Title
Cell-specific analysis of tracheobronchial secretory cells and secretions

Author
Finkbeiner, Walter E.

Publication Date
1989

Peer reviewed|Thesis/dissertation

CELL-SPECIFIC ANALYSIS OF TRACHEOBRONCHIAL SECRETORY CELLS AND SECRETIONS

by

Walter E. Finkbeiner, M.D.

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Experimental Pathology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

[redacted]

. . . . . .

Date **University Librarian**

Degree Conferred: March 26, 1989

To the memory of Edward A. Smuckler, M.D., Ph.D.

PREFACE

ACKNOWLEDGEMENTS

The major share of credit for the completion of these studies goes to my scientific advisor and colleague, Carol B. Basbaum, Ph.D. I thank her for her ideas, encouragement, support, and friendship. Thanks are also due to our collaborators, in particular, Jonathan H. Widdicombe, D.Phil., for his many contibutions. I also wish to express my thanks to Dr. Jay A. Nadel for his long-term support of and interest in my career. I gratefully acknowledge the technical assistance that I have received from Suzanne Carrier, Marianne Gallup, Jennifer Mann, Iris Ueki, and Manuel Uy. I would also like to express my thanks to the members of my thesis committee, Drs. Carol B. Basbaum, Susan J. Fisher, Daniel S. Friend, Jay A. Nadel, and Mary C. Williams. I am grateful to these individuals for their interest in my scientific progress. I would also like to acknowledge the support that I have received from the Chairs of the Department of Pathology, Drs, Edward A. Smuckler (1983-1986), Dr. Daniel S. Friend (1986-1987) and Dr. Dorothy F. Bainton (1987-present), during the tenure of my graduate studies. Special thanks are also due many members of the administrative and technical staffs of the Department of Pathology and the Cardiovascular Research Institute

Portions of this thesis have been previously published in the following papers:

1. Finkbeiner WE, Nadel JA, Basbaum CB: Establishment and characterization of a cell line derived from tracheal glands. In Vitro Cell Develop Biol 22:561-567, 1986.

2. Finkbeiner WE, Basbaum CB: Monoclonal antibodies directed against human airway secretions: Localization and characterization of antigens. Am J Pathol 131:290-297, 1988.

3. Paul A, Picard J, Mergey M, Veissiere D, Finkbeiner WE, Basbaum CB: Glycoconjugates secreted by bovine tracheal serous cells in culture. Arch Biochem Biophys 260:75-84, 1988.

4. Madison JM, Basbaum CB, Brown JK, Finkbeiner WE: Characterization of ß-adrenergic receptors in cultured bovine tracheal gland cells. Am J Physiol 256 (Cell Physiol 25):C310-C314, 1989.

CELL-SPECIFIC ANALYSIS OF TRACHEOBRONCHIAL SECRETORY CELLS AND SECRETIONS

by

Walter E. Finkbeiner, M.D.

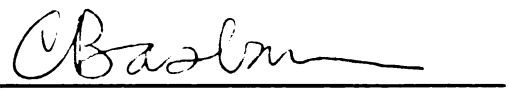
ABSTRACT

Tracheobronchial secretions are a complex mixture of molecules that originate from a heterogeneous group of secretory cells. Our understanding of tracheobronchial secretory mechanisms and secretory products has been hampered by an absence of homogeneous preparations of each tracheobronchial secretory cell type and by a lack of cell-specific biochemical markers of their secretions. In these studies, two methods (cell culture and monoclonal antibody production) that allowed cell-specific analysis of tracheobronchial secretion were used.

Bovine tracheal submucosal gland cells were isolated, placed into culture and serially propagated. In culture, the cells maintained features of serous cells. The cells incorporated ^{35}S into high molecular weight molecules. ß-adrenergic agonists stimulated release of radiolabeled molecules and elevations in intracellular cAMP levels, responses that could be blocked by the ß-adrenergic antagonist propranolol. Cyclic AMP appeared to be involved in the stimulus-secretion coupling events in serous cells since the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine potentiated the effects of isoproterenol on the secretory response and the elevation of intracellular cAMP levels. Furthermore, cAMP analogues elicited a secretory response in the absence of cAMP. The phosphorylation state of several cytosolic and particulate phosphoproteins was altered by cAMP-activated kinase activity. These data suggest that ß-adrenergic activation

and cAMP-mediated alterations in phosphoproteins may play a regulatory role in tracheal gland serous cell secretion.

Monoclonal antibodies were produced against human airway secretions. Twenty-nine hybridomas were selected for expansion because they selectively stained a single cell type or displayed another interesting distribution of staining. Antigens were further characterized by their sensitivity to periodate oxidations, selective affinity for three fraction peaks obtained by Sepharose 4B chromatography, and reactivity with molecules of various sizes, as estimated by solid phase radioimmunoassay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis with immunoblotting. The antigenic determinants for these antibodies included both carbohydrate and protein moieties associated with molecules of varying molecular weights. These antibodies will be useful for (1) quantitative detection of antigens in sputum or lavage samples by immunoassay and (2) purification and biochemical characterization of molecular constituents of airway secretions in health and disease.



Carol B. Basbaum, Ph.D.
Dissertation Committee Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Human tracheobronchial mucus is a complex mixture of water, ions, proteins and carbohydrates. It originates primarily from a heterogeneous group of secretory cells that either line the airway lumen (ciliated and goblet epithelial cells) or lie within airway walls (serous and mucous gland cells). Smaller contributions come from neuroendocrine cells (Kultschitzky cells) and inflammatory cells (primarily mast cells and plasma cells) that reside within the tracheobronchial walls and sometimes within the secretions themselves. Serum transudates may add ions, water and low molecular weight components to the secretions. Additionally, secretions arising from the distal lung make their way into the proximal airways.

In many chronic obstructive pulmonary diseases, tracheobronchial secretions increase [65, 151] and mucociliary clearance decreases [215]. Treatment for these diseases is often directed towards symptoms rather than causes. However, before pathologic changes in airway secretion in obstructive lung diseases can be treated mechanistically, mucus secretion in healthy human airways must be better understood.

Most studies of tracheobronchial secretion in both humans and animals have been performed using short-term organ cultures radiolabeled with precursors of glycoconjugates, and most work characterizing tracheobronchial secretory cells has been morphological and histochemical. Neither approach has permitted mechanistic analysis of the cell types involved in mucus production. To establish stimulus-response parameters for these cells requires methods to analyze them separately. Such methods can be categorized as (1) those involving the purification and culture of individual cell types and (2) those involving the identification of endogenous products or

the development of exogenous markers which can be used to quantitate the secretory activity of a particular cell type. Our understanding of tracheobronchial secretory mechanisms and secretory products has been hampered by an absence of homogeneous preparations of each tracheobronchial secretory cell type and a serious lack of cell-specific biochemical markers of their secretions. Experiments in which the secretion evoked by pharmacological stimulation is measured as output of nondialyzable or trichloroacetic acid-precipitable radiolabeled molecules are not specific enough to interpret the particular role of individual secretory cells since no radiolabeled precursor has been identified that is incorporated selectively by serous, mucous, or goblet cells [60, 92, 137]. Therefore, radiolabeled molecules secreted in response to a given drug represent the collective output of several cell types. Each cell type might be affected differently by a given stimulus, but differences cannot be resolved using radiolabels that are incorporated into heterogeneous products which are released from a heterogeneous cell population.

The objectives of this work were two: (1) development of preparations of tracheobronchial secretory cells composed of single cell types and (2) development of cell-specific markers of tracheobronchial secretory products. Before discussing the approaches used to achieve these objectives, the anatomy, physiology and biochemistry of the tracheobronchial secretory apparatus will be briefly reviewed.

THE CONDUCTING AIRWAYS

The conducting airways of the human respiratory system include the nasal passages, cartilaginous airways (trachea and bronchi) and noncartilaginous airways (bronchioles) to the level of the respiratory bronchioles. The conducting airways are functionally similar in that they sustain life by providing a conduit through which air is moved between the environment and the gas exchange regions of the lung. They are lined with respiratory mucosa composed primarily of pseudostratified, ciliated columnar epithelial cells and variable numbers of secretory (goblet) cells which overlie submucosal tissues containing submucosal glands. Respiratory mucosa and submucosa occupy a variable amount of the anterior third of the nasal cavity, nearly all of the middle and posterior third of the nasal cavity, the entire lining of the paranasal sinuses and the tracheobronchial airways. The trachea and bronchi of most species contain submucosal seromucous glands and these make up the majority of secretory tissue in tracheobronchial airways [203]. In normal bronchioles, the epithelium consists of ciliated cells and nonciliated bronchiolar epithelial (Clara) cells. There are no submucosal glands and goblet cells are rare [46].

SECRETORY APPARATUS OF THE TRACHEA AND BRONCHI

Ciliated epithelial cells

Ciliated epithelial cells are the airway cell type involved in active ion transport recent evidence suggests that they may contribute macromolecules to the respiratory fluid by constitutive secretion [252]. There is little species variation in their structure. They are columnar-shaped cells which rest on a basement membrane and extend to the epithelial surface. They have large nuclei and contain scattered

rough endoplasmic reticulum, free ribosomes, keratin tonofilaments, well-developed Golgi complexes and lysosomal aggregates within their cytoplasm. The apical cytoplasm contains numerous mitochondria which presumably provide energy for ion transport and ciliary movement. Their luminal surface contains from 200-300 cilia and 100-150 microvilli [204].

Goblet cells

Goblet cells are the major exocrine cell of the human tracheobronchial epithelium and are primarily invoved in secretion of mucin. There are approximately 6800 goblet cells per mm^2 in the epithelium of the human trachea [79]. Goblet cells are rare in pathogen-free rats [117], mice [183] and the trachea of ferrets [211].

The goblet cells of the airway epithelium vary in appearance with the cycle of secretory activity. With light microscopy after chemical fixation, cells in the resting phase have a goblet-like shape. However, with electron microscopy after freeze-substitution fixation, colonic goblet cells, cells which are analogous to the tracheobronchial goblet cells, have a columnar shape [108]. This suggests that the light microscopic "goblet" appearance may represent an artifactual change in cell shape due to swelling of secretory granules during fixation. The cells have a basally oriented nucleus and their perinuclear cytoplasm contains a network of rough endoplasmic reticulum and a prominent Golgi apparatus. The apical cytoplasm is filled with electron-lucent, membrane-bound secretory granules, 500-1500 μm in diameter [205]. An electron-dense core is occasionally seen within the granules. A three-dimensional reconstruction of pig goblet cell ultrastructure reveals that the secretory granules are arranged in discrete clusters and that each cluster is associated with a Golgi apparatus [2]. The membranes of individual secretory granules often appear discontinuous

particularly those nearest the airway lumen. This may represent a fixation artifact due to fragility of granule membranes [231]. However, intergranular coalescence is occasionally seen in colonic goblet cells, even in studies employing rapid freezing and freeze-substitution fixation [108].

Epithelial serous cells

These cells are rarely seen except in pathogen-free animals [118], during development [118] and in animals lacking submucosal glands.[183, 191]. They have irregularly-shaped, basally oriented nuclei, a perinuclear zone containing rough endoplasmic reticulum and a well-developed Golgi apparatus. The apical portion of the cells contains variable numbers of electron-dense secretory granules, 600 nm in diameter [117].

Submucosal glands

Tracheobronchial submucosal glands are branched tubuloalveolar structures, most numerous in the tissues between the cartilaginous rings, which empty their contents to airway lumena via a series of ducts [171]. The glands contain serous and mucous cells which are generally located in separate tubules but in some species may occur interspersed in the same tubule. In submucosal glands serous tubules arise distal to mucous tubules as lateral or terminal buds forming demilunes [171]. Species differences exist with respect to the presence, number and size of submucosal glands [99, 178]. Myoepithelial cells, which presumably aid secretion by a contractile mechanism [224] are found surrounding gland acini [28].

Mucous cells are columnar in shape with basally situated nuclei, prominent rough endoplasmic reticulum, well-developed Golgi apparatus and numerous electron-lucent granules which

range in size from 300-1800 nm [170]. With transmission electron microscopy, one can see fusion of adjacent granule membranes near the apical surface in resting cells, but as mentioned above, this may represent a fixation artifact.

Pyramidal-shaped serous cells have small, round nuclei located within the basal region of the cell. Their supranuclear cytoplasm is rich in rough endoplasmic reticulum and Golgi complexes. The apical portion of the cells contains numerous secretory granules and scattered lipid granules [170]. The granules are electron dense and range between 100 to 1800 nm in diameter and adjacent granules remain clearly separated [170].

INNERVATION OF THE TRACHEA AND BRONCHI

Mucosal and submucosal afferent nerves

Nerves in tracheobronchial epithelium are seen both close to the airway lumen and near the basement membrane at the base of epithelial cells in humans [133], cat [72], rat [82, 116], mouse [107], and chicken [39, 122]. In humans, axons are found near the basement membrane in trachea, lobar bronchi and segmental bronchi while luminal nerves for the most part occurred only in trachea and lobar bronchi [133]. The intraepithelial axons have small endings which are of two types ultrastructurally: (1) those containing microtubules, smooth endoplasmic reticulum and free nerve endings similar to sensory nerves seen in other organs and (2) those containing numerous mitochondria similar to mechanoreceptive nerves seen in other organs [17, 53]. Submucosal afferents have not been adequately studied though mechanoreceptor-type nerve endings are present within the submucosa [17].

Mucosal and submucosal efferent nerves

Parasympathetic nerves originate in nuclei in the medulla oblongata and synapse in paratracheal ganglia [7]. Postganglionic fibers innervate airway smooth muscle, vasculature, submucosal glands or epithelium [207]. There is considerable species variability in the innervation of submucosal glands [136]. Mann [158] has identified nerve fibers to the submucosal glands in sheep and goat but not in calf, pig or rabbit. Murlas et al. [176] have determined the distribution of cholinergic and adrenergic axons innervating cat submucosal glands. They found approximately nine times more cholinergic nerves than adrenergic nerves, and they noted that the density of innervation of serous cells was slightly greater than that of mucous cells. Cholinergic innervation to human submucosal glands has been confirmed by ultrastructural and histochemical studies [28, 185].

Sympathetic nerves arise from the superior cervical and stellate ganglia and synapse in the inferior cervical ganglia and upper thoracic ganglia [11]. Postganglionic fibers reach the respiratory tract and intermingle with cholinergic nerves to supply the airways. Evidence of innervation of submucosal glands has been obtained in sheep [158] cat [176, 226] and human [134, 184, 185].

Neuropeptide-containing nerves have been identified in the epithelium and near submucosal glands in many species [55, 76, 97, 135, 153, 154, 222, 247, 248, 249]. Epithelial axons with agranular vesicles have been described in humans [114], rats [116] and cats [72]. Histochemical studies have not identified neurotransmitters within these nerves [136]. The source and function of these axons is not known [17].

NEUROTRANSMITTER RECEPTORS OF THE TRACHEA AND BRONCHI

Because of the diffuse innervation of the airway, it is difficult to predict which pharmacological types of axons make functional synaptic connections with individual epithelial and gland cells. Therefore it is necessary to know the distribution of neurotransmitter receptors on these cells. The reversible antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) has been used to analyze the characteristics and distribution of muscarinic receptors in ferret airway [14]. Specific binding of [^{3}H]QNB was to a single high affinity site and which was localized prodominently to receptors over tracheobronchial smooth muscle and submucosal glands. Using the irreversibly binding muscarinic antagonist [^{3}H]propylbenzilycholine mustard, Basbaum and colleagues [20] found that muscarinic receptors in ferret submucosal glands are localized primarily to basolateral membranes where they have a mean receptor density of 5 binding sites/μm^2. Serous and mucous cells had identical receptor densities. Culp and Marin [70] prepared homogenates from isolated cat tracheal submucosal gland cells and found high affinity (K_D = 36 pM) and high density (35,000 receptors/cell) muscarinic receptors.

The distribution of adrenergic receptors as detected by autoradiography has been studied in ferret airways. Both α- and ß-adrenergic receptors are present in airway epithelium and submucosal gland cells [12, 13, 17]. However, α_1-adrenergic receptors were more numerous on serous than mucous cells, and ß-adrenergic receptors were more numerous on mucous than serous cells. By autoradiographic mapping, ß-adrenergic receptors have been identified in rat bronchial epithelium [67]. Carstairs et al. [52] found that bronchial submucosal glands in human lung contained 90% $ß_2$- and 10% $ß_1$-adrenergic receptors.

Limited studies of the autoradiographic localization of nonadrenergic, noncholinergic receptors have shown that vasoactive intestinal peptide (VIP) receptors are associated with airway epithelium in rat [145], guinea pig [51] and human [51]. In human airways, submucosal glands are particularly rich in VIP receptors [51]. In guinea pig lung, Substance P (SP) is associated with airway epithelium although not to as great an extent as that seen with airway smooth muscle [50].

CONTROL OF TRACHEOBRONCHIAL SECRETION

Resting airway secretion

Perry and Boyd [189] showed that rabbits and cats produce approximately 2 ml/kg body weight/day of respiratory tract fluid. Ueki et al. [250] collected secretions from individual cat tracheal submucosal glands using micropipettes and showed that mean resting secretion was ~9 nl/min. Estimations of total resting gland secretion were 24 ml/day. A study of laryngectomized patients provided evidence that humans produce lung secretions at a rate of 0.1-0.3 ml/kg body weight/day [244]. Although the mechanism responsible for resting airway secretion is unknown, it is clearly independent of neural control since it is unaffected by cutting extrinsic nerves [85, 92, 209, 235].

Parasympathetic control

Cholinergic is the predominant excitatory influence on tracheobronchial mucus secretion and is ubiquitous in all species investigated. In 1896, Kokin [128] showed that when dog vagus nerve was stimulated, drops of secretion appeared

on the tracheal mucosal surface. Later, Florey et al. [85] repeated these experiments in cats and dogs and found that stimulation of vagus or recurrent laryngeal nerves led to secretion from submucosal glands but not from epithelial goblet cells. The muscarinic receptor antagonist atropine inhibited this response. Since then, stimulation of parasympathetic nerves has been shown to increase airway secretions in other species including humans. Gallagher et al. [92] developed an *in situ* preparation to collect radiolabeled secretions from cat trachea. Parasympathetic nerve stimulation increased release of radiolabel, and this effect could be blocked by atropine. Secretory rates of tracheal submucosal glands increased after cervical vagal stimulation as determined by formation of tantalum "hillocks" in dogs [73] and micropipette measurements in cats [250]. Electrical field stimulation of parasympathetic nerves stimulated mucus secretion from excised ferret trachea [36, 37] and human bronchi [6].

Florey et al. [85] were the first to demonstrate that a parasympathetic agonist (pilocarpine) could stimulate tracheal submucosal gland secretion *in vivo*. Stimulation of airway secretions *in vivo* with parasympathomimetic drugs has also been confirmed in rabbit cat [43, 189] dog [43, 92] guinea pig [43], goose [123] and chicken [43]. Modern *in vitro* studies of airway secretion began in 1972 when Sturgess and Reid [235] showed that acetylcholine induced secretion of radiolabel, as assessed by light microscopic autoradiography, from both serous and mucous submucosal gland cells of human bronchi maintained in organ culture. Since then, stimulatory effects of cholinergic drugs on airway secretion *in vitro* have been confirmed in human by several investigations [6, 34, 60, 219]. Studies also demonstrated potent *in vitro* effects of parasympathetic agonists in cat [196], dog [54, 73], ferret [36, 37], pig [102], and hamster [180]. A summary of

studies demonstrating parasympathetic control of airway secretion is included in Table 1.

Sympathetic control

Adrenergic is generally a less potent influence on tracheobronchial mucus secretion and its presence and importance vary with the species examined. Kountz and Koenig [129] found that stimulation of cut sympathetic nerves either decreased or had no effect on bronchial secretion in dogs. After cervical sympathetic nerve electrical stimulation Boyd et al. [42] found negligible increases in respiratory tract fluid production by rabbits. Electric field stimulation of human bronchi mounted in Ussing chambers and radiolabeled with $^{35}SO_4$ could be completely blocked with atropine, suggesting that only cholinergic nerves mediate mucin secretion in human bronchi [6]. However, stimulation of stellate ganglia of cats caused a marked increase in tracheal mucus secretion [92]. This effect was blocked by ß-adrenergic but not α-adrenergic antagonists. In ferrets both α- and ß-adrenergic effects contributed to the release of radiolabeled macromolecules after electrical field stimulation [36].

Studies of the effects of sympathetic agonists on airway secretion have also been controversial. In their *in vivo* studies Boyd et al. [42] did not detect increased respiratory tract fluid secretion in guinea pigs and rabbits after systemic administration of sympathomimetic agents. Sturgess and Reid [255] and Boat and Kleinerman [34] failed to detect release of radiolabled secretions from organ cultures of human airways. Sympathetic agonists also failed to stimulate release of macromolecules by dog tracheal organ cultures [54]. However, Iravani, and Melville [109] found increased secretions on the mucosal surface of rat trachea after $ß_2$-adrenergic stimulation. Gallagher et al. [92] found increased secretion of mucus in cat tracheal organ cultures after

Table 1. Effects of parasympathetic, sympathetic, and nonadrenergic, noncholinergic control of tracheobronchial secretion. Numbers in parenthesis refer to text references.

	Human	Rat	Hamster	guinea pig	rabbit	Cat	Dog	Ferret	Pig	goose	chicken
Parasympathetic nerve stimulation	+ (6)					+ (85) + (92) + (250)	+ (128) + (129) + (85) + (73)	+ (37) + (36)			
Parasympathetic agonists	+ (60) + (235) + (34) + (219) + (6)		+ (180)	+ (43)	+ (189) + (43)	+ (189) + (43) + (92) + (196)	+ (43) + (54) + (123)	+ (22) + (242) + (36) + (18) + (94)	+ (102)	+ (190)	+ (172)
Sympathetic nerve stimulation	NE (6)				NE (42)	+ (92) + (187)	NE/- (129)	+ (37) + (36)			
Alpha-adrenergic agonists	NE (235) + (192) + (193) + (219)		- (180)	NE (42)	NE (42)	+ (250)	NE (54)	+ (22) + (242) + (36) + (94)			
Beta-adrenergic agonists	NE (235) NE (219) + (192) + (193)	+ (109)		NE (42)	NE (42)	+ (92) + (147)	NE (54)	+ (36) + (18) + (94)			
Nonadrenergic, noncholinergic nerve stimulation	NE (6)							+ (36)			
nonadrenergic, noncholinergic agents	NE (235) - (66)[a]					+ (155)[b] NE (210)[c]	+ (5) NE (5)[e] + (61)[a,f] NE (61)[g]	+ (188[a]) + (93[a,e]) + (38[e])			

[a] Vasoactive Intestinal peptide (VIP)
[b] endorphin peptides
[c] serotonin
[d] kallidin, met-lys-bradykinin
[e] Substance P (SP)
[f] physalaemin, eledoisin, eledoisin-related peptide
[g] Bombesin

treatment with norepinephrine, epinephrine, or isoproterenol and this effect could be abolished with propranolol. In other studies, investigators found convincing evidence of sympathetic control of secretion in cat [146, 196, 250], and ferret [36, 37]. Likewise, studies of human bronchi provided support for adrenergic control of secretion. Metabolically radiolabeled human bronchi, maintained in Ussing chambers, released radiolabeled macromolecules after α-, β_1- or β_2-adrenergic stimulation [192, 193]. However, mucus secretion from organ cultures of human bronchi increased with α- but not ß-adrenergic stimulation [219]. Additional studies of human tracheobronchial tissues are needed to resolve this inconsistency. In hamster tracheal ring organ cultures, ß-adrenergic stimulation had an inhibitory effect on glycoprotein secretion, but α-adrenergic agonists were not tested [180]. A summary of experiments demonstrating sympathetic control of airway secretion is included in Table 1.

Nonadrenergic, noncholinergic control

This category includes a variety of neurotransmitters that influence tracheobronchial secretion in a species-dependent manner. A role for nonadrenergic, noncholingergic nerves in regulation of tracheal secretion was suggested by investigators studying electrical field stimulation of cat [188] and ferret trachea [36]. In both studies, approximately one third of the stimulatory response due to electrical field stimulation remained despite the combined presence of atropine, phentolamine and propranolol. Presumably the residual stimulatory effect is due to nonadrenergic, noncholinergic mediators. Various neuropeptides have been shown to be stimulators of airway secretion in animals. Exogenous VIP stimulated release of ^{35}S-labeled macromolecules from ferret tracheal organ cultures [188]. In dog VIP, SP, physalaemin, eledoisin and eledoisin-related peptide,

met-lys-bradykinin-ser-val-gln, and kallidin all increased release of radiolabeled glycoproteins, while bombesin and bradykinin had no effect [5, 61]. Serotonin had no direct effect on tracheal mucus secretion in cat [210]. However, in dogs it potentiated secretion after vagal nerve stimulation [195]. Recently, Borson et al. [38] have obtained evidence indicating that the membrane-bound enzyme, neutral endopeptidase, modulates tachykinin-stimulated secretion from ferret trachea via its ability to degrade SP.

Endorphin peptides (dynorphin A, α-endorphin and morphine sulfate) have been shown to increase release of acid-precipitable radiolabeled molecules from cat tracheal organ cultures [155]. Naloxone blocked the effect of dynorphin A, providing evidence that dynorphin A stimulated mucus secretion via activation of opiate κ-receptors.

The role of neuropeptides in control of normal human airway secretion is not well understood. In human bronchi, the response to electrical field stimulation was completely inhibited with atropine [6]. Interestingly, VIP inhibited both baseline and methacholine-stimulated release of lysozyme and radiolabeled macromolecules from normal human bronchi, but not from bronchi obtained from patients with chronic bronchitis [66]. Thus, neuropeptides may play a modulating role in the control of human airway secretions, a mechanism which may become altered in hypersecretory disease. A summary of studies demonstrating nonadrenergic, noncholingergic control of airway secretion is included in Table 1.

Reflex control

Reflexes that control airway secretion have been identified from animal studies and generally involve direct stimulation of the airway [208]. Mechanical stimulation of sensory nerve endings in the nose, the nasopharynx and the

larynx increases mucus secretion in the cat [190]. Irritation of lower tracheal epithelium with ammonia vapor causes secretion in nonexposed segments of cat trachea via largely parasympathetic pathways [190]. Bronchial artery injections of capsaicin or bradykinin at doses that selectively stimulate bronchial C-fibers induces tracheal secretion in dogs, an effect which is abolished by cutting or cooling the vagus nerves [74]. Stimulation of cough receptors reflexly stimulates mucus secretion, a response that is beneficial in clearing the airways of unwanted material [188, 256]. Airway secretion is reflexly stimulated by hypoxia of carotid body chemoreceptors in dogs [74] and irritation of gastric mucosa in rabbits [189] or cats [96]. However, adaptive advantages of these responses are unclear.

Control by non-neural mediators

Numerous mediators synthesized by respiratory tract parenchymal cells, resident or "fixed" inflammatory cells of the airways and circulating inflammatory cells affect airway secretion [177]. Some of these have been shown to exert direct effects on airway secretion. However, little is known about possible indirect effects (i.e. via stimulation of an intermediate cells) which might probably contribute to *in vivo* secretory responses. Effects of various mediators are discussed below and a summary is provided in Table 2.

Arachidonic acid and metabolites. Arachidonic acid generated by inflammatory cells or injured tissues may be converted into prostaglandin (PG) or thromboxane (Tx) compounds by action of cyclooxygenase enzymes or into leukotriene (LT) or eicosanoid (HETE) compounds by lipoxygenase enzymes. These derivatives, along with arachidonic acid itself, have been shown to stimulate mucus secretion from airways. Sputum production increased in normal subjects after inhalation of

Table 2. Effects of non-neural mediators on tracheobronchial secretion. Numbers in parenthesis refer to text references.

	Human	Rat	Cat	Dog	Goose
Arachidonic acid	+ (164)				
PGA_2	+ (164)				
PGD_2	+ (164)		+ (210)		+ (210)
PGE_1	NE (206) NE (164)		+ (210)	NE (264)	+ (210)
PGE_2	NE (206) - (164)		+ (210)	NE (15)	+ (210)
$PGF_{1\alpha}$	NE (164)		+ (210)		+ (210)
$PGF_{2\alpha}$	+ (150)		+ (210)	+ (264) + (15)	+ (210)
PGI_2	NE (164)				
TxA_2		+ (265			
monoHETE	+ (220)				
LTC_4	+ (220) + (163) + (64)		+ (187)	+ (119)	
LTD_4	+ (220) + (163) + (64)	+ (265)			
PGF-A	+ (165)				
Histamine	NE (235) + (220)		+ (210)	NE (54)	+ (210)

$PGF_{2\alpha}$ [150]. Arachidonic acid, PGA_2, PGD_2, $PGF_{2\alpha}$ and monoHETEs increased while PGE_2 decreased mucus secretion from human bronchial organ cultures [162, 164]. Rich et al. [206] confirmed a stimulatory role for $PGF_{2\alpha}$ but could not reproduce an inhibitory effect of PGE_2. Prostaglandin E_1, $PGF_{1\alpha}$, and PGI_2 caused statistically insignificant increases in mucus secretion [164, 206]. Prostaglandins also increased airway secretion in cats [210] and in dogs [15, 264]. Intravenous injections of thromboxane A_2 into rats produced an increase in rat tracheal mucus gel thickness [265].

Leukotriene (LT) C_4 and LTD_4 stimulated release of glycoprotein [64, 163, 220] but not lysozyme [64] from human airways *in vitro*. Leukotriene C_4 had similar effects in the cat [187] and the dog [119]. Prostaglandin-generating factor of anaphylaxis (PGF-A), a peptide composed of 13 amino acids and derived from mast cells, stimulated mucus secretion from human bronchial organ cultures by stimulating the synthesis of lipoxygenase products [165]. Platelet activating factor (PAF) increased the release of acid-precipitable radiolabeled molecules from guinea pig, rat, rabbit and ferret tracheal explants via activation of synthesis of lipoxygenase products by airway epithelial cells [3].

Histamine. Inflammatory cells residing in airways synthesize numerous mediators in addition to arachidonic acid metabolites. Release of these mediators may influence airway secretion. Histamine, which is contained in the secretory granules of mast cells, failed to affect human bronchial secretion in one study [235]. However, in a later study histamine stimulated secretion by a histamine H_2-receptor mediated mechanism [219]. Histamine has been shown to stimulate secretion in cat and goose [210], but not dog [54] trachea *in vitro*.

Proteases. Recently, effects of proteases on secretory function of airways have been examined. Klinger et al. [124] detected release of radiolabeled macromolecules and presented evidence of accompanying goblet cell apocrine secretion from rabbit tracheal ring organ cultures exposed to *Pseudomonas aeruginosa* elastase or porcine pancreatic elastase. Niles et al. [180] found that human neutrophil elastase and porcine pancreatic trypsin increased release of radiolabeled macromolecules from hamster tracheal ring organ cultures. In studies of primary cultures of hamster tracheal epithelial cells, treatment with human neutrophil elastase released radiolabeled mucin molecules from the apical cell surfaces rather than from secretory granules [45, 121]. Varsano et al. [252] determined that monolayer cultures of dog tracheal epithelial cells spontaneously released $^{35}SO_4$-labeled glycoconjugates from their apical surfaces and that extracellular proteases including trypsin, Pseudomonas *aeruginosa,* elastase, thermolysin, Staphytlococcus *aureus* protease, mast cell chymase, plasmin, and kallikrein increased the release of sulfated products. The sulfated products released by trypsin had an apparent molecular weight of $\geq 10^6$ daltons as determined by gel filtration on Sepharose CL-4B. Digestion of the radiolabeled products with endo-ß-galactosidase or with keratanase suggested that they are glycoconjugates containing poly(N-acetyllactosamine)-type carbohydrate chains. Additional studies have demonstrated that the cell surface glycoconjugates are synthesized rapidly and constantly, pooled on the cell surface, and released continuously as an intact macromolecule [111].

Intracellular secretory control mechanisms

Investigations using whole animals or tracheobronchial organ cultures models have demonstrated numerous neurohumoral mechanisms capable of affecting airway secretion. However,

these models are inadequate to study the intracellular processes mediating tracheobronchial secretion.

Cyclic AMP. General information has been obtained by Whimster and Reid [255] using autoradiographic techniques. They determined that the adenosine 3',5'-cyclic monophosphate (cAMP) analogue $N^6,O^{2'}$-dibutyryladensosine 3',5'-cyclic monophosphate (Bt_2cAMP) and the phosphodiesterase inhibitor theophylline both increased release of radiolabeled macromolecules from human bronchial organ cultures. These agents likely mimicked the action of ß-adrenergic activation since Liedtke and coworkers [147] found that isoproterenol stimulated cAMP production and secretion from cat tracheal organ cultures. Additionally, they detected reduced binding of the photoaffinitylabel 8-N_3[^{32}P]cAMP to three major tracheal mucosa-submucosal proteins (49, 54 and 59 kDa) after endogenous cAMP levels were increased by ß-adrenergic stimulation. The 49 and 54 kDa proteins correspond in electrophoretic mobility to regulatory subunits of type I and type II cAMP-dependent kinases, and the 59 kDa protein may be the phosphorylated form of the regulatory subunit of type II cAMP-dependent protein kinase [147].

The role of cAMP as an important intracellular messenger in airway secretory cells received additional support from immunocytochemical studies. Using dog and cat trachea organ cultures and immunocytochemical techniques, Lazarus et al. [140, 141] detected increased amounts of intracellular cAMP in epithelial cells and submucosal glands after stimulation with ß-adrenergic agonists or PGE_1. Cyclic AMP was also increased in submucosal glands of organ cultures of ferret trachea after incubation with VIP [139].

Cyclic GMP. In 1972, Ball et al. [9] showed that cellular guanosine 3',5'-cyclic monophosphate (cGMP) increased in human tissues after cholinergic or α-adrenergic stimula-

tion. Administration of the cGMP analogue 8-bromo-cGMP to human airway organ cultures stimulated release of radiolabeled molecules suggesting that cGMP may be an important intracellular messenger in cholinergic and alpha-adrenergic induced airway secretion [219].

Calcium ion. An increase in cytosolic calcium ion concentration has been shown to play a major role in stimulus-secretion coupling in many endocrine and exocrine cells [77]. An effect of calcium in airway secretion was first noted by Bogart et al. [35] and Conover and Conod [68] in studies that showed that the calcium ionophore A23187 caused mucus secretion by rabbit tracheal explants. Balfre [8] found a similar effect of A23187 on chicken trachea, but also noted that depletion of extracellular calcium stimulated mucus secretion. Coles and coworkers [63] found that calcium was not involved in resting secretion of dog tracheal organ cultures but was an essential requirement for cholinergically-induced secretion. Later studies suggested that the increase in mucus secretion occurring after depletion of extracellular calcium resulted from clearance of mucus from secretory ducts of submucosal glands [61]. A calcium channel blocker, verapamil, failed to inhibit basal or cholinergically-induced secretion, but an intracellular calcium antagonist TMB-8 blocked cholinergically-induced mucus secretion from dog trachea [8]. This supported an hypothesis advanced earlier by Coles et al. [63] that intracellular calcium was more important than extracellular calcium during stimulation of mucus secretion.

Posterior membranes of dog trachea displayed decreased rates of mucus secretion when calcium was removed from the bathing fluid [161]. Since posterior membranes of dog trachea contain few if any submucosal glands this suggested that calcium may be involved in mucus secretion by epithelial goblet cells [160]. However, the methods used in this study

to quantitate secretion (detection of radiolabeled glycoconjugates) do not exclude glycoconjugates that could have been released from surface epithelial cells [252]. Mian et al. [172] described effects of calcium on mucus secretion from chicken trachea, another tissue which has sparse submucosal glands. Application of A23187 to either serosal or mucosal sides of chicken trachea stimulated secretion of radiolabeled macromolecules [172]. The effect of calcium on mucus secretion appears directional since only low luminal and high serosal calcium ion concentration stimulated secretion [172].

BIOCHEMISTRY OF TRACHEOBRONCHIAL MUCUS

The biochemical analysis of tracheobronchial mucus has often focused on mucin glycoproteins which are primarily secreted by goblet and mucous cells [169]. However, many other molecular components known to derive from airway cells have been detected in mucus secretions. Our understanding of the structure and specific function of many of these molecules is incomplete.

Mucin

Our knowledge of mucin structure comes from studies of various organs from different species. Mucins are large macromolecules (>1 x 10^6) that consist of a polypeptide chain or "core protein" which is partially covered by oligosaccharide side chains. These oligosaccharide side chains, which number in the hundreds, are linked to the protein core by O-glycosidic bonds. In mucins, the O-glycosidic bonds are always between N-acetylgalactosamine (GalNAc) and the serine and threonine residues of the protein core [27]. The oligosaccharide side chains average 10 sugars in length and occupy approximately 60% of the core protein [225].

About one half of the amino acid residues in this glycosylated region are either serine or threonine; however, not all of these residues are glycosylated [225]. The glycosylated portion of the protein core also contains many proline residues, and these residues may be important in determining whether or not nearby serine and threonine residues are glycosylated [104]. The oligosaccharide side chains contain galactose, fucose, sialic acid, N-acetyl glucosamine (GlcNAc) and GalNAc, and they often terminate in either a fucose or sialic acid [33, 103]. There is considerable variability (microheterogeneity) in not only the length, but also the sugar composition and complexity of these side chains [200, 216]. The purpose of these various glycoforms is unknown.

The nonglycosylated portion of the peptide chain or so called "naked" peptide region(s) contains many charged amino acids and almost all of the cysteines in the chain [225]. This region of the core protein is susceptible to proteolytic attack while the glycosylated region is resistant. It is not known whether there are two naked peptides flanking the glycosylated region or whether there is only one naked peptide end [225]. Mucins may form a polymeric structure through disulfide bonds via cysteine residues on the naked peptide chain [228], via "linking" peptides [4] or via interaction between oligosaccharides. However, there is considerable controversy over the nature of these mucin polymeric structures [87, 142, 227].

Non-mucin components

Other important molecules including lysozyme [41, 125, 166, 233], secretory IgA [233, 243], lactoferrin [40, 168], proline-rich proteins [253], low molecular weight protease inhibitor (antileukoprotease) [173], peroxidase [56], kallikreins [167], proteoglycans [62] and lipids [261] are also present in tracheobronchial mucus. Although it is known

that some of these products, e.g., secretory IgA [101], peroxidase [56], low molecular weight protease inhibitor [130] and lipids [258] may originate from more than one type of tracheobronchial secretory cells, some, including lysozyme [242], lactoferrin [40, 168], and proline-rich proteins [253] appear to originate only from serous cells. The importance of the nonmucin components of airway secretion is emphasized by recent analysis of "normal" secretions from dog and human which show absence of typical mucins, but the presence of high molecular weight glycoconjugates in the form of proteoglycans or perhaps a hybrid proteoglycan-mucin species [29, 30].

CELL-SPECIFIC STUDIES OF TRACHEOBRONCHIAL MUCUS SECRETION

Since serous and mucous gland cells have morpholocical, biochemical and histochemical differences [19], it is not surprising that their secretory functions are individually regulated. Quantitative morphologic studies provided the first direct evidence of cell-specific secretory regulation. The volume density of ferret tracheal serous gland cell secretory granules was reduced by α-adrenergic and cholinergic stimulation, but not ß-adrenergic stimulation [22]. Measurements of secreted lysozyme, a serous but not mucous cell product, after stimulation of ferret tracheal organ cultures supported the morphometric studies [242]. On the other hand, mucous cells, as assessed by surface density of their apical membranes responded to cholinergic and to ß-adrenergic, but not to α-adrenergic stimulation [18, 95]. Stimulation of ferret trachea with SP and VIP produced a definite secretory response in serous gland cells; however, effects of these agents on mucous cell secretion could not be adequately assessed [93]. Webber and Widdicombe [254] studied the effects of VIP on cholinergic and a-adrenergic on lysozyme secretion from ferret trachea. Vasoactive intestinal peptide signifi-

cantly increased the absolute amount of lysozyme secreted in response to phenylephrine. Vasoactive intestinal peptide had no effect on the absolute amount of lysozyme secreted in response to methacholine but it did produce a significant increase in the concentration of lysozyme when it was administered with methacholine. Hence, these investigators postulated that under these conditions, VIP might selectively inhibit secretion from mucous cells resulting in decreased volume flow and increased lysozyme concentation [254].

Cell-specific control of airway secretions may also explain the findings of Leikauf et al. [143]. Using micropipettes to collect secretions directly from cat tracheal gland ducts, these investigators were able to measure the secretory rate and characterize the viscoelastic properties of the secretions under resting conditions and after the administration of cholinergic and adrenergic agonists. When compared to resting secretions, all agonists increased the secretory rate. However, cholinergic stimulation produced secretions of identical viscoelastic properties, α-adrenergic stimulation produced secretions of lower viscosity and similar elasticity, while ß-adrenergic stimulation produced secretions of higher viscosity and lower elasticity.

Thus, the immunocytochemical localization of components of tracheobronchial mucus to specific cells, the morphological evidence indicating different responses to receptor activating drugs and the rheologic measurements indicating drug-dependent influences on the viscoelastic properties all support the hypothesis that tracheobronchial secretion is regulated in a cell-specific manner. To understand the cellular processes involved in the synthesis and secretion of respiratory mucus requires approaches for analyzing the major

secretory cell types individually on cellular and molecular levels. This dissertation describes two such approaches: (1) development of a cell line of bovine tracheal submucosal gland serous cells and (2) development of a panel of monoclonal antibodies directed against human airway secretions.

MATERIALS AND METHODS

MATERIALS

All media, phosphate buffered saline (PBS), and antibiotics were obtained from the Cell Culture Facility, University of California, San Francisco. Fetal calf serum (FCS) lots (HyClone Laboratories, Logan UT) were tested by the Cell Culture Facility for the ability to support colony formation and growth of selected standard cell lines. SP2/0 murine myeloma cells were obtained from the Cell Culture Facility. Tissue culture flasks, dishes and plates were obtained from either Becton Dickinson Labware (Oxnard CA), Corning Glass Works (Corning, NY), Nunc, Inc. (Naperville, Il) or Costar (Cambridge, MA). Serological pipettes were obtained from Becton Dickinson Labware or Costar. Sterile, conical centrifuge tubes were obtained from Corning. Filters were obtained from either Millipore Corporation (Bedford, MA) or Nalge Company (Rochester, NY). Microscopic slides and coverslips were obtained from Becton Dickinson Labware. Sodium cacodylate, uranyl acetate, propylene oxide, sodium maleate, methanol, glacial acetic acid, Schiff reagent, Giemsa stain concentrate, Permount, H_2O_2, Spectro/por dialysis tubing and scintillation vials were obtained from Fisher Scientific (Fair Lawn, NJ). Veroneal acetate (sodium barbitol) was obtained from Merck (Rahway, NJ). Oil red O, copper EM grids, Epon 812, DDSA, DMP-3, and NMA were obtained from Ted Pella, Inc. (Tustin, CA). Glutaraldehyde, osmium tetroxide, and toluidine blue were obtained from Electron Microscopy Sciences (Fort Washington, PA). Ethyl alcohol was obtained from Gold Shield Chemical Co. (Hayward, CA). OCT compound was obtained from Miles, Inc. (Elkart, IN). Normal goat and horse sera were obtained from Antibodies Incorporated (Davis, CA). Goat anti-mouse IgG-fluorescein isothiocyanate was obtained from Cappel (Westchester, PA). Dabco (1,4-diazabicy-

clo[2.2.2]octane) was obtained from Aldrich Chemicals (Milwaukee, WI). Glycol methacrylate monomer, benzol peroxide, polyethylene glcol 400, N,N-dimethlanilin were obtained form Polysciences, Inc (Warrington, PA). Biotinylated horse antimouse IgG and avidin biotin-peroxidase complex were obtained from Vector Laboratories, Inc. (Burlingame CA). Polyethelene glycol 1500 was obtained from the J.T. Baker Chemical Co. (Phillipsburg, NJ). Microtiter plates were obtained from Dynatech Laboratories, Inc. (Chantilly, VA). Sepharose 4B and CL4B were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Acrylamide-bis (37.5:1), sodium dodecyl sulfate, ammonium persulfate, bromophenol blue, and glycine were obtained from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH). Polystyrene beads were obtained from Precision Ball Co. (Chicago, IL). Sheep anti-mouse ^{125}I-labeled Ig (Fab')$_2$ fragments (6.5 μCi/mg) were obtained from New England Nuclear (Boston, MA). $Na_2[^{35}S]O_4$ (carrier free, 43 Ci/mg) was obtained from either Amersham (Arlington Heights, IL) or New England Nuclear. 1[^{14}C]-glucosamine HCl (50 mCi/mM) was obtained from either Amersham or ICN Biochemicals. γ-[^{32}P]-ATP (32 Ci/mM) was obtained from New England Nuclear. Hydrofluor was obtained from National Diagnostics (Manville, NJ). Ecolite was obtained from ICN Biochemicals. Chondroitinase ABC lyase was obtained from ICN Biochemicals. X-ray film was obtained from Eastman Kodak Co. (Rochester, NY). Mice were obtained from Bantin and Kingman, Inc. (Fremont, CA). *l*-Propranolol and *d*-propranolol were a gift from J.M. Madison, Department of Medicine, University of California, San Francisco, Fort Miley Veterans Administration Hospital. All other chemicals and the polyclonal antibodies against lactoferrin were obtained from Sigma Chemical, St Louis, MO.

BOVINE TRACHEAL GLAND SEROUS CELLS

Establishment and characterization of cell cultures

Isolation of tracheal submucosal gland cells. Bovine tracheas, obtained from a local abattoir, were rinsed twice in sterile PBS containing penicillin (10^6 U/l), streptomycin (100 mg/l), gentamicin (100 mg/l), and amphotericin B (2.5 mg/ml) at 4°C. The tracheas were opened longitudinally along the posterior membrane and mounted in a stretched position on a sterile dissecting tray. The epithelium at one end of the trachea was lifted free of the underlying submucosa by sharp dissection and the epithelium was removed in strips, exposing the underlying submucosal tissues. With a fine scalpel and forceps, the gland-rich submucosal tissues were removed from the tracheal cartilage and adventitia and placed in a 50 ml conical centrifuge tube containing Hanks' balanced salt solution (HBSS) with 20 mM HEPES buffer (pH 7.4), bovine serum albumin (1 mg/ml), and penicillin, streptomycin, gentamicin, and amphotericin B at the same concentrations used in the rinse solution (HBSS-A). After all of the submucosal tissues were collected, the medium was removed and the tissues were minced finely with scissors. The tissue pieces were transferred into trypsinizing flasks containing disaggregation medium (2 ml/mg tissue) composed of HBSS-A containing crude collagenase type IV (0.5 U/ml), elastase (6 U/ml), hyaluronidase (200 U/ml), and DNase (10 U/ml). The flasks were agitated on an orbital shaker (200 rpm, Bellco Biotechnology, Vineland, NJ) for 12-18 h. After this procedure, the disaggregation medium was replaced (4 ml/mg tissue). Agitation was continued, and the medium was collected and replaced every 30 min for 120 min. Collected medium was centrifuged (150 x *g*, 10 min) to obtain cell pellets of isolated gland cells. Isolated cells were resuspended in a 1:1 mixture of Dulbecco's modified Eagles' medium (4.5 g glucose/l) and Ham's F12 nutrient medium (DF12) supplemented with

antibiotics and FCS (20%), hereafter referred to as DF12/20% FCS. Cell yield and viability at each collection was assessed. Erythrosin B, made up as a 0.4% solution, was diluted 1:10 with cell suspension. This mixture was placed in a hemacytometer, and counts of viable and nonviable cells were obtained. After the last 30-min collection, the cells were pooled, and the yield of viable cells determined.

Initiation of cell culture, cryopreservation and growth kinetics. Freshly isolated cells in DF12/20% FCS were preplated for 1 h to remove fibroblasts and then plated at 5 x 10^5 cells/cm^2 onto T-25 culture flasks coated with human placental collagen as described by Coleman et al. [59]. Flasks were incubated at 37°C in 5% CO_2:95% air. The cells were observed daily with a phase contrast inverted microscope. Medium was changed at 24 and 48 h and every 3 d thereafter. At the time of the first change of medium (24 h), attachment of numerous gland cells and a few fibroblastic cells was apparent. When the monolayer of mixed cells reached 70 to 80% confluence, cells were removed from the flasks by gentle trypsinization with STV (0.9% NaCl, 0.1% trypsin, 0.2% EDTA) in two stages: the first stage removed easily detached fibroblasts, and the second stage removed the gland cells. The time required to remove the fibroblasts was determined by observing the cultures continuously with a phase microscope. Detached fibroblastic cells were discarded, flasks were rinsed with PBS, and fresh STV was added. After gland cells detached, they were resuspended in complete medium and subcultured. The two-step trypsinization procedure was repeated for 4 serial passages until a homogeneous population of gland cells was obtained, hereafter referred to as bovine tracheal gland or BTG cells. After the 2nd passage, antibacterial additives to the medium consisted only of gentamicin (50 µg/ml). Cells were maintained by routine subculturing at approximately weekly intervals.

Cells were routinely cryopreserved. Following trypsinization, cells were centrifuged (100 x *g*, 10 min, room temperature). Cell pellets were resuspended in a solution containing growth medium, FCS, dimethylsulfoxide (40:50:10). Aliquots of the cell suspension were frozen slowly (-1°C/min) to -70°C and then transferred to liquid nitrogen for long-term storage.

To study growth kinetics of BTG cells grown in serum-free DF12 or DF12 supplemented with either 10 or 20% FCS, cells (5×10^{5}) in their 25th passage were seeded onto human placental collagen-coated or uncoated T25 flasks. Triplicate cell counts from three flasks of each culture condition were made using a hemacytometer and a light microscope. Growth curve experiments were analyzed using a Newman-Keuls test.

Determination of chromosome number. Chromosome preparations were prepared from cells in the 18th passage by the method of Worton et al. [262]. Cells were seeded (4×10^{3} to 1×10^{4} cells/cm^2) in T75 culture flasks. After 5 days when the cells were in log growth phase, colcemid was added to each flask to a final concentration of 1×10^{-7} M. After 6 h, medium was removed and cells were trypsinized. Next, cells were centrifuged (100 x *g*, 10 min, room temperature), and the cell pellet was resuspended at room temperature in 5 ml of hypotonic solution (0.04 M KCl, 0.025 M Na citrate). After 20 minutes, 5 ml of ice cold acetic methanol (1 part glacial acetic acid, 3 parts methanol), freshly made, was added with constant mixing. The mixture was centrifuged (100 x *g*, 2 min, room temperature), the supernatant discarded and the pellet was mixed using a vortex mixer. Acetic methanol (5 ml) was added slowly and the sample was left on ice for 10 min. Next, the sample was centrifuged (100 x *g*, 2 min, room temperature), the supernatant discarded and the pellet resuspended in 0.2 ml of acetic methanol using a vortex mixer. Small amounts of the suspension, drawn into the tip of Pas-

teur pipette, were dropped onto cold glass microscopic slides. The slides were held vertically and the drop was allowed to run down the slides as it spread. Slides were dried rapidly over a beaker of boiling water and stained with Giemsa concentrate (1:10, v/v) in 0.01 M phosphate buffer pH 6.5 [90]. Slides were stained for 5 min, rinsed thoroughly in tap water, and rinsed briefly in deionized water before examination. Chromosomes of 50 metaphases were counted using a light microscope (Carl Zeiss, Inc., Thornwood, NY) and an oil immersion objective.

Ultrastructure. Electron microscopy was performed on cells grown on glass coverslips or on the surfaces of the culture vessels. For fixation, culture medium was replaced with a solution of 2.5% glutaraldehyde, 0.08 M Na cacodylate, 5 mM $CaCl_2$, and 1% sucrose (pH 7.4, 4°C). After 12 to 18 h, the cells were postfixed with 1.5% osmium tetroxide in 0.2 M phosphate buffer (pH 7.4) for 2 h. The cells were then rinsed in 0.025 M sodium maleate buffer (pH 6.0) and stained with uranyl acetate (1.5% in 0.025 M sodium maleate buffer, pH 5.2). Cells were either dehydrated and then infiltrated with Epon 812 on the culture vessel surface or scraped from the culture vessels and transferred into microcentrifuge tubes for processing. The cells grown on coverslips were embedded by placing embedding capsules on top of the monolayers and filling them with Epon 812. After polymerization was complete, the resin and the cells were separated from the coverslip surfaces. Semi-thin (0.5 μm) sections were cut with glass knives on an LKB Nova ultramicrotome, mounted on microscope slides, and stained with toluidine blue before examination with a light microscope. Specific areas were selected, and thin sections having a silver interference color were cut with a diamond knife and mounted on Formvar-coated copper slot grids. These sections were stained with uranyl acetate and lead citrate before examination in an electron microscope (JEOL 100S, JEOL USA Inc., Peabody, MA).

Histochemistry. Cells grown on glass coverslips or cultured cells trypsinized and spun onto glass slides with a cytospin centrifuge (Shandon Inc., Pittsburgh, PA) were stained with Alcian blue (pH 2.5/periodic acid-Schiff (AB/PAS) [175]. Staining was performed both with and without prior formalin (10%) fixation. The percentage of cells containing AB or PAS staining granules, or both, was determined (300 cells counted from two cytospin preparations). The number of AB/PAS granules per unit cytoplasmic area was determined for at least 100 stimulated and control cells using an ocular grid in a light microscope with a 100x oil immersion objective. The numbers of granules present in control and stimulated cells were analyzed by two-sample *t*-test. Cells grown on coverslips were also stained with oil red O [148] and acid phosphatase [25].

Immunofluorescence immunocytochemistry. Monoclonal antibodies directed against tracheal antigens were produced as described below. An antibody directed against human tracheal submucosal gland serous cells (B7E5) that cross-reacted with bovine tracheal submucosal gland serous cells was used to stain BTG cells. Unfixed cytospin preparations were stored at -20°C before staining. Immunofluorescence staining was carried out using a double antibody method [21]. Monoclonal antibody diluted 1:1 with phosphate buffered saline (PBS) containing 2% normal goat serum/0.6% Triton X-100 was applied to cells for 2 h at room temperature. Then they were rinsed with PBS containing 1% normal goat serum/0.3% Triton X-100 and incubated with goat anti-mouse IgG-fluorescein isothiocyanate for 30 min at room temperature. Following a rinse with PBS, they were covered with Dabco (1,4-diazabicyclo[2.2.2]octane) solution and glass coverslips before examination with a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). For negative controls, primary and secondary

antibodies were omitted, and cells were incubated in PBS containing 2% normal goat serum/0.6% Triton X-100, alone.

ß-Adrenergic regulation of bovine tracheal gland serous cell secretion

Radiolabeling of glycoconjugates, pulse chase, and pharmacologic stimulation. Cells in their 15th-25th passage were studied. Tissue culture flasks (25 or 75 cm^2) were seeded with 2 x 10^4 cells/cm^2. Medium was changed every 3rd day. On day 9, medium was replaced with 10 ml of DF12/20% FCS containing $Na_2[35S]O_4$ (7.5 µCi/ml). After 24 h, medium containing radiolabel was removed, and flasks were washed three times with PBS. Serum-free medium (DF12) was added to each flask, and every 30 min for 210 min the medium was collected and replaced. Agonists were added to medium during the 210- to 240-min period. Antagonists or the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine were added to medium during the 30-min collection period (180-210 min) before addition of agonists as well as during the period of agonist stimulation. At 240 min, cells were typsinized from culture flasks and counted. Viability was assessed by exclusion of erythrosin B. The collected samples were dialyzed (Spectra/por tubing; MW cutoff, 12,000-14,000 daltons) against 25 volumes of distilled water containing 0.1% (wt/vol) sodium azide. Aliquots of the dialysates from each collection were prepared for scintillation spectrometry by addition of scintillant (Hydrofluor, 2x vol). Samples were counted in a scintillation counter (Beckman Instruments, Inc., Irvine, CA). Receptor agonists tested included bethanechol (10^{-5} M), phenylephrine (10^{-5} M), *l*-isoproternol (10^{-8} to 10^{-3} M), *l*-epinephrine (10^{-8} to 10^{-4} M), *l*-norepinephrine (10^{-8} to 10^{-4} M), and *d,l*-isoproterenol (10^{-8} to 10^{-3} M) alone or with 1-methyl-3-isobutylxanthine (10^{-5} M). Additionally, cyclic AMP analogs 8-bromo-adenosine

3',5'-cyclic monophosphate (8-bromo-cAMP) and $N^6,O^{2'}$-dibutyryladensosine 3',5'-cyclic monophosphate (Bt_2cAMP) either alone (both 10^{-3} M) or with MIX (10^{-5} M) were each tested for their effect on BTG secretion. Antagonists tested included *l*-propranolol or *d*-propranolol both (10^{-9}-10^{-4} M) added with *l*-isoproterenol (10^{-5} M).

The degree of stimulation-induced secretion was evaluated by comparing the release of nondialyzable radiolabel (cpm) for the periods immediately after (samples collected after 240 min) and immediately preceding (samples collected after 210 min) drug exposure. The ratio of radiolabel release immediately after the period of drug exposure to release during the immediately preceding period (secretory index) was determined for each flask. To control for variations from flask to flask and day to day, a relative secretory index was obtained by dividing the secretory index obtained from flasks receiving drugs by the secretory index obtained from control flasks not receiving drugs. Thus,

$$\text{Relative Secretory Rate} = \frac{\text{cpm}_{240}/\text{cpm}_{210} \text{ of flasks receiving drugs}}{\text{cpm}_{240}/\text{cpm}_{210} \text{ of control flasks}}$$

where cpm_{240} = counts per min of the samples collected immediately after the period of drug exposure and cpm_{210} = the counts per min of the samples collected immediately preceding the period drug exposure. A relative secretory rate significantly greater than 1.0 indicates the presence of a secretory response. Pharmacologic effects in secretory experiments were evaluated using a paired t-test and a Newman-Keuls test was applied when multiple comparisons were made [267].

Cyclic AMP assay. Cyclic AMP content of cells was determined at 0.5, 1, 5, 10, 20 and 30 min after incubation of cells with *d,l*-isoproterenol, *d,l*-isoproterenol and MIX, *d,l*-isoproterenol and *d,l*-propranolol or no drug. The medium

containing drugs and the control medium were removed at the various time intervals and replaced rapidly with ice cold 10% trichloroacetic acid (TCA). Cells were scraped from culture vessels and sonicated (Branson Ultrasonics Corp., Danbury, CT). An aliquot of this material was used to determine the protein content of each dish by the method of Lowry et al. [152]. From the remainder of the sonicated sample, the TCA was extracted five times in water-saturated ether. The samples were placed in a water bath at 60°C, the ether evaporated under a stream of air and the residue dissolved in acetate buffer. Aliquots of sample were assayed for cAMP by ^{125}I-radioimmunoassay. Measurements were corrected for a recovery of 82% as determined using a tritiated cAMP marker.

Cyclic AMP-dependent kinase assay. Protein kinases were assayed using a modification of the method of Burnham and Williams [47]. Tracheobronchial gland cell cultures were washed thoroughly with PBS, scraped from culture dishes and centrifuged (1000 x *g*). The cell pellet was resuspended in 1 ml of homogenization buffer consisting of 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EGTA, 1 mM dithiothreitol, 2 mM benzamidine, 1µg/ml leupeptin, and 0.1 µg/ml pepstatin. The resuspended cells were sonicated for 1 min following which they were centrifuged (1000 x *g*) to remove cells and large cell fragments. Soluble and particulate fractions were obtained by high speed centrifugation (47,000 x *g*, 1.5 h). Protein content of the samples was determined by the method of Lowry et al. [152]. The separated fractions were stored at -90°C.

The kinase assay was carried out at 30°C. Under control conditions (addition of histone alone) the reaction mixture contained 20 mM PIPES buffer (pH 7.0), histone type II (0.5 mg/ml), 0.1 mM dithiothreitol, 5 mM $MgCl_2$, and 0.1 M EGTA. Experimental conditions included addition of soluble proteins (30 µg/ml) and particulate proteins (60 µg/ml), both with and without cAMP (10^{-6} M). After preincubation of the mixture for

30 s at 30°C, γ-[^{32}P]-ATP (25 µM, 15,000 cpm/µl) was added to initiate the reaction. Samples (100 µl) were removed at timed intervals (1,2,4,8 min) and added to 4 ml of ice-cold 5% TCA, 1.5% sodium pyrophosphate and 1% sodium phosphate (monobasic). Precipitated samples were filtered on 45 µm filters (HATF, Millipore Corp. Bedford, MA), and filters were washed with 8 rinses of the TCA mixture. Next, filters were immersed in aqueous scintillation fluid (EcoLite) and counted in a scintillation counter. Phosphorylation was expressed in nmol/mg protein.

Cyclic AMP-dependent protein phosphorylation. The same incubation conditions as used in the kinase assay were used to detect phosphorylation of endogenous proteins except that protein concentration was 0.2 mg/ml and 1 mM 1-methyl-3-isobutylxanthine was added to the mixture. The assay was run with and without cAMP (10^{-6} M). After preincubation of the mixture for 30 s at 30°C, γ-[^{32}P]-ATP (2.5 µM, 70 µCi/ml) was added to initiate the reaction. After 30, 60, 90 and 120 s incubations, samples (100 µl) were removed from the reaction mixture and placed in 50 µl of "stop" solution consisting of 30 mM Tris-Cl (pH 7.4), 15% glycerol, 9% SDS, 0.05% bromophenol blue, and 5 mM ATP [47]. The samples were immediately boiled in water for 5 min and allowed to cool. Fifty µl of 8% 2-mercaptoethanol was added to each sample prior to storage overnight at -90°C. Next, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 4% stacking and 10% separating) was performed according to the method of Laemmli [132]. Gels were fixed in 50% methanol, 10% glacial acetic acid for 1h, incubated in fresh fix solution for 2h, and transferred to a 10% glycerol solution for 1-2 h. Gels were dried at 80°C under vacuum for 2 h and allowed to cool for 30 min while still under vacuum. Finally, gels were exposed to X-ray film (Kodak X-Omat AR) for 1-2 days at -90°C before

developing. Autoradiographs were scanned with a densitometer (Gilson Medical Electronics, Inc., Middleton, WI), and peak height in densitometric tracings was measured.

Physiology and biochemistry of bovine tracheal gland organ cultures

Organ culture, radiolabeling of glycoconjugates, pulse chase and pharmacologic stimulation. Submucosal gland-rich tissues were obtained from a bovine trachea and placed in incubation medium consisting of Ham's F12 nutrient medium containing penicillin (10^6 U/l), streptomycin (100 mg/l) and gentamicin (100 mg/l). The tissues were equilibrated with 5% CO_2:95% O_2 in a Dubnoff metabolic shaking incubator (Precision Scientific, Inc., Chicago, IL) and radiolabeled with either 50 µCi 1-[^{14}C]-glucosamine or 1 mCi $Na_2[^{35}S]O_4$ for 20 h. Tissues labeled with [^{14}C]-glucosamine or $[^{35}S]O_4$ were divided equally into 5 and 11 beakers, respectively. All beakers contained 10 ml of Ham's F12 nutrient medium. Tissues were transferred to beakers containing fresh medium at 30-min intervals for 3.5 h. At 210 min the beakers containing [^{14}C]-glucosamine-labeled tissues contained either isoproterenol (10^{-5} M, n = 3) or Ham's F12 nutrient medium alone (n = 2). Similarly at 210 min, the tissues labeled with $[^{35}S]O_4$ were placed into beakers containing medium alone (n = 2) or isoproternol (n = 3), phenylephrine (n = 3) or bethanechol (n = 3), all at 10^{-5} M. Medium from each collection was dialyzed as described above. Incorporated radioactivity in 1 ml aliquots of the samples was determined by scintillation spectroscopy and relative secretory rates in response to drugs were determined. The remainder of the dialysate was lyophilized and stored at -20°C.

Gel filtration chromatography and enzymatic digestion of the void volume material. Medium collected from [^{14}C]-glucosamine-labeled tissues during the 30 min prior to and 30 min after administration of isoproterenol (10^{-5} M) was concentrated and applied to a Sepharose CL-4B column (1.5 x 60 cm). The eluting buffer was 0.1 M sodium acetate (pH 6.0) containing 4 M guanidine chloride and 0.5% chaps. Fractions (1 ml) were collected and an aliquot of each fraction was counted for radioactivity. The high molecular weight void volume fractions were pooled, dialyzed and concentrated. Aliquots this material were digested with chondroitinase ABC from *Proteus vulgaris*, 0.4 unit/ml at 37°C for 18 h in 0.1 M Tris acetate, pH 7.3.

MONOCLONAL ANTIBODIES DIRECTED AGAINST HUMAN AIRWAY SECRETIONS

Preparation of monoclonal antibodies

Fusions. Sputum from a patient with cystic fibrosis, blood group B, Le (a-b+), was dialyzed against distilled water (Spectro/por MW cutoff 12,000-14,000) and concentrated by centrifugal evaporation (Speed Vac, Savant Instruments, Inc., Farmingdale, NY). Balb/C mice, 5 weeks of age, were given intraperitoneal injections of 100 µg of the evaporated sample dissolved in 100 µl of sterile phosphate buffered saline (PBS) emulsified with an equal volume of complete Freund's adjuvant. The intraperitoneal injections were separated by 3 weeks. We stimulated the immune response 2 weeks after the second injection with an intravenous injection of the same material but without the Freund's adjuvant. Three days later, the spleens were removed from the immunized mice and homogenized between frosted glass microscope slides. Homogenates were rinsed in DME, resuspended in the same medium and fused with azaguanine-resistant murine myeloma cells (SP 2/0 Ag14) using polyethelene glycol [126, 127]. The fused cells were placed in medium containing thymocyte feeder cells obtained from 4-6 week-old Balb/C mice, DME, 20% FCS, and hypoxanthine/aminopterin/thymidine. The cell suspension was distributed into 96-well plates and incubated at 37°C in 7% CO_2:93% air. Hybridoma cells producing antibodies chosen for further analysis were cloned twice using the limiting dilution cloning method [58, 144].

Screening of antibodies. Tissue sections for screening antibodies produced by hybridomas were obtained by fixing pieces of normal human trachea in 0.1 M PO_4 buffer (pH 7.4) containing 4% paraformaldehyde (2h, 4°C). Tissue was cryoprotected by an 18 h incubation in 30% sucrose/0.1 M PO_4 buffer (pH 7.4) before being frozen in OCT compound.

Sections (5 μm), made using a cryostat (Bright, Hacker Instruments, Inc., Fairfield, NJ), were placed on glass slides and stored at -20°C until used for immunofluorescence. All incubations were at room temperature. Slides were rinsed in PBS to remove OCT. Hybridoma supernatant was diluted 1:1 with PBS containing 2% normal goat serum/0.6% Triton X-100, and the diluted supernatant was applied to the sections for 2 h. Sections were rinsed in PBS containing 1% normal goat serum/0.3% Triton X-100. Next, the slides were incubated with goat anti-mouse IgG-fluorescein isothiocyanate for 30 min. They were rinsed briefly in PBS (3 min); before being covered with Dabco solution and glass coverslips. The sections were examined in a fluorescence microscope.

Localization of antigens in plastic-embedded tissue

Normal human tracheal tissues were processed by the method of Beckstead [24]. Thin pieces of trachea, cut longitudinally, were fixed at 4°C for 2 h using 1% paraformaldehyde in 0.1 M PO_4 buffer (pH 7.4). Additional processing, all at 4°C, was carried out using an Autotechnicon Tissue Processor (Technicon, Chauncey, NY). The tissues were fixed for an additional 6 h using 4% paraformaldehyde in 0.1 M PO_4 buffer (pH 7.4); washed twice for 1 h in 0.1 M PO_4 buffer (pH 7.4) containing 2% sucrose and 50 mM NH_4Cl; washed twice for 1 h in 0.1 M PO_4 buffer (pH 7.4) containing only 50 mM NH_4Cl; dehydrated in 75% acetone for 1 h, 100% acetone for 2 h, 50% acetone/glycol methacrylate monomer for 1 h; and infiltrated with 100% glycol methacrylate monomer for 1 h, then 4 h. The tissues were embedded under vacuum (15-20 mm Hg) overnight in a mixture of glycol methacrylate monomer (20 ml), benzol peroxide (0.09 g) and polyethylene glycol 400 with N,N-dimethlanilin (0.5 ml). Sections were cut at 2 μm with glass knives using a Sorvall JB-4 microtome (Du Pont Co., Wilmington DE), and the sections were transferred to glass coverslips and air-dried.

Immunoperoxidase staining of plastic-embedded tracheobronchial tissues with murine monoclonal antibodies was performed using a modification of a biotin-avidin procedure [24, 105]. Before incubation with monoclonal antibody, plastic-embedded sections were digested with 0.25% porcine trypsin in 0.09% saline, pH 7.6, for 10 min at 37°C and incubated in Ca^{2+}- and Mg^{2+}-free PBS (CMF-PBS) containing 2% Tween 20 for 30 min at 37°C. After three rinses in CMF-PBS, sections were incubated in 3% normal horse serum in CMF-PBS (30 min, 37°C). Following three rinses in CMF-PBS, sections were incubated overnight at 4°C with undiluted supernatant from hybridoma cultures. This step, as well as other antibody incubations, was accomplished by placing 50 µl drops of antibody solution on parafilm and laying the coverslips gently onto the drops. On the following day, coverslips were rinsed with CMF-PBS and incubated at room temperature for 1 h in CMF-PBS containing 3% normal horse serum and 0.5% biotinylated horse anti-mouse IgG. After three rinses in CMF-PBS, endogenous peroxidase was blocked by incubation 40% methanol, 1.5% H_2O_2 (5 min, room temperature). Following an additional three rinses in CMF-PBS, coverslips were incubated for 1 h at room temperature with avidin-biotin-peroxidase complex. After 3 rinses, the peroxidase reaction was developed by incubating coverslips for 10 min in CMF-PBS containing 0.05% 3,3'-diaminobenzidine (DAB), 0.1 M imidazole and 0.1 M Na azide and then for an additional 15 min in CMF-PBS with 0.05% DAB, 0.1 M imidazole, 0.1 M Na azide and 0.1% H_2O_2 at room temperature and in the dark; and enhanced by incubation in 0.5% $NiCl_2$. Finally, sections were counterstained with Gill's hematoxylin #2 for 1.5 min followed by blueing with Scott's water (0.2% $NaHCO_3$, 1% $MgSO_4$). After a final rinse, coverslips were air dried and mounted with Permount. Negative controls consisted of incubations in which primary and secondary antibodies were omitted.

Determination of immunoglobulin subclasses

An enzyme linked immunoassay (Calbiochem, La Jolla, CA) was used to identify antibody subclass. Goat anti-mouse immunoglobulins were immobiliized on microtiter plates. Plates were incubated for 1 h with hybridoma supernatants, diluted 1:2-1:10 in PBS. Negative controls were incubated with nutrient medium alone. Plates were rinsed three times with PBS, then incubated (1h) with rabbit anti-mouse typing sera (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, and IgA). Following three PBS rinses, the plates were incubated with peroxidase conjugate (1 h) and after three more PBS rinses, with 1% urea peroxide. Positive wells developed an amber color within 20 minutes while control wells became pale yellow.

Periodate sensitivity of antigens

Sodium periodate in 50 mM sodium acetate buffer (pH 4.5) was applied to frozen sections of trachea mounted on glass microscope slides, and the slides were incubated in the dark. Three conditions of periodate oxidation were used: mild (10 mM, 10 min, 4°C), moderate, (50 mM, 1 h, 4°C) and harsh (100 mM, 12 h, room temperature). Sections were rinsed in 10 mM sodium borohydride in PBS for 30 min at room temperature to reduce peroxide groups generated during periodate oxidation and to prevent nonspecific cross-linking of antibody to antigen through Schiff base formation. Next, sections were rinsed five times in PBS containing 1% normal goat serum/0.3% Triton-X-100, and monoclonal antibodies were applied to the frozen sections as described above. Specific fluorescence of the periodate-treated sections was compared to control sections incubated in buffer alone.

Gel filtration chromatography

Endotracheal secretions were collected from a second cystic fibrosis patient. These secretions were dialyzed

against 8 volumes of sodium acetate (0.01 M, pH 5.5) containing 1 mM EDTA, 0.02% sodium azide, and 0.5 mM phenylmethylsulfonyl fluoride for 24 h at 4°C. The dialyzed material was centrifuged (6000 x g) for 15 min. The supernatant was passed through a column (5 cm x 80 cm) of Sepharose 4B equilibrated with sodium acetate (0.1 M, pH 5.5) containing 0.02% sodium azide. Fractions (12 ml) were collected at room temperature, monitored for protein, and pooled. Pooled fractions, dialyzed against distilled water (30 h, 4°C) and lyophilized, were used in either radioimmunoassays or in SDS-PAGE.

Solid-phase radioimmunoassay

The solid-phase sandwich radioimmunoassay developed by Podolsky and coworkers [194] was used. Sepharose 4B fraction peaks were solubilized in bicarbonate buffer (pH 9.2) at a concentration of 5 µg/ml. Polystyrene beads (6.35 mm) were coated with material from individual fractions (40 beads/10 ml) by overnight incubation at room temperature with gentle agitation. Next, nonspecific binding sites were blocked by incubating beads for 1 h in buffer containing supernatant from non-antibody producing myeloma cells (SP 2/0). Then, beads were incubated with monoclonal antibodies (hybridoma supernatant diluted 1:10) for 1 h at 37°C. Following three rinses in distilled water, beads were incubated for 1 h at 60°C with ^{125}I-labeled sheep anti-mouse Ig $(Fab')_2$ fragments (200,000 cpm per assay mixture; specific activity 6.5 µCi/mg) in 150 µl of buffer containing 0.01 M Tris-HCl (pH 7.5), EDTA at 2.0 µg/ml, and thimerisol. Finally, beads were washed five times in distilled water before bound radioactivity was measured using a gamma counter (Hewlett-Packard Co., Palo Alto, CA). Antibodies were assayed in duplicate and each antibody was assayed in at least three separate experiments. Activity was compared to a negative control

consisting of SP 2/0 supernatant or supernatant obtained from unrelated hybridoma cultures by the Scheffé's F test [267].

SDS-PAGE and immunoblots

Fractions recovered from Sepharose 4B chromatography were solubilized in Tris buffer (0.0625 M) containing SDS (2%) and mercaptoethanol (5%). SDS-PAGE was performed according to Laemmli [132]. For fraction I (V_o) material we used 3-10% acrylamide gradient gels and for fractions II and III, 4% stacking gels and 7.5% separating gels. Components were stained with Coomassie brilliant blue and molecular weight was estimated by determining migration relative to high molecular weight protein standards. Samples were transferred from the gels to nitrocellulose using the method of Towbin and coworkers [246] using an electroblotting system (Bio-Rad, Richmond, CA) operating at 20 V for 17 h. Successful transfers were confirmed by the absence of Coomassie staining in gels and by the presence of amido black staining [49] in nitrocellulose.

The nitrocellulose was cut into strips and Western blot reactions were performed at room temperature with constant shaking. First the strips were incubated for 1 h in PBS containing 1% normal horse serum/0.05% Tween 20. Then strips were transferred to PBS/0.05% Tween 20 containing monoclonal antibody (hybridoma supernatant diluted 1:10) for 1 h. Strips were rinsed six times in PBS/0.05% Tween 20, incubated for 1 h in PBS containing 1% horse serum/0.05% Tween 20, and for 30 min in PBS containing 1% normal horse serum and 0.5% biotinylated horse antimouse IgG was applied to the strips for 30 min. After rinsing the strips (6x) in PBS, they were incubated in PBS, 0.1% Tween 20 and avidin-biotin complex (0.5%) for 30 min, washed with PBS (6x), and soaked in 0.3% $COCl_2$ for 5 min. The peroxidase reaction was developed by incubating the strips for approximately 1 min in 0.1% DAB,

0.1% imidazole and 0.1% H_2O_2. The reaction was stopped by transferring the strips to distilled water.

RESULTS

BOVINE TRACHEAL GLAND SEROUS CELLS

Establishment and characterization of cell cultures

Isolation of tracheal submucosal gland cells, establishment of a cell line, and growth kinetics. Cell yield/gm of submucosal tissue was 2.90 ± 0.25 cells (mean ± SE). Viability, as assessed by exclusion of vital dye, was consistent among preparations (63 ± 1.9%, mean ± SE; n = 4). Microscopic examination revealed that the isolated cell preparations contained numerous red and white blood cells. The majority of nonhematogenous cells were epithelial. These cells, as well as any contaminating fibroblasts adhered to culture vessels. Fibroblasts were reduced in number by the preplating step. Blood cells, nonviable cells, and other cells that failed to attach were removed in the first few changes of medium. The rapid growth of bovine tracheal gland (BTG) cells after subculturing and their greater resistance than fibroblasts to trypsinization permitted the development of an apparently homogeneous cell line by the 4th passage. Examination of cultures using an inverted phase contrast microscope revealed that the BTG cells have a polygonal shape, with large, oval nuclei and moderately abundant cytoplasm (Figure 1).

Cell proliferation in medium containing 0%, 10%, or 20% FCS was compared when cells were grown on collagen-coated vs. uncoated flasks (Figure 2). Most rapid growth occurred when bovine tracheal gland cells were plated on collagen in medium containing 20% FCS. On uncoated tissue culture plastic, cells grew better in medium containing 20% than 10% FCS ($P < 0.05$). Serum-free medium failed to support cell growth, regardless of the presence of collagen.

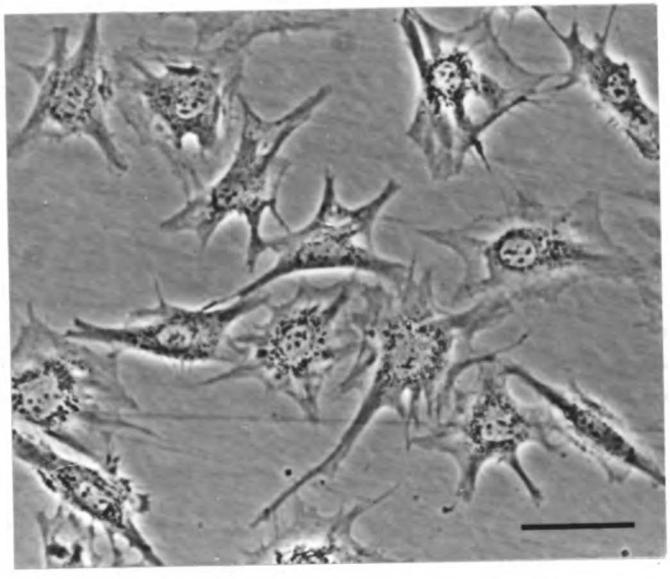


Figure 1. Phase contrast micrograph of bovine tracheal gland serous cells grown on a collagen-coated culture dish. Bar = 50 μm. From Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.

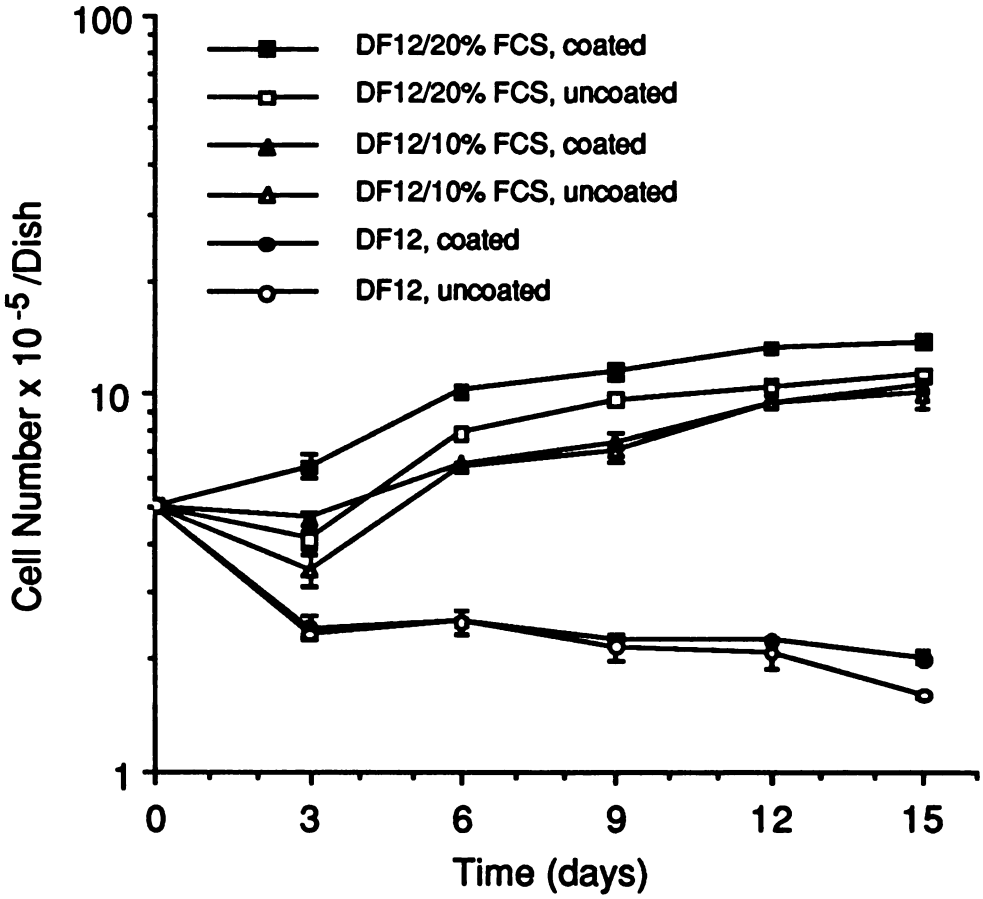


Figure 2. Growth kinetics of bovine tracheal gland cells. Cells (5 x 10^5) were seeded into T25 flasks, with or without collagen coating in serum-free DF12 or DF12 supplemented with 10 or 20% FCS. Triplicate cell counts were made using a hemacytometer. Values shown are the mean (±SE) of three experiments. From Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.

Chromosome number. The bovine diploid chromosome number of 60 [106] was maintained (Figure 3).

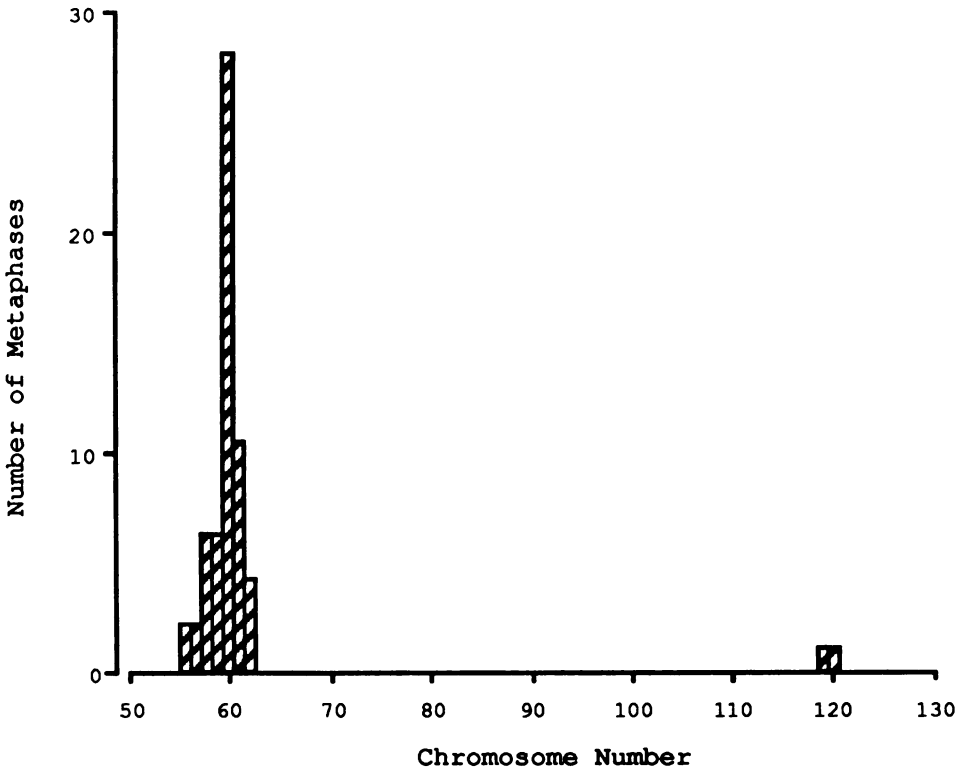


Figure 3. Chromosome numbers of bovine tracheal gland cells in the 18th passage. The diploid bovine chromosome number is maintained. From Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.

Ultrastructure. Ultrastructural examination revealed cells with discrete electron-dense granules (Figure 4A). Granule morphology was comparable to that seen in bovine tracheal gland serous cells (Figure 4B). Fusion of adjacent granular membranes was not seen. Nuclei were round or oval with dispersed chromatin and often contained dense nucleolar structures. Rough endoplasmic reticulum, free ribosomes, and Golgi complexes were present. Numerous large mitochondria,

scattered lysosomes and lipid droplets could be identified adjacent to the secretory granules. Junctional complexes connected the lateral membranes of confluent cells, and apical membranes contained microvilli (Figure 5).

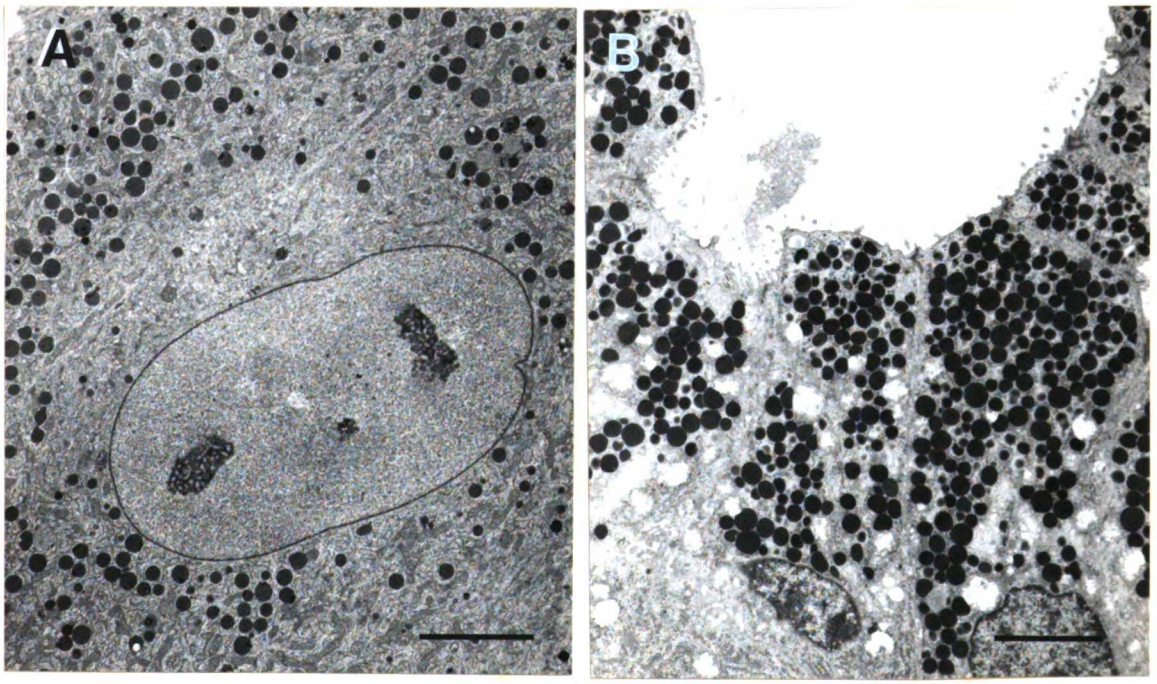


Figure 4. Electron micrographs comparing bovine tracheal gland cells with serous cells of a bovine tracheal submucosal gland acinus. **A**-bovine tracheal gland cells in culture. **B**-bovine tracheal submucosal gland acinus. Note the similarity between the secretory granules in A and B. Bar = 5 μm. From Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.

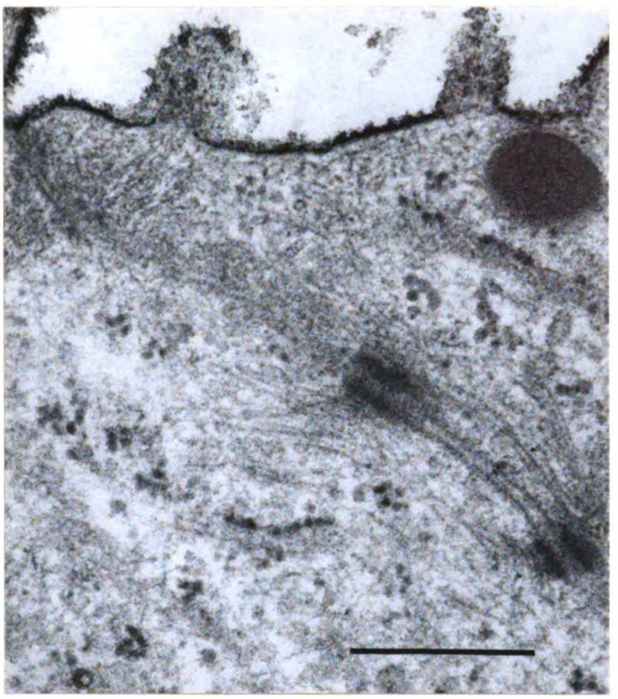


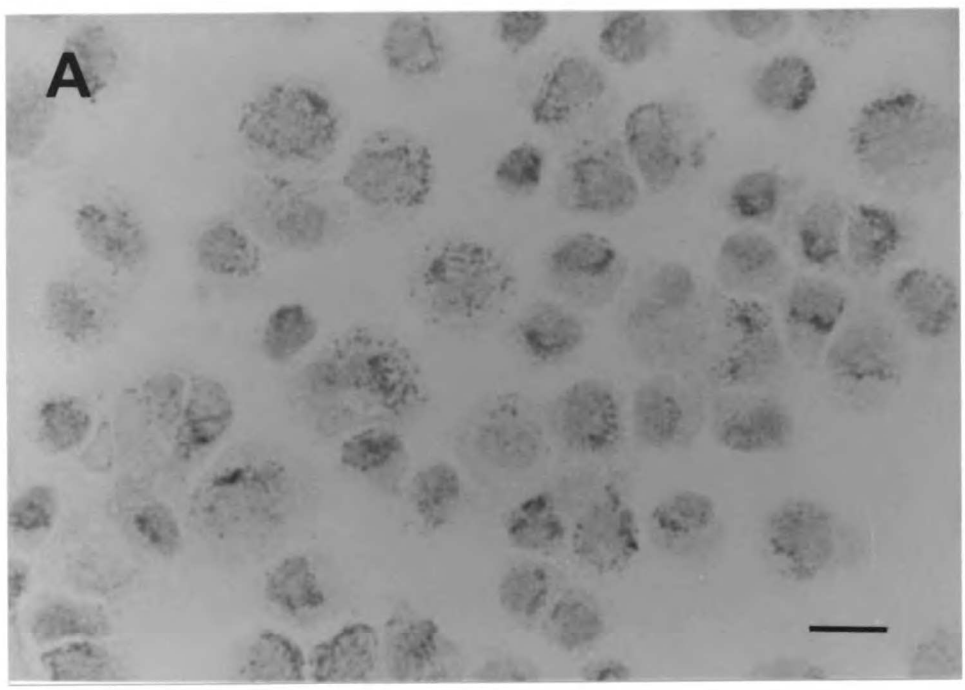
Figure 5. Electron micrograph of bovine tracheal gland cells in culture. A junctional complex connects the lateral membranes of adjacent cells. Note the presence of microvilli on the apical membrane. Bar = 1 µm. From Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.

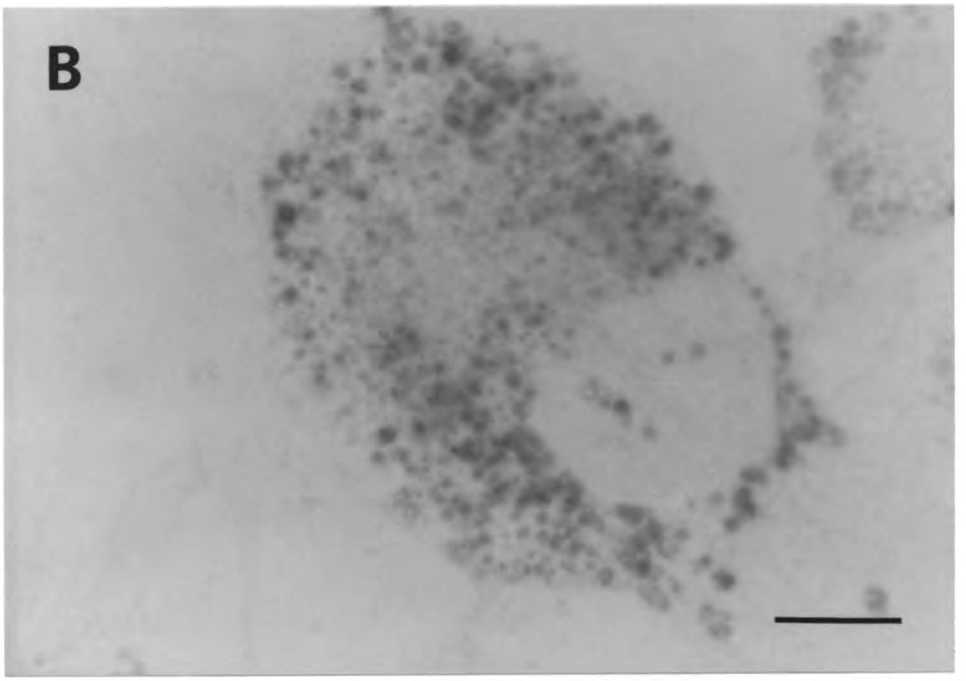
Histochemistry. Cells grown on glass coverslips with or without a collagen coating were identical by phase contrast and electron microscopy to cells grown on collagen-coated plastic. On glass, however, the cells grew more slowly. Virtually all cells contained Alcian blue (AB)- or (periodic acid Schiff (PAS)-positive granules or both (Figure 6). The ratio of AB- to PAS-positive granules was reduced by prior formalin fixation. Fixation also led to apparent granule aggregation. The proportion of granules showing alcianophilia varied from cell to cell under both fixed and nonfixed conditions. At pH 2.5, the presence of AB positivity indicates the presence of polysaccharides carrying a negative charge which could be conferred either by sulfate or sialic acid

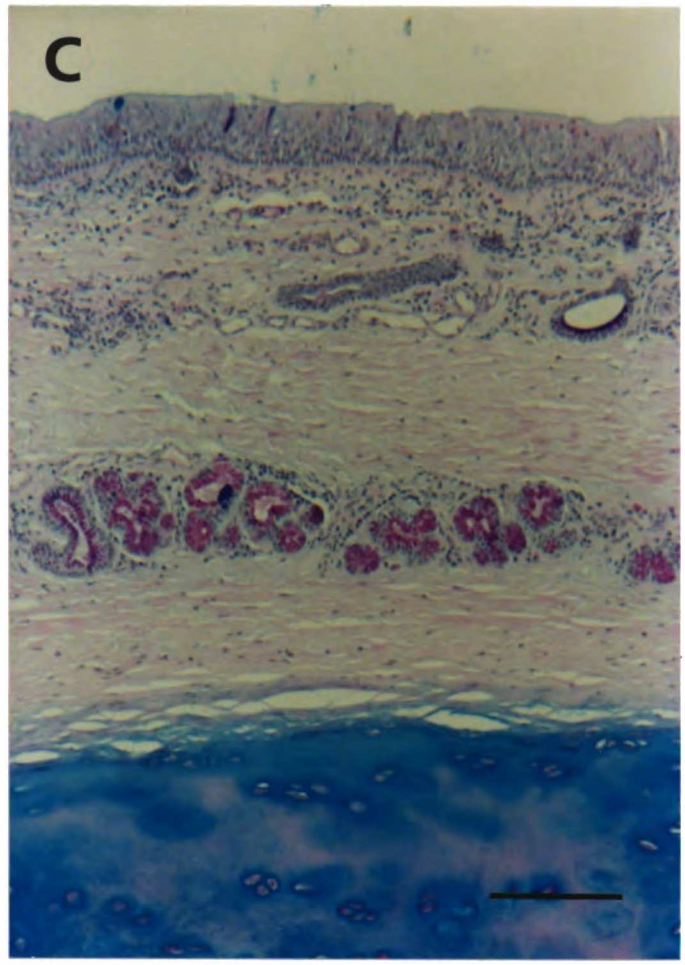
residues. The concentration of AB/PAS-staining granules was reduced after stimulation with *d,l*-isoproterenol (10^{-5} M; control:2.05 ± 0.08 granules/μm^2, n = 101; stimulated: 1.55 ± 0.07 granules/μm^2, n = 107; mean ± SE; $P<0.001$).

The cells also contained droplets of triglyceride as indicated by a positive staining reaction with oil red O (Figure 7). These droplets could be discriminated from the glycoconjugate-containing granules at the level of the phase contrast microscope by their extremely refractile appearance. In the electron microscope, lipid droplets could be discriminated from secretory granules by reduced osmiophilia in the lipid droplet interior (due to extraction during processing). Many cells contained lysosomes as determined by staining for the presence of acid phosphatase (Figure 8). Lysosomal distribution was confined to the perinuclear region, whereas AB/PAS-positive secretory granules were distributed extensively throughout the cells.

Figure 6. (next 3 pages) Alcian blue pH 2.5/periodic acid-Schiff staining of bovine tracheal gland cells. **A**-Cytospin preparation of freshly trypsinized cells. Nearly all cells contain AB- or PAS-positive granules or both. Bar = 30 μm. **B**-Direct staining of cell grown on a collagen-coated glass coverslip. Both AB- and PAS-positive granules are seen. Bar = 2 μm. **C**-Formalin-fixed, paraffin-embedded section of bovine trachea for comparison. PAS-positive serous gland cells predominate over mucous cells in this species. Bar = 20 μm. A and B from Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.


A


B


C

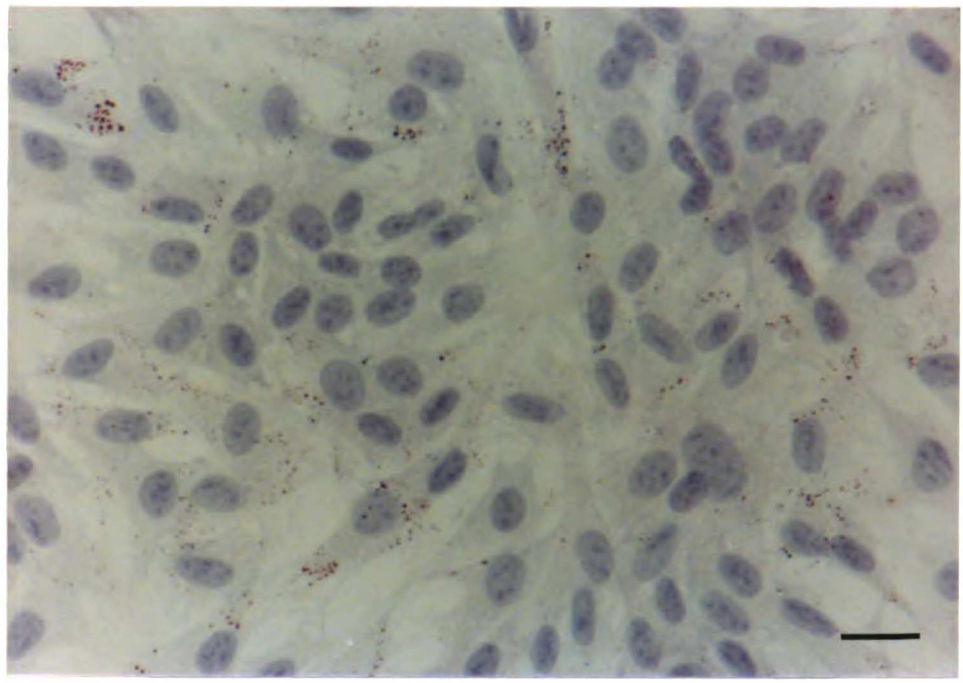


Figure 7. Oil red O staining of bovine tracheal gland cells. Scattered granules containing lipid are present within the cytoplasm. **Bar** = 30 µm.

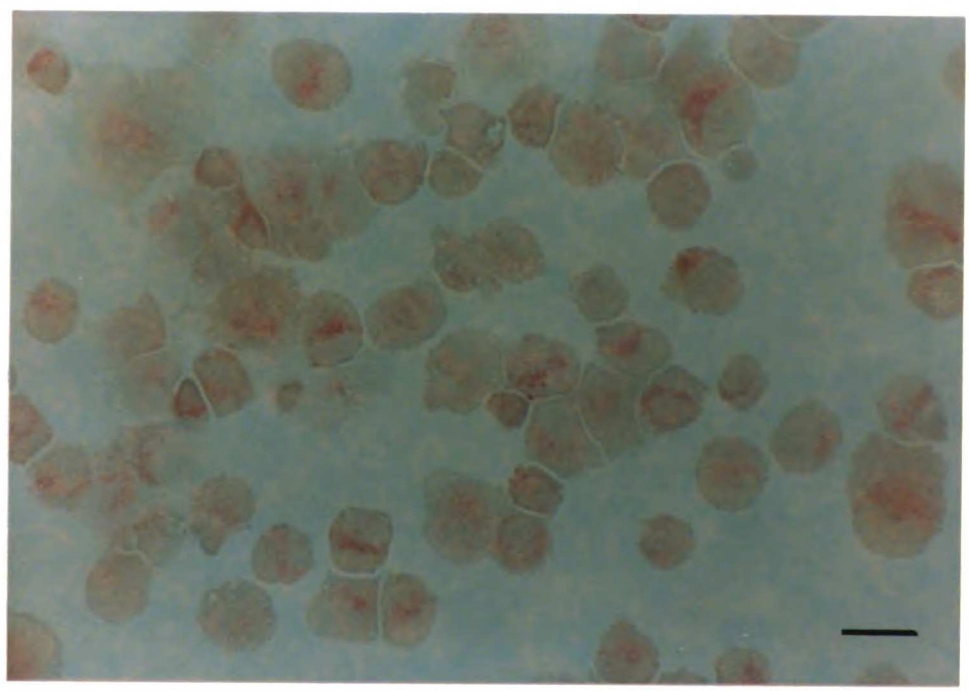


Figure 8. Acid phosphatase enzyme histochemical staining of bovine tracheal gland cells. Reaction product is predominantly located in the perinuclear region. Bar = 30 µm.

Immunocytochemistry. Controls in which primary and secondary antibodies were omitted were negative. The monoclonal antibody B7E5, which stains bovine tracheal serous cells, also stained the BTG cells. Fluorescence was detected in nearly all cells. The pattern of staining was punctate, suggesting localization within granules (Figure 9).

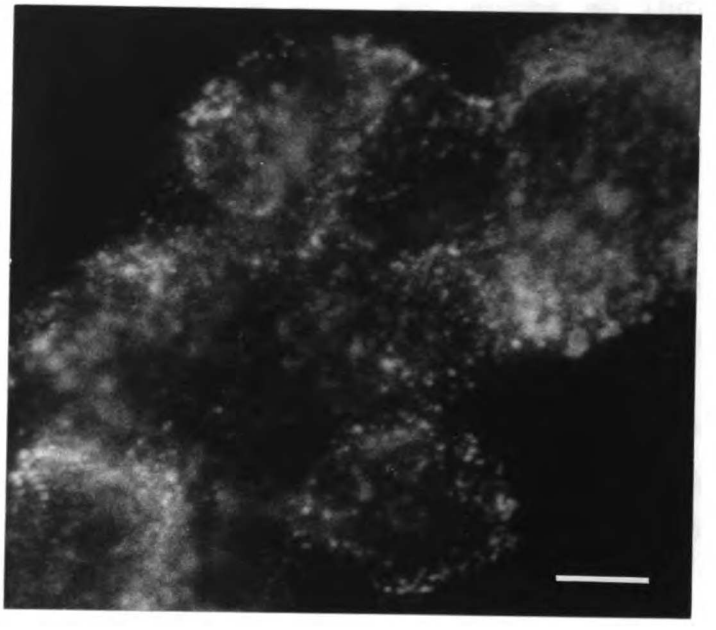


Figure 9. Fluorescence micrograph showing staining pattern obtained using antibody B7E5 on a cytospin preparation of bovine tracheal gland cells. The fluorescence is punctate, suggesting localization within granules. Bars = 10 μm. From Finkbeiner, W.E., Nadel, J.A., and Basbaum, C.B.: Establishment and characterization of a cell line derived from bovine tracheal glands, In Vitro Cell. Devel. Biol. 22:561-567, 1986. Reproduced with permission.

ß-Adrenergic regulation of bovine tracheal gland serous cell secretion

Physiology. Sensitivity of cultured cells to adrenergic and cholinergic receptor activation was evaluated by measuring the capacity of specific receptor agonists and antagonist to modulate release of ^{35}S-labeled glycoconjugates. Cell viability at the termination of experiments with each drug or drug combination was greater than 90% and did not differ between control and drug-treated samples. Neither bethanechol (10^{-5} M) nor phenylephrine (10^{-5} M) caused an increase in the relative secretory rate. The *l*-isomers of isoproterenol, epinephrine and norepinephrine (all at 10^{-4} M) each increased the rate of ^{35}S release from bovine tracheal gland cells by factors of 1.7 ± 0.1, 1.7 ± 0.1, 1.6 ± 0.1 (mean ± SE, n = 7-9), respectively (Figure 10). However, at 1 x 10^{-6} M, these adrenergic agonists stimulated ^{35}S release from BTG cells with significantly different ($P < 0.05$) potencies; the rank order of potency was *l*-isoproterenol > *l*-epinephrine > *l*-norepinephrine. Stimulation by *l*-isoproterenol (10^{-5} M) was inhibited to a greater degree by *l*-propranolol than by *d*-propranolol indicating the stereoselectivity expected for receptor-mediated responses (Figure 11).

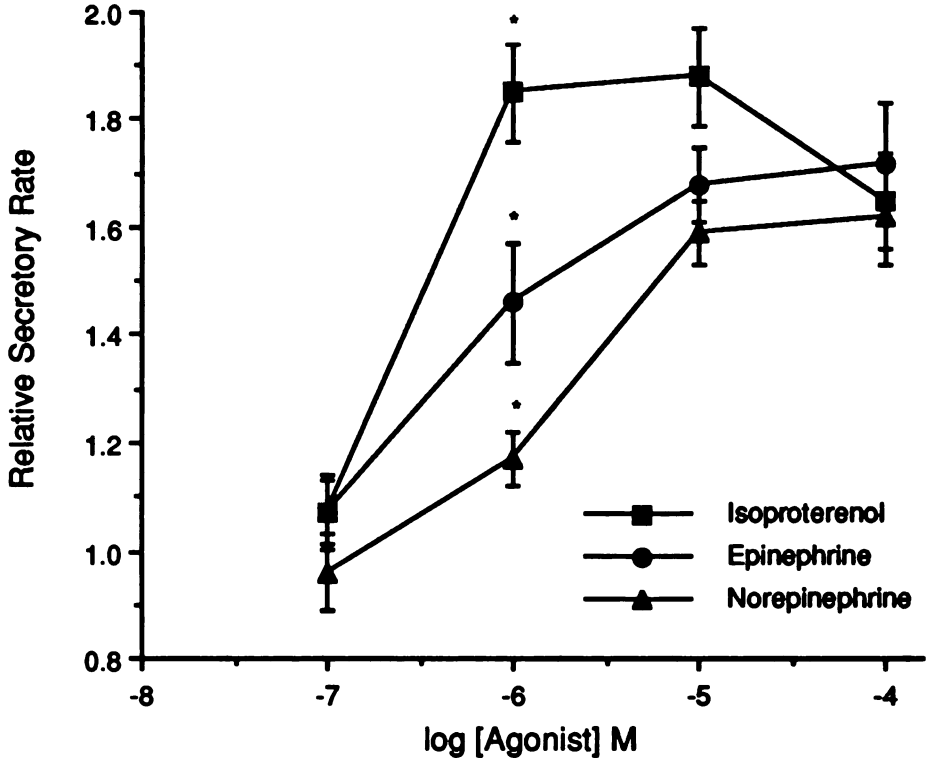


Figure 10. Response of bovine tracheal gland cells to adrenergic agonists *l*-isoproterenol, *l*-epinerphrine and *l*-norepinephrine. Each data point is mean ± SE ($n \geq 7$). (*) = values that are significantly different ($P < 0.05$) from each other. From Madison J.M., Basbaum, C.B., Brown, J.K. and Finkbeiner, W.E.: Characterization of ß-adrenergic receptors in cultured bovine tracheal gland cells. Am. J. Physiol. 256 (Cell Physiol. 25):C310-C314, 1989. Reproduced with permission.

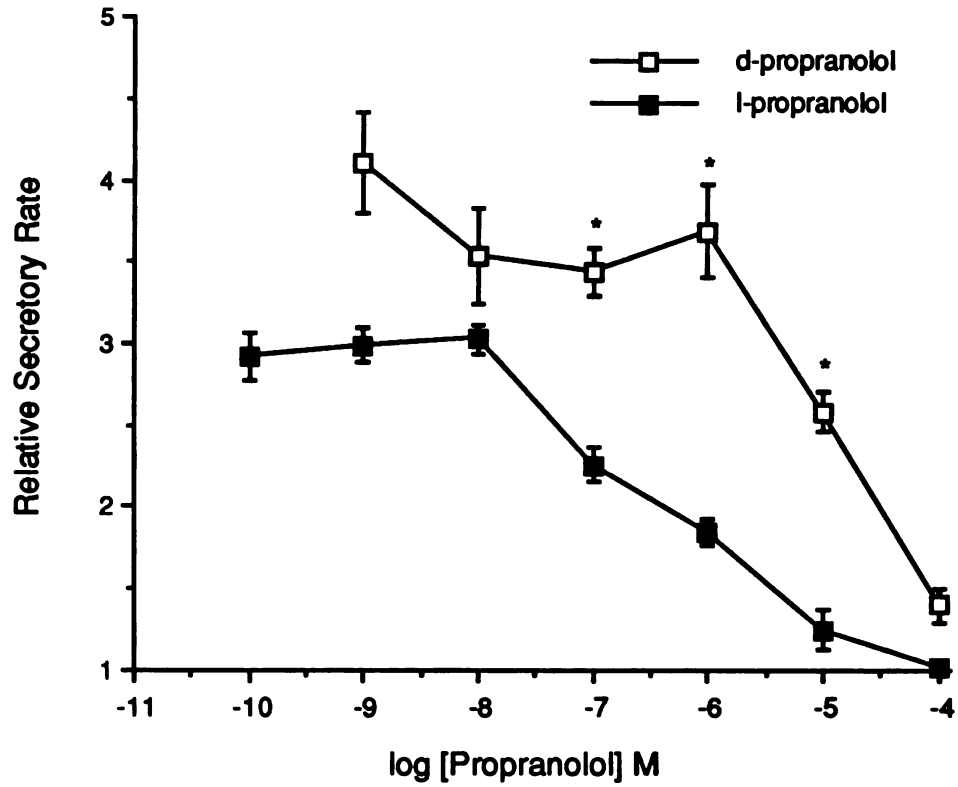


Figure 11. Effects of propranolol on secretory response to isoproterenol (10^{-5} M). Isoproterenol-induced secretion of ^{35}S-labeled molecules from bovine tracheal gland cells was quantified in the presence of either *l*-propranolol (10^{-10}-10^{-4} M) or *d*-propranolol (10^{-9}-10^{-4} M). Values are mean ± SE for n ≥ 5 separate determinations except in the case of *l*-propranolol (10^{-4} M) in which one determination was made. (*) = values that are significantly different ($P < 0.05$) from those observed at the same concentration of *l*-propranolol. Modified from Madison J.M., Basbaum, C.B., Brown, J.K. and Finkbeiner, W.E.: Characterization of ß-adrenergic receptors in cultured bovine tracheal gland cells. Am. J. Physiol. 256 (Cell Physiol. 25):C310-C314, 1989. Reproduced with permission.

Based on previous work with other exocrine cells [48], it seemed likely that ß-adrenergic receptor dependent secretion in bovine tracheal gland cells would be mediated by alterations in the level of intracellular cAMP. If so, then the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine

should augment the secretory response and analogues of cAMP should mimic the secretory response in the absence of ß-adrenergic receptor activation. Figure 12 shows the dependence of relative secretory rate on the concentration of *d,l*-isoproterenol in the presence and absence of 1-methyl-3-isobutylxanthine. The relative secretory rate was augmented by 1-methyl-3-isobutylxanthine as reflected by a leftward shift of the dose-response curve. This finding indirectly suggested that stimulus-secretion coupling in bovine tracheal gland cells was mediated by cAMP.

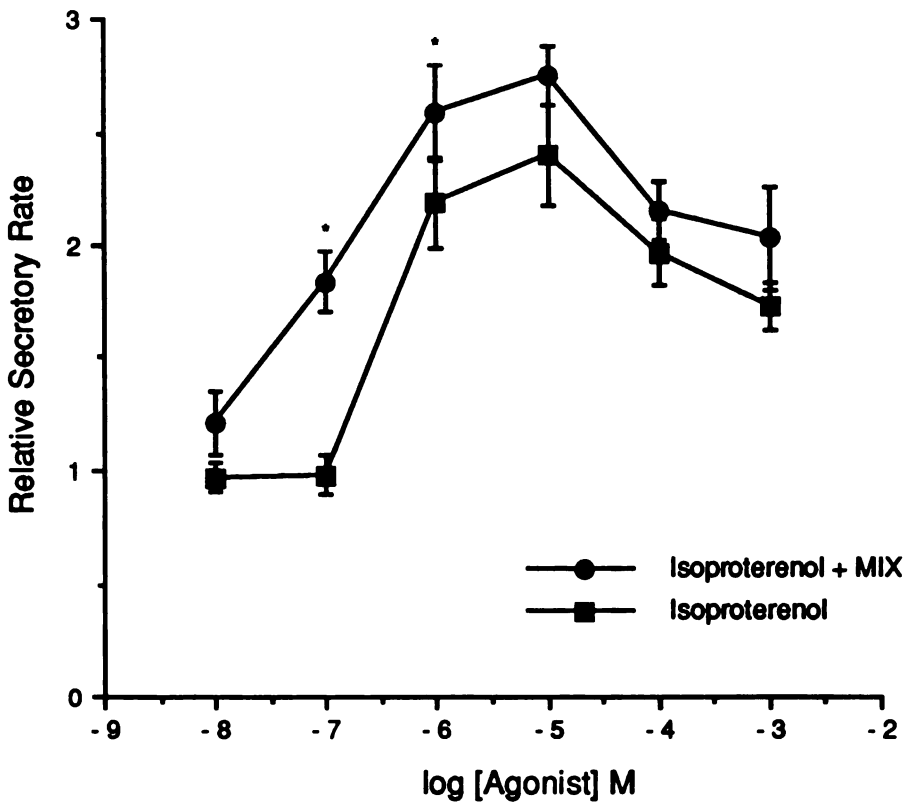


Figure 12. Effect of a phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (MIX) on the relative rate of isoproterenol-stimulated secretion of ^{35}S-labeled molecules (relative secretory rate) from bovine tracheal gland cells. Values are mean ± SE (n = 5). (*) = values that are significantly different ($P < 0.05$) from those observed at the same concentration of isoproterenol.

To determine whether alterations in the level of intracellular cAMP are triggered by ß-adrenergic receptor stimulation, I measured intracellular levels of cAMP at various time points after stimulation with *d,l*-isoproterenol (Figure 13). Intracellular cAMP levels rose tenfold within 1 minute then decreased slowly. Thirty minutes after stimulation, cAMP levels still remained at least fourfold greater than the untreated control levels. Furthermore, when the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (10^{-5} M) was present in the medium, the cAMP rise was increased at all but the 30 second time point. The cAMP response was completely inhibited by the addition of *d,l*-propranolol (10^{-5} M). These studies confirmed directly that cAMP was elevated after ß-adrenergic stimulation.

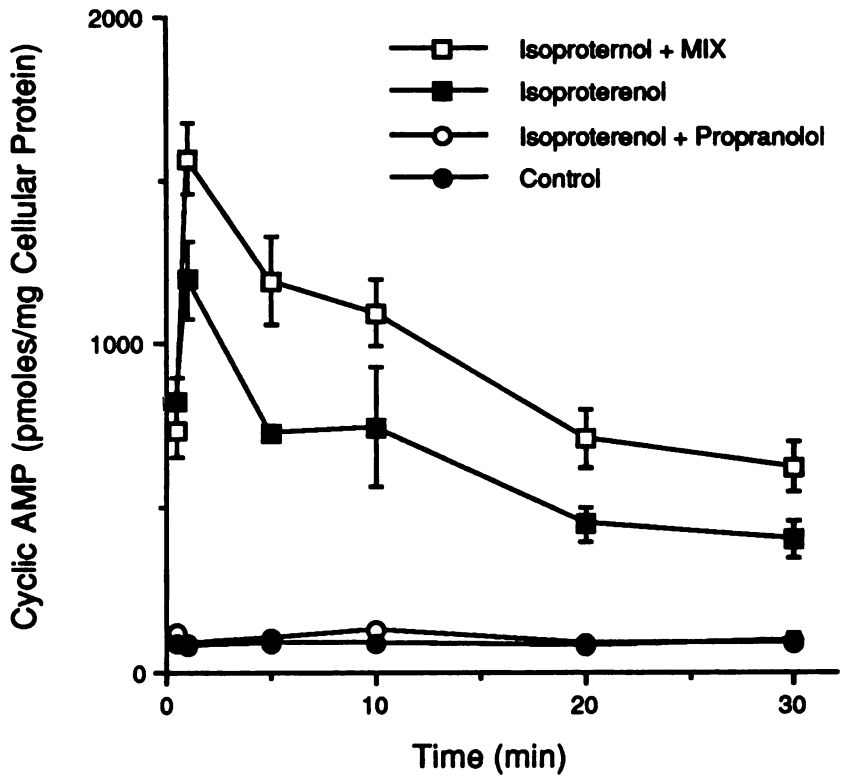


Figure 13. Time courses of changes of intracellular cAMP in bovine tracheal gland cells. Values after addition of drugs are means ± SE ($n \geq 7$). Control values are means ± SE of ($n \geq 5$).

As shown in Figure 14, cAMP production by bovine tracheal gland cells was maximal between 10^{-4} to 10^{-6} M *d,l*-isoproterenol. These doses corresponded to those evoking the maximal secretion.

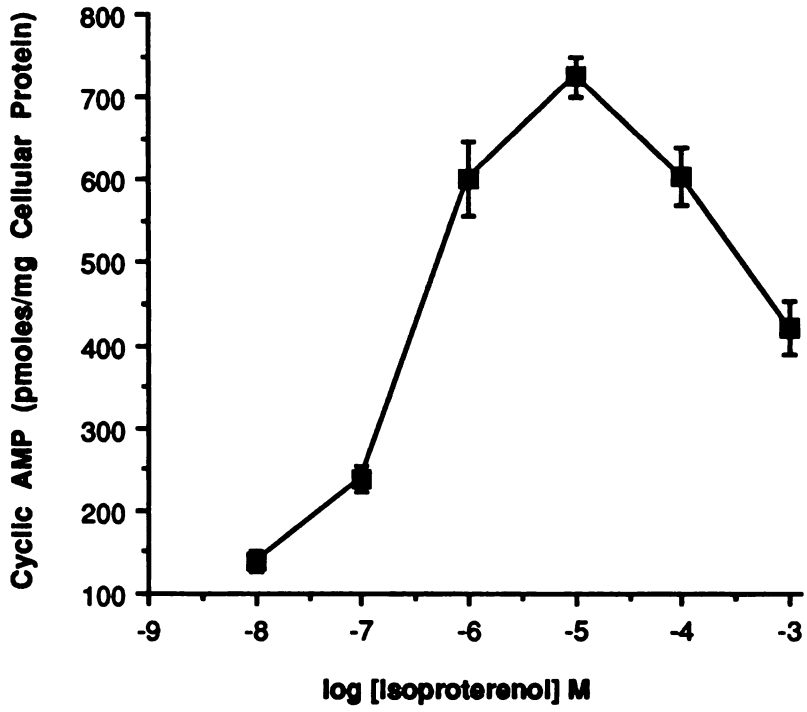


Figure 14. Dose-dependent effect of *d,l*-isoproterenol on generation of intracellular cAMP in bovine tracheal gland cells. Cultures were exposed to isoproterenol for 5 min. Values are means ± SE ($n \geq 7$).

To directly test the hypothesis that cAMP is a second messenger mediating secretion in bovine tracheal gland cells, I measured the relative secretory rate of cells incubated with cAMP analogues. Figure 15 shows the relative secretory rate observed after administration of 8-bromo-cAMP (10^{-3} M) and Bt_2-cAMP (10^{-3} M) in the presence and absence of 1-methyl-3-isobutylxanthine (10^{-5} M). In the absence of the phosphodi-

esterase inhibitor 1-methyl-3-isobutylxanthine, cAMP analogues did not increase the release of ^{35}S-labeled molecules. However, when 1-methyl-3-isobutylxanthine was present, these agents significantly ($p < 0.05$) increased the release of the ^{35}S-labeled molecules. These studies confirmed that elevations of intracellular cAMP are capable of causing secretion of ^{35}S-labeled molecules from bovine tracheal gland serous cells. Therefore, cAMP is a second messenger in the stimulus-secretion coupling events in these cells.

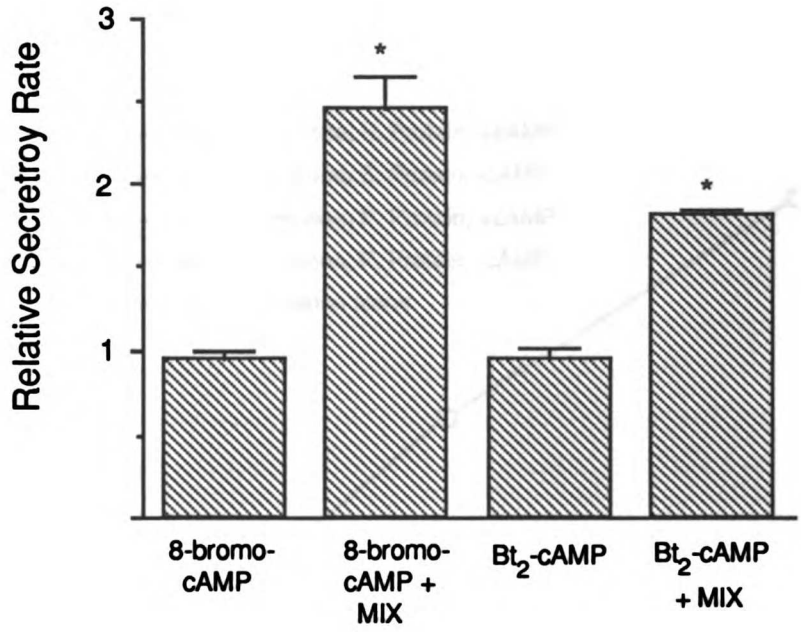


Figure 15. Effect of cAMP analogues 8-bromo-cAMP (10^{-3} M) and Bt_2-cAMP (10^{-3} M) in the absence or the presence of 1-methyl-3-isobutylxanthine (MIX; 10^{-5} M) on the relative rate of secretion of ^{35}S-labeled molecules (relative secretory rate) from bovine tracheal gland cells secretion. A relative secretory rate of 1 indicates basal secretory levels. Values are means ± SE (n = 5). (*) = values that are significantly ($P < 0.05$) different from control.

Cyclic AMP-dependent kinase activity. Cyclic AMP elicits its biological responses by kinase-dependent alterations in phosphorylation levels of one or more cellular proteins. I have performed experiments to identify the kinases and phosphoproteins involved in the cAMP-dependent serous cell secretory pathway. Cyclic AMP-dependent kinase activity, as measured by cAMP-stimulated transfer of $[^{32}P]PO_4$ from γ-$[^{32}P]$-ATP to the exogenous phosphate acceptor histone was linear during the 8-min period in which it was measured (Figure 16). Most of the cAMP-dependent kinase activity was found in the soluble protein fraction.

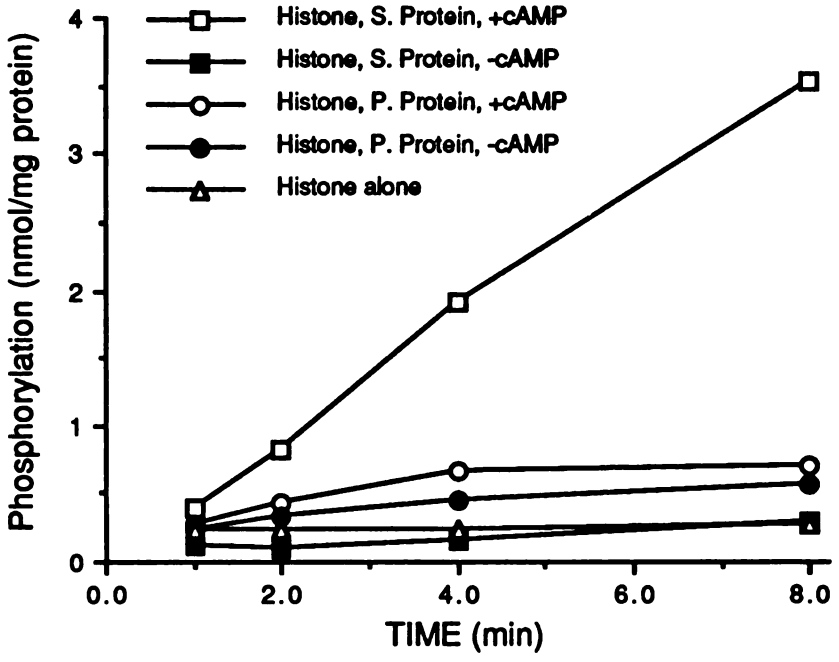


Figure 16. Phosphorylation of histone by cell extracts from bovine tracheal gland cells. Values are from one representative experiment and are normalized to the amount of soluble or particulate protein present. When histone was added alone, values were normalized to the concentration added to the other samples.

Cyclic AMP-dependent phosphorylation of endogenous proteins. As shown in Figures 17 and 18 and as expected from the data on histone phosphorylation, cAMP-dependent kinase activity towards endogenous acceptors was greater in the cytosolic than the membrane fraction. In the membrane fraction only two proteins, approximate molecular weights 49 kDa and 55 kDa showed cAMP-dependent phosphorylation (Figure 18). The effects of cAMP in the cytosolic fraction were more complex. Analysis of densitometric tracings revealed essentially three patterns of phosphorylation (Figure 19). In the first pattern (Figure 19 A,G) phosphorylation of a 200 kDa protein and a 100 kDa protein was independent of cAMP. More commonly (Figure 19 B,C,E,F,H,L,M,N,O), cAMP increased protein phosphorylation. In one case phosphorylation of a 25 kDa protein (Figure 19 N) reached near maximal levels by 30 s. The third pattern was seen with a 49 kDa protein and a 55 kDa protein (Figure 19 J,K). After incubation with cAMP, these proteins, which were maximally phosphorylated within 30 s under control conditions, showed a decreased rate of phosphorylation in the presence of cAMP, but reached the same equilibrium levels of phosphorylation as the control.

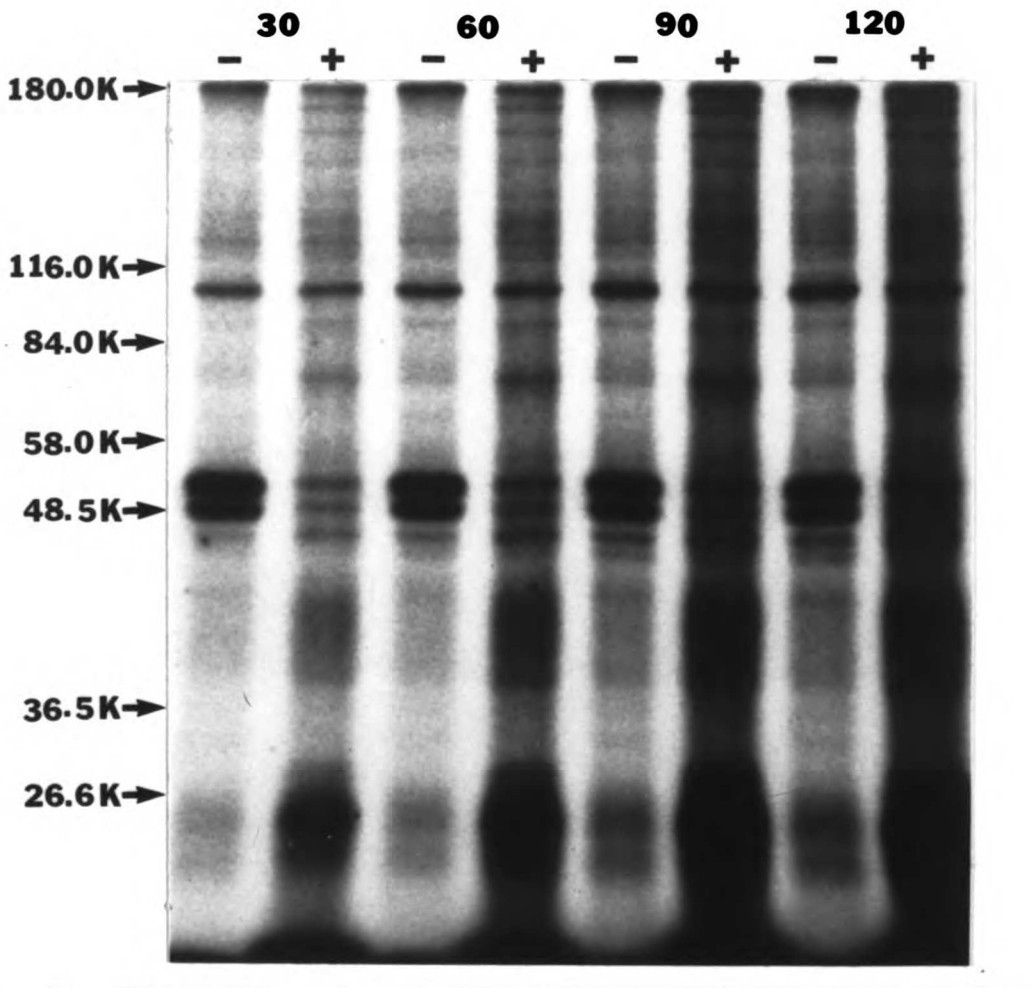


Figure 17. Autoradiogram of phosphorylated proteins from bovine tracheal gland serous cells. Soluble cell extracts were incubated in the presence γ-[^{32}P]-ATP, in the absence (-) and presence (+) of cAMP (10^{6} M). The reaction was terminated at 30, 60, 90 and 120 s.

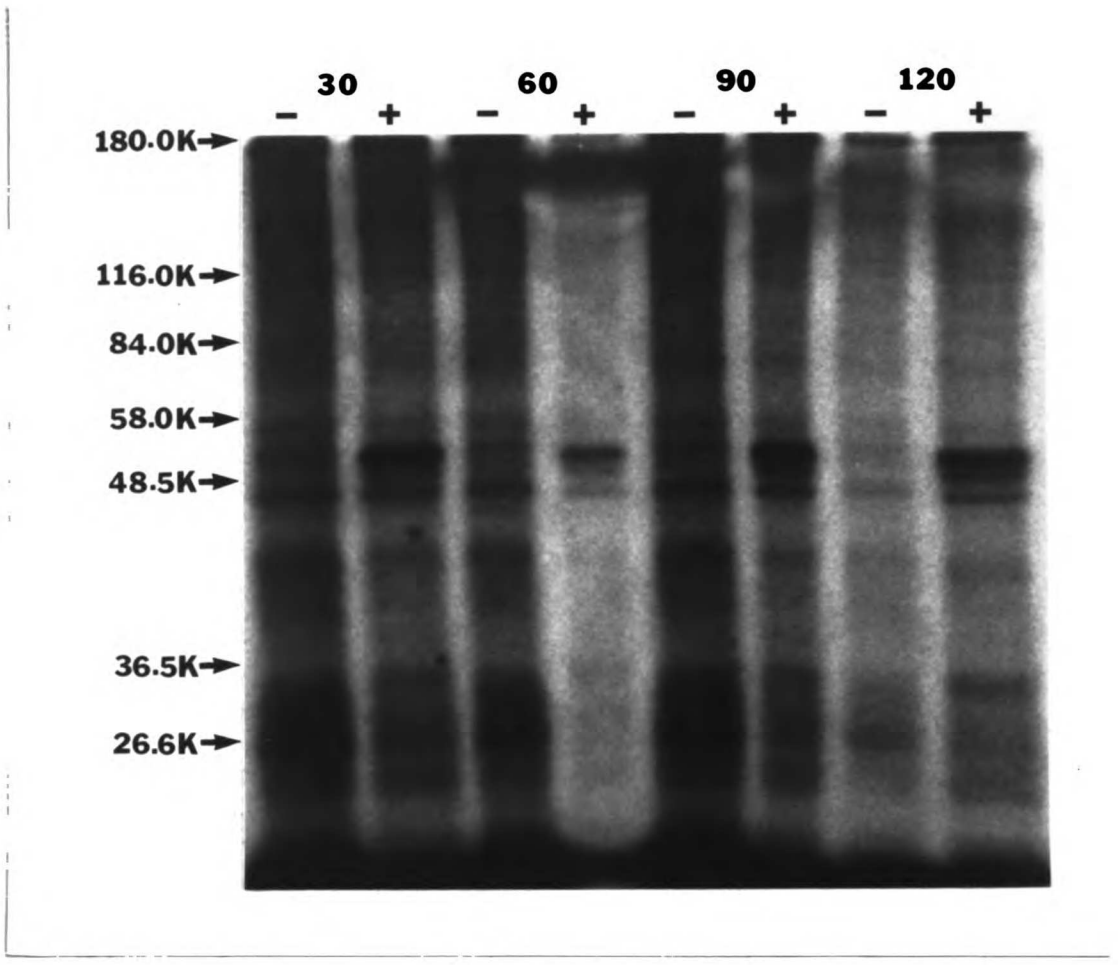


Figure 18. Autoradiogram of phosphorylated proteins from bovine tracheal gland serous cells. Particulate cell extracts were incubated in the presence γ-[^{32}P]-ATP, in the absence (-) and presence (+) of cAMP (10^{6} M). The reaction was terminated at 30, 60, 90 and 120 s.

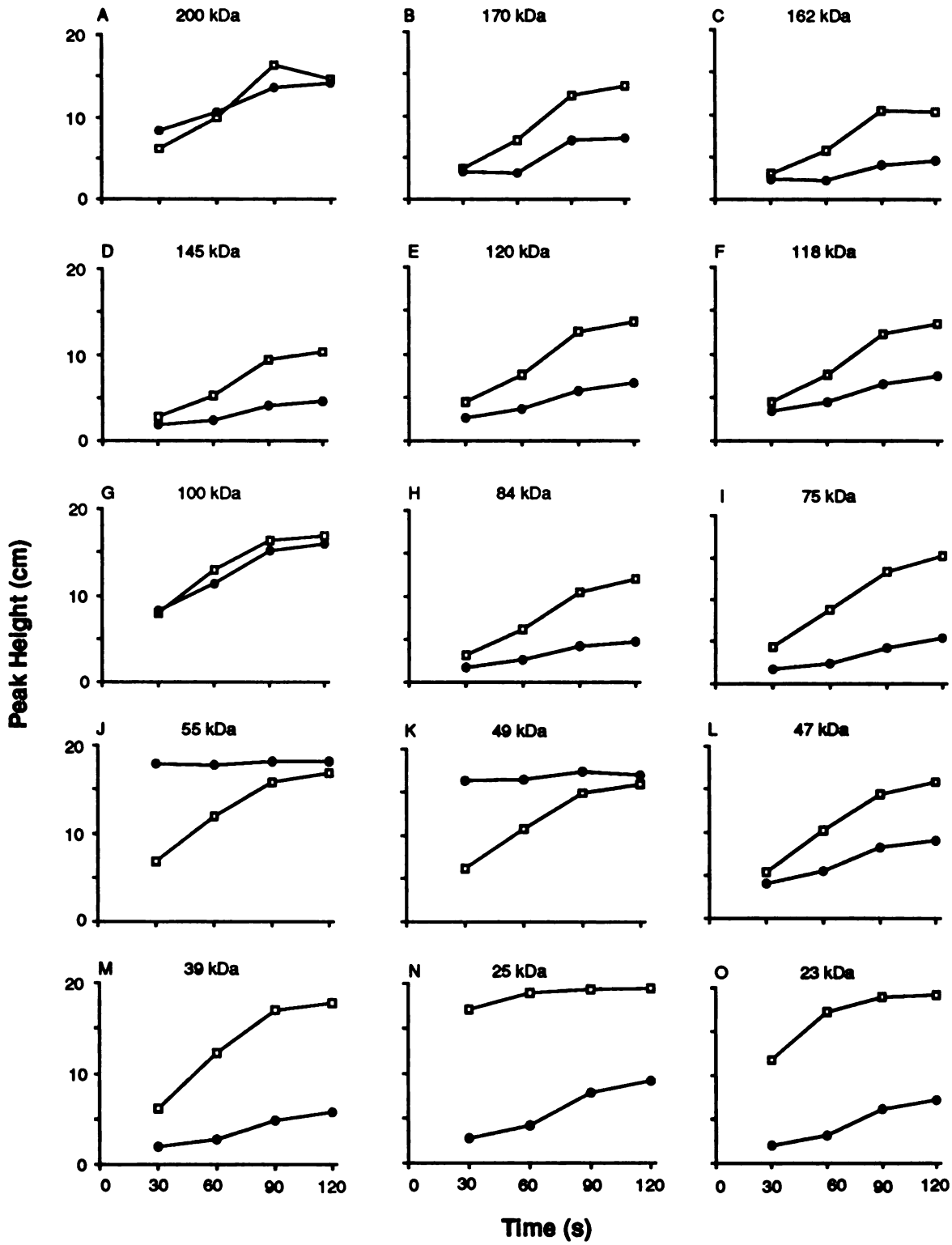


Figure 19. Comparison of peak heights obtained from autoradiographic density profiles of endogenous phosphorylation of soluble proteins with (—□—) and without (—●—) cAMP.

Physiology and biochemistry of bovine tracheal gland organ cultures

Radiolabeling of glycoconjugates, pulse chase, and pharmacologic stimulation. To verify that cultures of bovine tracheal gland serous cells represented a model that could be compared with the serous cells of intact tracheal glands, I studied the secretory responses of bovine tracheal submucosal tissues maintained in organ culture. The effects of cholinergic, α-adrenergic, and ß-adrenergic stimulation on bovine tracheal submucosal glands secretion maintained in organ culture revealed that each was effective in stimulating the release of ^{35}S-labeled molecules (Table 3). The rank order of potency was isoproterenol > bethanechol > phenylephrine.

Table 3. Effect of cholinergic, α-adrenergic and ß-adrenergic drugs on bovine tracheal submucosal gland organ cultures.

DRUG	RELATIVE SECRETORY RATE
Bethanechol	1.45 ± 0.09*
l-Phenylephrine	1.24 ± 0.01*
d,l-Isoproterenol	1.64 ± 0.17†

Values are means ± SE of 3 determinations. Submucosal gland tissues were exposed to each agonist at 10^{-5} M for 30 min. *P<0.05. †P<0.1.

Gel filtration chromatography and enzymatic digestion of void volume. These studies were performed to verify that material secreted by bovine tracheal serous cell cultures was similar to material secreted by tracheal submucosal glands. When nondialyzable molecular components from isoproterenol-stimulated BTG secretions were analyzed by gel filtration chromatography on Sepharose CL-4B, significant amounts of ra-

diolabeled material were present in the void volume (Figure 20). Chondroitinase ABC lyase significantly degraded the void volume material (Figure 21).

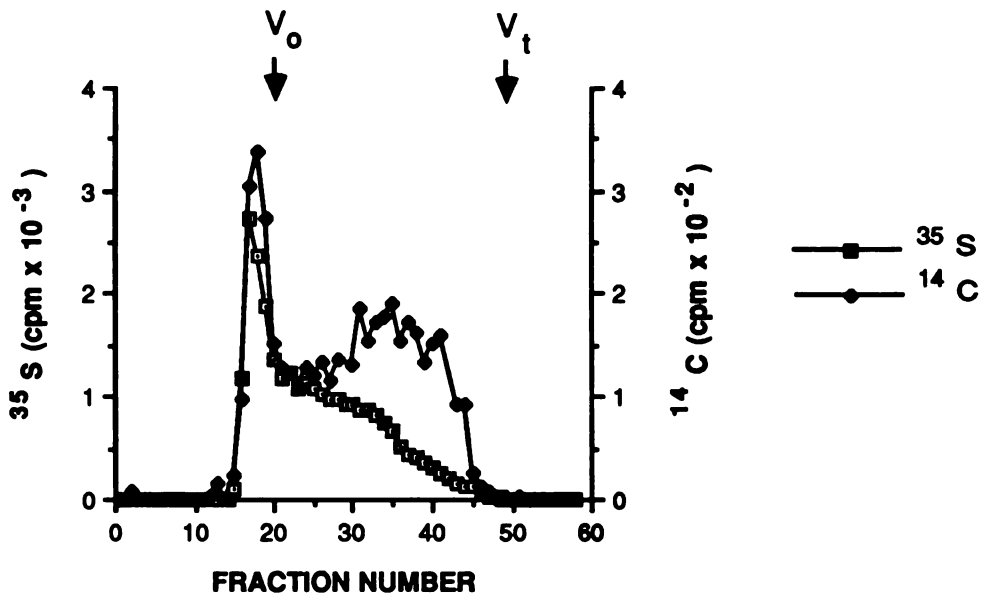


Figure 20. Sepharose CL-4B chromatography of medium from bovine tracheal organ cultures. Submucosal tissues were incubated with $Na_2[^{35}S]O_4$ or [1-^{14}C]-glucosamine and stimulated with *d,l*-isoproterenol as described in Material and Methods. Collected medium was dialyzed, lyopholized and applied to a Sepharose CL-4B column (1.5 x 60 cm). Fractions (1.4 ml) were collected and radioactivity counted. Fractions 15-21 were pooled and used for further characterization. V_o and V_t refer to the elution positions of Blue Dextran 2000 and [1-^{14}C]-glucosamine, respectively.

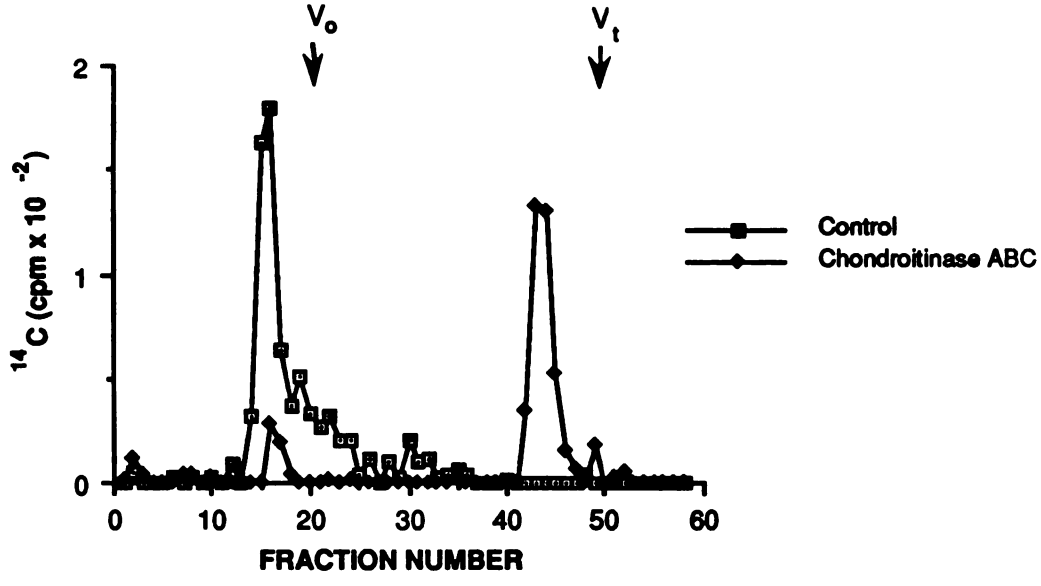


Figure 21. Sepharose CL-4B chromatography of [1-^{14}C]-glucosamine-labeled V_o obtained from pooled fractions. Material was fractionated as in Figure 20 before and after treatment with chondroitinase ABC lyase. V_o and V_t refer to the elution positions of Blue Dextran 2000 and [1-^{14}C]-glucosamine, respectively. From Paul A., Picard, J., Mergey, M., Veissiere, D., Finkbeiner, W.E. and Basbaum, C.B.: Glycoconjugates secreted by bovine tracheal serous cells in culture. Arch. Biochem. Biophys. 260:75-84, 1988. Reproduced with permission.

MONOCLONAL ANTIBODIES DIRECTED AGAINST HUMAN AIRWAY SECRETIONS

Preparation of monoclonal antibodies

Fusions and screening of antibodies. Two fusions yielded 401 wells containing hybridoma clones (35% growth). Supernatant from these wells was screened on paraformaldehyde-fixed frozen sections of normal human trachea. One hundred and fifty supernatants stained human tracheal submucosal gland cells alone or in combination with the surface goblet cells. Twenty-nine hybridomas were selected on the basis of their staining pattern for expansion and further characterization.

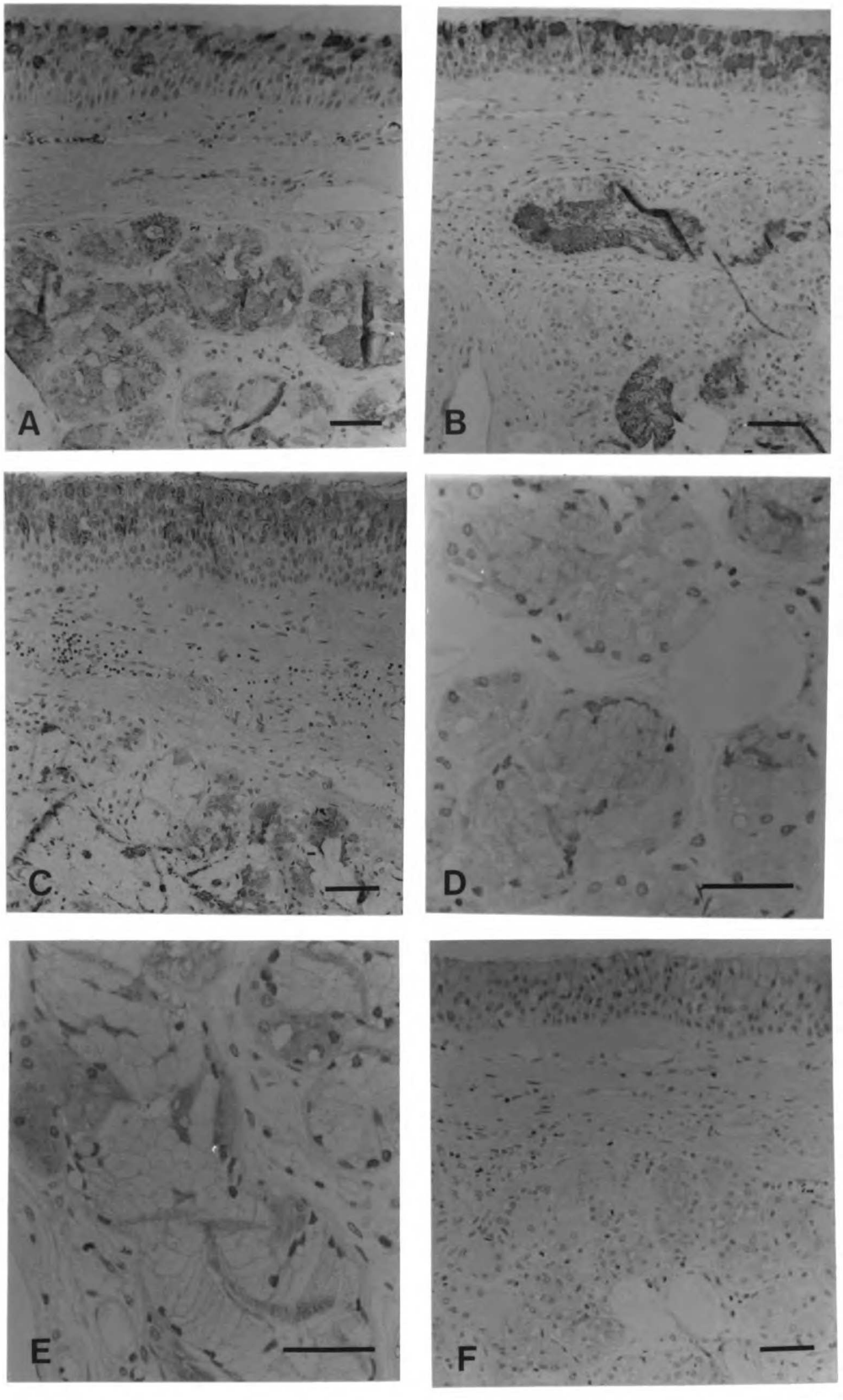
Localization of antigens in plastic-embedded tissue

To better localize the cellular antigens, immunocytochemistry was performed on plastic-embedded tracheal tissues. Mild aldehyde fixation and cold processing of the tissues preserved antigens and thinly sectioning the tissues allowed their precise localization (Figure 22). All antibodies selected by immunofluorescence on frozen sections also stained the plastic-embedded tissue. Four antibodies stained epithelial goblet cells and submucosal serous and mucous cells, twelve antibodies stained goblet and mucous cells, four stained goblet and serous cells, four stained only mucous cells and five only serous cells. In some cases, antibodies recognized only subpopulations of a given cell type. Localization of antibody staining is summarized in Table 4.

Determination of immunoglobulin subclasses

Antibodies of the IgG_1, IgG_{2b}, IgG_3, and IgM subclasses were obtained. A summary of immunoglobulin classification is presented in Table 4.

Figure 22. (next page) Types of staining of human trachea by antibodies directed against secretory cell antigens. **A**-Antibody A1D3; goblet, serous, and mucous cells are stained. **B**-Antibody A2F3; goblet and mucous cells are stained. **C**-Antibody B8C3; goblet and serous cells are stained. **D**-Antibody B8C4; only mucous cells are stained. **E**-Antibody B7E5; only serous cells are stained. **F**-Omitted primary negative control. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.


A
B
C
D
E
F

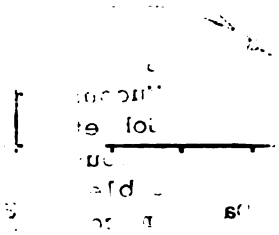


TABLE 4. IMMUNOCYTOCHEMICAL LOCALIZATION AND IMMUNOGLOBULIN CLASSIFICATION OF MONOCLONAL ANTIBODIES. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.

Antibody	Immunocytochemical Localization	Ig Subclass
A1D3	Goblet, mucous & serous cells	M
A1D7	Mucous cells	G_1
A1E11	Goblet & mucous cells	G_1
A1F11	Mucous	G_1
A1F11	Goblet cells, subpopulations of mucous & serous cells	G_1
A2E7	Serous cells	G_1
A2F3	Goblet & mucous cells	G_1
A3B7	Serous cells	G_{2b}
A3G11	Goblet cells, subpopulation of mucous cells	M
A6D8	Goblet cells, subpopulation of mucous cells	G_3
A8E4	Mucous cells	G_1
A10F5	Serous cells	G_1
A10G5	Goblet cells, subpopulations of mucous & serous cells	G_1
B1D8	Serous cells	G_1
B2B8	Goblet, mucous & serous cells	G_1
B3D11	Goblet cells, subpopulation of mucous cells	G_1
B3E8	Goblet & mucous cells	M
B3F2	Goblet & mucous cells	M
B3F10	Serous cells, subpopulation of goblet cells	G_1
B4C11	Goblet & mucous cells	M
B4F7	Goblet, mucous & serous cells	M
B5D5	Goblet & mucous cells	M
B5D7	Goblet cells, subpopulation of mucous cells	G_1
B5E9	Mucous cells	G_1
B6E8	Goblet & mucous cells	M
B6G6	Goblet cells, subpopulation of mucous cells	G_1
B7E5	Serous cells	M
B8C3	Goblet & serous cells	G_1
B8E10	Goblet cells, serous cells, subpopulation of mucous cells	G_1

Periodate sensitivity of antigens

Fluorescence was abolished or considerably reduced for three antibodies after mild periodate treatment, three antibodies after moderate treatment, and nine antibodies after harsh treatment. Fourteen antibodies were unaffected by periodate treatment. For antibodies whose reactivity was reduced by mild treatment, more severe treatment resulted in complete loss of reactivity.

Gel filtration chromatography

Fractionation of endotracheal secretions from a cystic fibrosis patient on Sepharose 4B yielded three peaks which were pooled as shown in Figure 23. The apparent molecular weights of fractions I, II, and III were > 1,000,000, 300,000, and <100,000 daltons, respectively. These fractions were used in the solid phase radioimmunoassay and for SDS-PAGE with immunoblotting to determine the apparent molecular weights of the antigens recognized by the monoclonal antibodies.

Solid-phase radioimmunoassay

The reactivity of each antibody with molecular weight fractions is shown in Table 5. Sixteen antibodies had highest or exclusive affinity for material eluting in fraction I (void volume), one for material in fraction II, and one for material in fraction III. One antibody showed equal affinity for fractions I and II. Ten antibodies that reacted immunocytochemically with tracheal tissue sections failed to react with fractionated airway secretions in the solid phase RIA.

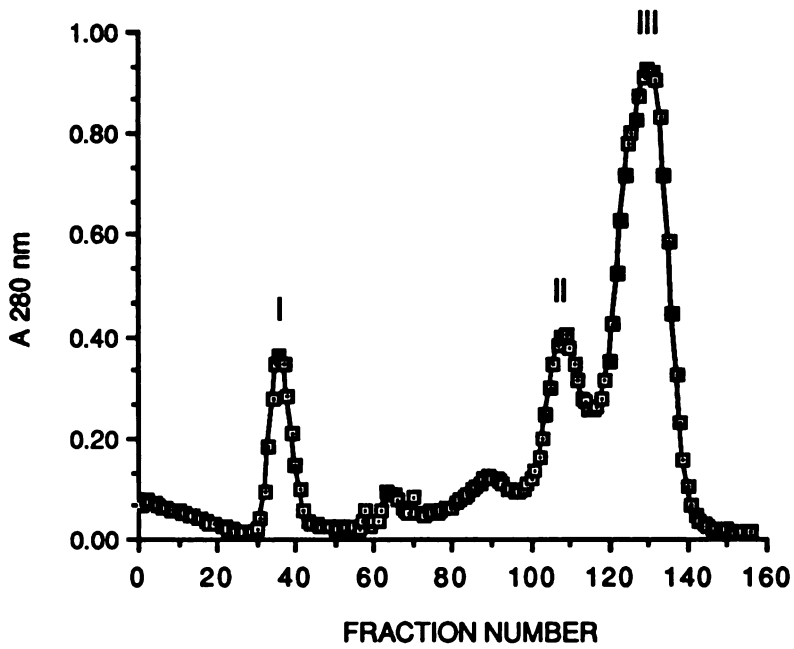


Figure 23. Gel filtration chromatography of endotracheal secretions collected from a patient with cystic fibrosis. Dialyzed secretions were applied to a column of Sepharose 4B in sodium acetate (0.1 M, pH 5.5) containing 1 mM EDTA, 0.02% sodium azide, and 0.5 mM phenylmethylsulfonyl fluoride and collected and monitored as described in Materials and Methods. Pooled Fractions I, II, and III are indicated. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.

TABLE 5. Reactivity of antibodies (expressed as percent of negative control ± SE) with pooled Sepharose 4B fractions. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.

ANTIBODY	FRACTION I	FRACTION II	FRACTION III
A1D3	306 ± 2*	118 ± 9	119 ± 18
A1D7	137 ± 5	145 ± 5	123 ± 6
A1E11	300 ± 11*	132 ± 2	149 ± 28
A1F8	241 ± 8	318 ± 43*	166 ± 38
A1F11	133 ± 6	121 ± 5	143 ± 17
A2E7	310 ± 22*	125 ± 14	116 ± 22
A2F3	392 ± 42*	130 ± 21	148 ± 30
A3B7	192 ± 6	250 ± 17	282 ± 15*
A3G11	279 ± 29	150 ± 18	160 ± 15
A6D8	363 ± 19*	305 ± 22*	273 ± 16
A8E4	321 ± 12*	203 ± 12	184 ± 4
A10F5	150 ± 20	166 ± 21	131 ± 26
A10G5	455 ± 18*	194 ± 12	161 ± 2
B1D8	126 ± 13	196 ± 32	212 ± 40
B2B8	168 ± 1	154 ± 13	170 ± 18
B3D11	336 ± 26*	114 ± 20	122 ± 19
B3E8	331 ± 2*	182 ± 12	177 ± 22
B3F2	182 ± 18	151 ± 18	164 ± 12
B3F10	309 ± 15*	104 ± 18	115 ± 15
B4C11	311 ± 14*	162 ± 19	164 ± 21
B4F7	322 ± 8*	206 ± 23	177 ± 27
B5D5	290 ± 11	149 ± 3	127 ± 9
B5D7	415 ± 7*	200 ± 3	177 ± 2
B5E9	294 ± 4*	115 ± 6	143 ± 8
B6E8	311 ± 30*	149 ± 16	158 ± 20
B6G6	493 ± 18*	165 ± 15	165 ± 13
B7E5	139 ± 19	184 ± 15	238 ± 14
B8C3	401 ± 17*	165 ± 15	133 ± 17
B8E10	184 ± 12	152 ± 19	184 ± 23

*Significant at 95%

SDS-PAGE and immunoblots

By immunoblotting, 18 of 29 antibodies reacted with components of Sepharose 4B fraction peaks further separated by SDS-PAGE. Representative immunoblots are shown in Figure 24 and approximate molecular weights of the antigens are shown in Table 6. Whereas some antibodies reacted with single discrete bands (e.g. A1D3, B3D11, A2F3 and A1E11, lanes A,B,C, and G, respectively, Figure 24), others (e.g. B6G6, lane E, Figure 24) reacted with single broad zones, or multiple discrete bands and/or zones (e.g. B6E8, B4C11, B1D8 and B7E5, lanes E,F, H and I, respectively, Figure 24). Two antibodies, B3D11 and A1E11, (lanes B and G, respectively, Figure 24) may be identical since they both react with a band having a molecular weight of approximately 377 kDa on immunoblots, stain goblet and mucous cells, recognize an antigen that is insensitive to periodate oxidation, and elute in the same Sepharose 4B fraction peak. Another pair, B1D8 and B7E5 (lanes H and I, respectively, Figure 24) show similar immunoblot staining of Fraction II material, immunocytochemical localization, and Sepharose 4B fraction peak affinity. However, after SDS-PAGE of the Fraction 3 material, A1D8, but not B7E5, reacts with a 14 kDa molecular weight band (Table 6).

TABLE 6. MOLECULAR WEIGHTS OF ANTIGENS ESTIMATED BY IMMUNOBLOT ANALYSIS. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.

Antibody	Molecular Weights (k Daltons)
A1D3	394
A1E11	377
A2E7	119-131
A2F3	401
A3G11	222-435
A6D8	422;313-414;263-305;116-145;71;61;42;31;17-21
B1D8	86-114;77;66;14
B3D11	377
B3E8	353-435
B3F10	323-350
B4C11	367-435;310-342;174-200
B4F7	435; 92;64-74;39-45;17-32
B5D5	385-435;303-350;222-282;178-211;128-151;93-103
B5D7	376-435;296-342;222-269;67-81
B6E8	362-414;308-355;129-146
B6G6	372-414
B7E5	86-114;77;66
B8C3	356

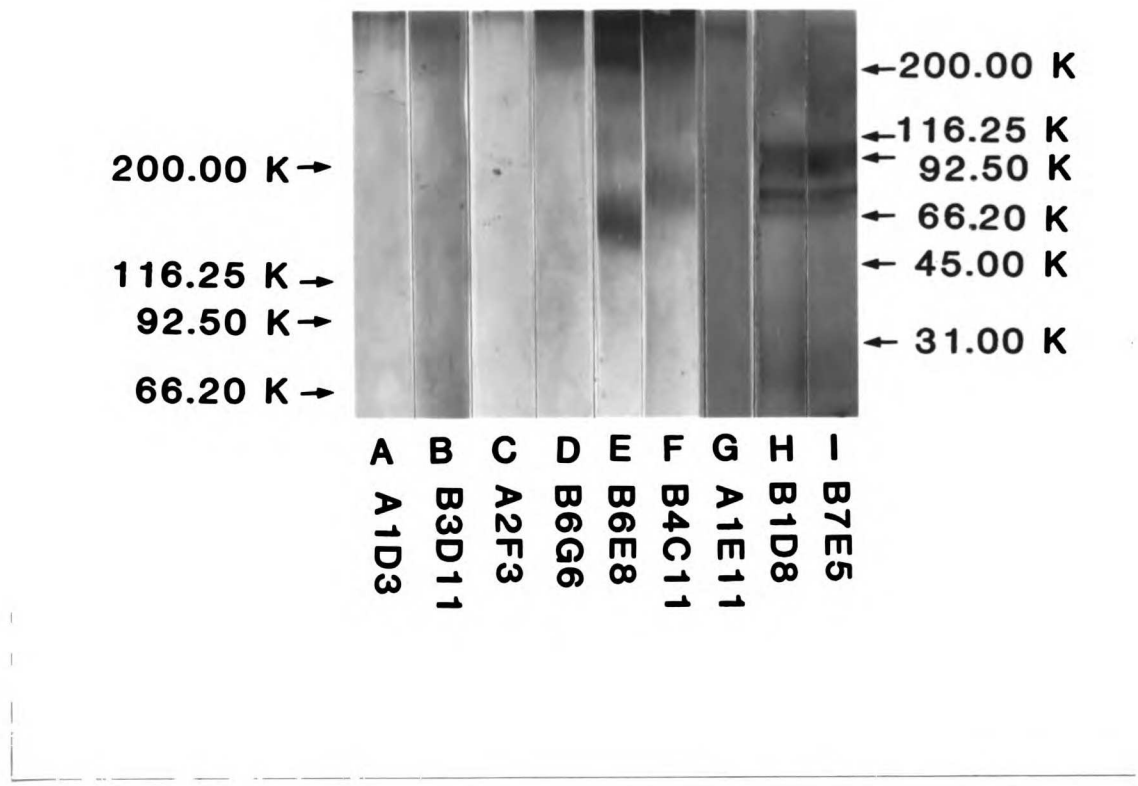


Figure 24. Representative SDS-PAGE and immunoblot analysis of Sepharose 4B material. Lanes A-G, Fraction I material. Lanes H-I, Fraction II material. Left, molecular weight standards for lanes A-G; right, molecular weight standards for lanes H-I. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.

DISCUSSION

In the past, studies of tracheobronchial secretion have been limited by an inability to effectively study the individual secretory cells, their products and the cellular mechanisms that control them. The objective of this dissertation project was the development of experimental approaches for the cell-specific analysis of tracheobronchial secretory cells on a biochemical and molecular level. This objective was realized using two cell biologic techniques, cell culture and monoclonal antibody production, and by applying these techniques in the analysis of tracheobronchial cells and their secretions. In this dissertation are described the first cultured cell line established from tracheal submucosal gland cells (bovine) and the first panel of monoclonal antibodies raised against human tracheobronchial secretions.

BOVINE TRACHEAL GLAND SEROUS CELLS IN CULTURE

The cells of the tracheal glands derive from plaques of the surface epithelium that invaginate into the submucosa before birth [239, 245]. These cells differentiate to form the acinar and duct cells that remain in continuity with the tracheal lumen throughout life, and from whose secretions the mucus blanket covering the epithelial surface is maintained and renewed continuously.

The gland cells are morphologically distinct from the goblet cells of the surface epithelium and have different responses to secretagogues [223]. Although cultures of cells from the tracheal surface epithelium were developed and studied earlier [59, 100, 120, 174, 260, 263] it is not possible to extend findings obtained from surface epithelial cultures (even when they contain mucin-secreting subpopulations) to questions involving secretion from the submucosal glands. For

this reason we sought to develop a preparation of functional tracheal gland cells.

One of the main problems encountered during the development of tracheal submucosal gland cell cultures was the separation of tracheal submucosal gland acinar cells from the dense connective tissue framework that surrounds them. Culp et al. [71] approached this problem by using an animal species (cat) which had a relatively large ratio of gland cell tissue to connective tissue. However, their methods required a large number of experimental animals in order to obtain a sufficient yield of cells. To overcome these problems, we attempted to increase yield by isolating cells from larger species, i.e. sheep and dog. Although it was possible to obtain higher cell yields from the tracheas of larger animals, viability was compromised considerably due to the difficulty in isolating cells from the denser connective tissue present in the tracheas of larger animals [83]. Despite the problems associated with the use of larger animals, I was eventually able to develop a method for obtaining consistent preparations of relatively high yield and satisfactory viability from bovine tracheas. The method relied on the use of an enzyme solution containing collagenase, hyaluronidase, and elastase; avoided mechanical methods of cell isolation; and extended the period of digestion to as long as 24 h. Although these preparations contained viable serous and mucous cells, they were contaminated by red and white blood cells, connective tissue cells and nonviable cells of glandular and mesenchymal origin. In this state, the preparation was not suitable for functional studies. In order to obtain a pure preparation of tracheal gland cells, a method for placing these cells in culture was developed.

When placed in culture, epithelial cells often lose characteristics of differentiation or even fail to proliferate [32, 213]. The cultured tracheal serous cells described

in this study maintain many important characteristics of differentiation and proliferate in culture. Even with serially propagation these properties remain intact. Although the proliferative capacity of these cells is unusual for epithelial cells which have not undergone transformation [214], there is no evidence to suggest that the bovine tracheal gland serous cells have transformed spontaneously. Transformed cells generally exhibit genetic abnormalities such as changes in ploidy and increases in the frequency of individual chromosomes [31], express anchorage independence as exemplified by growth of cells detached from the substrate [156], show disorientated growth and foci of rounded cells within the regular monolayer due to loss of contact inhibition [1, 89], demonstrate lower serum and growth factor dependence than their normal counterparts [238, 240], exhibit tumorigenesis [98], are able to grow in semi-solid media [88] and are immortal [90, 241]. The preservation of the normal diploid bovine chromosome number reveals that these cells are indeed derived from bovine tissue and does not suggest that spontaneous transformation of these cells is responsible for their adaptability to the culture environment. Further evidence against transformation of these cells is their inability to sustain growth in the absence of serum or specific growth factors and the maintenance of contact inhibition. Additionally, the cells are not immortal since they undergo progressive senescence and eventual crisis after approximately 35-40 passages as an increasing number of nondividing, enlarged, nonproliferating cells appear in the cultures. We have not investigated whether or not a transformed clone of cells will arise spontaneously from a senescent culture.

In situ, the bovine tracheal submucosal glands contain mainly serous cells, and in culture, the cells isolated from these glands retain many differentiated features of serous cells. By electron microscopy, the cultures are found to be composed of epithelial cells having junctional complexes.

The secretory granules are intensely osmiophilic and clearly demarcated from adjacent granules. Histochemically, the granules are found to contain large quantities of polysaccharides, a feature consistent with their identification as serous cells [137]. Polysaccharide-containing granules (as revealed by AB/PAS staining) were present throughout the cytoplasm of virtually all the cultured cells. Although the cells also contain lysosomes, their distribution (as revealed by acid phosphatase staining) was confined to the perinuclear region. This is consistent with observations made on cultures of hamster tracheal epithelial cells [174]. The presence of serous cell-specific antigen (detected by monoclonal antibody B7E5) in cultured bovine tracheal gland cells corroborates the localization of the AB/PAS-positive material to specific secretory granules. Furthermore, these cells release another serous cell marker, lysozyme, into the culture medium [112].

The cells in culture retain not only the morphological, histochemical and biochemical features of tracheal gland serous cells, but also the ability to take up radiolabeled sulfate, incorporate it into macromolecules, and release it upon physiological stimulation. The cells respond physiologically by secreting glycoconjugates after stimulation with ß-adrenergic agonists, a response described previously for tracheobronchial organ cultures from a variety of species including human [192, 193], cat [92, 147, 196, 250], rat [109] and ferret [36, 94, 95]. Evidence that the cell response to ß-adrenergic stimulation is physiological and not due to toxicity is provided by vital dye exclusion and demonstration that ß-adrenergic stimulation is blocked stereoselectively at the level of the receptor by the adrenergic receptor antagonist propranolol. Additionally, evidence is provided that bovine tracheal submucosal tissues, maintained as organ cultures, also secrete radiolabeled glycoconjugates after ß-adrenergic stimulation.

Despite the identification of a secretory response of bovine tracheal submucosal organ cultures to muscarinic and α-adrenergic agonists, the cultured serous cells failed to display a similar response. We cannot rule out that α-adrenergic and muscarinic receptors observed in organ cultures were due to mucous cells. However, due to the small numbers of mucous cells in bovine submucosal glands and the finding that the majority of high molecular weight glycoconjugates released in organ culture were sensitive to chondroitin sulfate, it is more likely that the α-adrenergic and muscarinic receptors were more likely due to activation of serous cell receptors, and that these receptors are not retained in culture. The loss of muscarinic responsiveness may relate to the culture conditions rather than the isolation procedure since Culp and Marin [70] demonstrated functionally intact muscarinic receptors in enzymatically disaggregated cat tracheal submucosal gland cells. Preliminary studies suggest that the loss of muscarinic responsiveness is due to absence of the muscarinic receptor rather than to post-receptor modifications (Madison, J.M. and Basbaum, C.B., University of California, San Francisco, personal communication).

Culture-induced alteration of receptor function has been reported by many other investigators. For example, when placed in primary culture, hepatocytes shift from α- to ß-adrenergic control of glycogenolysis [182]. This shift is associated with an increase in $ß_2$-adrenergic receptor density [179, 217] and a decrease in α_1-adrenergic receptor density [110, 217]. The mechanism of this change is unknown [201]; however it has been suggested that it may involve the suppression of a guanine nucleotide regulatory protein [110].

In addition to ß-adrenergic agonists, other agents effective in stimulating airway mucus secretion stimulate the release of ^{35}S-labeled molecules from the cultured serous

cells. These include several cyclooxygenase products including PGE_1, PGE_2, PGA_1, and PGD_2 [230], histamine [229], bradykinin (Sommerhoff, unpublished observations), and the mast cell protease, chymase [229]. Although its role the stimulus-secretion coupling mechanisms of bovine tracheal gland serous cells has not been explored, changes in intracellular calcium ion concentration are likely to be important since addition of the calcium ionophore A23187 results in the release of significant amounts of radiolabel from the cultured cells [84].

The rank order of adrenergic agonist potency (isoproterenol > epinephrine > norepinephrine) suggests that the secretory response to isoproterenol is mediated by the $ß_2$-adrenergic receptor subtype [138]. Had the response been mediated by $ß_1$-adrenergic receptors, epinephrine and norepinephrine would be equipotent in stimulating secretion. Additionally, studies of BTG cells by Madison et al. [157] which included radioligand binding experiments using a ^{125}[iodo]cyanopindolol to identify ß-adrenergic receptors and a specific $ß_2$-adrenergic receptor antagonist, ICI 118.551, confirm a $ß_2$-adrenergic receptor density of greater than 1000 per cell.

Light microscopic, autoradiographic studies show that both $ß_1$- and $ß_2$-adrenergic receptors are present on human submucosal glands with $ß_2$-adrenergic receptors predominating by a 9:1 ratio [52]. However, at the light microscopic level it is impossible to determine whether there are differences in the distribution of these receptor subtypes between serous and mucous gland cells. The results of Madison et al. [157] suggest that cultured bovine submucosal gland serous cells contain only $ß_2$-adrenergic receptors. Together these studies suggest that $ß_1$-adrenergic receptors may be restricted to mucous gland cells, but before this can be confirmed

differences due to species and effects of cell culture on expression of ß-adrenergic receptor subtype must be addressed.

Studies of exocrine organs such as the parotid gland have demonstrated that following activation of the ß-adrenergic receptor, adenylate cyclase converts adenosine triphosphate into cAMP, and cAMP serves as a second messenger in the regulation of secretion and other cellular processes [48]. Tracheobronchial submucosal gland secretion is likely to be regulated in a similar fashion. Consistent with this hypothesis, ß-adrenergic activation increases cAMP levels in cat tracheal explants [147] and increases reactivity of the submucosal gland serous cells in dog and cat tracheal tissue sections stained with antisera against cAMP [140]. However, in neither of these studies could intracellular cAMP levels be quantified.

Bovine tracheal gland serous cell cultures provide a convenient system for quantitatively analyzing the effects of ß-adrenergic receptor stimulation on intracellular cAMP concentration. Within 1 min, cultured bovine serous cells exposed to isoproterenol increase intracellular cAMP levels tenfold. The levels decline slowly over 30 min and remain at least fourfold greater than control levels. The rapid rise in the concentration of intracellular cAMP and its slow return to control levels is similar to that seen in the rat parotid gland [75], rat submandibular gland [197], and rat hepatocytes [110]. However, it is different from that reported for primary cultures of human tracheal epithelial cells in which isoproterenol stimulation causes a rapid increase of intracellular cAMP levels, and attainment of new steady state levels after 20 min [257, 259]. In cat tracheal explants containing both epithelial and glandular tissue, isoproterenol causes cAMP levels to rise to maximal levels within 10 minutes and then remain stable for 90 minutes

[147]. This time course of cAMP elevation during ß-adrenergic receptor stimulation is more consistent with those reported for cultured tracheal surface epithelial cells [257, 259] than for tracheal gland serous cells.

The absolute intracellular concentration of cAMP of bovine tracheal serous cells 1 min after stimulation with isoproterenol is similar to that reported for primary cultures of epithelial cells from human trachea [257], but approximately fivefold greater than that reported for isolated rat submandibular cells [197]. However, in the presence of 1-methyl-3-isobutylxanthine, isoproterenol stimulation produces nearly identical intracellular cAMP levels in cultured tracheal serous cells and rat submandibular cells. This suggests that the submandibular gland cells may have greater phosphodiesterase activity thereby attenuating the rise in intracellular cAMP after ß-adrenergic stimulation.

Indirect evidence for the involvement of cAMP in bovine serous cell secretion is revealed by the findings that the dose-response relationship for isoproterenol-stimulated generation of cAMP correlates with the dose-response relationship for isoproternol-stimulated secretion of ^{35}S-labeled molecules. Further, the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine potentiates both isoproterenol-evoked secretion of ^{35}S-labeled molecules and the production of intracellular cAMP. Finally, the ß-adrenergic receptor antagonist propranolol completely blocks both effects and cAMP analogues can mimic the effects of ß-adrenergic activation. The finding that the cAMP analogues are effective only in the presence of 1-methyl-3-isobutylxanthine suggests that these analogues are rapidly metabolized by bovine tracheal serous cell phosphodiesterase.

Cyclic AMP is thought to exert most of its effects by activation of cAMP-dependent protein kinase [237]. Cyclic

AMP-dependent kinase consists of two distinct subunits, a regulatory subunit (R) which is a receptor for cAMP, and a 40.9 kDa catalytic (C) subunit. In the absence of cAMP the enzyme exists as an inactive tetramer, R_2C_2. However, in the presence of cAMP, the R subunit binds to the cyclic nucleotide causing a decrease in the affinity of the R subunit for the C subunit. This results in the formation of an $R_2(cAMP)_4$ dimer and 2 free catalytically active C subunits [26, 44]. These catalytic subunits then catalyze the phosphorylation of a diverse group of substrate protein molecules, the functional consequences of which are still largely unknown [221].

Reversible changes in protein phosphorylation due to kinases and phosphatases are thought to play a major role in the regulation of secretion in exocrine cells. However, the mechanism by which phosphorylation and dephosphorylation of substrate proteins controls secretion is not known. However, by evaluating the kinetics of phosphorylation and phosphate turnover with respect to phosphate turnover, it may be possible to focus investigations on phophoproteins which are involved in exocytosis [198]. Purification and characterization of such proteins should provide important insights into stimulus-secretion coupling mechanisms.

In cultured bovine tracheal gland serous cells most of the cAMP-dependent protein phosphorylation was found in the soluble fraction cell extracts. Although activity in the soluble fraction was greater than that of the particulate fraction this may not reflect the physiological distribution of the cAMP-dependent kinase since the association of the enzyme may be altered by conditions in which the assay was run [69]. The levels of phosphorylation present in bovine tracheal gland serous cells were nearly identical to the levels reported in human tracheal surface epithelial cells maintained in primary culture [16].

Analysis of cAMP-dependent endogenous phosphorylation by SDS-PAGE of soluble (cytosolic) and particulate (membrane) fractions of bovine tracheal serous cells reveals that the particulate fraction contains only two proteins, approximate molecular weights 49 kDa and 55 kDa, showing cAMP-dependent phosphorylation. On the other hand, the soluble fraction contains at least thirteen proteins demonstrating a cAMP-dependent change in phosphorylation. Most of these showed increased phosphorylation with cAMP, most dramatically a 25 kDa protein was near maximal levels after just 30 seconds. This protein may be analogous to a rapidly phosphorylated 26 kDa protein that has been identified in rat parotid and submandibular glands [113, 198]. Because it is rapidly phosphorylated and then dephosphorylated in response to ß-adrenergic stimulation followed by ß-adrenergic blockade, it has been proposed to play a regulatory role in exocytosis [198, 199].

Two proteins found in the soluble cell fraction (49 kDa and 55 kDa) showed slower phosphorylation in the presence of cAMP. At this time it is not known whether the 49 kDa and 55 kDa found in the soluble cell fraction are analogous to the proteins of the same apparent molecular weight that are present in the particulate cell fraction. It is also not known whether these proteins which are found in both the soluble and particulate fractions correspond to the 49 kDa and 54 kDa proteins described by Liedtke et al. [147]. The molecular weights of these proteins are consistent with their identity as the regulatory subunits of cAMP-dependent kinase. There are two major isozymes of cAMP-dependent kinase, type I and type II [181]. On SDS-PAGE, the regulatory subunits of the type I and type II isozymes of bovine heart cAMP-dependent kinase have estimated molecular weights of 49 kDa and 55-58 kDa, respectively [26, 212]. However, molecular weights of the regulatory subunits as determined by SDS-PAGE may differ

with respect to species and tissue [26]. The type II, but not the type I regulatory subunit is autophosphorylated [80]. Although in most cells and tissues the enzyme is predominantly in the soluble fraction, membrane bound cAMP-dependent kinase activity has been identified [149].

Thus, one hypothesis to explain the findings in bovine tracheal submucosal gland serous cell is that these cells contain both the type I and type II isozymes of cAMP-dependent kinase and these isozymes reside in both cytosol (soluble fraction) and membranes (particulate fraction). Further, our findings suggest that in the cytosol, these proteins exist predominantly as phosphorylated species in the cytosol and dephosphorylated species in the membrane. This may be due to either autophosphorylation or *in vitro* phosphorylation effects perhaps via activation of cGMP-dependent kinase [26, 78, 218]. In any case, an effect of cAMP in modulating the phosphorylation state of these 49 kDa and 55 kDa proteins is seen. Further studies including labeling of intact, living cultures with $^{32}PO_4$ and 8-azido [^{32}P]cAMP are required to clarify these findings.

Phosphorylation of specific tracheal gland cell proteins may be involved not only in the phasic phenomena following neurotransmitter receptor activation, but also in regulation of gene expression. The growth and differentiation of submucosal gland cells have been shown to be altered by ß-adrenergic agonists. For example, chronic administration of isoproterenol to pigs has been shown to increase submucosal gland cell mass and shift glycoconjugate content to the acidic glycoproteins [23]. The molecular mechanisms of alterations in the growth and differentiation of tracheobronchial glands due to chronic ß-adrenergic stimulation and presumably protein kinase activation have not been explained. Regulation of transacting factors that control gene transcription have been

shown to occur via changes in protein phosphorylation; and such mechanisms could operate to control the growth and differentiation of tracheobronchial gland cells. Mechanisms by which cAMP-dependent kinase subunits translocate to the nucleus or are activated there could act on DNA elements to regulate genes [221]. Continuous cultures of bovine tracheal gland serous cells could be an excellent system in which to examine the role of protein phosphorylation in the control of gene expression.

The biochemical nature of glycoconjugates secreted by cultured bovine tracheal serous cells has been investigated [186]. Cells were allowed to incorporate [1-^{14}C]glucosamine before stimulation of secretion with isoproterenol. Analysis of nondialyzable molecular components by gel filtration on Sepharose CL-4B, revealed that significant amounts of radiolabeled material are present in the void volume, indicating the presence of high-molecular weight (>10^6 daltons) glycoconjugates. Characterization of these glcoconjugates by chemical analysis and specific enzymatic digestions with chondroitinase ABC and AC revealed that the major glycoconjugates secreted by these cells are chondroitin sulfate proteoglycans, hyaluronic acid, and asparagine-linked glycoproteins. In addition, small amounts of chondroitinase-resistant material representing either O-linked glycoproteins such as mucins or O-linked oligosaccharides carried on proteoglycans are also present. Studies of secretions harvested from organ cultures of bovine trachea reveal an identical sensitivity of the high molecular weight material to chondroitinase ABC confirming the similarity of secretions regardless of whether they are derived from short-term organ cultures of bovine tracheal submucosa or continuous cultures of bovine tracheal gland serous cells.

The identification of glycosaminoglycans as the major carbohydrate secreted by serous cells represents a major new

finding in the biochemistry of trachobronchial mucus. Glycosaminoglycans had been identified in respiratory tract secretions collected *in vivo* by aspiration and *in vitro* by recovery from organ cultures [62, 91, 115]. However, the cellular source of these materials was previously unknown. Recently, Bhaskar et al. [29, 30] found that the glycoconjugates present in human respiratory tract secretions changes from one dominated primarily by glycosaminoglycans in health, to one dominated by mucin in disease. This may signify that serous cells are the major contributors to "healthy" tracheobronchial secretions and that the contribution of mucous cells is recruited by injury or disease.

That glycosaminoglycans are released by stimulus-secretion coupling mechanisms in bovine tracheal gland serous cells indirectly shows that these molecules are stored and released from serous cell granules. Direct evidence for this is provided by immunocytochemical localization of chondroitin sulfate proteoglycan in secretory granules of bovine tracheal gland cells *in vivo* [81] and in culture [229].

Glycosaminoglycans have been identified in secretory granules of other protein-secreting exocrine cells such as the pituitary [266], adrenal medulla [159] and pancreas [202, 236]. Perhaps, in serous cells as in other exocrine cells, glycosaminoglycans act to bind and concentrate cationic secretory proteins within macromolecular aggregates, thereby helping to exclude water form the granule and reducing the need for energy dependent pumping mechanisms [236]

MONOCLONAL ANTIBODIES AGAINST HUMAN AIRWAY SECRETIONS

At least some components of tracheobronchial secretions appear to be very immunogenic based on the high proportion of hybridoma clones secreting antibodies directed against tracheobronchial cells. Table 7 summarizes the characteristics of these monoclonal antibodies. Since the secretory cells of the trachea derive embryologically from common precursor cells, it is not surprising that many antibodies stain more than one cell type [239, 245]. Although antigens exclusive to serous and mucous cells were detected, antigens exclusive to goblet cells were not. Monoclonal antibodies recognizing goblet cells also recognized mucous cells, serous cells or both. These results agree with those of St. George et al. [234] but differ from those of an earlier study in which monoclonal antibodies directed against sheep tracheal secretion were found to include goblet cell-specific antibodies [21].

Although some antibodies described in this study are strictly cell-specific, they do not necessarily stain all individual cells of a given type. In agreement with St. George et al. [234], and as expected from histochemical results from Spicer et al. [232], some antibodies identified subpopulations of airway secretory cell types. The significance of subpopulations is unknown at this time. Although staining differences may reflect true phenotypic differences, they might also simply reflect metabolic or maturational differences among members of a single cell type.

Based on the results of periodate oxidation experiments, the epitopes recognized by these monoclonal antibodies appear to include both carbohydrate and peptide determinants. Although periodate oxidation carried out at high temperature and long duration can potentially oxidize polypeptides the

TABLE 7. SUMMARY OF MONOCLONAL ANTIBODIES: LOCALIZATION, SENSITIVITY AND APPARENT MOLECULAR WEIGHT OF ANTIGENS. From Finkbeiner, W.E. and Basbaum, C.B.: Monoclonal antibodies directed against human airway secretions. Am. J. Pathol. 131:290-297, 1988. Reproduced with permission.

Antibody	Localization*	Periodate Sensitivity†	Column Fraction††
A1D3	G,M,S	NS	I
A1F11	G,M,S	NS	-
A10G	G,M,S	harsh	I,II
B2B8	G,M,S	harsh	-
B4F7	G,M,S	NS	I,II,III
B8E10	G,M,S	NS	-
A1E11	G,M	NS	I
A2F3	G,M	moderate	I
A3G11	G,M	mild	I
A6D8	G,M	harsh	I,II
B3D11	G,M	NS	I
B3E8	G,M	moderate	I
B3F2	G,M	mild	-
B4C11	G,M	moderate	I
B5D5	G,M	NS	I
B5D7	G,M	NS	I
B6E8	G,M	NS	I
B6G6	G,M	mild	I
B3F10	G,S	harsh	I
B8C3	G,S	harsh	I
A1F8	M	NS	II
A8E4	M	harsh	I
B1D7	M	harsh	-
B5E9	M	NS	I
A2E7	S	harsh	I
A3B7	S	harsh	III
A10F5	S	NS	-
B1D8	S	NS	II,III
B7E5	S	NS	II

*G, goblet cells; M, mucous cells; S, serous cells.

†Mild, 10 mM $NaIO_4$, 10 min, 4°C; moderate, 50 mM $NaIO_4$, 1 h, 4°C; harsh, 100 mM $NaIO_4$, 12 h, room temperature. NS, not sensitive.

††Column Fraction results from RIA and SDS-PAGE immunoblots.

oxidation conditions used in this study are probably mild enough to affect only carbohydrate structures. Van Lenten and Ashwell [251] have shown that periodate oxidation corresponding to our mild treatment selectively cleaves between 7-8-9-hydroxyl positions of terminal unsubstituted sialic acid residues. Periodate oxidation corresponding to the moderate treatment cleaves terminal, nonreducing sugars, as well as some glycosidically linked hexoses containing adjacent, unsubstituted hydroxyls. Harsh periodate oxidation probably destroys carbohydrate residues having unsubstituted hydroxyls incapable of forming inter-residue hemiacetals [86]. Harsh oxidation may also affect polypeptides [57, 131]. The fact that three of the antibodies (A3G11, B3F2 and B6G6) were sensitive to mild periodate oxidation suggests the involvement of carbohydrate epitopes containing terminal sialic acid residues.

Solid phase RIA and immunoblot analysis demonstrated that a majority (65%) of antibodies recognized determinants in Fraction I (Table 7). It is likely that the molecules carrying these epitopes are of relatively high molecular weight. Although elution in Fraction I under non-dissociating conditions does not in itself constitute evidence for the large size of the antigenic molecules, the limited migration of these molecules on SDS gels strongly indicates they are of large size. High molecular weight antigens were identified in all three cell types. However, "low" molecular weight antigens (Fractions II and III) were most frequently associated with serous cells, either exclusively or in combination with goblet and mucous cells. These "low" molecular weight antigens were resistant to mild and moderate periodate treatments. Additional studies involving enzymatic digestion are needed to determine whether these antigens are associated with protein or carbohydrate moieties.

Ten antibodies that reacted immunocytochemically with tracheal tissue sections failed to react with statistical significance in the solid phase RIA. This phenomenon was also observed for a panel of monoclonal antibodies directed against colonic mucin, and it was suggested that this may reflect unfavorable conformations of antigens after binding to the beads [194]. Although this could explain the failure of most antibodies to react it may not account for all of the negative reactions observed. Antibodies A3G11 and B3F2 were both sensitive to mild periodate oxidation suggesting a terminal carbohydrate epitope, unlikely to be affected by conformational changes resulting from binding to the beads. It should also be noted that in the solid phase RIA, antibodies A3G11 and B5D5 did react to a greater degree with Fraction I than the other fractions and antibody B1D8 reacted with Fractions II and III to a greater degree than Fraction I. All three of these antibodies reacted positively with these respective fractions when tested by SDS-PAGE and immunoblot analysis. Of the 11 antibodies that did not react by immunoblot analysis, five demonstrated a significant reaction with the fractionated airway secretions in the solid phase RIA. Negative reactions in immunoblots after SDS-PAGE might be due to sensitivity after treatment with SDS or ß-mercaptoethanol.

CONCLUSIONS AND FUTURE DIRECTIONS

In this work, it has been shown that the isolation and culture of cells from tracheobronchial submucosal glands is feasible. In culture, bovine tracheal submucosal gland serous cells propagate and maintain partial differentiation. This has allowed analysis of some of the secretory regulatory mechanisms operating in these cells to be investigated. Additionally, it has allowed more complete biochemical studies to be performed resulting in the identification of glycosaminoglycans as the major carbohydrate secreted by serous

cells. Monoclonal antibodies directed against human tracheobronchial secretions have been shown to be useful in identifying products, both protein and carbohydrate, secreted by tracheobronchial secretory cells. These antibodies have identified groups of antigens that are unique to specific cell types and groups of antigens that are shared among the tracheobronchial secretory cells.

Thus, the characterization of the cultured cells and antigens recognized by the monoclonal antibodies has already shed light on some aspects of airway secretion. It is likely that additional knowledge will be gained by using these systems to answer the many remaining questions. For example, the establishment of a continuous cell line of bovine tracheal gland serous cells will allow a detailed analysis of the biochemical products synthesized and secreted by this cell type. As is often the case with cultured cells some of the *in vivo* functions are lost or altered. Careful manipulations of the the cell culture environment should not only improve the differentiation of serous cells in culture but should indicate which factors are responsible for establishing and maintaining the serous cell phenotype. These cells should continue to be useful for studying the effects of protein phosphorylation, both short-term effects such as those involved in the control of exocytotic events and long-term effects such as those involved in the control of growth and differentiation.

Monoclonal antibodies directed against human airway secretions that show cellular specificity can serve as useful markers of differentiation of human tracheobronchial gland cells in culture. They can be used to identify differentiated features of lung-derived tumor cell lines and perhaps be used to distinguish lung-derived malignancies from those derived from other organs. Monoclonal antibodies can be used to purify their respective antigens for detailed biochemical

and structural analysis. Monoclonal antibodies directed against protein epitopes may be useful in molecular cloning. Those monoclonal antibodies showing cellular specificity can be used as probes for the detection of antigen in sputum or lavage samples. Comparisons can be made between healthy and diseased individuals. Previously, the release of glyconjugates from human cells has been detected by incorporated radiolabeled precursors such as $[^{35}S]O_4$ and $[^{14}C]$- or $[^{3}H]$-glucosamine [66, 193, 219]. Results obtained from these studies, while shedding considerable light on the general control of airway secretion are unsatisfactory for determining the behavior of individual cell types. The most promising application of the antibodies described here may lie in the development of immunoassays to monitor cell activity *in vivo* in patients. Recent evidence suggests that the biochemistry of respiratory secretions is profoundly changed in patients with airway disease [29, 30]. Using monoclonal antibodies it should be possible to determine whether the normal ratio of serous:mucous:goblet cell antigen in bronchial lavage fluid is altered in disease, and whether there are abnormalities in the sensitivity of each cell type to drugs like atropine and isoprenaline. As new drugs become available, cell-specific monoclonal antibodies may provide useful tools for testing their effects at the cellular level in the treatment of chronic obstructive lung disease.

REFERENCES

1. Aaronson, S. A. and G. J. Todaro. Basis for the acquisition of malignant potential by mouse cells cultivated *in vitro*. Science. **162**: 1024-1026, 1968.

2. Adler, K. B., D. H. Hardwick and J. E. Craighead. Porcine tracheal goblet cell ultrastructure. Exp. Lung Res. **3**: 69-80, 1982.

3. Adler, K. B., J. E. Schwarz, W. H. Anderson and A. F. Welton. Platelet activating factor stimulates secretion of mucin by explants of rodent airways in organ culture. Exp. Lung Res. **13**: 25-43, 1987.

4. Allen, A., A. Bell, M. Mantle and J. P. Pearson. The structure and physiology of gastrointestinal mucus. In: *Mucus in Health and Disease*-II, Chantler, E. N., J. B. Elder and M. Elstein, eds., New York: Plenum Press, 1982, p. 115-133.

5. Baker, A. P., L. M. Hillegass, D. A. Holden and W. J. Smith. Effect of kallidin, substance P, and other basic polypeptides on the production of respiratory macromolecules. Am. Rev. Respir. Dis. **115**: 811-817, 1977.

6. Baker, B., A. C. Peatfield and P. S. Richardson. Nervous control of mucin secretion into human bronchi. J. Physiol. (Lond.). **365**: 297-305, 1985.

7. Baker, D. G. Parasympathetic motor pathways to the trachea: recent morphologic and electrophysiologic studies. In: *The Upper Airways*, Widdicombe, J. H. ed., Philadelphia: W.B. Saunders, Clin. Chest Med. 7:223-229, 1986.

8. Balfre, K. The effects of calcium and calcium ionophore A23187 on mucin secretion and potential difference in the isolated chicken trachea. J. Physiol. (Lond.). **275**: 80P-81P, 1978.

9. Ball, J. H., H. I. Kaminsky, J. G. Hardman, A. E. Broadus, E. W. Sutherland and J. W. Liddle. Effect of catecholamines and adrenergic blocking agents on plasma and urinary cyclic nucleotides man. J. Clin. Invest. **51**: 2124-2129, 1972.

10. Barbieri, E. J., E. Bobyock, W. S. Chernick and R. F. McMichael. The calcium dependency of mucus glycoconjugate secretion by canine tracheal explants. Br. J. Pharmacol. **82**: 199-206, 1984.

11. Barnes, P. J. Adrenergic regulation of airway function. In: *The Airways: Neural Control in Health and Disease*, Kaliner, M.A. and P.J. Barnes eds., New York: Marcel Decker, 1988, p. 57-85.

12. Barnes, P. J. and C. B. Basbaum. Mapping of adrenergic receptors in the trachea by autoradiography. Exp. Lung Res. **5**: 183-192, 1983.

13. Barnes, P. J., C. B. Basbaum, J. A. Nadel and J. M. Roberts. Localization of ß-adrenoreceptors in mammalian lung by light microscopic autoradiography. Nature (Lond.) **299**: 444-447, 1982.

14. Barnes, P. J., J. A. Nadel, J. M. Roberts and C. B. Basbaum. Muscarinic receptors in lung and trachea: autoradiographic localization using [^{3}H]quinuclidinyl benzilate. Eur. J. Pharmacol. **86**: 103-106, 1983.

15. Barsigian, C. and E. J. Barbieri. The effects of indomethacin and prostaglandins E_2 and $F_{2\alpha}$ on canine tracheal mucus generation. Agents Actions. **12**: 320-327, 1982.

16. Barthelson, R. and J. Widdicombe. Cyclic adenosine monophosphate-dependent kinase in cystic fibrosis tracheal epithelium. J. Clin. Invest. **80**: 1799-1802, 1987.

17. Basbaum, C. B. Innervation of the airway mucosa and submucosa. Semin. Respir. Med. **5**: 308-313, 1984.

18. Basbaum, C. B. Regulation of airway secretory cells. In: *The Upper Airways*, Widdicombe, J. H. ed., Philadelphia: W.B. Saunders, Clin. Chest Med. 7:231-237, 1986.

19. Basbaum, C. B. and W. E. Finkbeiner. Mucus-producing cells of the airways. In: *Lung Cell Biology*, Massaro, D., ed., New York: Marcel Dekker, 1989, p.37-79.

20. Basbaum, C. B., M. A. Grillo and J. H. Widdicombe. Muscarinic receptors: evidence for a nonuniform distribution in tracheal smooth muscle and exocrine glands. J. Neurosci. **4**: 508-520, 1984.

21. Basbaum, C. B., J. Mann, A. Chow and W. E. Finkbeiner. Monoclonal antibodies as probes for unique antigens in secretory cells of mixed exocrine organs. Proc. Natl. Acad. Sci. USA. **81**: 4419-4423, 1984.

22. Basbaum, C. B., I. Ueki, L. Brezina and J. A. Nadel. Tracheal submucosal gland serous cells stimulated in vitro with adrenergic and cholinergic agonists: a morphometric study. Cell Tissue Res. **220**: 481-498, 1981.

23. Baskerville, A. The development and persistence of bronchial gland hypertrophy and goblet cell hyperplasia in the pig after injection of isoprenaline. J. Pathol. **119**: 35-47, 1976.

24. Beckstead, J. H. Optimal antigen localization in human tissues using aldehyde-fixed plastic-embedded sections. J. Histochem. Cytochem. **33**: 954-958, 1985.

25. Beckstead, J. H. and D. F. Bainton. Enzyme histochemistry on bone marrow biopsies: reactions useful in the differential diagnosis of leukemia and lymphoma applied to 2-micron plastic sections. Blood. **55**: 386-394, 1980.

26. Beebe, S. J. and J. D. Corbin. Cyclic nucleotide-dependent protein kinases. In: *The Enzymes*, Boyer, P. D. and E. G. Krebs, eds., Orlando: Academic Press, 1986, p. 43-111.

27. Beeley, J. G. *Laboratory Techniques in Biochemistry and Molecular Biology*. Amsterdam: Elsevier, 1985.

28. Bensch, K. G., G. B. Gordon and L. R. Miller. Studies on the bronchial counterpart of the Kultschitzky (Argentaffin) cell and innervation of bronchial glands. J. Ultrastruct. Res. **12**: 668-686, 1965.

29. Bhaskar, K. R., D. D. O'Sullivan, J. Seltzer, T. H. Rossing, J. M. Drazen and L. M. Reid. Density gradient study of bronchial mucus aspirates from healthy volunteers (smokers and nonsmokers) from patients with tracheostomy. Exp. Lung Res. **9**: 289-308, 1985.

30. Bhaskar, K. R., D. D. O'Sullivan, J. Seltzer, T. H. Rossing, J. M. Drazen and L. M. Reid. Density gradient analysis of secretions produced in vitro by human and

canine airway mucosa: identification of lipids and proteoglycans in such secretions. Exp. Lung Res. **10**: 401-422, 1986.

31. Biedler, J. L. Chromosome abnormalities in human tumour cells in culture. In: *Human Tumour Cells in Vitro*, Fogh, J., ed., New York: Academic Press, 1976, p. 359-394.

32. Bissell, D. M. and P. S. Guzelian. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. Ann. N.Y. Acad. Sci. **349**: 85-98, 1981.

33. Boat, T. F., P. W. Cheng, R. N. Iyer, D. M. Carlson and I. Polony. Human respiratory tract secretions. Arch. Biochem. Biophys. **177**: 95-104, 1976.

34. Boat, T. F. and J. I. Kleinerman. Human respiratory tract secretions. 2. Effect of cholinergic and adrenergic agents on *in vitro* release of protein and mucous glycoprotein. Chest. **67**: 32S-34S, 1975.

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

36. Borson, D. B., M. Charlin, B. D. Gold and J. A. Nadel. Neural regulation of $^{35}SO_4$-macromolecule secretion from tracheal glands of ferrets. J. Appl. Physiol. **57**: 457-466, 1984.

37. Borson, D. B., R. A. Chinn, B. Davis and J. A. Nadel. Adrenergic and cholinergic nerves mediate fluid secretion from tracheal glands of ferrets. J. Appl. Physiol. **49**: 1027-1031, 1980.

38. Borson, D. B., R. Corrales, S. Varsano, M. Gold, N. Viro, G. Caughey, J. Ramachandran and J. A. Nadel. Enkephalinase inhibitors potentiate substance P-induced secretion of $^{35}SO_4$-macromolecules from ferret trachea. Exp. Lung Res. **12**: 21-36, 1987.

39. Bower, A. J., S. Parker and V. Molony. An autoradiographic study of afferent innervation of the trachea, syrinx and extrapulmonary primary bronchus of *Gallus gallus comesticus*. J. Anat. **126**: 169-180, 1978.

40. Bowes, D., A. E. Clark and B. Corrin. Ultrastructural localisation of lactoferrin and glycoprotein in human bronchial glands. Thorax. **36**: 108-115, 1981.

41. Bowes, D. and B. Corrin. Ultrastructural immunocytochemical localisation of lysozyme in human bronchial glands. Thorax. **32**: 163-170, 1977.

42. Boyd, E. M., S. Jackson and A. Ronan. The effect of sympathomimetic amines upon the output of respiratory tract fluid in rabbits. Am. J. Physiology. **138**: 565-568, 1943.

43. Boyd, E. M. and M. S. Lapp. On the expectorant action of parasympathomimetic drugs. J. Pharmacol. Exp. Ther. **87**: 24-32, 1946.

44. Bramson, H. N., E. T. Kaiser and A. Mildvan. Mechanistic studies of cAMP-dependent protein kinase action. CRC Crit. Rev. Biochem. **15**: 93-124, 1984.

45. Breuer, R., T. G. Christensen, R. M. Niles, P. J. Stone and G. L. Snider. Human neutrophil elastase causes glycoconjugate release from the epithelial cell surface

of hamster trachea in organ culture. Am. Rev. Respir. Dis. **139**: 779-782, 1989.

46. Bucher, U. and L. Reid. Development of the mucus-secreting elements in human lung. Thorax. **16**: 219-225, 1961.

47. Burnham, D. B. and J. A. Williams. Activation of protein kinase activity in pancreatic acini by calcium and cAMP. Am. J. Physiol. **246** (Gastrointest. Liver Physiol 9): G500-G508, 1984.

48. Butcher, F. R. and J. W. Putney Jr. Regulation of parotid gland function by cyclic nucleotides and calcium. Adv. Cyclic Nucleotide Res. **13**: 215-149, 1980.

49. Campbell, A. M. *Monoclonal Antibody Technology,* Amsterdam: Elsevier, 1984.

50. Carstairs, J. R. and P. J. Barnes. Autoradiographic mapping of substance P receptors in lung. Eur. J. Pharmacol. **127**: 295-296, 1986.

51. Carstairs, J. R. and P. J. Barnes. Visualization of vasoactive intestinal peptide receptors in human and guinea pig lung. J. Pharmacol. Exp. Ther. **239**: 249-255, 1986.

52. Carstairs, R., A. J. Nimmo and P. J. Barnes. Autoradiographic visualization of beta-adrenoceptor subtypes in human lung. Am. Rev. Respir. Dis. **132**: 541-547, 1985.

53. Cauna, N. Fine structure of the receptor organs and its probable functional significance. In: *Touch, Heat and Pain,* de Reuck, A. V. S. and Knight, J. eds., London: Churchill, 1966, p. 117-127.

54. Chakrin, L. W., A. P. Baker, P. Cristian and J. R. Wardell. Effect of cholinergic stimulation of the release of macromolecules by canine trachea in vitro. Am. Rev. Respir. Dis. **108**: 69-76, 1973.

55. Cheung, A., J. M. Polak, F. E. Bauer, A. Cadieux, N. D. Christofides, D. R. Sprinall and S. R. Bloom. Distribution of galanin immunoreactivity in the respiratory tract of pig, guinea pig, rat, and dog. Thorax. **40**: 889-896, 1985.

56. Christensen, T. G. and J. A. Hayes. Endogenous peroxidase in the conducting airways of hamsters. Morphologic evidence of synthesis and secretion. Am. Rev. Respir. Dis. **125**: 341-346, 1982.

57. Clamp, J. R. and L. Hough. The periodate oxidation of amino acids with reference to studies on glycoproteins. Biochem. J. **94**: 17-24, 1965.

58. Coffino, P., R. Baumal, R. Laskov. and J. D. Scharff. Cloning of mouse myeloma cells and detection of rare variants. J. Cell. Physiol. **79**: 429-440, 1972.

59. Coleman, D. L., I. K. Tuet and J. H. Widdicombe. Electrical properties of dog tracheal epithelial cells grown in monolayer culture. Am. J. Physiol. **246** (Cell Physiol. 15): C355-C359, 1984.

60. Coles, S. Regulation of the secretory cycles of mucous and serous cells in the human bronchial gland. In: *Mucous in Health and Disease*, Elstein, M. and D. V. Parke, eds., London: Plenum Press, 1977, p. 155-168.

61. Coles, S. J., K. R. Bhaskar, D. D. O'Sullivan, K. H. Neil and L. M. Reid. Airway mucus: composition and regu-

lation of its secretion by neuropeptides in vitro. In: *Mucus and Mucosa*. Nugent, J. and M. O'Connor eds., London: Pitman., 1984, p. 40-60. (CIBA Foundation Symposium 109.)

62. Coles, S. J., K. R. Bhaskar, D. D. O'Sullivan and L. M. Reid. Macromolecular compositions of secretions produced by human bronchial explants. In: *Mucus in Health and Disease*-II, Chantler, E. N., J. B. Elder and M. Elstein, eds., New York: Plenum Press, 1982, p. 357-360.

63. Coles, S. J., J. Judge and L. Reid. Differential effects of calcium ions on glycoconjugate secretion by canine tracheal explants. Chest. **81**: 34S-36S, 1982.

64. Coles, S. J., K. H. Neill, L. M. Reid, K. F. Austen, Y. Nii, E. J. Corey and R. A. Lewis. Effects of leukotrienes C_4 and D_4 on glycoprotein and lysozyme secretion by human bronchial mucosa. Prostaglandins. **25**: 155-170, 1983.

65. Coles, S. J. and L. Reid. Glycoprotein secretion *in vitro* by human airway: normal and chronic bronchitis. Exp. Mol. Pathol. **29**: 326-341, 1978.

66. Coles, S. J., S. I. Said and L. M. Reid. Inhibition by vasoactive intestinal peptide of glycoconjugate and lysozyme secretion by human airways in vitro. Am. Rev. Respir. Dis. **124**: 531-536, 1981.

67. Conner, M. W. and L. M. Reid. Mapping of ß-adrenergic receptors in rat lung: effect of isoproterenol. Exp. Lung Res. **6**: 91-101, 1984.

68. Conover, J. H. and E. J. Conod. The influence of cystic fibrosis serum and calcium on secretion in the rabbit

tracheal mucociliary apparatus. Biochem. Biophys. Res. Commun. **83**: 1595-1601, 1978.

69. Corbin, J. D., P. H. Sugden, T. M. Lincoln and S. L. Keely. Compartmentalization of adenosine 3':5'-monophosphate and adenosine 3':5'-monophosphate-dependent kinase in heart tissue. J. Biol. Chem. **252**: 3854-3861, 1977.

70. Culp, D. J. and M. G. Marin. Characterization of muscarinic cholinergic receptors in cat tracheal gland cells. J. Appl. Physiol. **61**: 1375-1382, 1986.

71. Culp, D., D. P. Penney and M. G. Marin. A techique for the isolation of submucosal gland cell from cat trachea. J. Appl. Physiol. **55**: 1034-1041, 1983.

72. Das, R. M., P. K. Jeffery and J. G. Widdicombe. The epithelial innervation of the lower respiratory tract of the cat. J. Anat. **126**: 123-131, 1978.

73. Davis, B., M. Marin, S. Fischer, P. Graf, J. Widdicombe and J. A. Nadel. New method for study of canine mucous gland secretion in vivo. Am. Rev. Respir. Dis. **113**: 257, 1976.

74. Davis, B., A. M. Roberts, H. M. Coleridge and J. C. G. Coleridge. Reflex tracheal gland secretion evoked by stimulation of bronchial C-fibers in dogs. J. Appl. Physiol. **53**: 985-991, 1982.

75. Deeg, M. A., R. M. Graeff, T. F. Walseth and N. D. Goldberg. A Ca^{2+}-linked increase in coupled cAMP synthesis and hydrolysis is an early event in cholinergic and ß-adrenergic stimulation of parotid secretion. Proc. Natl. Acad. Sci. USA. **85**: 7867-7871, 1988.

76. Dey, R. D., W. A. Shannon and S. I. Said. Localization of VIP-immunoreactive nerves in airways and pulmonary vessels of dogs, cats, and human subjects. Cell Tissue Res. **220**: 231-238, 1981.

77. Douglas, W. W. Aspects of the calcium hypothesis of stimulus-secretion coupling: electrical activity in adenohypophyseal cells and membrane retrieval after exocytosis. Methods Cell Biol. **23**: 483-501, 1981.

78. Edelman, A. M., D. K. Blumenthal and E. G. Krebs. Protein serine/threonine kinases. Ann. Rev. Biochem. **56**: 567-613, 1987.

79. Ellefsen, P. and M. Tos. Goblet cells in the human trachea. Quantitative studies of a pathological biopsy material. Arch. Otolaryng. **95**: 547-555, 1972.

80. Erlichman, J., D. Sarkar, N. Fleischer and C. S. Rubin. Identification of two subclasses of type II cAMP-dependent protein kinases. J. Biol. Chem. **255**: 8179-8184, 1980.

81. Escudier, E., L. S. Forsberg and C. B. Basbaum. Chondroitin sulfate is a secretory product of tracheal gland serous cells in vivo as well as in culture. J. Cell Biol. **105**: 330a, 1987.

82. Fillenz, M. and R. I. Woods. Sensory innervation of the airways. In: *Breathing. Proceedings of the Hering Breuer Centenary Symposium*, Porter, R. ed., London: Churchill, 1970, p.101-107.

83. Finkbeiner, W. E. and C. B. Basbaum. Evaluation of sheep and dog tracheas as a source of isolated submucosal gland cells. Physiologist. **26**: A125, 1983.

84. Finkbeiner, W. E., A. Paul and C. B. Basbaum. Isolation of bovine tracheal submucosal gland cells and establishment of a cell line. Fed. Proc. **44**: 642, 1985.

85. Florey, H., H. M. Carleton and A. Q. Wells. Mucus secretion in the trachea. Br. J. Exp. Pathol. **13**: 269-284, 1932.

86. Forsberg, L. S. and J. H. Pazur. The characterization of hemiacetal bonds formed after periodate oxidation of heteroglycans. Carbohydrate Res. **75**: 129-140, 1979.

87. Forstner, J. F., I. Jabbal, R. Oureshi, D. I. C. Kells and G. G. Forstner. The role of disulphide bonds in human intestinal mucin. Biochem. J. **181**: 725-732, 1979.

88. Freedman, V. H. and S. Shin. Cellular tumorigenicity in nude mice: Correlation with cell growth in semi-solid medium. Cell. **3**: 355-359, 1974.

89. Freshney, R. I. Culture of glioma of the brain. In: *Brain Tumours, Scientific Basic Clinical Investigation and Current Therapy*, Thomas, D. G. T. and D. I. Graham eds., London: Butterworths, 1980 p. 21-50.

90. Freshney, R. I. *Culture of Animal Cells. A Manual of Basic Technique*. New York: Alan R. Liss, 1987.

91. Gallagher, J. T. and P. W. Kent. Structure and metabolism of glycoproteins and glycosaminoglycans secreted by organ cultures of rabbit trachea. Biochem J. **148**: 187-196, 1975.

92. Gallagher, J. T., P. W. Kent, M. Passatore, R. J. Phipps and P. S. Richardson. The composition of tracheal mucus

and the nervous control of its secretion in the cat. Proc. R. Soc. Lond. [Biol.]. **192**: 49-76, 1975.

93. Gashi, A. A., D. B. Borson, W. E. Finkbeiner, J. A. Nadel and C. B. Basbaum. Neuropeptides degranulate serous cells of ferret tracheal glands. Am. J. Physiol. **251** (Cell Physiol. 20): C223-C229, 1986.

94. Gashi, A. A., J. A. Nadel and C. B. Basbaum. Autoradiographic studies of the distribution of ^{35}sulfate label in the ferret trachea: effects of stimulation. Exp. Lung Res.: **12**: 83-96, 1987.

95. Gashi, A. A., J. A. Nadel and C. B. Basbaum. Tracheal gland mucous cells stimulated *in vitro* with adrenergic and cholinergic drugs. Tissue Cell. (in press).

96. German, V. F., R. Corrales, I. F. Ueki and J. A. Nadel. Reflex stimulation of tracheal submucosal gland secretion by gastric irritation in cats. J. Appl. Physiol. **52**: 1153-1155, 1982.

97. Ghatei, M. A., M. N. Sheppard, D. J. O'Shaughnessy, T. E. Adrian, G. P. McGregor, J. M. Polak and S. R. Bloom. Regulatory peptides in the mammalian respiratory tract. Endocrinology. **111**: 1248-1254, 1982.

98. Giovanella, B. C., J. S. Stehlin and L. J. Williams. Heterotransplantation of human malignant tumors in "nude" mice. II. Malignant tumors induced by injection of cell cultures derived from human solid tumors. J. Natl. Cancer Inst. **52**: 921, 1974.

99. Goco, R. V., M. B. Kress and O. C. Brantigan. Comparison of mucus glands in tracheobronchial tree of man and animals. Ann. N.Y. Acad. Sci. **106**: 555-571, 1963.

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

101. Goodman, M. R., D. W. Link, W. R. Brown and P. K. Nakane. Ultrastructural evidence of transport of secretory IgA across bronchial epithelium. Am. Rev. Respir. Dis. **123**: 115-119, 1981.

102. Hartmann, J. F., C. F. Hutchison and M. E. Jewell. Pig bronchial mucous membrane: a model system for assessing respiratory mucus release in vitro. Exp. Lung Res. **6**: 59-70, 1984.

103. Havez, R. and P. Roussel. Bronchial mucus: physical and biochemical features. In: *Bronchial Asthma: Mechanisms and Therapeutis*, Weiss, E. B. and M. S. Segal eds., Boston: Little, Brown and Co., 1976, p. 409-422.

104. Hill, H. D., Jr., M. Schwyzer, H. M. Steinman and R. L. Hill. Ovine submaxillary mucin: primary structure and peptide substrates of UDP-N-acetylgalactosamine:mucin transferase. J. Biol. Chem. **252**: 3799-3804, 1977.

105. Hsu, S.-L. Raine and H. Fanger. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. J. Histochem. Cytochem. **29**: 577-580, 1981.

106. Hsu, T. C. and K. Benirschke. *An Atlas of Mammalian Chromosomes*, New York: Springer Verlag, 1967.

107. Hung, K. S., M. S. Hertwech, J. D. Hardy and C. G. Loosli. Ultrastructure of nerves and associated cells in

bronchiolar epithelium of the mouse lung. J. Ultrastruct. Res. **43**: 426-437, 1973.

108. Ichikawa, M., A. Ichikawa and S. Kidodoro. Secretory process of mucus-secreting cells in mouse colonic mucosa studied by rapid freezing and freeze-substitution. J. Electron Microsc. **36**: 117-127, 1987.

109. Irvani, J. and G. N. Melville. Mucociliary function of the respiratory tract as influenced by drugs. Respiration. **31**: 350-357, 1974.

110. Itoh, H., F. Okajima and M. Ui. Conversion of adrenergic mechanism from an α- to a ß-type during primary culture of rat hepatocytes. J. Biol. Chem. **259**: 15464-15473, 1984.

111. Iwamoto, I., J. A. Nadel, S. Varsano and L. S. Forsberg. Turnover of cell-surface macromolecules in cultured dog tracheal epithelial cells. Biochim. Biophys. Acta. **966**: 336-346, 1988.

112. Jacquot, J., E. Puchelle, C. Basbaum and W. Finkbeiner. Secretion of lysozyme by bovine tracheal serous cells in culture. Am. Rev. Respir. Dis. **133**: A296, 1986.

113. Jahn, R., C. Unger and H. D. Soling. Specific protein phosphorylation during stimulation of amylase secretion by ß-agonists or dibutyryl adenosine 3',5'-monophosphate in the rat parotid gland. Eur. J. Biochem. **112**: 345-352, 1980.

114. Jeffery, P. K. The innervation of bronchial mucosa. In: *Cellular Biology of the Lung*, Cumming, C., ed., New York: Plenum Press, 1982, p. 1-25.

115. Jeffery, P. K. The origins of secretions in the lower respiratory tract. Eur. J. Respir. Dis. **71** (Suppl. 153): 34-42, 1987.

116. Jeffery, P. K. and L. Reid. Intra-epithelial nerves in normal rat airways: a quantitative electron microscopic study. J. Anat. **114**: 35-45, 1973.

117. Jeffery, P. K. and L. M. Reid. New observations of rat airway epithelium: a quantitative and electron microscopic study. J. Anat. **120**: 295-320, 1975.

118. Jeffery, P. K. and L. Reid. Ultrastructure of airway epithelium and submucosal gland during development. In: *Lung Biology in Health and Disease. Development of the Lung*, Hodson, W. A., ed., New York: Dekker, New York, 1977, p. 87-134.

119. Johnson, H. G., R. A. Chinn, A. W. Chow, M. K. Bach and J. A. Nadel. Leukotriene-C_4 enhances mucus production form submucosal glands in canine trachea in vivo. Int. J. Immunopharmacol. **5**: 391-396, 1983.

120. Kim, K., J. I. Rearick, P. Nettesheim and A. M. Jetten. Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture. J. Biol. Chem. **260**: 4021-4027, 1985.

121. Kim, K. C., K. Wasano, R. M. Niles, J. E. Schuster, P. J. Stone and J. S. Brody. Human neutrophil elastase releases cell surface mucins from primary cultures of hamster tracheal epithelial cells. Proc. Natl. Acad. Sci. USA. **84**: 9304-9308, 1987.

122. King, A. S., J. McLelland, R. D. Cook, D. Z. King and C. Walsh. The ultrastructure of afferent nerve endings in avian lung. Respir. Physiol. **22**: 21-40, 1974.

123. King, M. and N. Vines. Effect of methacholine chloride on rheology and transport of canine tracheal mucous. J. Appl. Physiol. **47**: 26-31, 1979.

124. Klinger, J. D., B. Tandler, C. M. Liedtke and T. F. Boat. Proteinases of Pseudomonas aeruginosa evoke mucin release by tracheal epithelium. J. Clin. Invest. **74**: 1669-1678, 1984.

125. Klockars, M. and S. Reitamo. Tissue distribution of lysozyme in man. J. Histochem. Cytochem. **23**: 932-940, 1975.

126. Kohler, G. and C. Milstein. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. **256**: 495-497, 1975.

127. Kohler, G. and C. Milstein. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. **6**: 511-519, 1976.

128. Kokin, P. Uber die secretorischen Nerven der Kelhopfund Luftrohrenschleimdrusen. Pflugers Arch. Ges. Physiol. **63**: 622-630, 1896.

129. Kountz, W. B. and K. Koenig. Studies of bronchial secretion. J. Allergy. **1**: 429-433, 1930.

130. Kramps, J. A., C. Franken and J. H. Dijkman. ELISA for quantitative measurement of low molecular weight bronchial protease inhibitor in human sputum. Am. Rev. Respir. Dis. **129**: 959-963, 1984.

131. Krotoski, W. A. and H. E. Weimer. Peptide-associated and antigenic changes accompanying periodic oxidation of human plasma orosomucoid. Arch. Biochem. Biophys. **115**: 337-344, 1966.

132. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**: 680-685, 1970.

133. Laitinen, A. Ultrastructural organization of intraepitelial nerves in the human airway tract. Thorax. **40**: 488-492, 1985.

134. Laitinen, A., M. Partanen, A. Hervonen and L. A. Laitinen. Electron microscopic study on the innervation of the human lower respiratory tract: evidence of adrenergic nerves. Eur. J. Respir. Dis. **67**: 209-215, 1985.

135. Laitinen, A., M. Partanen, A. Hervonen, M. Pelto-Huikko and L. A. Laitinen. VIP like immunoreactive nerves in human respiratory tract. Histochemistry. **82**: 313-319, 1985.

136. Laitinen, L. A. Detailed analysis of neural elements in human airways. In: *The Airways: Neural Control in Health and Disease*, Kaliner, M.A. and P. J.Barnes eds., New York: Marcel Decker, 1988, p.35-96.

137. Lamb, D. and L. Reid. Histochemical types of acidic glycoprotein produced by mucous cells of the tracheobronchial glands in man. J. Pathol. **98**: 213-229, 1969.

138. Lands, A. M., A. Arnold, J. P. McAuliff, F. L. Ludnena and T. G. Brown. Differentiation of receptor systems

activated by sympathomimetic amines. Nature (Lond.) **214**: 597-598, 1967.

139. Lazarus, S. C., C. B. Basbaum, P. J. Barnes and W. M. Gold. cAMP immunocytochemistry provides evidence for functional VIP receptors in trachea. Am. J. Physiol. **251** (Cell Physiol.): C115-C119, 1986.

140. Lazarus, S. C., C. B. Basbaum and W. M. Gold. Localization of cAMP in dog and cat trachea: effects of ß-adrenergic agonists. Am. J. Physiol. **247** (Cell Physiol. 16): C327-C334, 1984.

141. Lazarus, S. C., C. B. Basbaum and W. M. Gold. Prostaglandins and intracellular cyclic AMP in respiratory secretory cells. Am. Rev. Respir. Dis. **130**: 262-266, 1984.

142. Le Treut, A., G. Lamblin, N. Houdret, P. Degand and P. Roussel. Reevaluation of the action of reducing agents on soluble mucus glycoproteins from human bronchial secretion. Biochimie. **63**: 425-434, 1981.

143. Leikauf, G. D., I. F. Ueki and J. A. Nadel. Autonomic regulation of viscoelasticity of cat tracheal gland secretions. J. Appl. Physiol. **56**: 426-430, 1984.

144. Lernhardt, W., J. Andersson, A. Coutinho and F. Melchers. Cloning of murine transformed cell lines in suspension culture with efficiences near 100%. Exp. Cell Res. **111**: 309-316, 1978.

145. Leroux, P., H. Vaudry, A. Fournier, S. St.-Pierre and G. Pelletier. Characterization and localization of vasoactive intestinal peptide receptors in the rat lung. Endocrinology. **114**: 1506-1512, 1984.

146. Liedtke, C. M., T. F. Boat and S. A. Rudolph. ß-adrenergic agonists increase cAMP levels and stimulate mucous glycoprotein secretion in cat tracheal explants. Chest. **81**: 20S-21S, 1982.

147. Liedtke, C. M., S. A. Rudolph and T. F. Boat. ß-adrenergic modulation of mucin secretion in cat trachea. Am. J. Physiol. **244** (Cell Physiol.): C391-C398, 1983.

148. Lillie, R. D. Various oil soluble dyes as fat stains in the supersaturated isopropranol technique. Stain Technol. **19**: 55-58, 1944.

149. Lohmann, S. M., U. Walter and P. Greengard. Identification of endogenous substrate proteins for cAMP-dependent protein kinase in bovine brain. J. Biol. Chem. **255**: 9985-9992, 1980.

150. Lopez-Vidriero, M. T., I. Das, A. P. Smith, R. Picot and L. Reid. Bronchial secretion from normal human airways after inhalation of prostaglandin $F_{2\alpha}$, acetylcholine, histamine and citric acid. Thorax. **32**: 734-739, 1977.

151. Lopez-Vidriero, M. and L. Reid. Chemical markers of mucus and serum glycoproteins and their relation to viscosity in mucoid and purulent sputum from various hypersecretory diseases. Am. Rev. Respir. Dis. **117**: 465-477, 1978.

152. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**: 265-275, 1951.

153. Lundberg, J. M., A. Anggard, J. Fahrenkrug, T. Hokelt and V. Mutt. Vasoactove intestinal polypeptide in

cholinergic neurons of exocrine glands: functional significance of coexisting transmitters for vasodilation and secretion. Proc. Natl. Acad. Sci. USA. **77**: 1651-1655, 1980.

154. Lundberg, J. M., E. Brodin and A. Saria. Effects and distribution of vagal capsaicin-sensitive substance P neurons with special reference to the trachea and lungs. Acta Physiol. Scand. **119**: 243-252, 1983.

155. Lundgren, J., M. A. Kaliner, C. Logun and J. H. Shelhamer. The effects of endorphins on mucous glycoprotein secretion from feline airways in vitro. Exp. Lung Res. **12**: 303-309, 1987.

156. Macpherson, I. and L. Montagnier. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology. **23**: 291-294, 1964.

157. Madison, J. M., C. B. Basbaum, J. K. Brown and W. E. Finkbeiner. Characterization of ß-adrenergic receptors in cultured bovine tracheal gland cells. Am. J. Physiol. **256** (Cell Physiol. 25): C310-C314, 1989.

158. Mann, S. P. The innervation of mammalian bronchial smooth muscle: the localization of catecholamines and cholinesterases. Histochem. J. **3**: 319-331, 1971.

159. Margolis, R. U. and R. K. Margolis. Isolation of chondroitin sulfate and glycopeptides from chromaffin of adrenal medulla. Biochem. Pharmacol. **22**: 2195-2197, 1973.

160. Marin, M. G. Pharmacology of airway secretion. Pharmacol. Rev. **38**: 273-289, 1986.

161. Marin, M. G., J. A. Estep and J. P. Zorn. Effect of calcium on sulfated mucous glycoprotein secretion in dog trachea. J. Appl. Physiol. **52**: 198-205, 1982.

162. Marom, A., J. H. Shelhamer, F. Sun and M. Kaliner. Human airway monohydroxyeicosatetraenic acid generation and mucus release. J. Clin. Invest. **72**: 122-127, 1983.

163. Marom, Z., J. H. Shelhamer, M. K. Bach, D. R. Morton and M. Kaliner. Slow-reacting substances, leukotriene C_4 and D_4, increase the release of mucus from human airways in vitro. Am. Rev. Respir. Dis. **126**: 449-451, 1982.

164. Marom, Z., J. H. Shelhamer and M. Kaliner. Effects of arachidonic acid, monohydroxyeicosatetraenoic acid and prostaglandins on the release of mucous glycoproteins from human airways in vitro. J. Clin. Invest. **67**: 1695-1702, 1981.

165. Marom, Z., J. H. Shelhamer, L. Steel, E. J. Goetzl and M. Kaliner. Prostaglandin-generating factor of anaphylaxis induces mucous glycoprotein release and the formation of lipoxygenase products of arachidonate human airways. Prostaglandins. **28**: 79-91, 1984.

166. Mason, D. Y. and C. R. Taylor. The distribution of muramidase (lysozyme) in human tissues. J. Clin. Res. **28**: 124-132, 1975.

167. Masson, P. L. and J. F. Heremans. Sputum proteins. In: *Sputum: Fundamentals and Clinical Pathology*, Dulfano, M. J. ed., Springfield, IL: Charles C. Thomas, 1973, p.412-475.

168. Masson, P. L., J. F. Heremans, J. J. Prignot and G. Wauters. Immunohistochemical localisation and bacterio-

static properties of an iron-binding protein from bronchial mucus. Thorax. **21**: 538-544, 1966.

169. Mazzuca, M., L. M., J. Lafitte and P. Roussel. Use of lectins for detection of glycoconjugates in the glandular cells of the human bronchial mucosa. J. Histochem. Cytochem. **30**: 956-966, 1982.

170. Meyrick, B. and L. Reid. Ultrastructure of cells in the human bronchial submucosal glands. J. Anat. **107**: 281-199, 1970.

171. Meyrick, B., J. M. Sturgess and L. Reid. A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. Thorax. **24**: 729-736, 1969.

172. Mian, N., C. E. Anderson, A. J. Pope, A. R. Smith, P. S. Richardson, K. Balfre and P. W. Kent. Directional Ca^{2+} effect on stimulation of mucin secretion from chicken trachea in vitro. Biochem. J. **208**: 425-433, 1982.

173. Mooren, H. W. D., C. J. L. M. Meyer, J. A. Kramps, C. Franken and J. H. Dijkman. Ultrastructural localization of the low molecular weight protease inhibitor in human bronchial glands. J. Histochem. Cytochem. **30**: 1130-1134, 1982.

174. Mossman, B. T., E. B. Ezerman, K. B. Adler and J. E. Craighead. Isolation and spontaneous transformation of cloned lines of hamster tracheal epithelial cells. Cancer Res. **40**: 4403-4409, 1980.

175. Mowry, R. W. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for

the colloidal iron stain, the use of Alcian blue G8x and their combinations with the periodic acid-Schiff reaction. Ann. N.Y. Acad. Sci. **106**: 402-423, 1956.

176. Murlas, C., J. A. Nadel and C. B. Basbaum. A morphometric analysis of the autonomic innervation of cat tracheal glands. J. Auton. Nerv. Syst. **2**: 23-37, 1980.

177. Nadel, J. A. and M. J. Holtzman. Regulation of airway responsiveness and secretion: role of inflammation. In: *Asthma: Physiology, Immunopharmacology, and Treatment*, Lichtenstein, L.M., A. B. Kay and K. F. Austen eds., London: Academic Press, 1984, p. 129-155.

178. Nadel, J. A., J. H. Widdicombe and A. C. Peatfield. Regulation of airway secretions, ion transport, and water movement. In: *Handbook of Physiology. The Respiratory System*, Fishman, A. P. and A.B. Fisher eds., Bethesda, MD: The American Physiological Society, 1985, p. 419-445.

179. Nakamura, A. T., A. Tomomura, C. Noda, M. Shimoji and A. Ichihara. Acquisition of ß-adrenergic response by adult rat hepatocytes during primary culture. J. Biol. Chem. **258**: 9283-9289, 1983.

180. Niles, R. M., T. G. Christensen, R. Breuer, P. J. Stone and G. L. Snider. Serine proteases stimulate mucous glycoprotein. J. Lab. Clin. Med. **108**: 489-497, 1986.

181. Nimmo, H. G. and P. Cohen. Hormonal control of protein phosphorylation. Adv. Cyclic Nucleotide. Res. **8**: 145-266, 1977.

182. Okajima, F. and M. Ui. Conversion of adrenergic regulation of glycogen phosphorylase and synthesis from an α-

to a ß-type during primary culture of rat hepatocytes. Arch. Biochem. Biophys. **213**: 658-668, 1982.

183. Pack, R. J., L. H. Al-Ugaily, G. Morris and J. G. Widdicombe. The distribution and structure of cells in the tracheal epithelium of the mouse. Cell Tissue Res. **208**(65-84): 1980.

184. Pack, R. J. and P. S. Richardson. The aminergic innervation of the human bronchus: a light and electron microscopic study. J. Anat. **138**: 493-502, 1984.

185. Partanen, M., A. Laitinen, A. Hervonen, M. Toivanen and L. A. Laitinen. Catecholamine- and actaylcholinesterase-containing nerves in human lower respiratory tract. Histochemistry. **76**: 175-188, 1982.

186. Paul, A., J. Picard, M. Mergey, D. Veissiere, W. E. Finkbeiner and C. B. Basbaum. Glycoconjugates secreted by bovine tracheal serous cells in culture. Arch Biochem. Biophys. **260**: 75-84, 1988.

187. Peatfield, A. C., P. J. Piper and P. S. Richardson. The effect of leukotriene C_4 on mucin output into the cat trachea. Br. J. Pharmacol. **77**: 391-393, 1982.

188. Peatfield, A. C. and P. S. Richardson. Evidence for noncholinergic nonadrenergic nervous control of mucous secretion into the cat trachea. J. Physiol. (Lond.). **342**: 335-345, 1983.

189. Perry, W. F. and E. M. Boyd. A method for studying expectorant action in animals by direct measurement of the output of respiratory tract fluids. J. Pharmacol. Exp. Ther. **73**: 65-77, 1941.

190. Phipps, R. J. and P. S. Richardson. The effects of irritation at various levels of the airway upon tracheal mucus secretion in the cat. J. Physiol. (Lond.). **261**: 563-581, 1976.

191. Phipps, R. J., P. S. Richardson, A. Corfield, J. T. Gallagher, P. K. Jeffery, P. W. Kent and A. Passatore. A physiological, biochemical and histological study of goose tracheal mucin and its secretion. Philos. Trans. R. Soc. London. (Biol.) **279**: 513-540, 1977.

192. Phipps, R. J., I. P. Williams, R. J. Pack and P. S. Richardson. Adrenergic stimulation of mucus secretion in human bronchi. Chest. **81**: 19S-20S, 1982.

193. Phipps, R. J., I. P. Williams, P. S. Richardson, J. Pell, R. J. Pack and N. Wright. Sympathomimetic drugs stimulate the output of secretory glycoproteins from human bronchi in vitro. Clin. Sci. **63**: 23-28, 1982.

194. Podolsky, D. K., D. A. Fournier and K. E. Lynch. Human colonic goblet cells: demonstration of distinct subpopulations defined by colonic mucin-specific monoclonal antibodies. J. Clin. Invest. **77**: 1263-1271, 1986.

195. Popovac, D., R. Chinn, P. Graf, J. Nadel and B. Davis. Serotonin potentiates nervous stimulation of mucus gland secretion in canine trachea in vivo. Physiologist. **22**: 102, 1979.

196. Quinton, P. M. Composition and control of secretions from tracheal bronchial submucosal glands. Nature. **279**: 551-552, 1979.

lar mucin secretion. Am. J. Physiol. **241** (Cell Physiol. 10): C76-C85, 1981.

198. Quissell, D. O., L. M. Deisher and K. A. Barzen. The rate limiting step in cAMP-mediated exocytosis in the rat parotid and submandibular glands appears to involve analogous 26-kDa integral membrane phosphoproteins. Proc. Natl. Acad. Sci. USA. **82**: 3237-3241, 1985.

199. Quissell, D. O., L. M. Deisher and K. A. Barzen. Role of protein phosphorylation in rat salivary gland exocytosis. J. Dent. Res. **66**: 596-598, 1987.

200. Rademacher, T. W., R. B. Parekh and R. A. Dwek. Glycobiology. Ann. Rev. Biochem. **57**: 785-838, 1988.

201. Refsnes, M., D. Sandnes and T. Christoffersen. The relationship between ß-adrenoceptor regulation and ß-adrenergic responsiveness in hepatocytes. Studies on acquisition, desensitization and resensitization of isoproterenol-sensitive adenylate cyclase in primary culture. Eur. J. Biochem. **163**: 457-466, 1987.

202. Reggio, H. A. and G. E. Palade. Sulfated compounds in the zymogen granules of the guinea pig pancreas. J. Cell Biol. **77**: 288-314, 1978.

203. Reid, L. Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. Thorax. **15**: 132-141, 1960.

204. Rhodin, J. A. G. The ciliated cell. Ultrastructure and function of the human tracheal mucosa. Am. Rev. Respir. Dis. **93**,Suppl.: 1-15, 1966.

205. Rhodin, J. and T. Dalhamn. Electron microscopy of the tracheal ciliated mucosa in rat. Z. Zellforsch. Mikrosk. Anat. **44**: 345-412, 1956.

206. Rich, B., A. C. Peatfield, W. I.P. and P. S. Richardson. Effects of prostaglandins E_1, E_2, and $F_{2\alpha}$ on mucin secretion from human bronchi in vitro. Thorax. **39**: 420-423, 1984.

207. Richardson, J. B. State of the Art. Nerve supply to the lungs. Am. Rev. Respir. Dis. **119**: 785-802, 1979.

208. Richardson, P. S. and A. C. Peatfield. The control of airway mucus secrtion. Eur. J. Respir. Dis. **71** [Suppl. 153]: 43-51, 1987.

209. Richardson, P. S. and R. J. Phipps. The anatomy, physiology, pharmacology and pathology of tracheobronchial mucus secretion and the use of expectorant drugs in human disease. In: *Respiratory Pharmacology*, Widdicombe, J.G., ed., Oxford: Pergamon Press, 1981, p. 437-475.

210. Richardson, P. S., R. J. Phipps, K. Balfre and R. L. Hall. The roles of mediators, irritants and allergens in causing mucin secrtion from the trachea. In: *Respiratory Tract Mucus*. Amsterdam: Elsevier/Excerpta Medica/North Holland, 1978, p. 111-131. Ciba Found. Symp. 54.)

211. Robinson, N. F., L. Venning, H. Kyle and J. G. Widdicombe. Quantitation of the secretory cells of the ferret tracheobronchial tree. J. Anat. **145**: 173-188, 1986.

212. Robinson-Steiner, A. M., S. J. Beebe, S. R. Rannels and J. D. Corbin. Microheterogeneity of type II cAMP-dependent protein kinase in various mammalian species and tissues. J. Biol. Chem. **259**: 10596-10605, 1984.

213. Rojkind, M., Z. Gatmaitan, S. Mackensen, M-A. Giambrone, P. Ponce and L. M. Reid. Connective tissue biomatrix: its isolation and utilization for long-term cultures of normal rat hepatocytes. J. Cell Biol. **87**: 255-263, 1980.

214. Ruben, R. L. and K. A. Rafferty, Jr. Limited *in vitro* replicative livespan of cultured human epithelial cells: a survey of recent literature. Growth. **42**: 357-368, 1978.

215. Santa Cruz, R. Tracheal mucous velocity in normal man and patients with obstructive lung disease; effects of terbutaline. Am. Rev. Respir. Dis. **109**: 458-463, 1974.

216. Schachter, H. Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. Biochem. Cell Biol. **64**: 163-181, 1986.

217. Schwarz, K. R., S. M. Lanier, E. A. Carter, C. J. Homcy and R. M. Graham. Rapid reciprocal changes in adrenergic receptors in intact isolated hepatocytes during primary cell culture. Mol. Pharmacol. **27**: 200-209, 1985.

218. Scott, C. W. and M. C. Mumby. Phosphorylation of type II regulatory subunit of cAMP-dependent protein kinase in intact smooth muscle. J. Biol. Chem. **260**: 2274-2280, 1985.

219. Shelhamer, J. H., Z. Marom and M. Kaliner. Immunologic and neuropharmacologic stimulation of mucous glycoprotein release from human airways in vitro. J. Clin. Invest. **66**: 1400-1408, 1980.

220. Shelhamer, J. H., Z. Marom, F. Sun, M. K. Bach and M. Kaliner. The effects of arachinoids and leukotrienes on

the release of mucus from human airways. Chest. **81**: 36S-37S, 1982.

221. Shenolikar, S. Protein phosphorylation: hormones, drugs, and bioregulation. FASEB J. **2**: 2753-2764, 1988.

222. Sheppard, M. N., J. M. Polak, J. M. Allen and S. R. Bloom. Neuropeptide tyrosine (NYP): a newly discovered peptide is present in the mammalian respiratory tract. Thorax. **39**: 326-330, 1984.

223. Sherman, J. M., P. W. Cheng, B. Tandler and T. F. Boat. Mucous glycoproteins from cat tracheal goblet cells and mucous glands separated with EDTA. Am. Rev. Respir. Dis. **124**: 476-479, 1981.

224. Shimura, S., T. Sasaki, H. Sasaki and T. Takishima. Contractility of isolated single submucosal gland from trachea. J. Appl. Physiol. **60**: 1237-1247, 1986.

225. Silberberg, A. and F. A. Meyer. Structure and function of mucus. In: *Mucus in Health and Disease*-II, Chantler, E. N., J. B. Elder and M. Elstein, eds., New York: Plenum Press, 1982, p. 53-74..

226. Silva, D. and G. Ross. Ultrastructural and fluorescence histochemical studies on the innervation of the tracheobronchial muscle of normal cats and cats treated with 6-hydroxydopamine. J. Ultrastruct. Res. **47**: 310-328, 1974.

227. Slayter, H. S., G. Lamblin, A. Le Treut, C. Galabaert, N. Houdret, P. Degand and P. Roussel. Complex structure of human bronchial mucus glycoprotein. Eur. J. Biochem. **142**: 209-218, 1984.

228. Snary, D., A. Allen and R. H. Pain. Structural studies on gastric mucoproteins: lowering of molecular weight after reduction with 2-mercaptoethanol. Biochem. Biophys. Res. Commun. **40**: 844-851, 1970.

229. Sommerhoff, C. P., G. H. Caughey, W. E. Finkbeiner, S. C. Lazarus, C. B. Basbaum and J. A. Nadel. Mast cell chymase: a potent secretagogue for airway gland serous cells. J. Immunol. **142**: 2450-2456, 1989.

230. Sommerhoff, C. P., W. E. Finkbeiner, J. A. Nadel and C. B. Basbaum. Prostaglandin D_2 and prostaglandin E_2 stimulate ^{35}S-labeled macromolecule secretion from cultured bovine tracheal gland serous cells. Am. Rev. Respir. Dis. **135**: A363, 1987.

231. Specian, R. D. and M. R. Neutra. Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. J. Cell Biol. **85**: 626-640, 1980.

232. Spicer, S. S., L. W. Chakrin, J. R. Wardell Jr. and W. Kendrick. Histochemistry of mucosubstances in the canine and human respiratory tract. Lab. Invest. **25**: 483-490, 1971.

233. Spicer, S. S., R. Frayser, G. Virella and B. J. Hall. Immunocytochemical localization of lysozymes in respiratory and other tissues. Lab. Invest. **36**: 282-295, 1977.

234. St. George, J. A., D. L. Cranz, S. C. Zicker, J. R. Etchison, D. L. Dungworth and C. G. Plopper. An immunohistochemical characterization of rhesus monkey respiratory secretions using monoclonal antibodies. Am. Rev. Respir. Dis. **132**: 556-563, 1985.

235. Sturgess, J. and L. Reid. An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland. Clin. Sci. **43**: 533-543, 1972.

236. Tartakoff, A., L. J. Greene and G. E. Palade. Studies on the guinea pig pancreas. J. Biol. Chem. **249**: 7420-7431, 1974.

237. Taylor, S. S. Protein kinases: a diverse family of related proteins. Bioessays. **7**: 24-29, 1987.

238. Temin, H. M. Studies on carcinogenesis by avian sarcoma viruses. III. The differential effect of serum and polyanions on multiplication of uninfected and converted cells. J. Natl. Cancer. Inst. **37**: 167-175, 1966.

239. Thurlbeck, W. M., B. Benjamin and L. Reid. Development and distribution of mucous glands in the fetal human trachea. Br. J. Dis. Chest. **550**: 49-53, 1961.

240. Todaro, G. J. and J. E. DeLarco. Growth factors produced by sarcoma virus-transformed cells. Cancer Res. **38**: 4147-4154, 1978.

241. Todaro, G. J. and H. Green. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. **17**: 299-313, 1963.

242. Tom-Moy, M., C. B. Basbaum and J. A. Nadel. Localization and release fo lysozyme from ferret trachea: effects of adrenergic and cholinergic drugs. Cell Tissue Res. **228**: 549-562, 1983.

243. Tomasi, T. B. J., E. M. Tan, A. Solomon and R. A. Prendergast. Characteristics of an immune system common to certain external secretions. J. Exp. Med. **121**: 101-124, 1965.

244. Toremalm, N. H. The daily amount of tracheobronchial secretions in man: a method for continuous tracheal aspiration in laryngectomized and tracheotomized patients. Acta Otolaryngol. (Stockh.) **Suppl. 158**: 43-53, 1960.

245. Tos, M. Development of the tracheal glands in man. Acta Pathol. Microbiol. Scand. **68** (Suppl. 185): 1-130, 1966.

246. Towbin, H., T. Staehelin and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. **76**: 4350-4354, 1979.

247. Uddman, R., J. Alumets, O. Densert, R. Hakanson and F. Sundler. Occurrence and distribution of VIP nerves in the nasal mucosa and tracheobronchial wall. Acta Otolaryngol. (Stockh.). **86**: 443-448, 1978.

248. Uddman, R., E. Moghimzadeh and F. Sundler. Occurrence and distribution of GRP-immunoreactive nerve fibres in the respiratory tract. Arch. Otorhinolaryngol. **239**: 145-151, 1984.

249. Uddman, R. and F. Sundler. Neuropeptides in the airways: a review. Am. Rev. Respir. Dis. **136**: 53-58, 1987.

250. Ueki, I., V. F. German and J. Nadel. Micropipette measurement of airway submucosal gland secretion: autonomic effects. Am. Rev. Respir. Dis. **121**: 351-357, 1980.

251. Van Lenten, L. and G. Ashwell. Studies on the chemical enzymatic modification of glycoproteins. J. Cell Biol. **246**: 1889-1894, 1971.

252. Varsano, S., C. B. Basbaum, L. S. Forsberg, D. B. Borson, G. Caughey and J. A. Nadel. Dog tracheal epithelial cells in culture synthesize sulfated macromolecular glyconjugates and release them from the cell surface upon exposure to extracellular proteinases. Exp. Lung Res. **13**: 157-183, 1987.

253. Warner, T. F. and E. A. Azen. Proline-rich proteins are present in serous cells of submucosal glands in the respiratory tract. Am. Rev. Respir. Dis. **130**: 115-118, 1984.

254. Webber, S. E. and J. G. Widdicombe. The effect of vasoactive intestinal peptide on smooth muscle tone and mucous secretion from the ferret trachea. Br. J. Pharmacol. **91**: 139-148, 1987.

255. Whimster, W. F. and L. Reid. The influence of dibutyryl cyclic adenosine monophosphate and other substances on human bronchial mucous discharge. Exp. Mol. Pathol. **18**: 234-240, 1973.

256. Widdicombe, J. G. Receptors in the trachea and bronchi of the cat. J. Physiol. (Lond.). **123**: 71-104, 1954.

257. Widdicombe, J. H. Cystic fibrosis and ß-adrenergic response of airway epithelial cell cultures. Am. J. Physiol. **251** (Regulatory Integrative Comp. Physiol. 20): R818-R822, 1986.

258. Widdicombe, J. G. Role of lipids in airway function. Eur. J. Respir. Dis. **71** (Suppl.): 197-204, 1987.

259. Widdicombe, J. H. and R. A. Barthelson. Altered chloride transport across primary cultures of tracheal epithelium in cystic fibrosis. In: *Cellular and Molecular Basis of Cystic Fibrosis*, Mastella, G. and P. M. Quinton eds., San Francisco: San Francisco Press, 1988, 377-382.

260. Widdicombe, J. H., D. L. Coleman, W. E. Finkbeiner and I. K. Tuet. Electrical properties of monolayers cultured from cells of human tracheal mucosa. J. Appl. Physiol. **58**: 1729-1735, 1985.

261. Woodward, H., B. Horsey, V. P. Bhavanandan and E. A. Davidson. Isolation, purification and properties of respiratory mucus glycoproteins. Biochemistry. **212**: 694-701, 1982.

262. Worton, R. G. and C. Duff. Karyotyping. In: *Methods in Enzymology, Vol. 58: Cell Culture*, Jakoby, W. B. and C. Pastan eds., New York: Academic Press, 1979, p. 322-344.

263. Wu, R. and D. J. Smith. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. In Vitro. **18**: 800-812, 1982.

264. Yamatake, Y. and S. Yanaura. New method for evaluating bronchomotor and bronchosecretory activities: effects of prostaglandins and antigen. Jpn. J. Pharmacol. **28**: 391-402, 1978.

265. Yanni, J. M., W. L. Smith and M. H. Foxwell. U46619 and carbocyclic thromboxane A_2-induced increases in tracheal mucous gel layer thickness. Prostaglandins Leukotrienes Essential Fatty Acids. **32**: 45-49, 1988.

266. Zanini, A., G. Giannattasio, G. Nussdorfer, R. K. Margolis, R. U. Margolis and J. Meldolesi. Molecular organization of prolactin granules. II. Characterization of glycosaminoglycans and glycoproteins fo the bovine prolactin matrix. J. Cell. Biol. **86**: 260-272, 1980.

267. Zar, J. H. "Biostatistical Analysis." 1974 Prentice-Hall, Inc. Englewood Cliffs.