable material. The cells were prelabeled in 0.5 ml F12 medium containing 5% FCS and 0.5 μCi/ml [^{14}C]threonine. After 1h the cells were washed and 0.5 ml F12 medium containing label and bound serum proteins from DEAE Affi-gel blue column were added at 20% concentration as indicated by the arrow. The cells were allowed to secrete for various times, the medium was collected and precipitated by the addition of alcian blue solution and processed for liquid scintillation spectrometry, as described in Methods. The results are expressed as the mean ± S.E. of triplicate plates and is representative of two independent experiments.


△ CF Bound
○ N Bound
CPM [14C]-Thr in Alcian Blue-precipitable Secreted Material (x 10³)
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
TIME (HR)

(Figure 30). To further document the stimulatory response observed in the presence of the bound CF proteins the secreted proteins were precipitated with time by the addition of alcian blue, as described in Methods. Alcian blue specifically precipitates polyanionic glycoproteins (Hall et al., 1980) and, therefore, the 'mucin-like' proteins secreted by these cells would be contained within the precipitates. Figure 31 shows the results obtained from such an experiment. It was found that the bound fraction from CF serum stimulated 'mucin-like' glycoprotein secretion approximately 2.5 fold over that of the bound fraction obtained from normal serum.

Analysis of Secreted Proteins by HTE Cells

The proteins secreted by HTE cells in response to stimulation were analyzed by SDS-PAGE (4-10% linear gradient) followed by fluorography, as described in Methods. Cells were prelabeled for 1h with 0.5 μCi/ml of [^{14}C]threonine, then fresh label in F12 medium plus either the bound serum fraction of CFS or NHS at 20% concentration were added. The cells were allowed to secrete for 3 and 7 hours, after which polyanionic glycoproteins were precipitated from the medium by the addition of alcian blue followed by processing for electrophoresis. Figure 32 shows the resultant fluorogram. It appears that

Figure 32. Fluorogram of [^{14}C]threonine labeled proteins [secreted] by HTE cells. Cells were prelabeled in 100 mm cell culture dishes containing 2.0 ml F12 medium and 2.0 μCi/ml [^{14}C]threonine. After 1hr CF or N bound proteins, obtained from DEAE Affi-gel blue column chromatography, were added at a concentration of 20%. The cells were allowed to secrete for 3 and 7h, then the medium was collected and precipitated by the addition of alcian blue solution, as described in Methods. The pellets were washed three times, resuspended in 0.125M Tris-HCl buffer, pH 6.8, containing 10% SDS, 5% mercaptoethanol, and 1% glycerol, and boiled for 5 min. before being applied to the gel. The precipitates were subjected to SDS-PAGE (4-10% linear gradient); the gel was stained and prepared for fluorography, as described in Methods. The arrows indicate the electrophoretic mobility of the molecular weight markers: 93 kd (phosphorylase A); 63 kd (bovine serum albumin); 48 kd (ovalbumin).

Lane 1. [^{14}C]threonine labeled proteins secreted in response to stimulation by normal fractionated serum proteins, 7h.

Lane 2. [^{14}C]threonine labeled proteins secreted in response to stimulation by CF fractionated serum proteins, 3h.

Lane 3. [^{14}C]threonine labeled proteins secreted in response to stimulation by CF fractionated serum proteins, 7h.

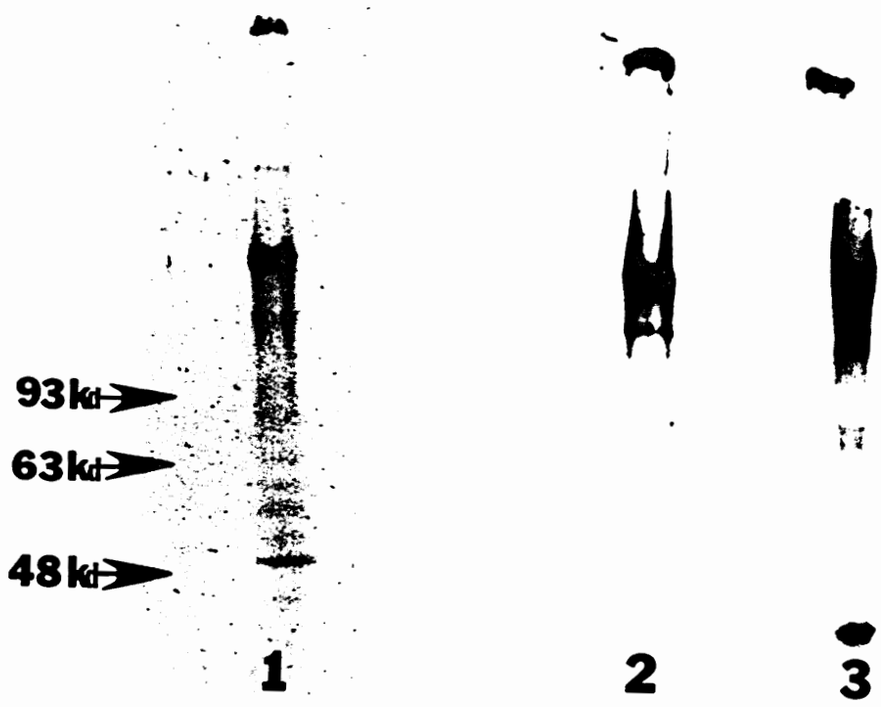

93kd
63kd
48kd
1
2
3

Figure 33. Flow chart of the purification scheme employed to obtain various serum fractions from either normal or CF serum.

SCHEME OF PURIFICATION

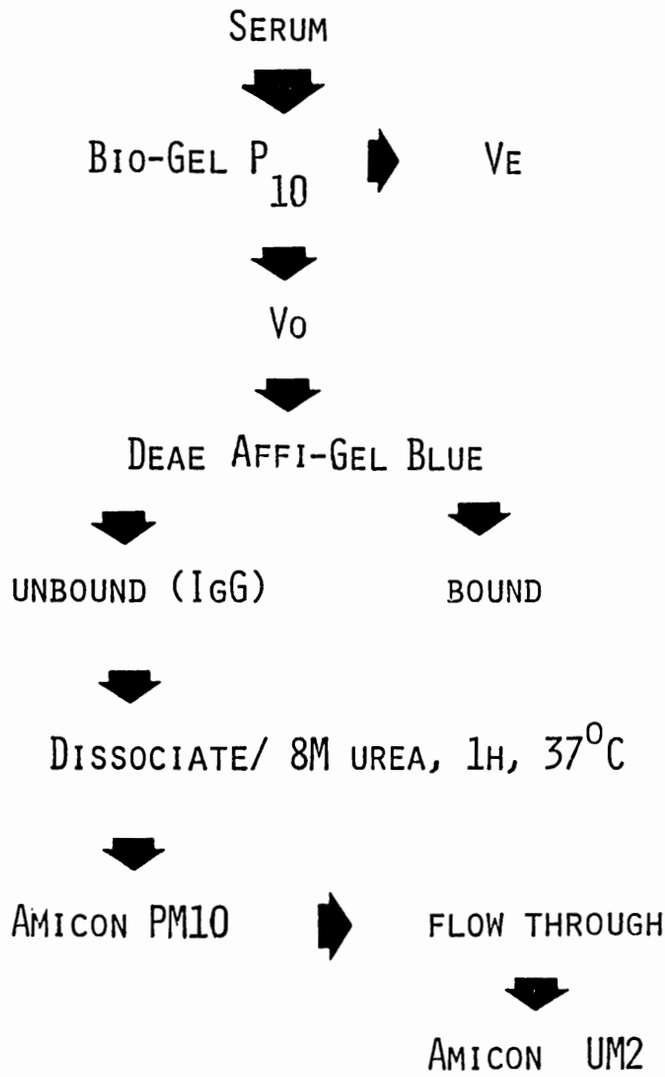
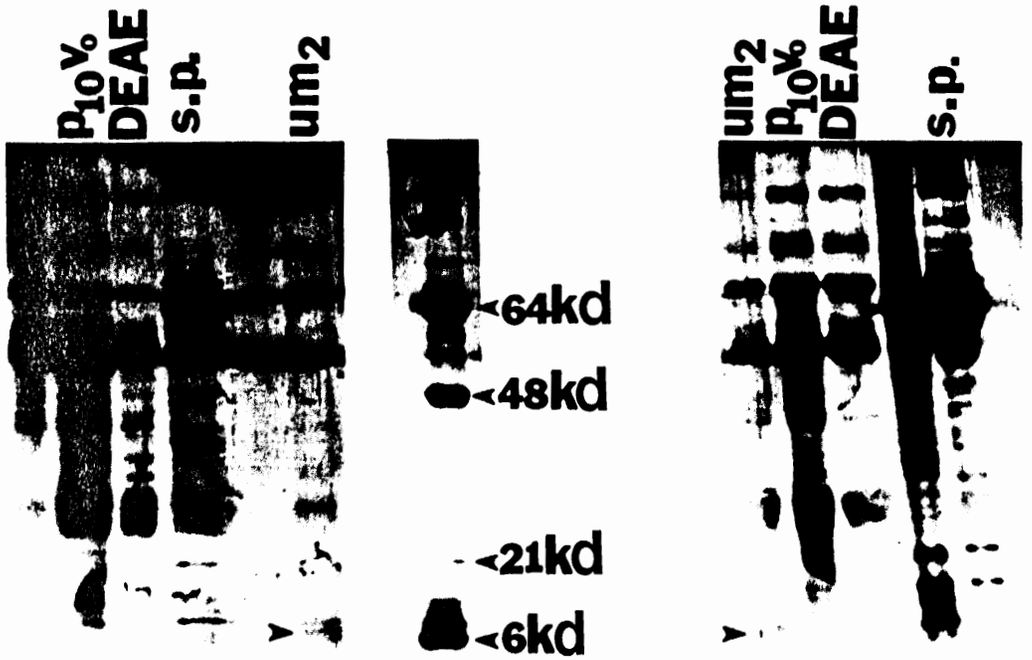


Figure 34. SDS-PAGE (7-20% linear gradient) of the proteins obtained during various steps of purification of factor.

Lanes 2 and 9:
Bio-Gel VoP10 (2.0 μg)
Lanes 3 and 10:
IgG serum fraction (2.0 μg)
Lanes 4 and 12:
total serum proteins (3.0 μg)
Lanes 6 and 8:
UM2 retained proteins (0.5 μg)
Lane 7:
molecular weight markers
64 kd:bovine serum albumin (0.5 μg)
48 kd:ovalbumin (0.5 μg)
21 kd:soybean trypsin inhibitor (0.5 μg)
6 kd:bovin trypsin inhibitor (0.5 μg)

The gel was stained for protein with the Bio-Rad silver stain. The arrow indicates the electrophoretic mobility of low molecular weight proteins bound to serum IgG.



1 2 3 4 5 6 7 8 9 10 11 12

CFS NHS

these cells constitutively secrete several polypeptides ranging widely in molecular weight (Figure 32, lane 1) in the presence of normal factor. After 3 and 7h of stimulation with CF factor the secretion of specific polypeptides was stimulated (Figure 32, lane 2 and 3). There was an appearance of high molecular weight radiolabeled material which did not enter the gel and also heightened synthesis of 200 kd and 30 kd species.

SDS-PAGE of Fractionated Serum Proteins

Figure 33 shows the purification scheme employed throughout fractionation of factor. During each step of fractionation SDS-PAGE was employed to check for relative purification of proteins from either normal or CF serum. Figure 34 shows the results of a 7-20% linear SDS-PAGE treated with the Bio-Rad silver stain, as described in Methods. Relative purity can be seen by comparing total serum protein (Lanes 4 and 12) to fractionated components. Lanes 3 and 10 contain the unadsorbed DEAE-Affi-gel blue fractions. Both the IgG heavy and light chains (50 kd and 23 kd) and contaminating molecules could be detected. This was to be expected due to the increased sensitivity of the silver stain over that of Coomassie Brilliant Blue. Lanes 6 and 8 represent the proteins obtained after dissociation, ultrafiltration and concentration. Contaminant

IgG molecules were also contained within this fraction, as well as low molecular weight species (6 kd) which were initially bound to IgG. The dissociation, ultrafiltration, and concentration steps were carried out once more to remove contaminant species prior to testing in the HTE assay. This treatment removed the majority of the IgG from this fraction as documented by subsequent SDS-PAGE of these proteins.

Ouchterlony Double-Diffusion Immunoprecipitation

The proteins which were unadsorbed to the DEAE Affi-gel blue affinity matrix were tested in Ouchterlony double-diffusion immunoprecipitation. The center well of the diffusion plate contained 10 mg/ml of sheep anti-human IgG and serial dilutions of the unadsorbed fraction from either normal or CF serum were made in adjacent wells starting with undiluted protein up to a dilution of 1:16 with PBS. Cross-reactivity between the unadsorbed proteins and sheep anti-human IgG was evident from the precipitin line which encompassed the center well (Figure 35).

Stimulus-Secretion Coupling as a Means of Factor Purification

The foregoing established that stimulus-secretion coupling could be used as a quantitative means of assessing the presence of factor and would provide a method for

Figure 35. Ouchterlony double-diffusion immunoprecipitation of normal or CF serum unbound fraction from DEAE Affi-gel blue column chromatography. IgG isolated by DEAE Affi-gel blue column chromatography was tested in Ouchterlony double-diffusion immunoprecipitation. The center well contained 10 mg/ml of sheep anti-human IgG. The arrow indicates the addition of 20 µl of undiluted IgG followed by 1:2, 1:4, and 1:16 dilution of IgG in PBS.

A. IgG fraction from CF serum.

B. IgG fraction from normal serum.

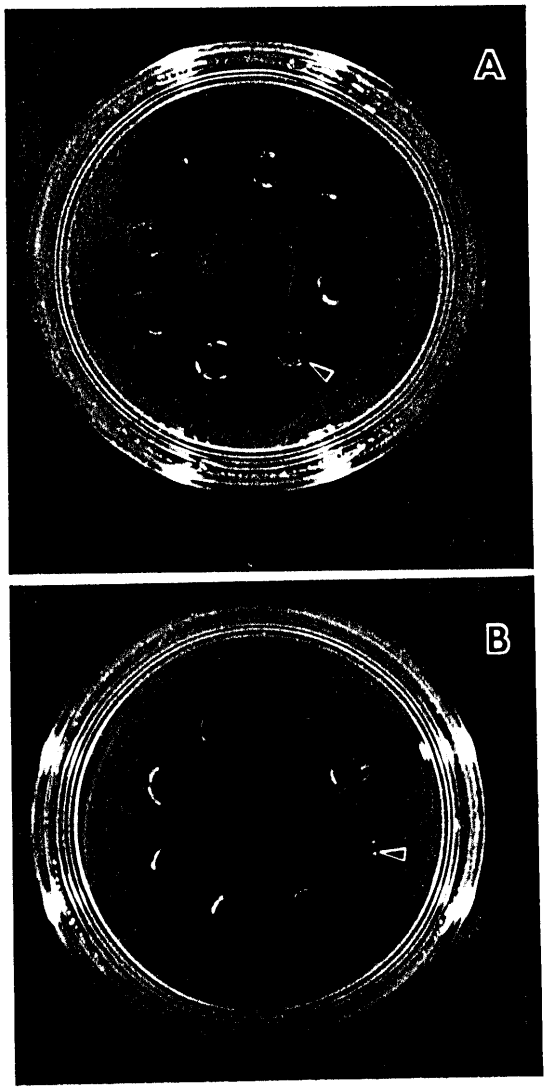

A
B

Figure 36. The effects of IgG and bound fraction from CF or normal serum on the uptake of calcium ion by HTE cells. Proteins were added at physiological concentration in 0.5 ml F12 medium containing 5 μCi/ml $^{45}Ca^{2+}$. Samples were taken with time by removing the medium and processing the cells for liquid scintillation spectrometry, as described in Methods. The data are expressed as the Σ mean ± S.E. of two independent experiments in which duplicate plates were tested. N-B, CF-B: Normal or CF serum fractionated on DEAE Affi-gel blue column chromatography; CF-IgG: cystic fibrosis serum IgG fraction.

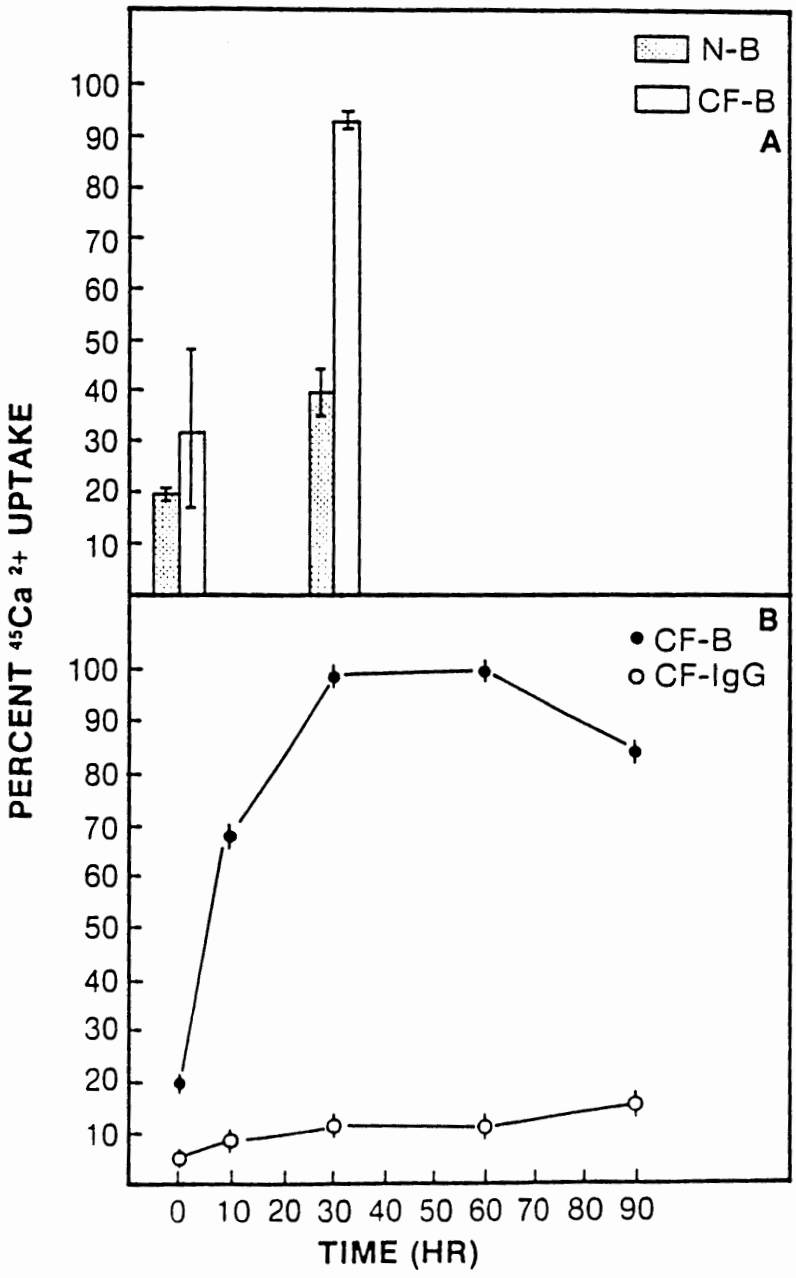
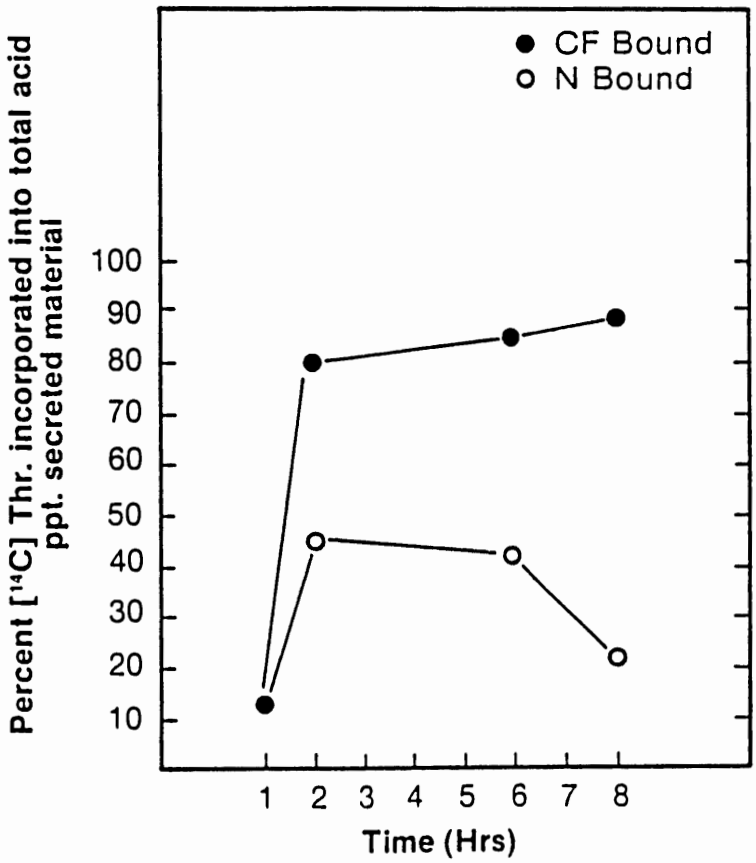

N-B
CF-B
A
B
CF-B
CF-IgG
PERCENT $^{45}Ca^{2+}$ UPTAKE
100
90
80
70
60
50
40
30
20
10
0 10 20 30 40 50 60 70 80 90
TIME (HR)

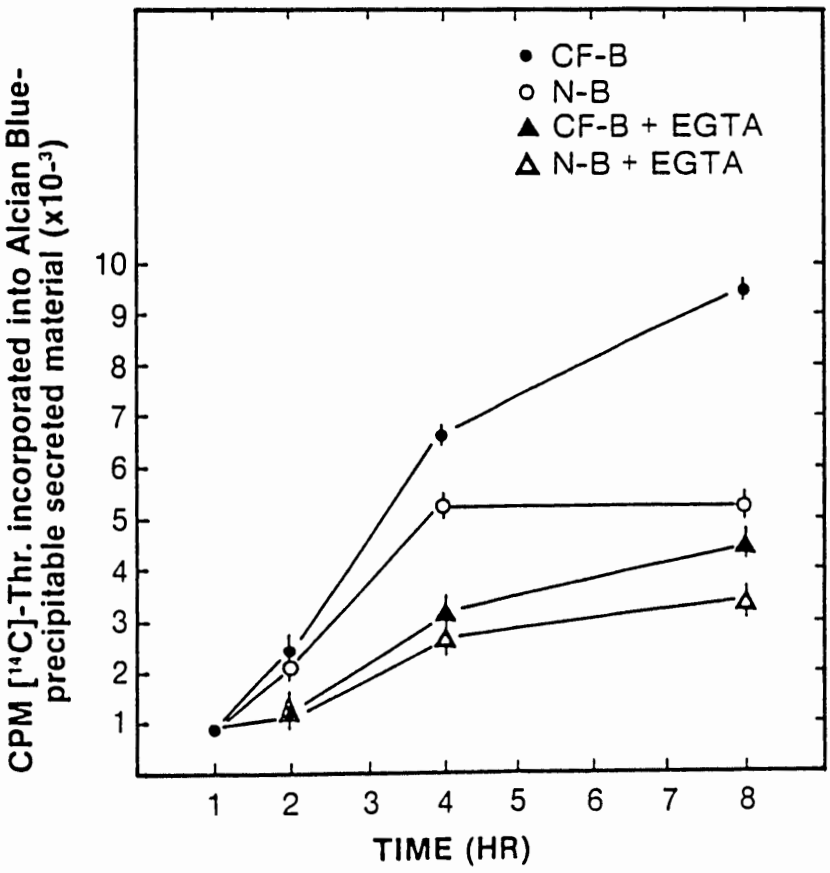
Figure 37. Effects of fractionated normal or CF serum of the secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium containing 0.3 μCi/ml of [^{14}C]threonine plus 5% FCS. After 1h the medium was removed and the cells were washed, 0.5 ml F12 was added which contained labed and 20% fractionated serum proteins. The cells were allowed to secrete with time and both cells and medium were collected and processed for liquid scintillation spectrometry, as described in Methods. Each point is the mean of triplicate plates and is representative of three independent experiments. CF-bound, N-bound: CF or normal serum fractionated on DEAE Affi-gel blue column chromatography.


CF Bound
N Bound
Percent [14C] Thr. incorporated into total acid ppt. secreted material
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
Time (Hrs)

further fractionation of the active stimulatory component in CF serum.

The proteins bound by the DEAE Affi-gel blue were tested in their ability to cause the cells to take up calcium and concomitantly cause an increase in cellular secretion. It was found that the CF proteins stimulated uptake of calcium ion over that of the corresponding normal proteins (Figure 36), and this response was similar to that observed with whole serum over that of the IgG fraction. Also this serum fraction stimulated the percentage of total acid precipitable material secreted by the cells approximately 2.5 fold over its normal counterpart (Figure 37). Not only was there a stimulation of secretory activity but also the types of proteins stimulated to be secreted were polyanionic, 'mucin-like', glycoproteins as revealed by precipitation with alcian blue (Figure 38). Once again there was approximately a 2.5 fold increase in secretion in the presence of CFS proteins over those of the normal counterpart. Chelation of available calcium ion with EGTA resulted in abolishment of the level of secretion obtained with the CFS proteins to the level obtained with the normal fraction.

Figure 38. The effects of EGTA on the secretion of [^{14}C]threonine labeled proteins by HTE cells. The cells were prelabeled in 0.5 ml F12 medium containing 0.5 μCi/ml [^{14}C]threonine plus 5% FCS. After 1h the medium was removed and the cells were washed and replaced with 0.5 ml F12 medium containing label and 20% normal or CF fractionated serum ± EGTA. The cells were allowed to secrete for various times and the medium was collected and precipitated with alcian blue and processed for liquid scintillation spectrometry, as described in Methods. The data are expressed as the mean of triplicate plates and are representative of two independent experiments. CF-B, N-B: cystic fibrosis or normal serum bound fraction chromatographed on DEAE Affi-gel blue.


CF-B
N-B
CF-B + EGTA
N-B + EGTA
CPM [14C]-Thr. incorporated into Alcian Blue-precipitable secreted material (x10-3)
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
TIME (HR)

Effect of Chymostatin on Secretion

Patients affected with CF harbor *Pseudomonas aeruginosa* (PA) in the respiratory tract. Some reports have indicated that the exotoxin A and elastase, which are proteases secreted by PA, will cause expulsion of mucus from the epithelium of rabbit tracheal explants. These proteases are serine type proteases (Klinger *et al*., 1978) and, therefore, their activity can be abolished by treatment with a serine protease inhibitor, chymostatin. The cells were exposed to various concentrations of chymostatin, 50 μg/ml and 250 μg/ml, in the presence of CFS and allowed to secrete for various times before being precipitated with alcian blue. Neither concentrations of inhibitor tested had any effect on the amount of alcian blue precipitable secreted material by HTE cells in response to stimulation by CFS (Table VIII).

Table VIII. Effects of Chymostatin on the Secretion of Alcian Blue-Precipitable Material by HTE Cells.[1]

Concentration of Protease Inhibitor (μg/ml)	CPM Incorporated Into Alcian Blue Precipitable Material ($\times 10^{-3}$)		
	1 h	3 h	7 h
0	2294 ± 719[2]	6618 ± 134	9591 ± 85
50	1621 ± 19	4708 ± 85	9420 ± 716
250	2581 ± 171	—	9049 ± 253

[1]Near confluent HTE cells were prelabeled with [^{14}C] thr (0.5 μCi/ml) in F12 medium containing 5% FCS. At one hour the cells were placed in 0.5 ml fresh F12 medium containing label plus 20% CF serum ± the protease inhibitor chymostatin. At the indicated times, the medium was precipitated with Alcian blue and processed for scintillation spectrometry as described in Methods.

[2]± S.E.

DISCUSSION

Fibroblast Secreted Proteins and Their Effect Upon Mucociliary Activity

Factors which alter ciliary rhythm of tracheal cilia were first described by Spock (1967). These factors have been reported to play a role in the etiology of CF by their apparent effect of the stimulation of mucus secretion by these tissues (Nagy *et al*., 1979). In this study hamster tracheal rings which were exposed to CF serum (CFS) showed an increased number of mucus droplets of the luminal surface, while normal (NHS), bronchitic (BHS), or fetal calf (FCS) serum did not elicit this response, as observed by SEM. This is in agreement with several other studies using rabbit tracheal explants (Nagy and Sturgess, 1976; Bogart *et al*., 1977; Bogart *et al*., 1978; Conover and Conod, 1978; Gabridge *et al*., 1979; Boat *et al*., 1982). Serum from the heterozygous parents also caused an increase in mucus release. However, it was difficult with this assay to determine if there was any difference in the amount of mucus released whem compared to CFS.

Several investigators have reported that CF fibroblasts could be used as *in vitro* sources of CF factor (Barnett *et al*., 1973a; Beratis *et al*., 1973; Bowman *et al*,

1973; Conover et al., 1976) because their secretions have a similar effect on the mucociliary activity of oyster gill and tracheal explants. The serum ciliary inhibitory activity has been shown to be IgG requiring (Carson et al., 1976; Carson and Bowman, 1982), although other reports indicate that the CF serum factor will induce ciliary dyskinesis without IgG being present (Blitzer and Shapira, 1982a). The ciliary dyskinesia factor secreted by fibroblasts has been assumed to share properties in common with the serum factor. Based upon this assumption the activity of the secreted factor has been shown to require the presence of IgG (Barnett et al., 1973a), while others do not find this to be the case (Conover et al., 1973; Tegner et al., 1981). Work by Kennedy et al. (1982) has suggested that the serum mucus stimulatory activity and the ciliary dyskinesia activity may reside in separate proteins. Moreover, several investigators have shown that ciliary-inhibition is a general serum induced phenomenon (Sanderson and Sleigh, 1981; Kennedy et al., 1982). This has been explained on the basis of the serum complement fraction causing 'leaky' membranes (Bhakdi and Jensen-Tranum, 1983) and, thus, causing an arrest in ciliary activity or alteration in ciliary rhythm due to an increased membrane permeability for calcium ion which would induce this effect (Satir, 1975; Walter and Satir, 1978). It was necessary

to determine optimal conditions for using the explant bioassay, prior to attempting to screen fibroblast secretions for mucus stimulatory activity. The data indicate that serum concentration plays a critical role in maintaining the integrity of the epithelium. Clear cut stimulatory effects between NHS and CFS could be detected with 10% serum. However, no differences between any of the CF-fibroblast and N-fibroblast proteins in their ability to induce secretion of mucus from tracheal rings, and no differences in the protein patterns following SDS-PAGE could be detected. It is possible that the fibroblasts secrete too little protein for detection of its activity. However, Blitzer and Shapira (1982a) reported clear-cut differences between normal and CF purified serum factor with concentrations as low as 1 μg/ml. Also Barnett et al. (1973a), Bowman et al. (1973), and Conover et al. (1974) reported that the ciliary inhibitory activity could be detected in unconcentrated conditioned medium. To determine whether or not there was a prerequisite for the mucus stimulatory activity IgG was added to the incubation medium. There were no observable differences between CF and normal fibroblast proteins in ability to induce mucus secretion from tracheal explants. This further supports the hypothesis that the mucus stimulatory activity and the ciliary-inhibition activity reside in separate proteins,

and it is the mucus stimulatory activity which can be related to one of the disease symptoms, mucus hypersecretion, while ciliary-inhibition activity bears no apparent relationship to the etiology of CF.

Stimulation of Cellular Secretion by CF Factor

The development of culture techniques for the isolation of pure populations of epithelial cells from hamster trachea (HTE) (Goldman and Baseman, 1980a) and the subsequent characterization of their secretory proteins as 'mucin like' (Goldman and Baseman, 1980b); Rudick and Rudick, 1982; Rudick *et al*., 1983) provided an *in vitro* system to study putative CF factor in relation to one of the disease symptoms, mucus hypersecretion. Could HTE cells be stimulated to hypersecrete by a CF serum factor in a manner analogous to the intact explant? The results indicate that this was the case. Sampling 19 CF patients and 12 normal individuals the putative factor could be detected by its stimulatory effect on cellular secretion. Of the heterozygous individuals tested they elicited responses intermediate betewen NHS and CFS. This would be consistent with CF being inherited as a single allele autosomal recessive. Treatment of the HTE cells with CFS resulted in: 1) increasing the apparent secretory rate upon exposure to CF factor, and also the percent of total synthesized protein secreted was heightened; 2) the cell

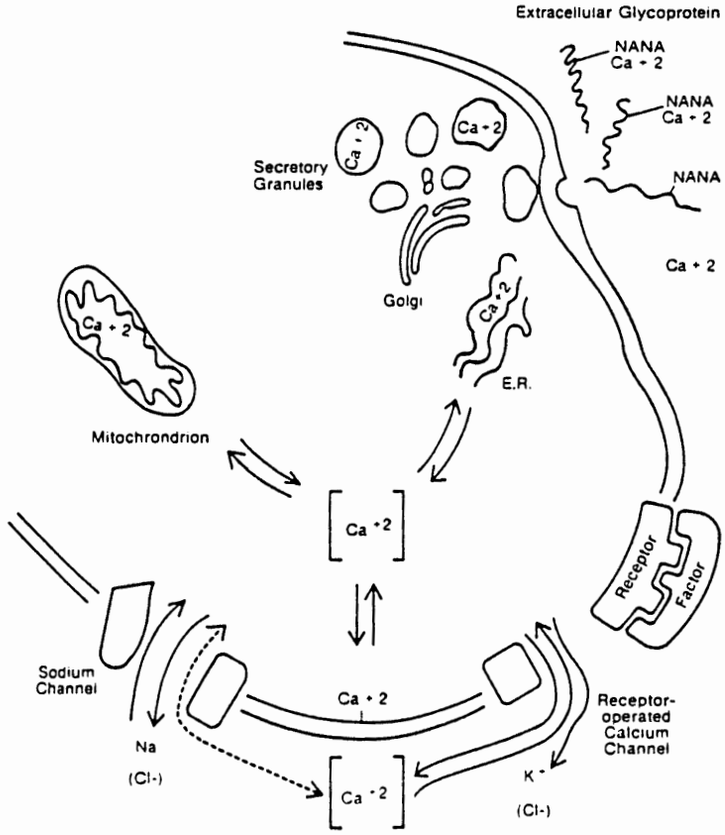
bound radioactivity decreased in CFS treated cells as compared to cells treated with NHS. These results can be explained by a CF factor causing more rapid exocytosis leading to release of cellular material, and possibly causing the channeling of precursor into synthesis of secretory proteins.

Mechanism of Hypersecretion:Stimulus-Secretion Coupling

Due to the extensively documented role calcium ion plays in cellular secretion (Cochran and Douglas, 1974; Eimerl *et al*., 1974; Douglas, 1981; Rubin, 1982) it seemed reasonable to determine whether calcium ion is important for the stimulation of secretion seen in the presence of CF factor, which would lead to investigations into its possible mode of action. Cells which were treated with CFS took up calcium ion rapidly, while HHS gave intermediate results and NHS elicited no response. Cells were also exposed to 10% FCS, the concentration normally used in propagating the cells, and 20% FCS, the concentration used in the uptake assays, but neither had an effect upon the influx of calcium ion. This indicated that the uptake observed in the presence of CFS was not merely an artifact of the type of serum or its concentration placed onto the HTE cells.

To extend the hypothesis of stimulus-secretion coupling in HTE cells exposed to CFS, it was important to establish a relationship between calcium ion entry and the degree of secretion. A correlation was in fact shown to exist between concentration of serum required for maximal uptake and subsequent secretion. As the concentration of CFS in the media increased there was greater influx of calcium ion with a concomitant increase in secretion. These data corroborate studies conducted in other systems (Foreman et al., 1973; Williams and Lee, 1974; Cochrane and Douglas, 1974; Eimerl et al., 1974; Quissell and Barzen, 1980) which have shown that calcium ion entry 'triggers' cellular secretion. Treatment of F12 medium plus CFS with 4 mM EGTA resulted in a 96% decrease in $^{45}Ca^{2+}$ uptake with a concomitant decrease in cellular secretion to the basal level obtain with NHS. More convincing evidence for the involvement of calcium ion in 'triggering' hypersecretion was obtained with the use of ionophore A23187. Ionophores are agents which increase the membrane permeability to certain ions (Rubin, 1982), and A23187 has a selectivity for calcium ion (Reed and Lardy, 1972). Hypersecretion was achieved with a level of ionophore which stimulated maximum calcium ion uptake (10 μg/ml) in either the absence of serum or with NHS in the incubation medium. This effect was independent of extracellular calcium ion concentration,

Figure 39. Model of HTE cells in response to stimullation by CF factor. The putative CF factor binds to a receptor located within the plasma membrane of HTE cells. This interaction results in the opening of a calcium ion channel. Once inside the cell calcium ion has several fates: 1) it can be sequestered and stored by either mitochondria or endoplasmic reticulum for subsequent retrieval; 2) it can associate with the plasma membrane via phospholipid interactions; 3) newly synthesized proteins destined for secretion can bind calcium ion via anionic residues of the oligosacchride chains; 4) the calcium can be actively pumped outward with a subsequent efflux of chloride. On the outside of the cell the calcium ion can bind to secretory proteins which have been released in response to the initial influx of calcium ion. This association is more than like via the abundant anionic sialic residues (NANA) found within mucins.


Extracellular Glycoprotein
NANA
Ca + 2
NANA
Ca + 2
NANA
Ca + 2
Secretory
Granules
Ca + 2
Ca + 2
Golgi
Ca + 2
E.R.
Ca + 2
Mitochrondrion
[Ca +2]
Receptor
Factor
Sodium
Channel
Ca + 2
Receptor-
operated
Calcium
Channel
Na
(Cl-)
[Ca +2]
K +
(Cl-)

ranging from 0.33 mM to 5.40 mM, indicating that calcium ion was not limiting and that an intracellular level is reached such that maximum stimulation is achieved. Moreover, chelation of the available calcium ion abolished the responses seen with ionophore. Using intact rabbit tracheal explants several workers (Bogart et al., 1977; Bogart et al., 1978) have proposed that CFS has an ionophore-like effect; these data would therefore support this idea. Cells stimulated in the presence of CFS plus verapamil, a calcium channel blocker (Lee and Tsien, 1983), exhibited a 50% decrease in calcium ion influx followed by a subsequent decrease in secretion. In light of this response it is plausible to suggest that HTE cells possess a receptor-mediated calcium ion channel which CF factor is responsible for opening via its interaction with receptor.

The model for stimulus-secretion coupling between factor and calcium ion influx resulting in hypersecretion is shown in Figure 39. If hypersecretion in vitro is a reflection of what occurs in vivo one could imagine that CF factor causes calcium ion influx into target epithelial cells and this would 'trigger' greater than normal quantities of mucus to be secreted by these cells, thus leading to the major symptom of CF: exocrine hypersecretion causing blockage of ducts, airways and digestive system. As calcium is actively pumped out of the cell it could become

bound to extracellular mucins via the abundant anionic sugar residues, thus increasing their viscosity. This in fact has been found to be the case by analysis of mucus secretions from CF patients (Gugler et al., 1967). The calcium ion which enters the HTC cell has several possible fates: 1) it could be sequestered for storage and subsequent release by endoplasmic reticulum or mitochondria; 2) it could associate with the internal plasma membrane via phospholipid interactions; 3) newly synthesized proteins destined for secretion could bind the calcium ion via anionic residues; or 4) the cell could actively pump the calcium ion out. The influx of calcium ion has also been linked to the efflux of sodium in a 2:1 exchange fashion (Rubin, 1982). This could provide a plausible explanation for elevated sweat electrolytes in the CF patient. In a similar fashion as calcium is actively pumped out of the cell chloride could also exit by cotransportation through the sodium channel, thus explaining the transient elevation of both sweat sodium and chloride levels. Or the sweat gland, as an exocrine gland, could be affected by CF factor causing electrolyte disturbances similar to those depicted with the respiratory epithelial cells. It would be of interest to see what effect CF-factor would have upon electrolyte transport and secretion in other systems.

After binding of factor to receptor it is probable that the factor-receptor complex is internalized. This phenomenon has been well documented in other systems (Brown et al., 1982; Miskimins and Shimizu, 1982). Depletion of surface receptors would signal the synthesis of new receptors and with the appearance of the free receptors on the plasma membrane new sites could be occupied by factor, thus signaling another round of stimulus-secretion and maintaining a chronic hypersecretory state in the CF patient.

Both in secretion and calcium ion uptake HHS produced half the maximum effects of CFS. Then how does HHS produce the in vitro effects but carriers do not display any clinical symptoms of the disease? It may be that HHS has only half the concentration of aberrant molecular as CFS, thereby producing half maximum uptake of calcium ion by opening half as many receptor operated channels, or, since a threshold amount of calcium entry may be required to 'trigger' secretion HHS having only half the concentration of factor would never stimulate the cells to take up the calcium ion required to cross the threshold. However, as the HHS concentration in contact with the HTE cells was increased, the receptor sites should eventually have been saturated, as they apparently were by CFS. Both in secretion and calcium uptake the least sums ratio of CFS/HHS

remained approximately two. This can be explained by the existence of a competing serum component, possibly the normal counterpart, which acts as factor antagonist. This could be experimentally tested by diluting CFS with NHS thereby introducing the suggested competitor.

This study was conducted on unmatched diseased individuals, regardless of disease severity or involvement. It could be that the variation of response observed with HHS and CFS is due to the genetic background of the individual. Thus, other serum components may modulate the activity of CF factor.

Partial Purification of the Mucus Stimulatory Activity

Based upon the observed secretory response of HTE cells exposed to CFS this system could be used as a means to assay for secretion stimulation activity during the process of fractionation. Previous studies have shown that the putative CF factor is associated with IgG in CFS (Barnett *et al*., 1973a; Blitzer and Shapira, 1982; Carson and Bowman, 1982) and can, in fact, be dissociated yielding a low molecular weight (4.5-11 kd) active protein (Carson and Bowman, 1982; Blitzer and Shapira, 1982a). Detection of CF factor in serum components resolved by Bio-Gel P10 with a molecular weight greater than 10 kd was not unexpected on the basis of previous reports of factor

associating with IgG, since this fraction would contain the IgG serum component.

To proceed to the next step, serum was further fractionated on DEAE Affi-gel blue. This affinity matrix binds serum proteins and under selective pH and ionic conditions most IgG appears in the flow-through peak. Testing the IgG from NHS or CFS in the secretion stimulation assay revealed no activity residing in this fraction, nor was there any effect upon calcium ion influx. A plausible explanation for this observation is that factor exists in serum as a free form and one which is associated with IgG, but it is the free protein molecule which retains that stimulatory activity and the complex (factor-IgG) itself is inactive. To test whether or not the putative factor was associated with IgG, treatment with 8 M urea was used to dissociate possible factor-IgG complexes. The peptides released and tested revealed the lack of any activity. This contradicts the work of Blitzer and Shapira (1982a) and others (Carson and Bowman, 1982) who reported the dissociation as a requisite for activity of factor. However, it does corroborate the study of Boat _et al_. (1982) in which they were unable to document any stimulation of mucus explusion by the serum IgG fraction. Moreover, Boat _et al_. (1982) reported a stimulatory component in a CF serum fraction not containing IgG which increased the synthesis of

high molecular weight [$^{35}SO_4$] = /[^{3}H]glucosamine labeled mucins by rabbit tracheal explants.

Elution of the remaining serum proteins from DEAE Affi-gel blue indicated that the stimulatory CF factor resided within this fraction. When tested in both calcium ion uptake and secretion stimulation assays, this fraction was stimulatory above IgG alone, above dissociated proteins from the IgG complexes, and above the NHS counterpart. Also, this activity was not attributed to either elastase or exotoxin A of Pseudomonas aeruginosa infection, since treatment with a protease inhibitor, chymostain, specific for these types of serine proteases had no effect upon the stimulatory activity.

Upon the analysis of the types of proteins secreted in response to stimulation by factor, a quite unexpected finding was that factor induces selective synthesis of two polypeptides. The HTE cells constitutively secrete various polypeptides, but factor halted the synthesis of a majority of these proteins and induced heightened synthesis of a 200 kd and 30 kd species, and also proteins with molecular weights so large that they are unable to enter the gel. This observation can be explained by entry of calcium ion which was shown to be a requisite for hypersecretion. White and Bancroft (1983) have reported that calcium regulates the levels of prolactin mRNA. In their system

TRH (thyrotropin-releasing hormone) required calcium ion to stimulate prolactin mRNA synthesis, and incubation with EGTA resulted in the absence of any such species. This would suggest a role for calcium in the regulation of gene expression.

The CF factor is most likely able to stimulate secretion via a receptor-mediated calcium channel, which means that the observed interaction is the coupling of an abnormal protein to a normal HTE cell receptor. Preliminary evidence suggests that this is an alpha-adrenergic receptor, since ionophore A23187 has been shown to stimulate the action of epinephrine (Selinger et al., 1974) and the use of propanolol, a beta-adrenergic receptor blocker, did not inhibit the stimulation of secretion seen in response to CFS. It may be that the CF patient contains elevated levels of a normal serum protein, or an altered protein with a higher affinity for receptor. The data support the idea that it is an altered protein which has a higher affinity for receptor. If the former is the case, increased levels of NHS should elicit the CFS response, but this was not found.

At this point one cannot assume that the stimulatory activity attributed to factor is a product of the actual mutant allele or is a secondary effect; this must await the purification of factor and a demonstration of its molecular

mechanism of action. However, the stimulatory activity can be related to many of the CF disease symptoms including the involvement of electrolyte disturbances and, therefore, must play some role in the etiology of CF.

SUMMARY

1. CF fibroblasts do not secrete a factor stimulatory for mucus secretion by hamster tracheal explants.
2. Stimulatory activity observed in response to CFS is not dependent upon IgG. The serum factor appears to be high in molecular weight ($\geq$ 10 kd). Also, mucus stimulatory activity and ciliary-inhibitory activity do not appear to reside within the same serum fraction.
3. CF factor appears to operate via a receptor-mediated calcium ion channel in the HTE cell plasma membrane. Hypersecretion is stimulated by an increased rate of calcium ion entry into the cell.
4. HHS causes half maximum calcium ion uptake and secretion, supporting the genetics of CF being inherited as a single allele autosomal recessive.
5. The model proposed on the basis of these results provides plausible explanations for many of the CF symptoms, including the involvement of electrolyte disturbances and mucus hypersecretion in the etiology of CF.

Areas for further research are numerous. The purification of factor is now being pursued. Also, an interest

would be to study the receptor and its interaction with factor at a molecular level.

LITERATURE CITED

Alhadeff, J. 1978. Glycoproteins and cystic fibrosis. Clinical Genetics. 14:189-201.

Barnett, D. R., Barranco, L. H., Lockhart, B. J., Meyne, J., and Bowman, B. H. 1973a. Cystic fibrosis: growth kinetics and production of the ciliary inhibitor by cultured fibroblasts. Texas Rep. Bio. Med. 31(4): 691-696.

_____, Schanfield, M. S., McCombs, M., and Bowman, B. H. 1973b. Isoelectric focusing and IgG allotyping of the serum fraction containing the cystic fibrosis ciliary inhibitor. Texas Rep. Bio. Med. 31(4):709-714.

Beratis, N. G., Conover, J. H., Conod, E. J., Bonoforte, R. J., and Hirschhorn, K. 1973. Studies on ciliary dyskinesia factor in cystic fibrosis. III. Skin fibroblasts and cultured amniotic fluid cells. Pediat. Res. 7:958-964.

Bhakdi, S. and Jensen-Tranum, J. 1983. Membrane damage by channel-forming proteins. TIBS. 8(4):134-136.

Blitzer, M. G. and Shapira, E. 1982a. A purified serum glycopeptide from controls and cystic fibrosis patients. I. Comparison of their mucociliary activity on rabbit tracheal explants. Pediat. Res. 16:203-208.

_____. 1982b. A purified serum glycopeptide from controls and cyctic fibrosis patients. II. Comparison of their immunologic properties using monoclonal antibodies. Pediat. Res. 16:938-942.

Boat, T. F., Cheng, P., and Wood, R. E. 1977. Tracheobronchial mucus secretion _in vivo_ and _in vitro_ by epithelial tissues from cystic fibrosis and control subjects. Mod. Probl. Pediat. 19:141-152.

_____, and Chang, P. 1978. Mucus glycoproteins in cystic fibrosis. In: Glycoproteins and glycolipids in disease processes. E. Walbo, ed. American Chamical Society, Wash., D.C. pp. 108-121.

_____, Polony, I., and Cheng, P. 1982. Mucin release from rabbit tracheal epithelium in response to sera from normal and cystic fibrosis subjects. Pediat. Res. 16:792-797.

Bogart, B. I., Conod, E. J., and Conover, J. H. 1977. The biologic activities of cystic fibrosis serum. I. The effects of cystic fibrosis sera and calcium ionophore A23187 on rabbit tracheal explants. Pediat. Res. 11:131-134.

_____, Conod, E. J., Gaerlan, P. F., and Conover, J. 1978. The biologic activities of cystic fibrosis serum. II. Ultrastructural aspects of the effect of cystic fibrosis sera and calcium ionophore A23187 on rabbit tracheal explants. Pediat. Res. 12:15-24.

Boucher, R. C., Knowles, M. R., Stults, M. J., and Gatzy, J. T. 1983. Epithelial dysfunction in cystic fibrosis. Lung. 161:1-17.

Bowman, B. H. 1976a. Minireview-current biochemical approaches in cystic fibrosis research. Life Sci. 19:1289-1296.

_____. 1976b. Factors related to cystic fibrosis. In: Cystic fibrosis projections into the future. Mangos, J. A. and Talano, R. C. eds. Pergamon Press: Stratton, N.Y. pp. 277-284.

_____ and Barnett, D. R. 1976c. Introduction: Current investigation and theories on the genetic defect in cystic fibrosis. Texas Rep. Bio. Med. 34(1):1-9.

_____, Barnett, D. R., Matalon, R., Danes, S. B., and Bearn, A. G. 1973. Cystic fibrosis: fractionation of fibroblast media demonstrating ciliary inhibition. Proc. Natl. Acad. Sci. U.S.A. 70:548-551.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brown, M. S., Anderson, R. G. W., and Goldstein, J. L. 1983. Recycling receptors: The round-trip itinerary of migrant membrane proteins. Cell. 32:663-667.

Carson, S. D., Harper, B. L., Barnett, D. R., Kurosky, B. J., and Bowman, B. H. 1976. Electrophoretic studies of the cystic fibrosis ciliary inhibitor and its interaction with immunoglobulin G. Texas Rep. Bio. Med. 34(1):209-219.

_____, and Bowman, B. H. 1982. Cystic fibrosis. I. Fractionation of the mucociliary inhibitor from plasma. Pediat. Res. 16:13-20.

Chen, T. R. 1977. In situ detection of mycoplasma contamination of mycoplasma contamination in cell cultures by fluroscent Hoechst 33258 stain. Exp. Cell. Res. 104:255.

Clamp, J. R. and Gough, M. 1979. Study of the oligosacchride units from mucus glycoproteins of meconium from infants and from cases of cystic fibrosis with mocenium ileus. Clin. Sci. 57:445-451.

Cochrane, D. E. and Douglas, W. W. 1974. Calcium-induced extrusion of secretory granules(exocytosis) in mast cells exposed to 40/80 or the ionophores A23187 and X537A. Proc. Natl. Acad. Sci. U.S.A. 71:408-412.

Collier, A. M. 1976. Techniques for establishing tracheal organ cultures. Procedure 43321. TCA Manual. 2:333.

Conover, J. H., Bonoforte, R. J., Hathaway, P., Paciuo, S., Conod, E. J., Hirschhorn, K., and Kopel, F. B. 1973. Studies on ciliary dyskinesia factor in cystic fibrosis. I. Bioassay and heterozygote detection in serum. Pediat. Res. 7:220-223.

_____, Conod, E. J., and Hirschhorn, K. 1974. Studies on ciliary dyskinesia factor in cystic fibrosis. II. Its possible identification as anaphylatoxin (C3a)-IgG complex. Life Sci. 14:253-266.

_____, Conod, E. J., and Hirschhorn, K. 1976. On the nature of the defect in cystic fibrosis. Texas Rep. Bio. Med. 34:45-50.

_____ and Conod, E. J. 1978. The influence of cystic fibrosis serum and calcium on secretion in the rabbit tracheal mucociliary apparatus. Biochem. Biophys. Res. Comm. 83(4):1595-1601.

Danes, B. S., Litwin, S. D., Hutteroth, T. H., Cleve, H., and Bearn, A. G. 1973. Characterization of cystic fibrosis factor and its interaction with human immunoglobulin. J. Exp. Med. 137:1538-1543.

Davis, P. B. and Laundon, S. C. 1979. Adenylate cyclase and β-adrenergic receptors and lymphocytes from patients with cystic fibrosis. Am. Rev. Resp. Dis. 119:302.

_____ and DiSant'Agnese, P. A. 1980. A review of cystic fibrosis at forty. Pediat. Res. 14:83-87.

DiSant'Agnese, P. A. and Davis, P. B. 1976. Research in cystic fibrosis. Part 1. New Engl. J. Med. 295:481-485.

Douglas, W. W. 1981. Aspects of the calcium-hypothesis of stimulus-secretion coupling:electrical activity in adenohyphseal cells, and membrane retrieval after exocytosis. In: Methods in Cell Biology, D. M. Prescott ed. Academic Press: New York, N.Y. 23:483-501.

Eimerl, S., Savion, J., Heichal, O., and Selinger, Z. 1974. Induction of enzyme secretion in rat pancreatic slices using the ionophore A23187 and calcium. J. Biol. Chem. 249(12):3991-3993.

Feigal, R. J. and Shapiro, B. L. 1979. Altered intracellular calcium in fibroblasts from patients with cystic fibrosis and heterozygotes. Pediat. Res. 13:764-768.

Fleming, N. and Sturgess, J. M. 1981. Stimulation of glycoprotein secretion in dispersed rat submandibular gland acini by cystic fibrosis serum. Experientia. 37:139-141.

Foreman, J. C., Mongar, J. L., and Gomperts, B. D. 1973. Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. Nature(London). 245:249-251.

Frates, R. C., Kaizu, T. T. and Last, J. A. 1983. Mucus glycoproteins secreted by respiratory epithelial tissue from cystic fibrosis patients. Pediat. Res. 17:30-34.

Gabridge, M. C., Bright, M. J., Agee, C., Nickerson, J. M., and Henderson, N. S. 1979. Development of an improved tracheal explant bioassay for the detection of the ciliary dyskinesia factor in cystic fibrosis serum. Pediat. Res. 13:31-35.

Goldman, W. E. and Baseman, J. B. 1980a. Selective isolation and culture of a proliferating epithelial cell population from the hamster trachea. In Vitro. 16(4):313-319.

_____. 1980b. Glycoprotein secretion by cultured hamster trachea epithelial cells: A model system for in vitro studies of mucus synthesis. In Vitro. 16(4):320-329.

Gugler, E., Pavllavicini, C. J., Swerdlow, H., and DiSant'Agnese, P. A. 1967. The role of calcium in submixillary saliva of patients with cystic fibrosis. J. Pediat. 71:585-588.

Hall, R. L., Miller, R. J., Peatfield, A. E., Richardson, P. S., Williams, I., and Lampert, I. 1980. A colorimetric assay for mucous glycoproteins using alcian blue. Biochem. Soc. Trans. 8:72.

Harpel, P. C. 1971. Studies on human plasma α_2-macroglobulin enzyme interactions. Evidence for protelytic modification of the subunit chain structure. J. Exp. Med. 138:508-521.

Harper, B. L., Barnett, D. R., and Bissett, J. D. 1976. Immunochemical studies of the plasma and cultured fibroblast media fractions containing the cystic fibrosis ciliary inhibitor. Texas Rep. Bio. Med. 34:73-82.

Impero, J. E., Harrison, G. M., and Nelson, T. E. 1981. Specificity of an isolated salivary factor material to cystic fibrosis. Pediat. Res. 15:940-944.

Kagayama, M. and Douglas, W. W. 1974. Electron microscope evidence of a calcium-induced exocytosis in mast cells treated with 40/80 or the ionophores A23187 and X537A. J. Cell. Bio. 52:519-526.

Katz, S. 1978. Calcium and sodium transport processes in patients with cystic fibrosis. Res. Comm. Chem. Path. Pharm. 19(3)491-503.

Kawai, Y. and Arinze, I. J. 1983. B-Adrenergic receptors in rabbit liver plasma membranes. J. Biol. Chem. 258:4364-4371.

Kennedy, J. R., Lin, K. D., and Duckett, K. E. 1982. Serum complement (C'3a and C'5)-induced inhibition of rabbit tracheal cilia. Cell Motility Supplement. 1:71-75.

Klinger, J. D., Straus, D. C., Hilton, C. B., and Bass, J. A. 1978. Antibodies to proteases and exotoxin A of Pseudomonas aeruginosa is patients with cystic fibrosis. Demonstration by radioimmunoassay. J. Inf. Diseases. 138(1):49-58.

Kurlandsky, L. E., Beringer, R. W., and Talamo, R. C. 1980. Mucus-stimulating activity in the sera of patients with cystic fibrosis: Demonstration and preliminary fractionation. Pediat. Res. 14:1263-1268.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London). 227:680-685.

Lee, K. S. and Tsien, R. W. 1983. Mechanism of calcium channel blockade by verapamil, D600, diltiazen, and nitrendipine in single dialysed heart cells. Nature (London). 302-790-794.

Mangos, J. A. 1976. Biochemistry of cystic fibrosis: an overview. In Cystic Fibrosis Projections into the Future, Mangos, J. A. and Talano, R. C. eds. Pergamon Press: Stratton, N. Y. pp. 157-164.

McNeely, M. C., Awasthus, Y. C., Barnett, D. R., Tatsuo, I., Schneider, L., Srivastava, S. C., and Bowman, B. H. 1982. Cystic fibrosis. II. The urinary mucociliary inhibitor. Pediat. Res. 16:21-29.

Miskimins, W. K. and Shimizu, N. 1982. Dual pathways for epidermal growth factor processing after receptor-mediated endocytosis. J. Cell. Phys. 112:327-338.

Moriarty, C. M., Barett, J. S., and Fontaine, M. 1982. Effects of normal and cystic fibrosis serum on ion transport and mucus glycoprotein secretion from the isolated rabbit trachea. J. Med. 13(4):257-274.

Nagy, C. E. and Sturgess, J. M. 1976. Cystic fibrosis: Effects of serum factors on mucus secretion. Lab. Invest. 34(6):588-595.

_____, Khan, S., and Sturgess, J. M. 1979. Serum factor in cystic fibrosis: correlation with clinical parameters. Pediat. Res. 13:729-732.

Ouchterlony, O. 1968. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor Science Publ., Inc., Ann Arbor, Mich. pp. 21-31.

Parsons, M. and Romeo, G. 1980. Cystic fibrosis α_2-macroglobulin protease interaction in vitro. Clin. Chim. Acta. 100:215-224.

Quinton, P. M. 1983. Chloride impermeability in cystic fibrosis. Nature (London). 301:421-422.

Quissell, D. O. and Barzen, K. A. 1980. Secretory response to dispersed rat submandibular cells. II. Mucin secretion. Am. J. Physiol. 238:C99-C106.

Reed, P. W. and Lardy, H. 1972. A23187: A divalent cation ionophore. J. Biol. Chem. 247:6970-6977.

Romeo, G., Parsons, M., Bossen, A., Moore, J. B., and Sforza, L. C. 1978. Trypsin binding IgG in cystic fibrosis. Nature (London). 274:909-911.

Rubin, R. P. 1982. Calcium and cellular secretion. Plenum Press: New York, N.Y. pp. 1-127.

Rudick, V. L. and Rudick, M. J. 1982. Glycoprotein secretion by cultured hamster tracheal epithelial cells. Fed. Proc. 41:1431.

Rudick, V. L., Wooten, M. W., and Rudick, M. J. 1983. Secretory activity of hamster trachea explants and isolated epithelial cells and the effects of cystic fibrosis serum. J. Cell. Phys. (submitted for publication).

Sanderson, M. J. and Sleigh, M. A. 1981. Serum proteins agglutinate cilia and modify ciliary coordination. Pediat. Res. 15:219-288.

Satir, P. 1975. Ionophore-mediated calcium entry induces mussel gill ciliary arrest. Science. 190:586-588.

Seale, T. W., Flux, M., Rennert, O. M., Quissell, D. O., Barzen, K. A., and Lafferty, J. L. 1980. CFF: Effect of serum on the secretory response of dispersed rat submandibular cells. Pediat. Res. 14:1398-1402.

Selinger, Z., Eimerl, S., and Schramm, M. 1974. A calcium ionophore stimulating the action of epinephrine on the α-adrenergic receptor. Proc. Natl. Acad. Sci. U.S.A. 71:128-131.

Shapira, E., Rao, G. J. S., Wessell, H. U., and Nadler, H. L. 1976a. Absence of an $\alpha_2$macroglobulin protease complex in cystic fibrosis. Pediat. Res. 10:812-817.

_____, Yoseph, Y. B., and Nadler, H. L. 1976b. Decreased formation of $\alpha_2$macroglobulin protease complexes in plasma of patients with cystic fibrosis. Biochem. Biophys. Res. Comm. 17(3):864-870.

_____, 1977a. Abnormal breakdown of $\alpha_2$macroglobulin trypsin complexes in cystic fibrosis. Clin. Chim. Acta. 78:359-363.

_____, Martin, C. L., and Nadler, H. L. 1977b. Comparison between purified $\alpha_2$macroglobulin preparations from normal controls and patients with cystic fibrosis. J. Biol. Chem. 252(22):7923-7929.

Sing, C. F., Risser, D. R., Howatt, W. F., and Erickson, R. P. 1982. Phenotypic heterogeneity in cystic fibrosis. Am. J. Med. Genetics. 13:179-195.

Spock, A., Heick, H. M. C., Cress, H., and Logan, W. S. 1967. Abnormal serum factor in patients with cystic fibrosis of the pancreas. Pediat. Res. 1:173-177.

Tegner, H., Cedar, O., Roomans, G. M., Kollberg, H., and Toremalm, N. G. 1981. Effects of cystic fibrosis serum and cell culture medium on the mucociliary activity of the respiratory tract. Acta. Pediat. Scand. 70:629-533.

_____ and Toremaln, N. G. 1981. On CFF and its proposed influence on mucociliary function. Rhinology. 19:115-120.

Walter, M. F., and Satir, P. 1978. Calcium control of ciliary arrest in mussel gill cells. J. Cell Bio. 79:110-120.

White, B. A. and Bancroft, F. C. 1983. Epidermal growth factor and thyrotropin releasing hormone interact synergistically with calcium to regulate prolactin mRNA levels. J. Biol. Chem. 258(7):4618-4622.

Williams, J. A. and Lee, M. 1974. Pancreatic acinar cells: use of a Ca^{++} ionophore to separate enzyme release from the earlier steps in stimulus-secretion coupling. Biochem. Biophys. Res. Comm. 60:542-548.

Wilson, G. B. and Fudenberg, H. H. 1976. Studies on cystic fibrosis using isoelectric focusing. II. Demonstration of deficient protelytic cleavage of $\alpha_2$macroglobulin in cystic fibrosis plasma. Pediat. Rés. 10:87-96.

_____, Fudenberg, H. H., Parise, M. T., and Floyd, E. 1981. Cystic fibrosis ciliary dsykinesia substances and pulmonary disease. Effects of ciliary dsykinesia substances on neutrophil movement *in vivo*. J. Clin. Invest. 68:171-183.