Respiratory
Pharmacology and
Pharmacotherapy

Series Editors:

Dr. David Raeburn
Discovery Biology
Rhône-Poulenc Rorer Ltd
Dagenham Research Centre
Dagenham
Essex RM10 7XS
England

Dr. Mark A. Giembycz
Department of Thoracic Medicine
National Heart and Lung Institute
Imperial College of Science, Technology and Medicine
London SW3 6LY
England

Airway Mucus: Basic Mechanisms and Clinical Perspectives

Edited by
D. F. Rogers
M. I. Lethem

Springer Basel AG

Editors:

Dr. Duncan F. Rogers
Department of Thoracic Medicine
National Heart and Lung Institute
Imperial College of Science,
Technology and Medicine
Dovehouse Street
London SW3 6LY
England

Dr. Michael I. Lethem
Department of Pharmacy
University of Brighton
Lewes Road
Brighton BN2 4GJ
England

Library of Congress Cataloging-in-Publication Data

Airway mucus : basic mechanisms and clinical perspectives / edited by
D.F. Rogers, M.I. Lethem
p. cm. – (Respiratory pharmacology and pharmacotherapy)
Includes bibliographical references and index.
ISBN 978-3-0348-9809-6 ISBN 978-3-0348-8874-5 (eBook)
DOI 10.1007/978-3-0348-8874-5
1. Airway (Medicine) – Secretions. 2. Mucus. 3. Airway (Medicine) – Pathophysiology. I. Rogers, D.F. (Duncan F.), 1953- II. Lethem, M. I. (Michael I.), 1957- . III. Series.
[DNLM: 1. Bronchial Diseases – physiopathology. 2. Mucus- – physiology. WF 500 A298 1997]
QP123.A35 1997
616.2' 3 – dc21
DNLM/DLC
for Library of Congress

Deutsche Bibliothek Cataloging-in-Publication Data

Airway mucus / ed by D. F. Rogers ; M. I. Lethem – Basel ; Boston : Berlin : Birkhäuser.
(Respiratory pharmacology and pharmacotherapy)
Basic mechanisms and clinical perspectives. – 1997

ISBN 978-3-0348-9809-6
NE: Rogers, Duncan F. [Hrsg.]

The publisher and editors cannot assume any legal responsibility for information on drug dosage and administration contained in this publication. The respective user must check its accuracy by consulting other sources of reference in each individual case.

The use of registered names, trademarks, etc. in this publication, even if not identified as such, does not imply that they are exempt from the relevant protective laws and regulations or free for general use.

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, re-use of illustrations, recitation, broadcasting, reproduction on microfilms or in other ways, and storage in data banks. For any kind of use the permission of the copyright holder must be obtained.

© 1997 Springer Basel AG
Originally published by Birkhäuser Verlag in 1997
Softcover reprint of the hardcover 1st edition 1997

Printed on acid-free paper produced from chlorine-free pulp. TCF ∞

Cover design: Markus Etterich

ISBN 978-3-0348-9809-6

9 8 7 6 5 4 3 2 1

Contents

List of Contributors

Bayan Abdul-Haq, Pharmacy Department, King's College London, London, UK
Eric W. Alton, Ion Transport Unit, National Heart and Lung Institute (Imperial College), London, UK
James N. Baraniuk, Division of Rheumatology, Immunology and Allergy, Georgetown University, Washington, DC, USA
Sophie de Bentzmann, Unité INSERM 314, Université de Reims, Reims, France
Ingemar Carlstedt, Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, Lund, Sweden
Julia R. Davies, Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, Lund, Sweden
C. William Davis, Departments of Physiology and Medicine, University of North Carolina at Chapel Hill, North Carolina, USA
Denis C. K. Fung, Peptide Therapeutics, Cambridge Science Park, Cambridge, UK
Dominique Gaillard, Unité INSERM 314, Université de Reims, Reims, France
Sandra J. Gendler, Department of Biochemistry and Molecular Biology, Mayo Clinic, Scottsdale, Arizona, USA
Stuart L. James, Department of Pharmacy, University of Brighton, Brighton, UK
Peter K. Jeffery, Lung Pathology Unit, National Heart and Lung Institute, Imperial College and Royal Brompton Hospital, London, UK
Michael I. Lethem, Department of Pharmacy, University of Brighton, Brighton, UK
Dechun Li, Lung Pathology Unit, National Heart and Lung Institute, Imperial College and Royal Brompton Hospital, London, UK
Christopher Marriott, Pharmacy Department, King's College London, London, UK
Gary P. Martin, Pharmacy Department, King's College London, London, UK
Terence M. Newman, The Secretory Mechanisms Group, Department of Physiology, University College London, London, UK
Gary J. Phillips, Department of Pharmacy, University of Brighton, Brighton, UK
Edith Puchelle, Unité INSERM 314, Université de Reims, Reims, France
Charlotte Rayner, Chest Clinic, St. George's Hospital, London, UK
Paul S. Richardson, Department of Physiology, St. George's Hospital Medical School, London, UK
Duncan F. Rogers, Thoracic Medicine, National Heart and Lung Institute (Imperial College), London, UK

Mary Callaghan Rose, Center for Molecular Mechanisms of Diseases, Children's Research Institute, Children's National Medical Center, Washington, DC, USA and Departments of Pediatrics and of Biochemistry and Molecular Biology, George Washington University, Washington, DC, USA

Michael J. Sanderson, Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts, USA

John K. Sheehan, Wellcome Trust for Cell-Matrix Research, University of Manchester, School of Biological Sciences, Manchester, UK

Myra Stern, Ion Transport Unit, National Heart and Lung Institute (Imperial College), London, UK

David J. Thornton, Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, School of Biological Sciences, Manchester, UK

Ursula M. Wells, Department of Physiology, St. George's Hospital Medical School, London, UK

John G. Widdicombe, Sherrington School of Physiology, UMDS, St. Thomas's Hospital, London, UK

Robert Wilson, Host Defence Unit, Imperial College of Science, Technology and Medicine, London, UK

Atsushi Yuta, Division of Rheumatology, Immunology and Allergy, Georgetown University, Washington, DC, USA

Jean-Marie Zahm, Unité INSERM 314, Université de Reims, Reims, France

ourselves would also like to thank our panel of critical reviewers: Carol Basbaum, Tony Corfield, Jack Harkema, Matthew Marin, Philipe Roussel, Bruce Rubin and Michael Sleigh. One factor which increases the impact of the book is the colour illustrations in a number of chapters. We are indebted to Allen & Hanbury's, Glaxo Wellcome UK for sponsoring the colour reproduction. Finally, we are grateful to David Raeburn and Mark Giembycz for their vision and involvement in initiating the *Respiratory Pharmacology and Pharmacotherapy* series which has allowed us to bring respiratory tract mucus to a wider audience of lung researchers.

Duncan F. Rogers and Michael I. Lethem
December 1996

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D.F. Rogers and M.I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 1
Airway Surface Liquid: Concepts and Measurements

John G. Widdicombe

Sherrington School of Physiology, UMDS, St. Thomas's Hospital, London, UK

1. Introduction

Airway surface liquid (ASL) is the thin layer of watery solution that lies between the airway epithelium and the gas in the lumen. It is essential for the healthy function of the epithelium; in its absence cilia would be unable to beat, and mucociliary transport would be absent. In addition, various defensive mechanisms in the airway mucosa would be defective. The volume of ASL at any time and at any site in the airways must depend on the balance between inputs of liquid from the epithelium and the submucosal glands and centrally directed mucociliary transport, and the loss of liquid due to absorption of water through the epithelium, evaporation and further mucociliary transport. Any liquid that reaches the larynx will be swallowed or expectorated. The balance sheet of ASL volume will vary with different parts of the airways, since there will be different rates of secretion from submucosal glands and by the epithelium, different rates of absorption of ASL through the epithelium, and different inputs and outputs due to mucociliary transport.

2. Functions of ASL

Rogers [1] has summarised the functions ASL (Table 1). It is clear that ASL has a crucial protective role for the airways and alveoli, and is a major barrier in protecting the lungs from any potentially deleterious invader entering via the airstream. In addition, its presence and chemical composition must be essential for the normal physiological function of the lungs.

3. Sol and Gel

It is usual to subdivide ASL into a sol, or periciliary liquid (PCL) surrounding the cilia, and an overlying layer of gel thought to be derived mainly from submucosal glands and possibly from epithelial goblet cells. This concept is a sensible one, since the cilia would have difficulty in beating in the relatively viscous secretion that is thought to come from glands, and one would expect mucociliary transport to be only effective of a rather viscous layer of mucus gel could carry material up the ciliary escalator. Even if PCL and gel had identical chemical characteristics, their physical properties might be different, since if the liquid is thixotropic [2], the beating cilia might make the PCL less viscous than the gel. Experimental support for the concept of sol and gel is weak. It consists mainly of histological and

Table 1. Functions of respiratory tract fluid*

Physical barrier to inhaled airborne organisms, particles and other irritants, and to aspirated foods and liquids
Entrapment of organisms, particles and irritants
Formation of the "vehicle" on which irritants are transported by mucociliary action for clearance from the airways
Provision of a "waterproof" layer over the epithelium to limit desiccation
Humidification of inspired gas
pH buffering capacity
Lubrication
Insulation
Neutralisation of toxic gases
Selective macromolecular sieve
Source of immunoglobulins and provision of extracellular surface for their activity
Source of antibacterial and other protective enzymes and provision of extracellular surface for their activity

* In bronchial diseases associated with abnormalities in respiratory tract fluid, many of the protective functions listed above may be lost. Modified from Rogers [1].

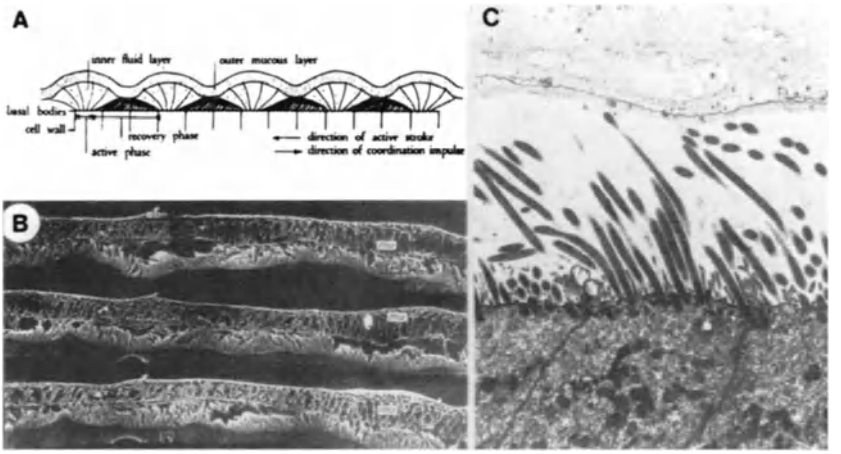


Figure 1. The two-layer hypothesis for airway secretions: (A) As originally proposed by Lucas and Douglas [3]; (B) Two layers as revealed by electron microscopy of rat trachea [4]; (C) Two layers seen in rapidly frozen preparations from rabbit tracheal cultures [7].

electron microscopic observations which show that the PCL stains more weakly for mucins and is less electronopaque than the overlying layer of secretion [3–7] (Figure 1). These results are supported by the appearance of ASL on freezing, when smaller "crystal voids" are seen in the gel, suggesting a higher mucin content [8]. Even in animals without submucosal glands, such as the rabbit, there does seem to be an overlying layer of gel, even if it is thinner than in other mammalian species [7] (Figure 1). Possibly this layer comes from epithelial goblet cells, and its density and viscosity may be influenced by epithelial ion-transport mechanisms. The other quoted evidence for separate sol and gel layers is usually derived from the fact that sputum can be centrifuged into components with high and low density, with viscosities and mucin contents also being high and low, respectively [9]. However, the relationships between sol and gel derived from sputum and their sources in the lungs have never been established, and most of the sputum specimens which have been analysed in this way come from patients with quite severe pulmonary disease. It would be interesting to see if sputum collected from healthy subjects by provocation with, for example, hyperosmolar saline aerosols could be easily separated into sol and gel components.

If the PCL were thicker than the length of the extended epithelial cilia, the sheet of gel would float above the cilia, and its transport by ciliary beat would be ineffective. If the PCL tended to contract below the tips of the cilia, the sheet of gel would be pulled tightly on the cilia, and again its transport might become ineffective. It is therefore theoretically plausible

that the thickness of the PCL is accurately controlled. One factor determining this thickness could be the capillary forces (surface tension) generated by the presence of the cilia [10, 11]. There are about eight cilia, each of diameter 200 nm, per square micrometre of apical epithelial membrane. In a cross-section through the ciliary bed at right angles to the ciliary shafts, for every epithelial square centimetre there would be 10^8 cilia, with circumference ($\pi \times$ diameter) 630 nm each, giving a total surface length of about 500 m per square centimetre. If we assume a zero contact angle and a surface tension of 25 dynes cm^{-1}, similar to body fluid, the force of capillarity for the liquid between the cilia should generate a pressure holding liquid in the PCL of about 625 cm H_2O, equivalent to a transepithelial osmotic gradient of about 25 mmol kg^{-1} (25 mOsm). This is considerably greater than the probable local osmotic pressure gradient generated by active absorption of sodium (see below), and suggests that as long as there are cilia, there will be liquid around them. However, this analysis is complicated by the presence of a mucus gel layer above the cilia. This layer would limit the production of a gas–liquid interface at the level of the cilia which is necessary for capillarity to exert its hydrostatic force. In the absence of a mucus gel layer, as is almost certainly true for bronchioles and smaller airways that lack submucosal glands, the influence of capillarity could be crucial.

4. ASL Thickness

Estimates of total ASL thickness give widely different values. It is generally agreed that the PCL is present and as deep as the length of the cilia, say 5–10 μm [4, 7]. The question is whether any mucus gel layer above the cilia is present and contributes to the total ASL thickness. Most light microscopic observations on gel thickness give values in the range 5–20 μm [4, 6, 10, 12]. With rapid freezing techniques for large airways from cows [8, 13] and monkeys [14], values as low as 0.1–2 μm have been obtained for tracheobronchial gel thickness. However, with these preparations the gel may have been cleared by mucociliary transport, a process that *in vitro* would take only a few minutes for a layer of mucosa of a few square centimetres. A similar method has given a total ASL thickness for cow trachea of 23 μm [8]. Physiological measurements give far higher values. Surface probing with optical or electrical resistance methods *in vivo* and *in vitro* give values of total ASL (PCL plus gel) thickness as high as 35 μm for sheep [15] and 100–200 μm [16, 17] or 250 μm [18] for guinea pig. A recent method of estimating tracheal ASL thickness with *in vitro* tracheas gives average values of 46 μm for ferret, 42 μm for rabbit [19] and 52 μm for guinea pig (S. Duneclift, U. Wells and J. Widdicombe, unpublished results), the rabbit and guinea pig values corresponding to estimates of 46 and 19 μm respectively, derived from a theoretical analysis of ASL thickness [12].

All these methods assess the combined PCL and gel thickness; some are *in vitro* observations, and it is difficult to say whether the thickness values apply *in vivo*. ASL thickness has usually been determined only for large airways. Rapid-freeze techniques with bronchioles (diameter 0.06–1.06 mm) in guinea pig suggest that the total ASL thickness may be as low as 0.5–2 μm [20]. Other values for rat bronchioles are 0.2–5 μm [21], 1–9 μm [22] and 0.2–0.4 μm above the cilia [23]. For humans a bronchiolar value of 1.8 μm has been determined [22]. At this airway level there are no submucosal glands and few goblet cells. We know little about ASL thickness in airways intermediate between bronchioles and primary bronchi, although one study gives values for segmental bronchi of 6.9 and 6.1 μm for humans and rats, respectively [22].

If the total ASL thickness in the larger airways is averaged to about 50 μm of which 40 μm is gel, this would correspond to 4 μl of gel per square centimetre of airway surface. For the healthy human trachea, if the mucociliary transport velocity is 5–10 mm min^{-1} towards the mouth (see later), a gel thickness of 40 μm would correspond to a tracheal mucus flow of about 2–4 μl min^{-1} per centimetre of circumference; this would be the equivalent of a total mucus flow of 18–36 ml day^{-1} if the tracheal diameter is 2 cm. This output would be far too small to measure or observe, and in fact the output of ASL at the laryngeal exit of the lungs has never been determined in healthy subjects. Estimates suggest that it is lower than 50 ml day^{-1} [24], which would fit with the estimate of 18–36 ml day^{-1} derived above.

However, deeper in the lungs one would expect a far greater flow of ASL. Kilburn [25] pointed out that the sum of the cross-sectional perimeters for all human airways of a given generation declines from about 1×10^6 cm in the 16^{th}-generation human bronchioles to 5 cm in the trachea. For a constant mucociliary transport velocity and in the absence of transepithelial liquid exchange or gland secretion, this decrease in total perimeter would in theory result in the depth of ASL being 200,000-fold greater in the trachea than in the bronchioles. In fact, from the figures quoted, the difference in thickness seems to be about 20- to 50-fold. Alternatively, mucus velocity should increase 200,000-fold from bronchioles to trachea and, although there is good evidence that mucociliary transport is slower in the smaller airways than in the larger ones, the difference is thought to the only about 10-fold [26]. Kilburn proposed that the airways actively absorb liquid from the flow of mucus, which enters them by mucociliary transport from the lower airways. If the values given above are accurate, about 99.75% of ASL should be absorbed during mucociliary transport. In support of this hypothesis, we now know that human airway epithelia absorb sodium actively (see Section 6.1), which activity carries water with it due to the creation of osmotic gradients and would tend to decrease ASL thickness (see below). The resultant effect of this absorption of water on the concentration of macromolecules has, however, not been evaluated. Their

concentrations in PCL at different airway levels has not been determined, and one has to suppose that the absorption of macromolecules takes place by an unknown mechanism in parallel with the absorption of sodium and water.

5. Composition of ASL

This has been extensively studied from analyses of sputum samples, of bronchoalveolar lavage and of direct collection of mucus gel from large airways *in vivo* and *in vitro* [27–29]. Separate analysis of the composition of PCL and the mucus gel layer does not seem to have been done, except in the case of sputum samples where the distinction between the two layers is uncertain. The only evidence of difference in chemical composition of the two ASL layers is, as already mentioned, the histological and electron microscopic differences in structure between PCL and mucus gel. The presence of a higher concentration of mucoglycoproteins in the gel layer compared with the PCL could lead to different ionic compositions, insofar as mucins bind ions and influence their free concentration. In addition, the mucins may absorb or bind various other macromolecules, such as albumin and DNA, which might not be found in the PCL. If the gel layer comes predominantly from submucosal glands, one might expect to find a higher concentration of other secretory substances from these glands, such as lysozyme and lactoferrin, than in the PCL, although this condition would depend on the ease of diffusion of substances from the gel into the PCL and whether or not they are bound in the gel.

5.1. Electrolytes

The list of chemical constituents measured in the ASL is long [27–29]. They include H^+, Na^+, K^+, Cl^-, Ca^{2+} and PO_4^{3-}, and the concentrations of these electrolytes may be quite different from those in interstitial liquid. For example, all studies agree that K^+ and probably Ca^{2+} concentrations are much greater in ASL compared with plasma. This must be partly due to the electrically negative luminal potential if these ions diffuse passively. There is no general agreement about the osmolality of ASL compared with that of interstitial liquid, although a number of studies suggest that it may be greater, with values as high as 350 mmol kg^{-1} [10, 30]. *In vivo* the osmolality would also be affected by evaporative water loss, at least during mouth breathing.

5.2. Macromolecules

The major macromolecule in ASL is mucoglycoprotein or mucin, although proteoglycans coming either from glycocalyx or from serous cells in sub-

mucosal glands are also conspicuous and probably contribute much to the physical properties of the secretions [31]. In addition, ASL contains a high content of lipids, derived partly from the transport of alveolar surfactant up into the airways, but also from secretions from the airway epithelial cells and submucosal glands [32, 33]. Albumin and other proteins derived from the plasma are present, with increased concentrations in inflammatory and other pathological conditions (see below). Immunoglobulins, derived both by plasma exudation and from tissues such as plasma cells and the epithelium, are an important component in airways defence. In addition, a large number of enzymes and antienzymes have been described, as well as many antibacterial substances and antioxidants. The constituents most studied in terms of airways defence are lactoferrin, lysozyme and secretory IgA [33]. Recent studies have concentrated on the importance of various proteases and peptidases [34], in particular neutral endopeptidase, and their role in breaking down tachykinins (such as substance P) and bradykinin, although this function may be more important in the mucosa than in the ASL. The products of cellular debris may be important in airway and lung pathological conditions, and the presence of DNA is both an important sign of cellular damage and also considerably changes mucus rheology [35].

5.3. pH

Measurements of the pH of ASL have given various values, but in general it seems to be on the acid side of neutrality [36]. The same is true for the thin layer of alveolar surface liquid [37]. However, pH will depend on the CO_2 content of the airway luminal gas, which oscillates between 0 and over 5.5%, and could change ASL pH from 7.4 to 6.3 transiently [36]. Studies on ferret trachea give a pH of 6.8 for ASL when luminal CO_2 is 5%, and this seems to be homeostatically controlled, presumably by epithelial secretion of bicarbonate and hydrogen ions [36]. The influence of the pH of ASL on, for example, the activity of the various enzymes and cellular systems in the ASL could be important, but has been little studied. Mucus rheology and ciliary actions are influenced by pH, but only when it deviates widely from normal values.

6. Determinants of ASL Volume

6.1. Ion and Water Transport

The mechanisms of ion transport by airway epithelia have been extensively studied and reviewed [38–41]. The main active ion transport mechanism throughout the airways of humans is absorption of sodium, which makes the airway lumen electrically negative compared with the submucosa by

Table 2. Water flows induced by active ion fluxes

Model	Flow rate ($\mu l\ cm^{-2}\ min^{-1}$)	Reference
Absorption		
Cow epithelium	0.12	[8]
Human epithelium	0.08	[42]
Sheep trachea	0.26	[43]
Ferret epithelium	0.20	[44]
Dog epithelium	0.17	[46]
Secretion		
Dog epithelium	0.14	[38]
Human epithelium	0.05	[42]
Ferret trachea	0.30	[44]
Dog epithelium	0.13	[45]

about 30 mV for the trachea, and induces net passive movement of chloride towards the submucosa, probably via the paracellular pathways. The transfer of salt into the mucosa across the epithelium creates osmotic gradients which result in water absorption. This absorption of sodium is inhibited by amiloride, which should therefore increase the volume of ASL. Active secretion of chloride into the lumen seems to be weak in humans, although stronger in dogs, but it can be enhanced by mediators such as isoprenaline, bradykinin, uridine triphosphate (UTP) and adenosine triphosphate (ATP). This active secretion of chloride should promote passage of water into the lumen.

The volume of water transported across the epithelium in response to active ion transport has been measured by a number of techniques (Table 2). Water flow has usually been estimated from active fluxes of ions, assuming that water flows passively to maintain isomolality, but direct volume measurements have also been made [38, 44]. Ion fluxes were promoted with various agonists. One result has been omitted [47] which gave a very low value for sodium absorption, probably for methodological seasons [39]. The mean inward flux due to sodium absorption is 0.17 $\mu l\ cm^{-2}\ min^{-1}$ or 1.7 $\mu m\ min^{-1}$. The corresponding mean values for promoted chloride secretion into the lumen are also 0.16 $\mu l\ cm^{-2}\ min^{-1}$ and 1.6 $\mu m\ min^{-1}$.

6.2. Passive Liquid Flow

Water will flow along osmotic gradients, and these have already been described when they occur in response to active ion transport across the epithelium. However, osmotic gradients may also be set up by, for example, evaporation of water from the ASL, or even by the absorption of hypo- or hyperosmolar solutions from aerosols. Hyperosmolality of ASL is

thought to be a major contributor to the bronchoconstriction and mucosal inflammation that occur during hyperventilation or cold, dry air inhalation especially in asthmatics, although the exact osmolality of ASL in these conditions has not been measured [48]. Estimates suggest it may be as high as a 1000 mmol kg^{-1} [48].

The effects of osmotic gradients on water flow have been determined for sheep trachea *in vivo*. When luminal liquid is controlled with an osmolality of 739 mmol kg^{-1}, water flows into the trachea at a rate of 0.8 µl cm^{-2} min^{-1}. Hyposmolar solutions (124 mmol kg^{-1}) cause water flow in the opposite direction of 1.2 µl cm^{-2} min^{-1} [49]. These values correspond to 0.18 and 0.71 µl cm^{-2} min^{-1} per 100 mmol kg^{-1} osmolality difference. Thus there seems to be unidirectional control of water flow under these conditions, flow into the lumen being more rapid. For bovine tracheal epithelial sheets, corresponding values are 0.71 and 0.75 µl cm^{-2} min^{-1} per 100 mmol kg^{-1} osmolality difference, showing little directional difference [50]. With canine tracheal epithelial sheets the water flux from submucosa to lumen in response to an osmotic gradient is 0.56 µl cm^{-2} min^{-1} per 100 mmol kg^{-1} osmolality difference [38].

6.2.1. Hydrostatic pressure: Hydrostatic pressure across the epithelium also determines water transport. It has been suggested that the interstitial liquid under the airway epithelium normally has a negative pressure [51], as has been described for other tissues, but the exact value of this pressure is uncertain for most of the models used. For dog tracheal epithelial sheets a submucosal pressure of 20 cm H_2O increased the rate of passive transport of ^{14}C-mannitol into the lumen more than 20-fold [52]. This transport should correspond to water flow, presumably through paracellular pathways from submucosal side to lumen, at a rate of 3.3 µl cm^{-2} min^{-1}, or 0.165 µl cm^{-2} min^{-1} per cm H_2O pressure, assuming linearity, which is uncertain. No water flow was detected with a 20-cm H_2O pressure gradient in the opposite direction (lumen to serosa). For guinea-pig trachea *in vitro*, submucosal pressures of 5 and 10 cm H_2O caused plasma equivalent fluxes of 0.039 and 0.075 µl cm^{-2} min^{-1} [53], assuming a surface area of 1 cm^2. These values correspond to 0.0078 and 0.0075 µl cm^{-2} min^{-1} per cm H_2O, suggesting linearity over this range. Why these values are 20 times smaller than for dog tracheal epithelium is not clear, although the applied pressures were smaller, and flux was across the entire tracheal wall. With the dog preparation submucosal to mucosal flow was slow at submucosal pressures at or under 10 cm H_2O, but increased dramatically when pressure was increased to 20 cm H_2O (J.H. Widdicombe, personal communication).

Thus, although quite small pressure gradients (less than 5 cm H_2O) directed towards the lumen will dilate the paracellular pathways and disrupt tight junctions of the airway epithelia, associated with large changes in hydraulic conductivity and permeability to hydrophilic molecules, the actual liquid fluxes seem to be rather small. If the epithelium is damaged,

however, fluxes could be considerably greater. For guinea-pig trachea application of inflammatory mediators such as histamine or bradykinin increased the plasma-equivalent flux to 0.18–0.22 μl cm^{-2} min^{-1} [54], assuming an exposure time of 12 min and a surface area of 1 cm^2.

When water fluxes due to osmolality and hydrostatic pressure are expressed in the same units (e.g. per cm H_2O), the former is about 25 times smaller than the latter. This may suggest that hydrostatic pressure gradients are more disruptive to the epithelium. Surface tension of ASL will cause a submucosal to mucosal hydrostatic pressure difference, greatest in the smallest airways. In bronchioles of diameter 0.5 mm with a surface tension of 25 dynes cm^{-1}, applications of Laplace's law to a cylinder gives a pressure difference of only 1 cm H_2O. For larger airways the value would be far smaller.

6.3. Submucosal Gland Secretions

The submucosal glands in the trachea and cartilaginous bronchi of most mammalian species including humans – rabbit and mouse are exceptions since they lack submucosal glands – have a density of about 1 per square millimetre. The glands contain mucous and serous acini, and because the latter are deeper, the liquid of low viscosity that they secrete probably washes the thick secretion of the mucous acini into the airway lumen [55]. The ionic composition of the secretions is similar to that of interstitial liquid [51]. Micropipetting individual glands allows estimates of secretion rates [56, 57]. At rest there may be no measurable secretion, but on stimulation by cholinergic and α-adrenergic agents the secretory rate increases by about 10 nl min^{-1}. This will give a total secretion of about 1 μl cm^{-2} min^{-1}. These figures can be compared with the total gland secretion of cow trachea, stimulated by methacholine, of 0.28 μl cm^{-2} min^{-1} [13], and of ferret trachea of about 0.33 μl cm^{-2} min^{-1} [58]. The last figure may be lower than the maximum since it is the average over 10 min and during this time the glands may have become exhausted. Measurements of the output of canine tracheal submucosal glands, stimulated reflexly and assessed from the rate of volume change of mucus displacing a thin layer of tantalum dust (hillocks), give individual gland values averaging 19 nl min^{-1}, or totalling 0.92 μl cm^{-2} min^{-1} [59]. Thus the range of submucosal gland secretion, at least for the trachea, seems to be 0–1 μl cm^{-2} min^{-1}.

6.4. Epithelial Goblet Cell Secretion

There has been no direct measurement of the volume of secretion from epithelial goblet cells, either at rest or during stimulation [60]. Based on the number of goblet and serous cells counted in the epithelium and compared

with the number of cells in mucous and serous acini in submucosal glands, it has been suggested that the secretory volume rate of the epithelial cells would be about 2.5% of that of the glands [61], which should give a maximum value of 0.025 μl cm^{-2} min^{-1}. However, it should be stressed that this value may be very inaccurate. For rabbit trachea, which lacks submucosal glands, the lysozyme output at rest is about 0.17 ng cm^{-2} min^{-1} [62], whereas for ferret trachea, which has abundant glands, the corresponding value is 25 ng cm^{-2} min^{-1} [63]. However, lysozyme secretion is an index of serous cell output and would not reflect volume changes due to mucous cells.

6.5. Evaporative Water Loss

It is generally agreed that during mouth breathing with either hyperventilation or exercise, with the inspired air colder than body temperature and/or with less than 100% humidity, there is considerable water loss from the trachea and main bronchi due to evaporation from the ASL. The exact amount of this water loss will depend on a number of factors, such as ventilation rate and inspired air temperature and humidity, but values of about 0.5 ml min^{-1} for adult humans are quoted by a number of authors [48, 64–67]. This water loss must come from the ASL, but it is not know how much comes from the trachea and various orders of bronchi, and in addition, water loss may be lessened by cooling of the respiratory gases during expiration.

If the water loss is from the first ten generations of the tracheobronchial tree [48], if they have a surface area of about 1000 cm^2 [68] and if the average ASL thickness in these airways is 10 μm, the volume of ASL would be 1 ml, so the average water loss would potentially halve the ASL thickness each minute. In addition it would potentially double ASL osmolality from 300 to 600 mmol kg^{-1}. From figures already quoted, setting up of such an osmotic gradient could draw water into the ASL at a rate of 2.1 μl cm^{-2} min^{-1}, or a total water flux of 2.1 ml min^{-1}, over four times the rate of evaporative water loss. Thus there seems to be a large margin to compensate for water loss. Put a different way, if the first ten generations of airways contributed 0.5 ml min^{-1} to water loss, and this was not compensated by water entering the ASL due to osmotic gradients, the average thickness of ASL would decrease by 5 μm min^{-1}.

6.6. Mucociliary Transport

This has been mentioned already and clearly varies enormously with the site in the tracheobronchial tree where it is present. Mucociliary transport velocity can be measured fairly easily in the large airways, including in

humans, by applying a suitable tracer in the mucus and measuring the rate at which it passes a detector. Measurements for human trachea give values varying from 5 to 20 mm min^{-1} [6, 69–73], and the differences seem to be partly methodological, with transport of large particle tracers being faster than that of small particles. Probably a thick sheet of gel would be transported faster than a thin one. If the thickness of the mucus sheet being transported is 40 μm (see earlier), the volume flow for a 5-mm min^{-1} velocity would be 2 μl cm^{-2} min^{-1}, and for a 20-min min^{-1} velocity, 8 μl cm^{-1} min^{-1}.

Of course, this liquid flow due to mucociliary transport would be compensated for by liquid input from the more peripheral airways, so in normal conditions one would expect no net change in ASL thickness due to mucociliary transport.

7. ASL and Drug Uptake

When an aerosol containing a drug is inhaled, the initial concentration in the ASL will depend on the relative volumes of aerosol and ASL, on the concentration of drug in the aerosol and on the distribution of aerosol in the airways. If there is a thick layer of ASL, the drug will be more diluted, and the initial concentration will be lower. The lower concentration results in an initial smaller rate of uptake of the drug, since uptake depends upon the concentration gradient of drug from ASL to submucosa. Thus, in conditions where ASL volume has increased, for example by mucus secretion in diseases such as cystic fibrosis and chronic bronchitis, drug concentration in ASL and rate of uptake will be smaller than if the ASL volume were less. The appropriate general equation is:

$$C_L = (C_A \cdot V_A)/(V_L + V_A) \quad (1)$$

where C_L is the concentration of drug in the ASL, C_A is the concentration of drug in the aerosol, V_A is the volume of aerosol deposited and V_L is the volume of the airway surface liquid.

If we assume that V_A is far smaller than V_L (e.g. with a particulate aerosol) and can be neglected, the equation simplifies to:

$$C_L = Q_A/V_L \quad (2)$$

where Q_A is the amount of drug deposited in the airways. Initial flux of drug (dQ/dt) into the mucosa can be derived from the permeability coefficient equation, namely:

$$dQ/dt = -P \cdot \Delta C \cdot S \quad (3)$$

where P is the permeability coefficient for the drug, ΔC is the concentration gradient across the epithelium, S is the airway surface area and the minus sign is directional.

Since $V_L = S \cdot T$ (or $S = V_L/T$), where T is the thickness of the ASL and $Q_A = C_L \cdot V_L$ (or $C_L = Q_A/V_L$), and if we assume that $\Delta C = C_L$ [12], then Eq. 3 becomes:

$$dQ/dt = -P(Q_A/V_L)/(V_L/T) \text{ or} \quad (4)$$

$$dQ/dt = -(P \cdot Q_A)/T \quad (5)$$

In other words, the initial flux of a drug into the airways is proportional to the permeability coefficient and the amount of drug deposited, and inversely proportional to the thickness of the ASL.

The percentage of uptake of a drug per unit time (%Cl) is 100 times the flux of the drug divided by the amount in solution, or:

$$\%Cl = 100(dQ/dt)/Q_A \quad (6)$$

Combining Eq. 5 and 6, it follows that

$$\%Cl = -100\ (P/T) \quad (7)$$

Thus the rate of clearance of a drug from the airway depends on the permeability of the epithelium and is inversely related to the thickness of the ASL (which determines the concentration of the drug), but is independent of amount delivered.

Similar equations can be derived if the non-simplified Eq. 1 is used.

It follows that if ASL thickness is high, deposition of a drug should be less effective, and this conclusion is supported by a number of experimental studies [74 – 77]. Furthermore, if drugs can be deposited in areas where the ASL thickness is small, for example the bronchioles and the alveoli, the rate of uptake into the body should be high. For example, if the permeabilities of a drug through the trachea and the alveoli are the same, and if the ASL thickness in the trachea is 50 μm and in the alveoli 0.15 μm [78], the deposition of a drug in the alveoli would result in over 300-times-greater uptake expressed as percent per unit time. However, this analysis, which has been developed elsewhere [12], does not take account of the role of mucociliary transport or the possible binding of drug to mucins in the ASL.

Taking values already used for evaporative water loss, if an aerosol is uniformally deposited throughout the first ten generations of airways in humans, and if the aerosol deposition rate is 0.1 ml min^{-1}, this volume will be entering a total ASL volume of 1 ml, with a resultant 10-fold dilution in the first minute. If the distribution of aerosol is restricted to larger airways with smaller surface area, then for the same ASL thickness and drug permeability the dilution will be smaller, the ASL concentration higher and the initial rate of uptake greater. As with considerations of evaporative water loss, there are few precise observations on which calculations can be based, and only general principles can be stated.

Table 3. Airway liquid flows and ASL thickness

Process	Liquid flow ($\mu l\ cm^{-2}\ min^{-1}$)	ASL thickness change ($\mu m\ min^{-1}$)
Active transport		
Sodium absorption	− 0.17	− 1.7
Chloride secretion	0.16	1.6
Inflammatory mediators	0.20	2
Secretion		
Submucosal glands	1	10
Epithelial cells	c.0.025	c.0.25
Osmolality		
Lumen to serosa	− 0.45 per 100 mmol kg^{-1}	− 4.5 per 100 mmol kg^{-1}
Serosa to lumen	0.67 per 100 mmol kg^{-1}	6.7 per 100 mmol kg^{-1}
Hydrostatic pressure	0.008 per cm H_2O	0.08 per cm H_2O
Evaporative water loss	− 0.5	− 5
Mucociliary transport	2–8	20–80

Values are averages of those given in the text. Liquid flow due to mucociliary transport would be replaced by flow from the periphery. − = loss of liquid from ASL.

8. Conclusions

It is becoming possible to consider a balance sheet for the volume of ASL, at least for the trachea of experimental animals. This is presented in Table 3. It is probably not surprising that the major items in the balance sheet are glandular secretion and mucociliary transport. Liquid movement in response to evaporative water loss, with water flux due to osmotic gradients, can also be large. The secretion of epithelial goblet and serous cells and water flux due to active ion transport are smaller components. It is important also to realise that by far the largest number of observations come from measurements on the trachea and, as indicated earlier, one would expect the balance sheet to be different for the smaller airways, where active liquid absorptive processes may dominate, permeability may be greater and ASL thickness less. The effect of such a balance of liquid flows on the concentrations of the different constituents in ASL could be extremely important, at least in relation to macromolecules. However, there is little information on this process.

Acknowledgements

I am grateful to J.H. Widdicombe for valuable and stimulating discussions, and to Jaymala Solanki for most efficient processing of the manuscript.

References

1. Rogers DF (1994) Influence of respiratory tract fluid on airway calibre. In: Raeburn D, Giembycz MA (eds). *Airways smooth muscle: Development and regulation of contractility.* Basel: Birkhäuser Verlag, 375–399.
2. King M, Rubin BK (1994) Rheology of airway mucus: Relationship with clearance function. In: Takishima T, Shimura S (eds). *Airway secretion: Physiological bases for the control of mucous hypersecretion.* New York: Marcel Dekker, 283–314.
3. Lucas AM, Douglas LC (1934) Principles underlying ciliary activity in the respiratory tract II: A comparison of nasal clearance in man, monkey and other mammals. *Arch Otolaryngol* 20: 518–541.
4. Yoneda K (1976) Mucous blanket of rat bronchus: An ultrastructural study. *Am Rev Respir Dis* 114: 837–842.
5. Wanner A (1977) Clinical aspects of mucociliary transport. *Am Rev Respir Dis* 116: 73–125.
6. Wanner A (1994) Possible control of airway hypersecretion. In: Takishima T, Shimura S (eds). *Airway secretion: Physiological bases for the control of mucous hypersecretion.* New York: Marcel Dekker, 629–646.
7. Sanderson MJ, Sleigh MA (1981) Ciliary activity of cultured rabbit tracheal epithelium: Beat pattern and metachrony. *J Cell Sci* 47: 331–347.
8. Wu DX-Y, Lee CYC, Uyekubo SN, Choi H, Bastacky SJ, Widdicombe JH. Physiological regulation of the depth of airway surface liquid. *J Gen Physiol.* In press.
9. Braga PC, Allegra L (1988) *Methods in bronchial mucology.* New York: Raven Press, 407.
10. Widdicombe JH, Widdicombe JG (1995) Regulation of human airway surface liquid. *Respir Physiol* 99: 3–12.
11. Widdicombe JG (1988) Force of capillarity tending to prevent drying of ciliary mucosa. In: Kaliner MA, Barnes PJ (eds). *The airways.* New York: Marcel Dekker, 597.
12. Widdicombe JG (1997) Airway and alveolar permeability and surface liquid thickness: Theory. *J Appl Physiol* 82: 3–12.
13. Wu DX-Y, Lee CYC, Widdicombe JH, Bastacky J (1996) Ultrastructure of tracheal surface liquid: Low-temperature scanning electron microscopy. *Scanning* 18: 1–4.
14. Dupuit F, Bout A, Hinnrasky J, Fuchey C, Zahm J-M, Imler J-L, Pavirani A, Valerio D, Puchelle E (1995) Expression and localization of CFTR in the Rhesus monkey surface airway epithelium. *Gene Therapy* 2: 156–163.
15. Seybold ZV, Mariassy AT, Stroh D, Kim CS, Gazeroglu H, Wanner A (1990) Mucociliary interaction *in vitro*: Effects of physiological and inflammatory stimuli. *J Appl Physiol* 68: 1421–1426.
16. Shephard KL, Rahmoune H (1994) Evaporation-induced changes in airway surface liquid on an isolated guinea pig trachea. *J Appl Physiol* 76: 1156–1165.
17. Rahmoune H, Shepard KL (1995) State of airway surface liquid on guinea pig trachea. *J Appl Physiol* 78: 2020–2024.
18. Yanni JM, Smith WL, Callaham EM, Foxwell MH, Alphin RS (1984) A method for measuring changes in tracheal mucus gel layer thickness as an indication of an immediate hypersensitivity response. *Int Arch Allergy Appl Immunol* 74: 351–355.
19. Duneclift S, Wells U, Widdicombe J (1997) Estimation of the thickness of airway surface liquid in the ferret trachea *in vitro. J Appl Physiol.* In press.
20. Yager D, Cloutier T, Feldman H, Bastacky J, Drazen JM, Kanm RD (1994) Airway surface liquid thickness as a function of lung volume in small airways of the guinea pig. *J Appl Physiol* 77: 2333–2340.
21. Ebert RV, Terraico MJ (1975) Observation of the secretion of the surface of the bronchioles with the scanning electron microscope. *Am Rev Respir Dis* 112: 491–496.
22. Mercer RR, Russell ML, Crapo JD (1992) Mucus lining layer in human and rat airways. *Am Rev Respir Dis* 145: 355.
23. Gil J, Weibel ER (1971) Extracellular lining of bronchioles after perfusion fixation of rat lungs for electron microscopy. *Anat Rec* 169: 185–200.
24. Richardson PS, Somerville M, Sheehan JK (1992) Airway mucus: What is it and how does it alter in disease? *Eur Resp Rev* 2: 263–266.
25. Kilburn KH (1968) A hypothesis for pulmonary clearance and its implications. *Am Rev Respir Dis* 98: 449–463.

26. Iravani J, Van As A (1972) Mucus transport in the tracheobronchial tree of normal and bronchitic rats. *J Pathol* 106: 81–93.
27. Boat TF, Cheng P-W, Leigh MW (1994) Biochemistry of mucus. In: Takishima T, Shimura S (eds). *Airway secretion.* New York: Marcel Dekker, 217–282.
28. Boat TF, Cheng PW (1980 Biochemistry of airway mucus secretions. *Fed Proc* 39: 3067–3074.
29. Richardson PS, Fung DCK (1992) Mucus and mucus-secreting cells in asthma. In: Barnes PJ, Rodger IW, Thomson NC (eds). *Asthma: Basic mechanisms and clinical management.* London: Academic Press, 157–190.
30. Robinson NP, Kyle H, Webber SE, Widdicombe JG (1989) Electrolyte and other chemical concentrations in the tracheal airway and mucus. *J Appl Physiol* 66: 2129–2135.
31. Kim KC, Opaskar-Hincman H, Bhaskar KR (1989) Secretions from primary hamster tracheal surface epithelial cells in culture: Mucin-like glycoproteins, proteoglycans and lipids. *Exp Lung Res* 15: 299–314.
32. Matthews LM, Spector S, Lemm J, Potter JL (1963) Studies on pulmonary secretions. *Am Rev Respir Dis* 88: 199–204.
33. Puchelle E, Girod-de Bentzmann S, Jacquot J (1992) Airway defence mechanisms in relation to biochemical and physical properties of mucus. *Eur Resp Rev* 2: 259–263.
34. Basbaum CB, Jany B, Finkbeiner WE (1990) The serous cell. *Annu Rev Physiol* 52: 97–113.
35. Mygind N, Brofeldt S, Ostberg B, Cerkez V, Tos M, Marriott C (1987) Upper respiratory tract secretions: Pathophysiology. In: Clarke SW, Lopez-Vidriero MT, Pavia D, Snell NJC, Widdicombe JG (eds). *Respiratory tract secretions. Eur J Resp Dis* Suppl 153, 71: 26–33.
36. Kyle H, Ward JPT, Widdicombe JG (1990) Control of pH of airway surface liquid of the ferret trachea. *J Appl Physiol* 68: 135–140.
37. Nielson DW, Goerke J, Clements JA (1981) Alveolar subphase pH in the lungs of unanesthetized rabbits. *Proc Nat Acad Sci USA* 78: 7119–7123.
38. Welsh MJ, Widdicombe JH, Nadel JA (1980) Fluid transport across the canine tracheal epithelium. *J Appl Physiol* 50: 905 – 909.
39. Widdicombe JH (1994) Ion and fluid transport by airway epithelium. In: Takishima T, Shimura S (eds): *Airway secretion.* New York: Marcel Dekker, 399–431.
40. Welsh MJ (1987) Electrolyte transport by airway epithelia. *Physiol Rev* 67: 1143–1162.
41. Widdicombe JH, Miller SS, Finkbinder WE (1994) Altered regulation of airway fluid content in cystic fibrosis. In: Rodge JA, Brock DJH, Widdicombe JH (eds). *Cystic fibrosis.* Chichester: John Wiley & Sons, 1111.
42. Jiang C, Finkbeiner WE, Widdicombe JH, McCray PB, Miller SS (1993) Altered fluid transport across airway epithelium in cystic fibrosis. *Science* 262: 424–427.
43. Phipps RJ, Torrealba PJ, Lauredo IT, Denas SM, Sielczak MW, Ahmed A, Abraham WM, Wanner A (1987) Bacterial pneumonia stimulates macromolecule secretion and ion water fluxes in sheep trachea. *J Appl Physiol* 62: 2388–2397.
44. Loughlin GM, Gerencser GA, Crowder MR, Boyd RL, Mangos JA (1982) Fluid fluxes in the ferret trachea. *Experientia* 38: 1451–1452.
45. Marin MG, Davis B, Nadel JA (1976) Effect of acetylcholine on Cl^- and Na^+ fluxes across dog tracheal epithelium *in vitro. Am J Physiol* 231: 1546–1549.
46. Boucher RC, Stutts ME, Gatzy JT (1981) Regional differences in bioelectric properties and ion flow in excised canine airways. *J Appl Physiol* 51: 706–714.
47. Nathanson IT, Widdicombe JH, Nadel JH (1983) Effect of amphotericin B on ion and fluid movement across dog tracheal epithelium. *J Appl Physiol* 55: 1257–1261.
48. Anderson SD (1992) Asthma provoked by exercise, hyperventilation and the inhalation of non-isotonic aerosols. In: Barnes PJ, Rodger IW, Thomson NC (eds). *Asthma.* Basic mechanisms and clinical management. London: Academic Press, 473–490.
49. Wells UM, Hanafi Z, Widdicombe JG (1994) Osmolality alters tracheal blood flow and tracer uptake in anaesthetized sheep. *J Appl Physiol* 77: 2400–2407.
50. Durand J, Durand-Arczynska W, Schoenenweid F (1986) Oxygen consumption and active sodium and chloride transport in bovine tracheal epithelium. *J Physiol* 372: 51–62.
51. Koller M-E, Woie K, Reed RK (1993) Increased negativity of interstitial fluid pressure in rat trachea after mast cell degranulation. *J Appl Physiol* 74: 2135–2139.
52. Kondo M, Finkbeiner WE, Widdicombe JH (1992) Changes in permeability of dog tracheal epithelium in response to hydrostatic pressure. *Am J Physiol* 262: 176–182.

53. Persson CGA, Erjefalt I, Gastafsson B, Luts A (1990) Subepithelial hydrostatic pressure may regulate plasma exudation across the mucosa. *Int Arch Allergy Appl Immun* 92: 148–153.
54. Erjefalt I, Persson CGA (1989) Inflammatory passage of plasma macromolecules into airway wall and lumen. *Pulm Pharmacol* 2: 93–102.
55. Meyrick B, Sturges JM, Reid L (1969) A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. *Thorax* 24: 729–736.
56. Quinton PM (1979) composition and control of secretions from tracheal bronchial submucosal glands. *Nature* 279: 551–552.
57. Ueki I, German VF, Nadel J (1980) Micropipette measurement of airway submucosal gland secretion: Autonomic effects. *Am Rev Respir Dis* 121: 351–357.
58. Webber SE, Widdicombe JG (1989) The transport of albumin across the ferret *in vitro* whole trachea. *J Physiol* 408: 457–472.
59. Davis B, Roberts AM, Coleridge HM, Coleridge JCG (1982) Reflex tracheal gland secretion evoked by stimulation of bronchial C-fibres in dogs. *J Appl Physiol* 53: 985–991.
60. Kim KC (1994) Epithelial goblet cell secretion. In: Takishima T, Shimura S (eds). *Airway secretion: Physiological bases for the control of mucous hypersecretion.* New York: Marcel Dekker, pp 433–449.
61. Reid L (1992) Measurement of the bronchial mucous gland layer: A diagnostic yardstick in chronic bronchitis. *Thorax* 91: 1590–1597.
62. Price AM, Webber SE, Widdicombe JG (1990) Transport of albumin by the rabbit trachea *in vitro*. *J Appl Physiol* 68: 726–730.
63. Price AM, Webber SE, Widdicombe JG (1993) Osmolality affects ion and water fluxes and secretion in the ferret trachea. *J Appl Physiol* 74: 2788–2794.
64. Smith CM, Anderson SD (1986) Hyperosmolarity as the stimulus to asthma induced by hyperventilation? *J Allergy Clin Immunol* 77: 729–736.
65. Solway J (1992) Respiratory air conditioning and the bronchial circulation. In: Butler J (ed). The bronchial circulation. New York: Marcel Dekker, 291–336.
66. Ray DW, Ingenito EP, Sterk M, Schumacker PT, Solway J (1989) Longitudinal distribution of canine respiratory tract heat water exchanges. *J Appl Physiol* 66: 2788–2798.
67. Tabka Z, Ben Jelria A, Guenard H (1987) Effect of breathing dry warm air on respiratory water loss at rest and during exercise. *Respir Physiol* 67: 115–125.
68. Weibel ER (1964) Morphometrics of the lung. In: Fenn WO, Rahn H (eds). *Handbook of physiology, respiration.* Washington DC: American Physiological Society, 285–307.
69. Yeates D, Aspin N, Levison H, Jones MT, Bryan AC (1975) Mucociliary tracheal transport rates in man. *J Appl Physiol* 39: 487–495.
70. Morrow PE, Gibb FR, Gazioglu KM (1967) A study of particulate clearance from the human lungs. *Am Rev Respir Dis* 96: 1209–1221.
71. Santa Cruz R, Landa J, Hirsch A, Sackner MA (1974) Tracheal mucous velocity in normal man and patients with obstructive lung disease: Effects of terbutaline. *Am Rev Respir Dis* 109: 459–463.
72. Wagner HN, Lopez-Majano V, Langan JK (1965) Clearance of particulate matter from the tracheobronchial tree in patients with tuberculosis. *Nature* 205: 252–254.
73. Annis P, Landa J, Lichtiger M (1976) Effects of atropine on velocity of tracheal mucus in anesthetized patients. *Anesthesiol* 44: 74–77.
74. King M, Kelly S, Costo M (1985) Alteration of airway reactivity by mucus. *Respir Physiol* 62: 47–59.
75. Kim CS, Eldridge A, Wanner A (1988) Airway responsiveness to inhaled and intravenous carbachol in sheep: Effect of airway mucus. *J Appl Physiol* 65: 2744–2751.
76. Abraham WM, Chapman GA, Marchette B (1983) Differences between inhaled and intravenous carbachol in detecting O_3 induced airway effects. *Environ Res* 35: 430–438.
77. Shore SA, Kariya ST, Anderson K, Skornik W, Feldman HA, Pennington J, Godleski J, Drazen JM (1987) Sulfur dioxide-induced bronchitis in dogs: Effects on airway responsiveness to inhaled and intravenously administered methacholine. *Am Rev Respir Dis* 135: 840–847.
78. Weibel ER, Gil J (1968) Electron microscopic demonstration of an extracellular duplex lining layer of alveoli. *Respir Physiol* 4: 42–57.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D.F. Rogers and M.I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 2
Structure and Biochemistry of Human Respiratory Mucins

David J. Thornton*, Julia R. Davies[1], Ingemar Carlstedt[1]
and John K. Sheehan

Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, School of Biological Sciences, Manchester, UK
[1] *Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, Lund, Sweden*

1. Introduction

Mucus is a highly hydrated, uniquely structured gel that, in conjunction with the ciliated cells in the surface epithelium, forms the mucociliary clearance system essential for the protection of the respiratory tract [1]. The polymer matrix of the mucus biofilm is provided by very large complex glycoproteins that were formerly known as mucus glycoproteins but which are now commonly referred to as mucins [2–7]. Mucins are high-M_r, extensively O-linked glycoproteins of exceptional mass, size and daunting complexity that are synthesised by cells in both the surface epithelium and in the underlying submucosal glands. In normal airways, mucus production

* Author for correspondence.

is maintained at a relatively low level, whereas in pathological conditions, such as asthma, cystic fibrosis and chronic bronchitis, mucus hypersecretion may cause major problems in airway clearance, resulting in impaired gas exchange and bacterial colonisation leading to infection and lung damage. In this chapter we will focus on the large polymeric mucins that are responsible for the formation of the gel-like protective barrier in human airways.

2. General Properties of Mucins

It is now well established that the gel-forming mucins from respiratory tract secretions, as well as those from cervical and gastrointestinal mucus, have similar physical and chemical properties and that all conform to the same structural design [4–9]. Respiratory mucins are high-M_r glycoproteins (Table 1) that are polydisperse in mass with values of M_r of the order $2-40\times10^6$ [4–7, 10–12]. In dilute solution, respiratory mucins behave as random coils, whilst under the electron microscope (Figure 1 A), they appear as long, linear and apparently flexible threads which are polydisperse in length (0.5–10 μm) [2–6, 9, 13–16]. A characteristic feature of mucins is their high content of carbohydrate, primarily in the form of short O-linked oligosaccharide chains, which can total as much as 80% of the weight of the macromolecule [17].

2.1. *Macromolecular Architecture*

Treating the large gel-forming mucins with reducing agents causes a dramatic decrease in their molecular mass and size [4–7, 10–12, 18]. The major products of reduction are the mucin monomer units that we term reduced mucin subunits, and in our hands these monomers have M_r

Table 1. Size of gel-forming respiratory mucins

Airway/disease	$M_r \times 10^{-6}$	Ref.
Normal	14–16	[4]
	15#	[11]
Asthma	11.5, 37	[6]
Cystic fibrosis	17*	[5]
	9.3	[10]
Chronic bronchitis	13, 14, 19, 20, 21, 29	[7]

Larger species from Sephacryl S-1000 chromatography.
* Void volume fraction from Sepharose CL-2B chromatography.

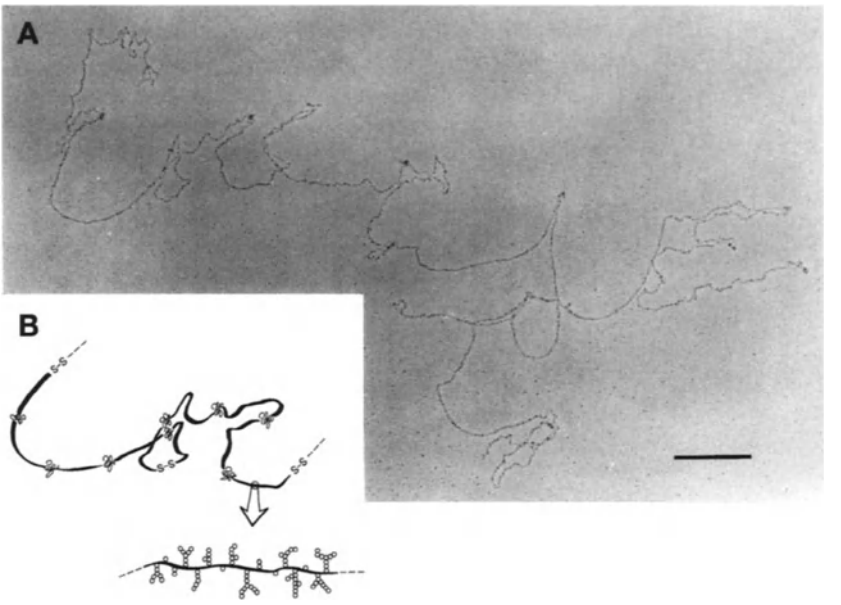


Figure 1. (A) Electron micrograph of large gel-forming respiratory mucins and (B) schematic representation of their macromolecular architecture. (A) Respiratory mucins visualised in the electron microscope [9] appear as long, entangled linear threads. The bar represents 300 nm. (B) Shown inset is an expanded schematic view of a portion of the mucin encompassing approximately two mucin subunits (monomers) each about 500 nm in length. The subunits are joined end to end via disulphide bonds (S-S in the diagram) and are comprised of oligosaccharide-rich regions (represented by the thickened line), approximately 100 nm in length, alternating with folded domains stabilised by disulphide bonds (represented by the knots). A blown-up representation of a section of the oligosaccharide-rich regions (not drawn to scale) highlights the diversity and density of the attached O-linked glycans (open circles).

$2-3\times10^6$ and size 400–600 nm. The reduction process not only disrupts intermolecular disulphide bonds but also results in unfolding of internal domains to expose previously cryptic regions of the mucin core protein [19]. The core protein of a monomer is likely to represent the primary gene product, but it cannot be ruled out that this has been post-translationally cleaved. In addition, reduction may release protein-rich fragments that are either proteolytically cleaved parts of the mucin apoprotein or attached (specifically or non-specifically) non-mucin protein. Proteinase treatment of reduced subunits yields high-M_r glycopeptides (M_r 300,000–500,000), and these fragments contain the majority of the O-linked oligosaccharides [4, 5, 7]. The protein backbone of the reduced subunit is thus composed of alternating oligosaccharide-rich, proteinase-resistant domains and proteinase-sensitive "naked" regions. The protein core of the oligosaccharide clusters is enriched in serine, threonine and proline (STP-rich regions), and

the former two amino acids are the sites of O-glycan attachment via the linkage sugar *N*-acetylgalactosamine. The cysteine residues appear to be confined to the "naked" domains, which are likely to be folded and stabilised by disulphide bonds. Electron microscopy of mucins before and after reduction [4, 5] together with a wealth of physical data [4–7] has led us to propose that intact mucins are polymers formed from disulphide bond-linked monomers (subunits) joined end to end in a linear array. A schematic representation of the model for mucin architecture is shown in Figure 1b).

The polydispersity in mass and size of the mucins arises, in part, from the oligomerisation of a variable number of their constituent monomers. It is clear from physical studies that these oligomeric mucins play the major role in determining the mechanical properties of the gel. However, it is not clear to what extent the rheological characteristics of the gel depend on entanglement of the large macromolecules, as opposed to molecular interactions, such as hydrogen bonds between the oligosaccharides [12]. The gel can be disrupted by non-covalent bond-breaking solvents, and this process is dramatically enhanced by the use of reducing agents [6], suggesting that entanglement is a key feature in the formation of mucus.

2.2. Mucin Apoproteins

Mucins are emerging as a large family of glycoproteins, and cDNA cloning has so far identified at least eight different human mucin genes [20–28]. Deduced amino acid sequences have confirmed the presence of STP-rich and cysteine-rich domains and highlighted some hitherto unexpected features of mucin apoprotein structure. For example, a characteristic feature of many, though not all, of the identified mucins is the repetitive nature of the glycan attachment STP-rich regions. These motifs are tandemly repeated along the mucin apoprotein, and their number can vary. The corresponding genes are polymorphic, and as a consequence of the variable number of tandemly repeated nucleotides, this region of the gene is known as the *v*ariable *n*umber *t*andem *r*epeat (VNTR) [29]. To date, the sequence of the tandem repeats has been found to be different for each of the mucins identified (for a review, see [30]. As predicted from biochemical data, these glycan attachment regions are flanked by "naked" cysteine-rich domains (see Figure 1b). Another feature to arise from cDNA cloning studies is the observed sequence homology between the "naked" protein cysteine-rich domains of two human mucins (MUC2 and MUC5AC) and the polymeric glycoprotein von Willebrand factor [31–33]. This latter glycoprotein is vital for normal haemostatic function, where it is required for adhesion of platelets to sites of vascular injury and for binding and stabilising blood coagulation factor VIII (for more detail, see [34 and 35]). Some of the cysteine-rich domains of von Willebrand factor are involved in its disulphide bondmediated oligomerisation to yield macromolecules that,

like mucins, are long, linear and polydisperse in both mass and size [34, 35], and it seems likely that these mucins may oligomerise in a similar fashion.

2.3. mRNA Expression

Mucin genes have been given the nomenclature "*MUC*" genes and have been numbered in order of discovery. In the case of *MUC5*, however, three groups of cDNA clones (designated 5A, 5B and 5C) were originally identified that all mapped to the same chromosomal band (11p15). Subsequently, the clones for 5A and 5C have been shown to be part of the same gene (*MUC5AC*), and there is now clear evidence that 5B clones are part of a distinct gene (*MUC5B*) [26]. For more detailed information on mucin genes see Chapter 3.

In situ hybridisation and Northern analyses have shown that a least seven mucin genes (*MUC1, 2, 3, 4, 5AC, 5B* and *7*) are expressed in the respiratory epithelium [24, 36–41], but the individual contribution of their products to the formation of the mucus gel is unclear. cDNA sequencing studies demonstrate that two of these (MUC1 and MUC7) are not large, disulphide bond-linked molecules [28, 42], and it is unlikely, given the deduced structure of these two mucins, that either makes a significant contribution to the physical properties of the gel. The expression of MUC3 mRNA has been localised to many cell types in both the surface epithelium and glands in human airways [38]. This has led to the suggestion that it may be a cell-surface/glycocalyx-located mucin [38] and, as such, would not be expected to contribute to the secretion. Of the other mucins, only MUC2 and MUC5AC have been demonstrated to be of the large gel-forming type [43–47], whereas this has yet to be shown for MUC4 and MUC5B.

2.4. Mucin Oligosaccharides

A characteristic feature of mucins is their high carbohydrate content, and the vast majority of their glycans are attached via O-glycosidic linkage between *N*-acetylgalactosamine (GalNAc) and the hydroxyamino acids serine and threonine in the STP-rich region of the mucin apoprotein. In addition to GalNAc, mucin oligosaccharides may also contain *N*-acetylglucosamine (GlcNAc), galactose (Gal), fucose, sialic acids and sulphate. Detailed studies of respiratory mucin oligosaccharides have revealed their astounding diversity (e.g. [48–50]) with, for example, as many as 88 different oligosaccharide structures identified so far from the mucins of a single individual [51]. It is clear, considering the number of different apomucins expressed in the respiratory tract, that these studies were probably performed on mixtures of mucins rather than on distinct populations. Thus,

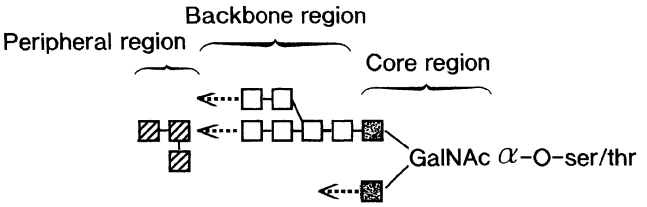


Figure 2. Schematic diagram of mucin O-linked oligosaccharides. The diagram shows the three regions (core, backbone and peripheral) of the oligosaccharide structure. The arrows represent regions of possible elongation of the structure.

the diversity of structures may appear greater than would be present on *individual* mucin species.

The oligosaccharide chains may contain as many as twenty (or more) monosaccharides and have been defined as consisting of three regions (Figure 2); the core region closest to the apoprotein, the backbone or extension region and the peripheral region [52]. The core region is formed by the addition of one or two sugars to the initial *N*-acetylgalactosamine residue, and at least five core structures have so far been identified in respiratory mucins [53]. The backbone, or extension region, contains repeating disaccharide units of Galβ1-3GlcNAc (type 1) or Galβ1-4GlcNAc (type 2), and both type 1 and type 2 backbones may be present in mucins from the same individual. Substitution at the 3 and 6 positions of the galactose residues may increase the complexity of the chains by the formation of branched structures. Chain elongation is terminated in the peripheral region by the addition, via an α-linkage, of GalNAc, GlcNAc, Gal, fucose or sialic acids. In addition, sulphate groups may be added [54]. A variety, of "determinants" are expressed in the peripheral region of mucin oligosaccharides, including the ABH blood group structures and the various Lewis structures (Le^a, Le^b, Le^x and Le^y).

In addition to the large numbers of O-linked oligosaccharide chains, it is becoming increasingly evident that small amounts of N-linked oligosaccharide are present in mucins. These appear to be of importance for the correct folding of the naked regions of the core protein essential for the initial stages of oligomerisation of the mucin monomers. Treatment of mucin-secreting cells or tissues in culture with tunicamycin abolishes oligomerisation [55].

The diversity of oligosaccharide structures on mucins reflects the expression of a large number of different glycosyltransferases in the secretory cells. If these transferases are expressed heterogeneously in cells producing the same apoprotein, it is likely that variants (glycoforms) with different glycosylation will appear. The resultant mucin glycoforms may have different properties which could, for example, have consequences for mucus gel formation [12] or interactions with respiratory pathogens.

3. Mucin Heterogeneity

Owing to the high level of substitution of mucins with O-linked glycans, many of the studies on the tissue distribution of these glycoproteins in the respiratory tract were performed with reagents reactive with carbohydrates [56–58]. Using classical histological stains for glycoproteins, i.e. periodate-Schiff's reagent (PAS), Alcian Blue (AB) at different pH values (pH 1.0 or pH 2.5), alone or in combination with PAS, and the high-iron diamine (HID) reagent, it has been suggested that several mucins are produced in the respiratory tract [56–58]. Lectin histochemistry also lends support to the idea that different families of mucins are present in the airways [59]. Stored within the mucin-producing cells in the surface epithelium and the underlying submucosal glands are both so-called neutral (PAS-positive) and acidic glycoproteins, the latter group being subdivided into sialylated (AB pH 2.5-positive) and sulphated (AB pH 1.0- or pH 2.5- and HID-positive) glycoconjugates, and the relative staining patterns with these reagents have been shown to change in disease [56–58]. By adapting some of the methods (e.g. PAS, AB and HID) used for the identification of glycoproteins in tissue sections, it has been possible to demonstrate different populations of mucins which may be described as PAS,-, AB- or HID-rich [60, 61] and that these account for at least some of the staining observed with these reagents in tissue sections. These studies also show that the so-called neutral PAS-rich mucins, in the nomenclature of the histologist, are in fact negatively charged molecules.

It is evident from mRNA expression and histological studies that respiratory secretions contain a mixture of mucin gene products and that the different apoproteins may exist in a number of glycoforms. In our most recent investigations, we have analysed respiratory secretions in order to assign a genetic identity to each of the gel-forming, oligomeric mucin species present and determine whether they are present as different glycoforms. To this end, we have employed density-gradient centrifugation, ion-exchange chromatography and agarose gel electrophoresis to identify three mucin populations which together comprise the bulk of the gel-forming species [46, 47, 62], and an example is shown in Figure 3. These three components are all high-M_r polymeric species that are fragmented into their constituent subunits by reducing agents, and as such all conform to the macromolecular design described earlier.

One of the mucins was identified as the product of the *MUC5AC* gene, whilst the genetic identity of the other two, which for the purpose of this article we will term MUCX and MUCY, has yet to be elucidated. On the basis of their lack of reactivity with a number of antisera raised against the MUC2 mucin, and the difference in their amino acid composition compared to that deduced for MUC2, we propose that they are not products of the MUC2 gene leading to the conclusion that this mucin is not a major gel-forming glycoprotein in the respiratory tract [46, 47]. It remains to be seen

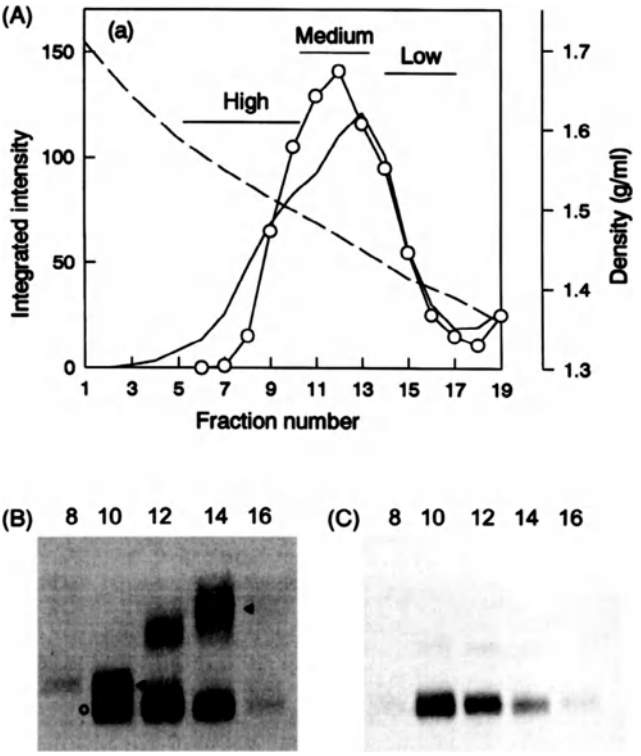


Figure 3 A–D. Identification of the oligomeric gel-forming mucins from respiratory secretions. Respiratory secretions were extracted with guanidinium chloride and mucins purified by CsCl density-gradient centrifugation [4]. (A) Shows an example of a density gradient performed in CsCl/0.2 M guanidinium chloride highlighting the total mucin (solid line) and MUC5AC mucin distributions (open circles). The MUC5AC mucin was detected with a polyclonal antiserum raised to a peptide sequence in non-TR regions [46, 47]. Reduced subunits from selected fractions across the total mucin distribution were analysed by agarose gel electrophoresis [85] followed by a Western blot and analysis with (B) a general mucin antiserum [19]. Three mucin subunit bands are seen, one of which was reactive with the MUC5AC antiserum (open circles) (C), whereas the other two mucins, as yet unidentified, are referred to here as MUCX (filled arrowhead) and MUCY (open arrowhead). (D) Mucins from the density gradient were pooled into high-, medium- and low-density fractions, as shown by the bars in (A), and reduced subunits were separated by ion-exchange chromatography (Mono Q). The distributions were analysed as in (A), and three peaks were resolved. The mucin of intermediate charge density is the MUC5AC mucin, and the least- and highest-charged components correspond to MUCX and MUCY, respectively. The bands corresponding to the three mucins after (B) electrophoresis and (D) ion-exchange chromatography are highlighted as follows: MUCX, filled arrowhead; MUC5AC, open circle; MUCY, open arrowhead.

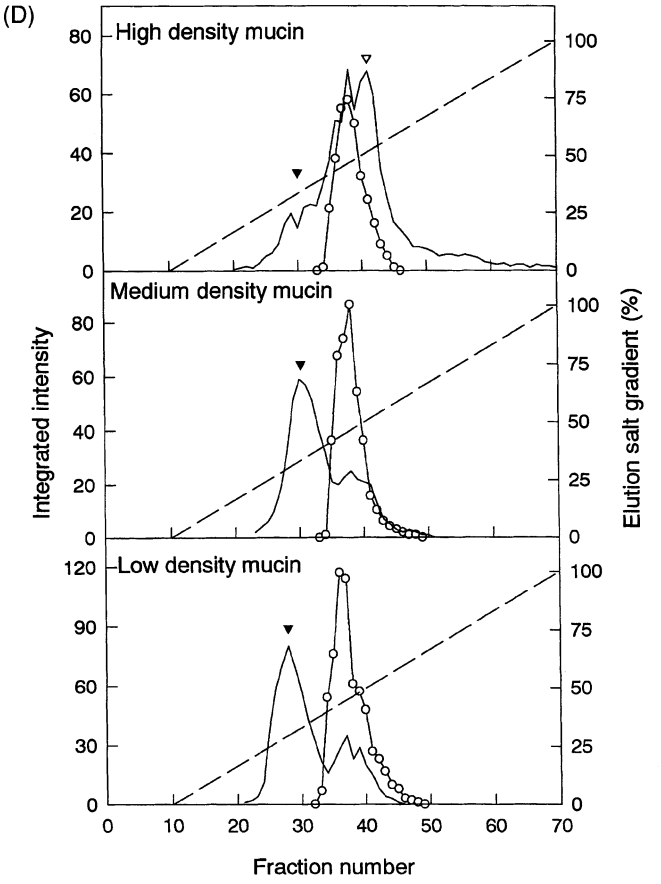


Figure 3 D.

whether the apoproteins of MUCX and MUCY are the products of new, as yet unidentified, mucin genes.

In studies of the normal and diseased respiratory tract, the mucus gel contains variable amounts of the three mucins identified, and it is interesting that in some samples one mucin may dominate whilst being virtually absent from another (Figure 4). In the normal secretions studied to date, the MUC5AC mucin appears always to be a prominent component [46, 47]. Of particular interest is the apparent conservation of the charge density of the three mucins, as judged by electrophoresis and ion-exchange chromatography [46, 47, 63], between healthy individuals and pathologic conditions (Figure 4). The distinctive pattern of glycosylation, at least at the level of charge density, associated with each of the mucins may reflect their different cellular origins.

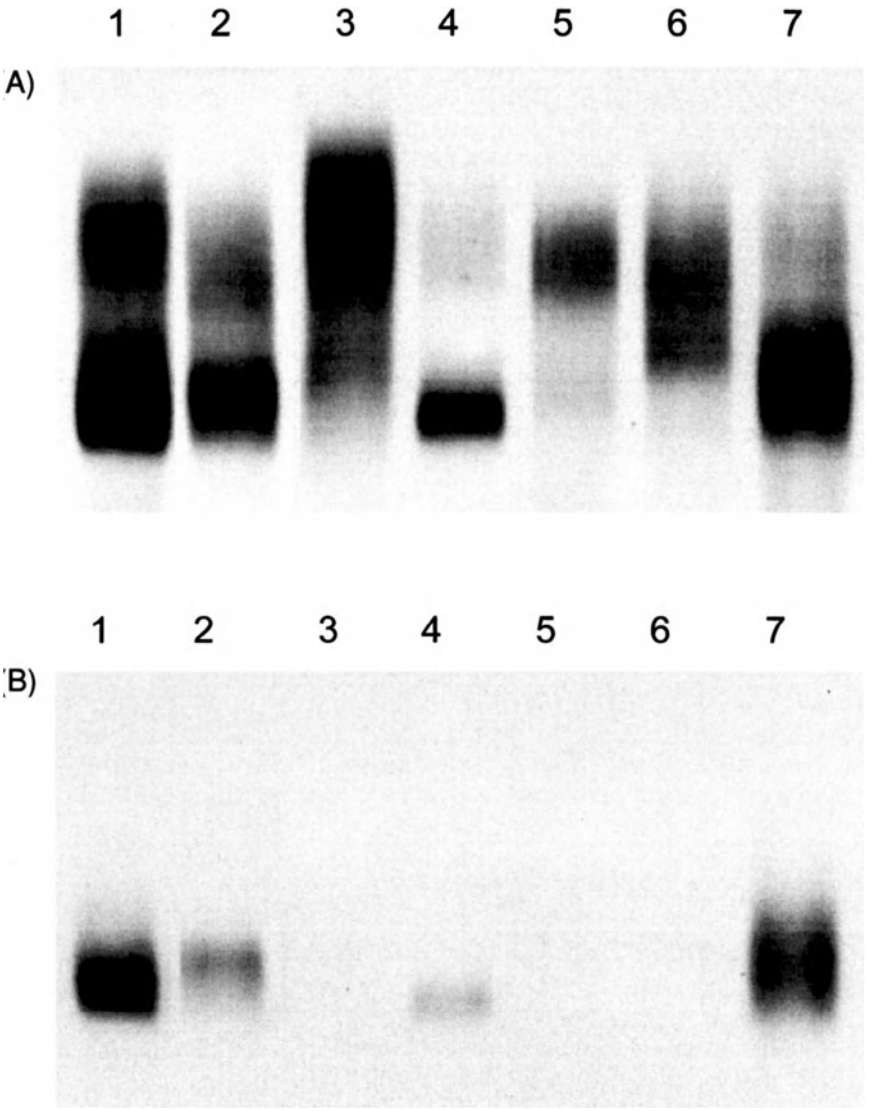


Figure 4. Agarose gel electrophoresis of gel-forming mucins from respiratory secretions. Reduced subunits from the gel-forming respiratory mucins were analysed by agarose gel electrophoresis [85]. Western blots of the gels were probed in (A) with a general mucin antiserum and in (B) with one specific for the MUC5AC mucin. Lane 1: Pooled secretions from normal individuals; lanes 2 and 4: asthma; lanes 3, 6 and 7: chronic bronchitis; lane 5: cystic fibrosis. It is clear that these samples contain variable amounts of the different mucins. However, due to the limited number of samples analysed to date, no conclusions can be drawn relating the level of any one of them to any pathological condition.

4. Goblet Cell and Submucosal Gland Mucins

In situ hybridisation data show that the goblet cells and mucous cells of the submucosal glands express different mucin apoprotein mRNAs, but until recently, there was no biochemical evidence to support the notion that the mucins produced by the two cells are different. However, Hovenberg and colleagues [63] have demonstrated that the MUC5AC mucin is a product of the goblet cells (Figure 5), and this is consistent with mRNA expression as shown by *in situ* hybridisation [40]. In addition, a second major mucin population that was less acidic than the MUC5AC mucin, probably corresponding to what was earlier designated MUCX, was enriched in the submucosal tissue and is thus likely to originate from the glands. Furthermore,

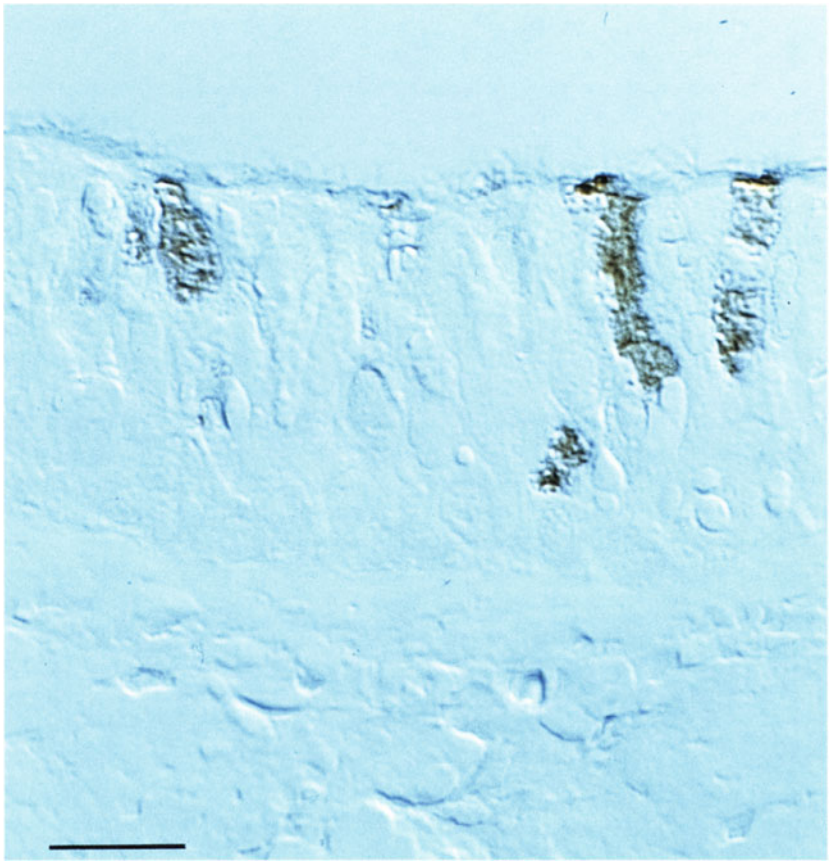


Figure 5. Immunolocalisation of the MUC5AC mucin in the respiratory epithelium. A section (4 µm) of human trachea probed with an antiserum to MUC5AC shows the mucin localised to the goblet cells, with little in the underlying submucosa. The section was visualised using a Nikon Optifot microscope with Nomarski optics. The bar represents 50 µm.

using antisera raised against synthetic peptides from the MUC2 apoprotein, this study failed to show any immunohistochemical staining over either the goblet cells or the submucosal glands, confirming the earlier suggestion that the MUC2 mucin is not a major component of the respiratory mucus gel.

Physiological and pharmacological investigations indicate that secretion from the goblet cells and submucosal glands is regulated differently. In human airways, the goblet cells do not appear to be innervated directly, although secretion can be induced by a range of substances, including irritant gases, bacterial products and adenosine triphosphate ATP (for a review, see ref. 64). In contrast, both sympathetic and parasympathetic nerves are associated with the submucosal glands [65].

The spatial restriction of two of the major gel-forming mucins in the respiratory tract, coupled with the fact that their cellular sources are under different physiological control, may have important consequences in regulating the properties of the mucus gel. The ability to alter the mucin composition of a secretion may provide a mechanism for fine-tuning the gel to cope with the rapidly changing circumstances to which the respiratory mucosa may be exposed. A possible consequence of this morphological separation of the different mucins is that they may not be intimately mixed in the secretion and that the gel is thus a composite of the different species. Although this has not been shown in the airways, laminated layers of different mucin types have been observed in histological studies of gastric mucus [66].

5. Mucins in Disease

While relatively small amounts of mucus are produced in the airways under normal conditions, diseases such as asthma, chronic bronchitis and cystic fibrosis are characterised by mucus hypersecretion. This overproduction is accompanied by hyperplasia and/or hypertrophy of the mucus-secreting cells. It is now firmly established that the major macromolecular components responsible for the rheological properties of the mucus gel in these diseases are mucins [5–7] and not, as has been suggested, proteoglycans [67]. The underlying causes of the three conditions are different, and the links between the primary defects and hypersecretion in each case are not exactly known. A clearer picture of the quality (character and size), type (gene products), cellular origin and quantity of the mucins present in health as compared with disease is required to determine their role in the pathological process.

5.1. "Acidity"

Many of the early studies of mucins in diseased airways were performed using the classical histological stains mentioned earlier, and in general

these investigations have suggested an increase in the "acidity" of the intracellular glycoproteins [68]. In cystic fibrosis, a more sulphated mucin population was predicted [69], and indeed a sulphated species, not observed to any great extent in secretions from apparently normal airways, has been isolated from CF sputum [60]. A sulphated population of mucins was also produced from cultured nasal epithelial cells from a person with cystic fibrosis [70]. In contrast, the expected increase in acidity of chronic bronchitic mucins [68] was not evident in the gel-forming species purified from the sputum of a number of individuals [7]. The majority of the molecules from sputum were rather less acidic than their normal counterparts, suggesting that the changes observed in the histochemical studies are not directly correlated to the properties of the mucins found in the gel. The major population of mucins may correspond to what we have termed MUCX, and as mentioned earlier, this component is likely to be a product of the submucosal glands. Thus, the mucus gel in chronic bronchitis may have an increased contribution of gland mucins as opposed to those from the goblet cells in the surface epithelium, and the increased acidity observed by histological investigation might thus have been obscured because mucins from the same cellular sources were not compared.

5.2. Gene Products

The three mucins MUC5AC, MUCX and MUCY are present in varying amounts in sputa from asthmatic, chronic bronchitic and cystic fibrotic airways (Figure 4). At present only a limited number of samples have been analysed, and it is not yet clear whether the level of any one of them can be related to the pathological process. Tumour necrosis factor α has been shown to increase mucin secretion in airway cells and upregulate the expression of MUC2, suggesting that this cytokine may contribute to pathogenesis of inflammatory airway diseases, in particular in asthma [71]. However, significant amounts of the MUC2 mucin were not found in the thick gel obstructing the airway of an individual who died in *status asthmaticus* [6] or in sputum from chronic bronchitic patients [46].

5.3. Size

In cystic fibrosis, mucin preparations contain a significant proportion of small species, and since the newly synthesised mucins are not inherently smaller [18], the decrease in size has been taken as evidence of proteolytic degradation [5]. In contrast, the majority (85% of the total weight) of the mucins isolated from a patient with asthma were much larger than those observed in the normal airways, leading to the speculation that these glycoproteins are abnormal in size and may lack some processing steps in

the macromolecular assembly pathway [6]. It should be stressed that all studies to date on the size distribution of the gel-forming mucins from the airways have been performed on what now appears to be a mixture of molecules, and no studies have focused on the individual species present. Recently, however, in normal airways, Hovenberg and colleagues [63] have demonstrated that submucosal gland mucins are larger than those produced in the goblet cells.

6. Mucin–Bacteria Interactions

Mucin–bacteria interactions are discussed in detail in Chapter 9 of this volume and are mentioned here only for completeness. Many investigators have shown binding of bacteria to respiratory mucus, and in the normal airway, where mucus is removed via mucociliary transport, this is thought to protect the mucosa from bacterial attachment. However, in hypersecretory diseases where mucus is not removed efficiently, binding may provide a mechanism by which pathogens may colonise and invade the mucosal surface. Several studies have demonstrated that respiratory pathogens including *Haemophilus influenzae* [72–74], *Pseudomonas aeruginosa* [75, 76] and *Pseudomonas cepacia* [77] bind to respiratory mucins. Mucin–bacteria interactions are considered to be mediated mainly via the vast range of oligosaccharide ligands presented by the mucins and receptors on the surface of the pathogen. For example, some of the carbohydrate recognition structures involved in the interaction with *P. aeruginosa* have been identified as type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) disaccharide units [78]. However, binding of this pathogen to mucins has been shown to be inhibited not only by GlcNAc but also by *N*-acetylneuraminic acid [79], highlighting the complex nature of the interaction. These glycan structures may also be present on cell surface glycoproteins and glycolipids, and it is thus likely that mucin oligosaccharides act as receptor analogues for sites used by the bacteria for colonisation. A possible consequence of this is that some individuals may have a "genetic susceptibility" to infection owing to the expression of oligosaccharide structures that may predispose them to bacterial colonisation. Changes in mucins in disease associated with their oligosaccharides have been observed both histologically and biochemically. A result of these alterations could be the colonisation of the respiratory tract by pathogens owing to an increased affinity for "new" carbohydrate receptors or lack of efficient competitors.

7. Mucin Biosynthesis

Studies of mucin biosynthesis and secretion have been carried out using *in vitro* models of human airway [80, 81] and human airway cells in culture

[70, 82, 83]. In such studies radiolabelled precursors have been used to label mucins, and the secreted macromolecules identified, quantified and characterised in terms of incorporated radiolabel. Since it is now clear that several different mucins are present in the airways, radiolabel will be selectively incorporated into those species with a high rate of synthesis, and secretion and measurements of release of radiolabel will reflect this bias. Recent evidence from human intestinal cell lines suggest that the large gel-forming species have long synthesis times, and it is therefore unlikely that these molecules become radiolabelled in such models. Indeed, the study by Sheehan *et al.* [44, 84], in which both radiolabelling and chemical detection methods were used in parallel, shows that both the size and quantity of the "mature" mucins would be underestimated by using radioactivity alone. Thus, the extent to which the results of investigations into mucin biosynthesis and secretion where radiolabels have been used as the sole markers for mucins are applicable to the large oligomeric mucins remains in doubt.

The macromolecular assembly of the large oligomeric gel-forming mucins in the respiratory tract is little studied or understood. Information on gastric mucins indicates that the assembly in the endoplasmic reticulum is dependent on prior N-linked glycosylation [55]. This process gives rise to dimers, trimers and tetramers which then undergo O-glycosylation before secretion. However, the degree of polymerisation of mucins (deca, dodecamer or larger) that we have observed in respiratory secretions (Table 1) does not fit this model, and to explain their existence, a further multimerisation process must be postulated. In a study of the biosynthesis of MUC2, it was shown that the oligomerisation of fully mature units (at least dimers) takes place after the addition of O-linked glycans in the Golgi or post-Golgi compartments [44]. Whether this process occurs in the assembly of the airway mucins is at present unknown, but sequence homologies in the cysteine-rich domains of the mucins MUC2 and MUC5AC [32, 33] suggest that at least MUC5AC may assemble in a similar fashion.

8. Summary and Future Perspectives

The primary function of airway mucus is clearance of particulate matter and protection against noxious agents in the air. This implies that the epithelium must be able to respond appropriately to a wide range of challenges, but, as yet, little is known about the pathway from insult to response in terms of which mucins, or other specific components of mucus, are secreted in any situation. The presence of two different mucus-producing cell types, the goblet cells and submucosal glands, that are under distinctly different physiological control and produce different mucins, provides the structural basis for tuning the properties of the secretion to a particular need. It may be speculated that the goblet cells are slowly feeding the

mucociliary escalator in the normal lung, whereas a larger secretion from the submucosal glands is used to dilute "irritants" and remove them by cough upon an acute challenge.

It is our contention that the rheological properties of mucus are derived primarily from the presence of the large, oligomeric, secreted mucins. The size, polydispersity, concentration and possibly also type of mucin present will dictate the properties of the gel. At least seven mucin genes are expressed in the respiratory tract, but it appears that only three mucins dominate within the gel. One of the three mucins is MUC5AC, but the two others could not be identified, although MUC1, MUC2 and MUC7 are ruled out on the basis of our data. These findings highlight the need to determine the actual amount of mucin in the tissue with biochemical techniques, rather than relying solely on the level of mucin apoprotein mRNA expression.

All three mucins conform to a similar structural design, i.e. they are polydisperse linear oligomers composed of subunits joined end to end. Furthermore, we have noted a distinctive "signature" for each of these mucins, regardless of individual, when subjected to ion-exchange chromatography or agarose electrophoresis. This implies that each mucin has a defined oligosaccharide profile, and that the differences between the three species is larger than interindividual variations. Thus, we would predict that the large heterogeneity of respiratory mucin oligosaccharides will even-tually be resolved into smaller subgroups associated with specific mucin gene products.

The role of mucin oligosaccharides remains to be fully understood. It has been demonstrated that certain bacteria bind specific oligosaccharide ligands, and it has been speculated that the primary function of oligosaccharide diversity is to enhance the possibility that bacteria bind to mucus and, thus, facilitate their removal by mucociliary transport. Furthermore, by providing competing "receptor analogues" for cell surface glycolipids and glycoproteins, mucins may trap bacteria and make them less successful in their attempts to colonise the mucosal surface. The "glycoprofiles" on the mucus-forming mucins may thus play a key role in governing the susceptibility to infection.

The identification of the MUC5AC and MUCX mucins as prominent products of the goblet cells and submucosal glands, respectively, and the availability of techniques to distinguish them in mixed secretions provides an opportunity to categorise hypersecretory disorders on the basis of the cellular origin of mucus. Such investigations would give new insight into the pathological process and direct attention to the afflicted cell type. Furthermore, since the two mucus-producing cell types are under different physiological control, it appears likely that different drugs are needed to modulate the secretory activity from goblet cells and submucosal glands.

Respiratory mucus is no longer just a messy material judged only by being purulent or not, but is slowly emerging as an elaborate gel formed by well-defined macromolecules whose biochemical and genetic identity, as

well as cellular origin, are about to be unravelled. The future prospect of defining the cellular origin of mucus hypersecretion in an individual patient on a routine clinical basis and then targeting the affected cell with a drug designed to interfere specifically with the secretory pathway of that cell would take respiratory hypersecretion into molecular medicine.

Added note

Since finalisation of this chapter we have obtained data that identifies MUCX and MUCY as different glycoforms of the *MUC5B* gene [86].

Acknowledgements

The authors wish to thank Mrs Marj Howard, Ms Annika Böök and Mr Anders Hansson for their expert technical assistance.

Work supported by grants from the Wellcome Trust, the Cystic Fibrosis Research Trust, the British Lung Foundation, the Swedish Medical Research Council (grant nos. 7902, 9823, 9711), the Medical Faculty of Lund, the Swedish National Association Against Heart and Chest Diseases, Centrala försöksdjursnämnden (CFN), the Smokeless Tobacco Research Council Inc. (USA), Greta and Johan Kocks Stiftelse, Stiftelsen Lars Hiertas Minne, Tore Nilssons Fund for Medical Research, Stiftelsen Riksförbundet Cystisk Fibros and Alfred Österlunds Stiftelse.

References

1. Sleigh MA, Blake JR, Liron N (1988) The propulsion of mucus by cilia. *Am Rev Respir Dis* 137: 726–741.
2. Rose MC, Voter WA, Brown CF, Kaufman B (1984) Structural features of human tracheobronchial mucus glycoprotein. *Biochem J* 222: 371–377.
3. Slayter HS, Lamblin G, LeTruet A, Galabert A, Houdret N, Degand P, Roussel P (1984) Complex structure of human bronchial mucus glycoprotein. *Eur J Biochem* 142: 209–218.
4. Thornton DJ, Davies JR, Kraayenbrink M, Richardson PS, Sheehan JK, Carlstedt I (1990) Mucus glycoproteins from normal human tracheobronchial secretions. *Biochem J* 265: 179–186.
5. Thornton DJ, Sheehan JK, Lindgren H, Carlstedt I (1991) Mucus glycoproteins from cystic fibrotic sputum: Macromolecular properties and structural architecture. *Biochem J* 276: 667–675.
6. Sheehan JK, Richardson PS, Fung DCK, Howard M, Thornton DJ (1995) Analysis of respiratory mucus glycoproteins in asthma: A detailed study from a patient who died in status asthmaticus. *Am J Respir Cell Mol Biol* 13: 748–756.
7. Davies JR, Hovenberg HW, Linden C-J, Howard R, Richardson PS, Sheehan JK, Carlstedt I (1996) Mucins in airway secretions from healthy and chronic bronchitic subjects. *Biochem J* 313: 431–439.
8. Carlstedt I, Sheehan JK (1984) Macromolecular properties and polymeric structure of mucus glycoproteins. *Ciba Found Symp* 109: 157–172.
9. Sheehan JK, Oates K, Carlstedt I (1986) Electron microscopy of cervical, gastric and bronchial mucus glycoproteins. *Biochem J* 239: 147–153.
10. Gupta R, Jentoft N, Jamieson AM, Blackwell J (1990) Structural analysis of purified human tracheobronchial mucins. *Biopolymers* 29: 347–355.
11. Gupta R, Jentoft N (1992) The structure of tracheobronchial mucins from cystic fibrosis and control patients. *J Biol Chem* 267: 3160–3167.

12. McCullagh CM, Jamieson AM, Blackwell J, Gupta R (1995) Viscoelastic properties of human tracheobronchial mucin in aqueous solution. *Biopolymers* 35: 149–159.
13. Jenssen AO, Harbitz O, Smidsrod O (1980) Electron microscopy of mucin from sputum in chronic obstructive bronchitis. *Eur J Respir Dis* 61: 71–76.
14. Mikkelsen A, Stokke BT, Christensen BE, Elgasaeter A (1985) Flexibility and length of human bronchial mucin studied using low-shear viscometry, birefringence relaxation analysis and electron microscopy. *Biopolymers* 24: 1683–1704.
15. Marianne T, Perini J-M, Lafitte J-J, Houdret N, Pruvot F-R, Lamblin G, Slayter HS, Roussel P (1987) Peptides of human bronchial mucus glycoproteins. *Biochem J* 248: 189–195.
16. Sheehan JK, Thornton DJ, Somerville M, Carlstedt I (1991) The structure and heterogeneity of respiratory mucus glycoproteins. *Am Rev Respir Dis* 144: S4–S9.
17. Rose MC (1992) Mucins: Structure, function, and role in pulmonary diseases. *Am J Physiol* 263: L413–L429.
18. Rose MC, Brown CF, Jacoby JZ, Lynn WS, Kaufman B (1987) Biochemical properties of tracheobronchial mucins from cystic fibrosis and non-cystic fibrosis individuals. *Pediat Res* 22: 545–551.
19. Sheehan JK, Boot-Handford RP, Chantler E, Carlstedt I, Thornton DJ (1991) Evidence for shared epitopes within the naked protein domains of human mucus glycoproteins. *Biochem J* 274: 293–296.
20. Gendler S, Lancaster CA, Tylor-Papidimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani E-N, Wilson D (1990) Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem* 265: 15286–15293.
21. Lightenberg MJL, Vos HL, Gennissen AMC, Hilkens J. Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. *J Biol Chem* 265: 5573–5578.
22. Gum JR, Byrd JC, Hicks JW, Toribara NW, Lamport DTA, Kim YS (1989) Molecular cloning of human intestinal cDNAs. *J Biol Chem* 264: 6480–6487.
23. Gum JR, Hicks JW, Swallow DM, Lagace RL, Byrd JC, Lamport DTA, Siddiki B, Kim YS (1990) Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. *Biochem Biophys Res Commun* 171: 407–415.
24. Porchet N, Van Cong N, Dufosse J, Audie JP, Guyonnet-Duperat V, Gross MS, Denis C, Degard P, Bernheim A, Aubert JP (1991) Molecular cloning and chromosomal localization of a novel human tracheobronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. *Biochem Biophys Res Commun* 175: 414–422.
25. Dufosse J, Porchet N, Audie JP, Guyonnet Duperat V, Laine A, Van-Seuningen I, Marrakchi S, Degard P, Aubert JP (1993) Degenerate 87-base pair tandem repeats create hydrophilic/hydrophobic alternating domains in human mucin peptides mapped to 11p15. *Biochem J* 293: 329–337.
26. Guyonnet-Duperat V, Audie JP, Debailleul V, Laine A, Buisine M-P, Galiegue-Zouitina S, Pigny P, Degard P, Aubert JP, Porchet N (1995) Characterization of the human mucin gene MUC5AC: A consensus cysteine-rich domain for 11p15 mucin genes? *Biochem J* 305: 211–219.
27. Toribara NW, Roberton AM, Ho SB, Kuo WL, Gum E, Hicks JW, Gum JR, Byrd JC, Siddiki B, Kim YS (1993) Human gastric mucin: Identification of a new species by expression cloning. *J Biol Chem* 268: 5879–5885.
28. Bobek LA, Tsai H, Biesbrock AR, Levine MJ (1993 Molecular cloning, sequence and specificity of expression of the gene encoding the low molecular weight salivary mucin (MUC7) *J Biol Chem* 268: 20563–20569.
29. Lancaster CA, Peat N, Duhig T, Wilson D, Taylor-Papdimitriou, Gendler S (1990) Structure and expression of the human polymophic epithelial mucin gene: An expressed VNTR unit. *Biochem Biophys Res Commun* 173: 1019–1029.
30. Gendler SJ, Spicer AP. Epithelial mucin genes (1993) *Annu Rev Physiol* 57: 604–634.
31. Gum JR, Hicks JW, Toribara NW, Siddiki B, Kim YS (1994) Molecular cloning of human intestinal mucin (MUC2) cDNA. *J Biol Chem* 269: 2440–2446.
32. Meerzaman D, Charles P, Daskal E, Polymeropoulos MH, Martin BM, Rose MC (1994) Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (MUC5). *J Biol Chem* 269: 12932–12939.

33. Lesuffleur T, Roche F, Hill AS, Lacasa M, Fox M, Swallow D, Zweibaum A, Real FX (1995) Characterization of a mucin cDNA clone isolated from HT-29 mucus-secreting cells. *J Biol Chem* 270: 1–9
34. Sadler JE (1991) von Willebrand factor. *J Biol Chem* 266: 22777–22780
35. Ruggeri ZM, Ware J (1993) von Willebrand factor. *FASEB J* 7: 308–316.
36. Pemberton L, Taylor-Papidimitriou J, Gendler S (1992) Antibodies to the cytoplasmic domain of the MUC1 mucin show conservation throughout mammals. *Biochem Biophys Res Commun* 185: 167–175.
37. Jany BH, Gallup MW, Yan PS, Gum JR, Kim YS, Basbaum CB (1991) Human bronchus and intestine express the same mucin gene. *J Clin Invest* 87: 77–82.
38. Dohrman A, Tsuda T, Escudier E, Cardone M, Jany B, Gum J, Basbaum C (1994) Distribution of lysozyme and mucin (MUC2 and MUC3) mRNA in human bronchus. *Exp Lung Res* 20: 367–380.
39. Aubert JP, Porchet N, Crepin M, Duterque-Coquillaud M, Vergnes G, Mazzuca M, Debuire B, Petiprez D, Degard P (1994) Evidence for different human tracheobronchial mucin peptides deduced from nucleotide cDNA sequences. *Am J Respir Cell Mol Biol* 5: 178–185.
40. Audie JP, Janin A, Porchet N, Chopin MC, Gosselin B, Aubert JP (1993) Expression of human mucin genes in respiratory, digestive and reproductive tracts ascertained by *in situ* hybridization. *J Histochem Cytochem* 41: 1479–1485.
41. Biesbrock AR, Bobek LA, Levine MJ (1995) Expression and biological activity of human salivary apo-mucin (MUC7). *Glycoconj J* 12: 599.
42. Hilkens J, Lightenberg MJL, Vos HL, Litvinov SV (1992) Cell membrane-associated mucins and their adhesion-modulating property. *Trends Biochem Sci* 17: 359–363.
43. Tytgat KMAJ, Buller HA, Opdam FJM, Kim YS, Einerhand AWC, Dekker J (1994) Biosynthesis of human colonic mucin: MUC2 is the prominent secretory mucin. *Gastroenterology* 107: 1352–1363.
44. Sheehan JK, Thornton DJ, Howard M, Carlstedt I, Corfield AC, Paraskeva C (1996) Biosnythesis of the MUC2 mucin: Evidence for a slow assembly of fully glycosylated units. *Biochem J* 315: 1055–1060.
45. Carlstedt I, Herrmann A, Hovenberg H, Lindell G, Nordman H, Wickstrom C, Davies JR (1995) Soluble and insoluble mucins-identification of distinct populations. *Biochem Soc Trans* 23: 845–851.
46. Hovenberg HW, Davies JR, Hermann A, Linden C-J, Carlstedt I (1996) MUC5AC, but not MUC 2, is a prominent mucin in respiratory secretions. *Glycoconj J* 13: 1–9.
47. Thornton DJ, Carlstedt I, Howard M, Devine P, Price M, Sheehan JK (1996) Respiratory mucins: Identification of core proteins and glycoforms. *Biochem J* 316: 967–975.
48. Breg J, Van Halbeek H, Vliegenthart JFG, Klein A, Lamblin G, Roussel P (1988) Primary structure of neutral oligosaccharides derived from respiratory-mucus glycoproteins of a patient suffering from bronchiectasis, determined by combination of 500-MHz ^{1}H-NMR spectroscopy and quantitative sugar analysis: Structure of 19 oligosaccharides having the GlcNAcβ(1-3)GalNAc-ol core (type 3) or the GlcNAcβ(1-3)[GlcNAcβ(1-6)]GalNAc-ol core (type 4). *Eur J Biochem* 171: 643–654.
49. Klein A, Lamblin G, Lhermitte M, Roussel P, Breg J, Van Halbeek H, Vliegenthart JFG (1988) Primary structure of neutral oligosaccharides derived from respiratory-mucus glycoproteins of a patient suffering from bronchiectasis, determined by combination of 500-MHz ^{1}H-NMR spectroscopy and quantitative sugar analysis: Structure of 16 oligiosaccharides having the Galβ(1-3)GalNAc-ol core (type 1) or the Galβ(1-3)[GlcNAcβ(1-6]GalNac-ol core (type 2). *Eur J Biochem* 171: 631–642.
50. Van Halbeek H, Breg J, Vliegenthart JFG, Klein A, Lamblin G, Roussel P (1988) Isolation and structural characterization of low-molecular-mass monosialyl oligosaccharides derived from respiratory-mucus glycoproteins of a patient suffering from bronchiectasis. *Eur J Biochem* 177: 443–460.
51. Klein A, Carnoy C, Lamblin G, Roussel P, Van Kuik A, Vliegenthart JFG (1993) Isolation and structural characterisation of novel sialylated oligosaccharide-alditols from respiratory mucus glycoproteins of a patient suffering from bronchiectasis. *Eur J Biochem* 211: 491–500.
52. Hounsell E, Feizi T (1982) Gastrointestinal mucins: Structures and antigenicities of their carbohydrate chains in health and disease. *Med Biol* 60: 227–236.

53. Scharfmann A, Lamblin G, Roussel P (1995) Interactions between human respiratory mucins and pathogens. *Biochem Soc Trans* 23: 836–839.
54. Lo-Guidice J-M, Wieruszeski J-M, Lemoine J, Verbert A, Roussel P, Lamblin G (1994) Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. *J Biol Chem* 269: 18794–18813.
55. Dekker J, Strous GJ (1990) Covalent oligomerization of rat gastric mucin occurs in the rough ER, is N-glycosylation dependent, and precedes initial O-glycosylation. *J Biol Chem* 265: 18116–18122.
56. Jeffry PK, Reid LM (1977) The respiratory mucus membrane. In: Brain JD, Proctor DF, Reid LM eds. *Respiratory defence mechanisms.* New York: Marcel Dekker, 193–245.
57. Lopez Vidriero MT, Reid LM (1978) Bronchial mucus in health and disease. *Med Bull* 34: 63–74.
58. Spicer SS, Schulte BA, Charkin LW (1983) Ultrastructural and histochemical observations of respiratory epithelium and gland. *Exp Lung Res* 4: 137–156.
59. Mazzuca M, Lhermitte M, Lafitte J-J, Roussel P (1982) Use of lectins for detection of glycoconjugates in the glandular cells of human bronchial mucosa. *J Histochem Cytochem* 30: 956–966.
60. Thornton DJ, Sheehan JK, Carlstedt I (1991) Heterogeneity of mucus glycoproteins from cystic fibrotic sputum. *Biochem J* 276: 677–682.
61. Sheehan JK, Thornton DJ, Carlstedt I (1990) Histochemical methods used in biochemical approaches to mucus glycoproteins. *Acta Histochemica* Suppl-Band XL, S. 133–135.
62. Thornton DJ, Devine PL, Hanski C, Howard M, Sheehan JK (1994) Identification of two major populations of mucins in respiratory secretions. *Am J Respir Crit Care Med* 150: 823–832.
63. Hovenberg JW, Davies JR, Carlstedt I (1996) Different mucins are produced by the surface epithelium and the submucosa in human trachea: Identification of MUC5AC as a major mucin from goblet cells. *Biochem J* 318: 319–324.
64. Rogers DF (1994) Airway goblet cells: Responsive and adaptable front-line defenders. *Eur Respir J* 7: 1690–1706
65. Partanen M, Laitinen A, Hervonen A, Toivanen M, Laitinen LA (1988) Catecholamine and acetylcholinesterase-containing nerves in the human lower respiratory tract. *Histochemistry* 76: 175–188.
66. Ota H, Katasuyama T (1992) Alternating laminated array of two types of mucin in the human gastric surface mucous layer. *Histochem J* 24: 86–92.
67. Bhaskar KR, O'Sullivan DD, Seltzer A, Rossing TH, Drazen TM, Reid LM (1985) Density gradient study of bronchial mucus aspirates from healthy volunteers (smokers and non-smokers) and from patients with tracheostomy. *Exp Lung Res* 9: 289–308.
68. DeHaller R, Reid LM (1965) Adult chronic bronchitis, morphology, histochemistry and vascularisation of bronchial mucous glands. *Med Thorac* 22: 549–567.
69. Boat TF, Cheng PW, Iyer RN, Carlson D, Polony Y (1976) Human respiratory tract secretion: Mucous glycoproteins of non-purulent tracheobronchial secretions, and sputum of patients with bronchitis and cystic fibrosis. *Arch Biochem Biophys* 177: 95–104.
70. Cheng PW, Boat TF, Cranfill K, Yankaskas JR, Boucher RC (1989) Increased sulfation of glycoconjugates by cultured nasal epithelial cells from patients with cystic fibrosis. *J Clin Invest* 84: 68–72.
71. Levine SJ, Larivee P, Logun C, Angus CW, Ognibene FP, Shelhamer JH (1995) Tumor necrosis factor-α induces mucin hypersecretion and MUC2 gene expression by human airway epithelial cells. *Am J Respir Cell Mol Biol* 12: 196–204.
72. Reddy MS, Scannapieco FA, Levine MJ (1988) Tracheobronchial mucin: Interactions with nontypeable *Haemophilus influenzae*. *Am Rev Resp Dis* 137: A317.
73. Davies JR, Carlstedt I, Nilsson A-K, Hœkansson A, Sabharwal H, Van Alpen L, Van Ham M, Svanborg C (1995) Binding of *Haemophilus influenzae* to purified mucins from the human respiratory tract. *Infect Immun* 63: 2485–2492.
74. Kubiet M, Ramphal R (1995) Adhesion of nontypeable *Haemophilus influenzae* from blood and sputum to human tracheobronchial mucins and lactoferrin. *Infect Immun* 63: 899–902.
75. Rhamphal R, Houdret N, Koo L, Lamblin G, Roussel R (1989) Differences in adhesion of *Pseudomonas aeruginosa* to mucin glycopeptides from sputa of patients with cystic fibrosis and chronic bronchitis. *Infect Immun* 57: 3066–3071.

76. Reddy MS (1992) Human tracheo-bronchial mucin: Purification and binding to *Pseudomonas aeruginosa*. *Infect Immun* 60: 1530–1535.
77. Sajjan US, Corey M, Karmali MA, Forstner JF (1992) Binding of *Pseudomonas cepacia* to normal intestinal mucin and respiratory mucin from patients with cystic fibrosis. *J Clin Invest* 89: 648–656.
78. Ramphal R, Carnoy C, Fieure S, Michalski JC, Houdret N, Lamblin G, Streeker G, Roussel P (1991) *Pseudomonas aeruginosa* recognises carbohydrate chains containing type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) disaccharide units. *Infect Immun* 59: 700–704.
79. Vishwanath S, Ramphal R (1985) Tracheobronchial mucin receptor for *Pseudomonas aeruginosa*: Predominance of amino sugars in binding sites. *Infect Immun* 48: 331–335.
80. Coles SJ, Reid LM (1978) Glycoprotein secretion *in vitro* by human airway: Normal and chronic bronchitis. *Exp Molec Pathol* 29: 326–341.
81. Phipps RJ, Williams IP, Richardson PS, Pell J, Pack RJ, Wright N (1982) Sympathomimetic drugs stimulate the output of secretory glycoproteins from human bronchi *in vitro*. *Clin Sci* 63: 23–28.
82. Shelhamer JH, Marom Z, Logun C, Kaliner M (1984) Human respiratory mucous glycoproteins. *Exp Lung Res* 7: 149–162.
83. Wu R, Martin WR, Robinson CB, St. George JA, Plopper CG, Kurland G, Last JA, Cross CE, McDonald RJ, Boucher R (1990) Expression of mucin synthesis and secretion in human tracheobronchial epithelial cells grown in culture. *Am J Respir Cell Mol Biol* 3: 467–478.
84. Sheehan JK, Hanski C, Corfield AP, Paraskeva C, Thornton DJ (1995) Mucin biosynthesis and macromolecular assembly. *Biochem Soc Trans* 23: 819–821.
85. Thornton DJ, Howard M, Devine PL, Sheehan JK (1995) Methods for the separation and deglycosylation of mucin subunits. *Anal Biochem* 227: 162–167.
86. Thornton DJ, Howard M, Khan N, Sheehan JK (1997) Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. *J Biol Chem* 272: 9561–9566.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D.F. Rogers and M.I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 3
Airway Mucin Genes and Gene Products

Mary Callaghan Rose[1] and Sandra J. Gendler[2]

[1] *Center for Molecular Mechanisms of Diseases, Children's Research Institute, Children's National Medical Center, Washington DC, USA, and Departments of Pediatrics and of Biochemistry and Molecular Biology, George Washington University, Washington DC, USA*
[2] *Department of Biochemistry and Molecular Biology, Mayo Clinic, Scottsdale, Arizona, USA*

1. Introduction

MUC genes and their MUC gene products have been the subject of several reviews in the last few years [1–4], as expected in a field where the molecular tools to address fundamental and disease-related questions are becoming available. A description and analysis of human mucin genes *MUC1* through *MUC7* and of porcine, canine, murine, rat and bovine mucin genes

were published in 1995 [4]. In this review, we have focused on *MUC* genes and gene products expressed in human airways and highlighted our current limited understanding of their role in health and disease. We have summarized the available information on *MUC8* and updated the available information on *MUC5/5AC*, *MUC5B* and animal mucin genes.

1.1. Mucin Glycoproteins

Mucin glycoproteins (mucins) are the major macromolecular components of mammalian mucus, the viscoelastic gel that coats and lubricates the epithelium of respiratory, gastrointestinal and reproductive tracts, and protects against infectious and environmental agents [1, 5–8]. Mucins constitute a family of large, highly glycosylated macromolecules that impart physical (aggregation, viscosity, viscoelasticity, lubrication) and biological (protection) properties to mucus. These multifunctional properties likely reflect specific, but not yet well-defined, structural determinants in mucin protein backbones (encoded by *MUC* genes) and in mucin O-glycosidic oligosaccharide chains (generated by glycosyltransferases). The MUC protein backbone and oligosaccharide chains of these complex glycoproteins are shown schematically in Figure 1.

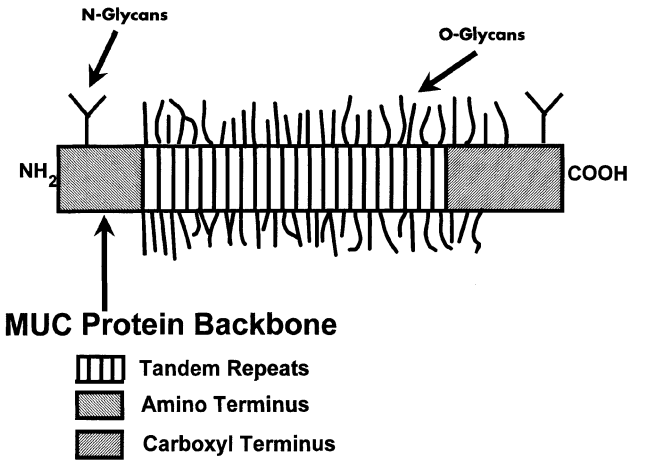


Figure 1. A schematic model of a glycosylated mucin glycoprotein. A MUC protein backbone, encoded by its *MUC* gene, is generally characterized by tandem repeats of unique sequences containing serine and/or threonine and proline and unique amino and carboxyl termini domains. Hundreds of O-glycans, attached to serine and threonine residues in the MUC backbone, are synthesized in a stepwise manner by specific glycosyltransferases [11]. Some N-glycosylation sites (NXS/T) in MUC protein backbones can be occupied (see text).

Table 1. Tandem repeats in human MUC mucins

Gene	Chromosome localization	Amino acids per repeat	Repeating sequence
*MUC1**	1q21	20	GSTAPPAHGVTSAPDTRPAP
*MUC2**	11p15.5	23	PTTTPITTTTTVTPTPTPTGTQT
MUC3	7q22	17	HSTPSFTSSITTTETTS
MUC4	3q29	16	TSSVSTGHATSLPVTD
MUC5/5AC	11p15.5	8 5	TTSTTSAP TTVGP/S
MUC5B	11p15.5	29	SSTPGTAHTLTVLTTATTPTATGSTATP
MUC6	11p15.5	169	SPFSSTGPMTATSFQTTTTYPTPSHPQTTLPTHVPPFSTSLVTPSTG TVITPTHAQMATSASIHSTPTGTIPPPTTLKATGSTHTAPPMTPTTS GTSQAHSSFSTAKTSTSLHSHTSSTHHPEVTPTSTTTITPNPTSTGTS TPVAHTTSATSSRLPTPFTTHSPPTGS
*MUC7**	4	23	TTAAPPTPSATTPAPPSSSAPPE
MUC8	12q24.3	13 41	TSCPRPLQEGTRV TSCPRPLQEGTPGSRAAHALSRRGHRVHELPTSSPGGDTGF

* Complete cDNA sequences reported.

1.2. Glycosylation of Airway Mucins

MUC proteins provide the scaffolding for the extensive post-translation O-glycosylation that results in mature mucins (Figure 1). Little is yet known about the specificity of O-glycosylation in airway epithelial cells, although numerous O-glycosidic oligosaccharides (O-glycans) have been isolated from airway mucins [9]. O-glycans are structurally heterogeneous [10] and are categorized by four major core type structures [11] to which galactose and/or N-acetylglucosamine are sequentially attached to produce elongated or branched oligosaccharides. The O-glycans are terminally glycosylated with sialyl, fucosyl and/or sulfo determinants.

O-glycans with core types 1, 2, 3 and 4 have been identified in airway mucin O-glycans (reviewed in ref. 1). A specific O-glycan core type may be unique to each of the seven *MUC* gene products expressed in the airways (Section 4.2; Table 2) and exhibit elongated O-glycans that are similar in size and in terminal glycosylation. If so, then only seven mature MUC mucins – each with O-glycans of a specific core type and some heterogeneity in size and charge – would be expected in airway secretions. However, the specificity of O-glycosylation may not be highly regulated by MUC primary structures. Each MUC may be able to be O-glycosylated and yield all four major core types, depending on the glycosylation status of the cell. The possibilities inherent in O-glycosylation illustrate the complexities of correlating specific O-glycans to specific serine and threonine residues in each MUC and ultimately to biological functions.

Identification of the structure of O-glycans attached to specific MUC proteins, i.e. phenotypic analysis of mucins, will be an important step in unraveling the roles of specific mucins in health and disease. This task will be complicated if specific domains in each MUC backbone (Figure 2) have different O-glycosylation determinants. For example, one of the four major

Table 2. Tissue specificity of *MUC* genes

Gene	Mucin	Major tissue localization	Respiratory tract expression
MUC1	Pan-epithelial	Breast, pancreas	Yes
MUC2	Intestinal, acidic	Jejunum, ileum, colon	Yes
MUC3	Intestinal, neutral	Colon	±
MUC4	Airway	Airways, colon	Yes
MUC5/5AC	Tracheobronchial	Airways, stomach	Yes
MUC5B	Airway	Airways, submaxillary glands	Yes
MUC6	Gastric	Stomach, ileum, gall bladder	±
MUC7	Salivary	Sublingual and submandibular glands	Yes
MUC8	Airway	Airways	Yes

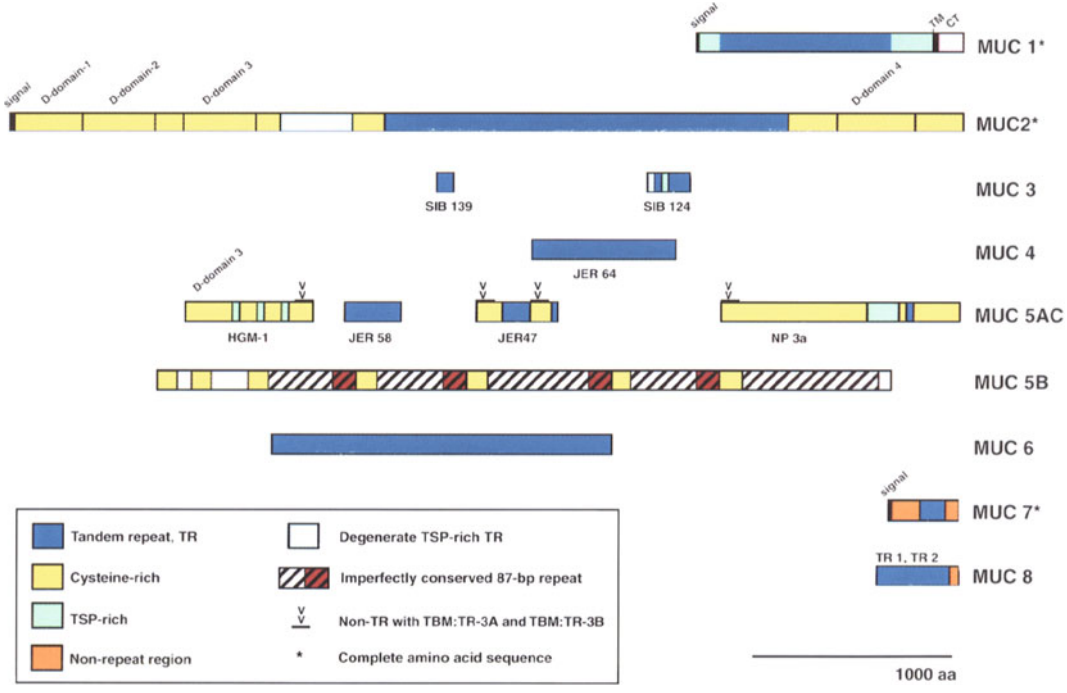


Figure 2. Models of MUC primary structures deduced from nucleotide sequences of completed *MUC* genes (*) or cDNA clones. Specific motifs – TRs, degenerate TRs, TSP-rich domains, cysteine-rich domains transmembrane (TM) and cytoplasmic tail (CT) domains – are depicted. The number of amino acids in MUC-specific TRs is given in Table 1. The number of TRs in a specific MUC or clone is given in the text and/or in references: MUC1 [13, 25, 26], MUC2 [24], MUC3 [15], MUC4 [16], MUC5/5AC [17, 18, 48], MUC5B [20, 39], MUC6 [21], MUC7 [22] and MUC8 [23, 30]. MUC2 is shown with 86 TRs, instead of the more frequently observed 100 TRs [24].

O-glycan core types may be biosynthetically elongated at serine and/or threonine residues in tandem repeat (TR) domains; a different core structure may be elongated in threonine/serine/proline-rich (TSP) domains; and yet a third core structure may be generated in cysteine-rich (CR) or other domains.

Each MUC protein may exhibit a unique cell or tissue-specific O-glycosylation, and it may be altered by disease. For example, MUC1 proteins isolated from human milk have core type-2 O-glycosides, while MUC1 mucins from BT-20 breast cancer cells have core type-1 O-glycosides [12].

2. The Gene Products of Human *MUC* Genes

2.1. Overview

Nine human *MUC* genes – *MUC1* [13], *MUC2* [14], *MUC3* [15], *MUC4* [16], *MUC5/5AC* [17–19], *MUC5B* [20], *MUC6* [21], *MUC7* [22] and *MUC8* [23] – have been identified, and their chromosomal localization determined (Table 1). Only three *MUC* genes (*MUC1, MUC2* and *MUC7*) have been completely sequenced. Their open reading frames (ORF) (Figure 2) and transcripts exhibit a vast range in size. The most frequent *MUC2* transcripts have 100 TRs and are 15 kilobases (kb) [24]. A commonly occurring *MUC1* transcript, with about 40 TRs, is 4.4 kb [25, 26]. The *MUC7* transcript, with 6 TRs, is 2.4 kb [22]. Northern blot analyses indicate that *MUC3, MUC4, MUC5/5AC, MUC5B, MUC6* and *MUC8* transcripts are greater than 9.5 kb, perhaps as large as *MUC2.*

MUC structures, based on amino acid sequences deduced from *MUC* genes or cDNA clones, are depicted in Figure 2. Primary structures have been directly confirmed for MUC2 [27], MUC5/5AC [17, 19] and MUC7 [22], as these gene products contain amino acid (aa) sequences determined by Edman analysed of peptides isolated from purified mucins. The validity of MUC identities has also been shown by immunochemical reactivity of antibodies raised either against MUC fusion proteins or specific MUC protein sequences. Antibodies raised against fusion protein expressed by cDNA clones encoding *MUC4, MUC5AC, MUC5B* [28, 29] and *MUC8* [30] exhibit immunoreactivity to mucous secretory granules in airway cells. Antibodies to specific peptide domains in MUC1 [31], MUC2 and MUC3 [32], MUC5 [33] and MUC6 [34] are immunoreactive in respiratory epithelial tissues or cells.

2.2. Characteristic Features of MUC Protein Backbones

A comparison of the primary structure of MUC mucins requires completion and confirmation of the ORF of known *MUC* genes. Nevertheless,

based on available information from completed and partial cDNA clones (Figure 2), certain characteristic features of the MUC family are emerging. These are described below, along with the increasing number of variations on a theme.

a) *Variable number of repeats.* A characteristic feature of *MUC* genes is a variable number of TRs of nucleotide sequences high in codons that encode serine and/or threonine (Table 1). However, as described below, partial cDNA clones encoding *MUC5B* (Section 2.4) and *MUC8* (Section 2.5) contain degenerate or imperfect TR nucleotide sequences, resulting in polypeptide structures that differ from the more classical TR primary structure depicted in Figure 1. Four partial cDNA clones of *MUC5B* exhibit irregular 87-bp TRs because of additions and deletions in their nucleotide sequence. The encoded polypeptides lack TRs, but have irregular repeats of 29 aa that are high in serine and threonine [20]. *MUC8* has imperfect 41-bp TRs which encode two types of consensus repeat peptides [23].

b) *Tandem repeats.* The TR of each MUC protein, as well as the non-tandem repeats of MUC5B, is a unique aa sequence high in threonine and/or serine residues and at least one proline (Table 1). Interestingly, a cysteine residue is present in the MUC8 TRs. TR sizes range from 5 and 8 aas in MUC5/5AC to 169 aa in MUC6. Both MUC5/MUC5AC (Section 2.3) and MUC8 (Section 2.5) have more than one type of consensus peptide TR.

c) *Cysteine-rich domains.* CR domains appear to be present in several secreted mucins (Figure 2). MUC2 contains CR domains that flank both the central TR domain and the TSP-rich degenerate TR domain in the amino terminus [24]. Three of the cDNA clones that encode MUC5/5AC yield CR domains that alternate with TR and/or with cysteine-free TSP-rich domains (Section 2.3). The carboxyl terminus of the high molecular weight sublingual mucin MG1, encoded by the pSM2-1 clone, also contains a CR domain [35]. MGM1 has recently been identified as tracheobronchial mucin MUC5B [98].

The CR domains in MUCs have sequence similarity to CR domains in von Willebrand factor (vWF), a large secreted, endothelial glycoprotein [36], that mediates platelet adhesion and thrombus formation at the site of vascular injury and is a carrier for procoagulation factor VIII. The structure/function relationships of specific domains in vWF have been elucidated (see ref. 37 for review). The CR domains of vWF include four D-domains and a 151-aa carboxyl-terminal sequence. The 151-aa C-terminal sequence of vWF is requisite for tail-to-tail dimerization and trafficking from the endoplasmic reticulum to the Golgi [38]. The high degree of sequence similarity at the carboxyl termini of vWF, MUC2, MUC5, MG1, pig submaxillary and bovine submaxillary mucins [17, 35] suggests that this CR domain contains sequences and motifs requisite for trafficking of large macromolecules, destined for glycosylation and secretion, through the endoplasmic reticulum to the Golgi.

The D-domains of vWF are involved in its multimerization and packaging into Palade bodies for storage and secretion in endothelial cells. Regions homologous to all four D-domains of vWF have been identified in the complete primary structure of MUC2 [24]. It is not yet known whether MUC2 D-domains 1 and 2, like those of prepro–vWF [37], are cleaved prior to its packaging into secretory granules. Cysteine-rich motifs may impart physical properties to secreted MUCs and facilitate their trafficking through intracellular organelles, packaging into secretory granules and movement in mucosal gels. Whether this is so will become evident as the primary structures of MUCs are completed and functionally analyzed. Indeed, a 108-aa CR motif in MUC5B, MUC5/5AC and MUC2 has recently been identified [39]. In contrast, the protein backbone of MUC7/MG2, a non-gel-forming secreted mucin, lacks CR domains and contains only two cysteines in its primary structure [22].

d) *Glycosylation.* MUCs have a high number of potential O-glycosylation sites because of a large number of threonine and serine residues in their protein backbones. These sites are mainly, but perhaps not exclusively, in the TR domains, as threonine and serine residues are also present in non-TR domains. No consensus sequences for O-glycosylation have yet emerged; parameters that influence O-glycosylation of specific TR sequences of specific serine or threonine residues are being delineated in several laboratories [40–42].

MUC proteins, unexpectedly, also contain several *N*-glycosylation sites (NXS/T) in their amino and/or carboxyl termini domains. MUC2 has 20 *N*-glycosylation sites; MUC1 and MUC7 have 5. Eleven *N*-glycosylation sites are present in clone NP3a, which encodes the 3′ end of MUC5/5AC. Some *N*-glycosylation sites of MUCs are occupied: MUC1 [43, 44] and MUC2 [45, 46] have been shown to undergo *N*-glycosylation during biosynthesis.

e) *Transmembrane domain.* Classically, mucins have been considered to be secreted macromolecules. The three completed *MUC* ORFs encode leader sequences, which direct nascent protein chains into the endoplasmic reticulum for processing as membrane or secreted glycoproteins. Uniquely, *MUC1* encodes a hydrophobic transmembrane domain within its carboxyl terminus domain and presents as a type-1 membrane glycoprotein. If proteolysis occurs in the extracellular milieu, MUC1 glycopeptide is released into the secretions [4].

2.3. MUC5/5AC, an Airway Mucin Gene and Gene Product

A current model of MUC5/5AC (Figure 2) is deduced from partial cDNA clones reported by several laboratories [17–19, 48]. JER 47 and JER 58 clones were isolated from a tracheal mucosa cDNA library [28], clone NP3a from a nasal polyp cDNA library [17] and clone HGM-1 from a

gastric cDNA library [19]. *MUC5/5AC* is sometimes referred to as *MUC5/5AC*; three groups of partially sequenced cDNA clones for *MUC5A, B, C* colocalized to chromosome 11p15 and thus were originally proposed to be on the same gene [29]. However, further analyses demonstrated that cDNA clones encoding *MUC5A* and *MUC5C*, but not *MUC5B*, were on the same gene [18] and encoded the TR-3A and TR-3B peptides [47] that serve as markers for tracheobronchial mucin (TBM) and are encoded by *MUC5* [17].

The deduced structures of JER 47, HGM-1 and NP3a suggest that MUC5/5AC contains CR domains interspersed with TR and/or TSP domains. This motif is illustrated in the primary structure deduced from the nucleotide sequence of clone JER 47, which encodes in the following order: $(TR)_2$, CR domain, $(TR)_{21}$, CR domain, $(TR)_4$ [18]. Both of the CR domains encodes by clone JER 47 contain an almost identical 130-aa sequence which includes the TBM:TR-3A and TBM:TR-3B peptide sequences [47]. This 130-aa sequence is also present once in clone NP3a, and clone HGM-1 terminates at the 122nd aa in the same 130-aa sequence. If the proposed model of MUC5/5AC is correct, then *MUC5/5AC* encodes two types of TR: (1) an eight-aa TR, with a consensus sequence TTSTTSAP, present 34 times in JER 58, almost 5 times in JUL32, and a total of 27 times in clone JER 47 [18], and (2) the pentapeptide TTVGP/S, which tandemly repeats 5 times in clones NP3a [17] and L31 [48].

Two cDNA clones, NP3a and L31, contain a polyadenylation signal sequence and poly A tail and thus encode the 3′ end of *MUC5*. The 3.6-kb NP3a clone was isolated from a nasal polyp cDNA library [17] and the 3.1 cDNA L31 clone from an HT29 colon adenocarcinoma cell cDNA library [48]. These clones are 98% identical at the nucleotide level, but changes in the ORF of L31 yield major differences in aa sequences in four domains. Restriction fragment analyses revealed some differences between the two clones, which may reflect allelic variation or alternative splicing.

HGM-1 [19] currently represents the most 5′ sequence of *MUC5/5AC*. Its localization is suggested by its sequence similarity to D-domain 3, which is localized near the amino terminus of MUC2 and vWF [24]. Northern blot analyses suggest that the *MUC5* transcript may be as large as that of *MUC2*.

2.4. MUC5B, an Airway Mucin Gene and Gene Product

Clones encoding *MUC5B* have been reported [29, 20, 39], and restriction fragment analyses have demonstrated that *MUC5B* sequences are not on the same gene as *MUC5/5AC* [18]. Recent information indicates that the genomic sequence of *MUC5B* encompasses about 40 kb with a large central exon of 10.7 kb which encodes a 3,570-aa peptide. Four super-repeats of 528 aa residues are detected within this large exon. In addition, 19 subdomains alternate with the imperfectly conserved STP-rich repeats

previously reported in clones JER 57, JUL 7, JER 28 and JUL 10. Within these subdomains are seven CR domains of 108 aas. These CR domains may represent a motif for secretory mucins, as similar domains are present in MUC5/5AC, MUC2, mouse Muc-5ac and rat Muc-2 [39].

2.5. MUC8, an Airway Mucin Gene and Gene Product

The recently identified *MUC8* gene [23] maps to chromosome 12q24.3 [30]. pAM1, a 941-bp cDNA clone [23], was isolated from a normal tracheal cDNA library in UniZAP by screening with affinity-purified polyclonal antibodies to deglycosylated human tracheal mucin, HTM-1 [49]. Bases 3-892 in pAM1 contain 22 degenerate 41-bp tandem repeats at the 5′ end with a short non-repeat sequence at the 3′ end. The degenerate TR nucleotide sequence encodes two types of consensus repeats. The 41-aa peptide sequence TSCPRPLQEGTPGSRAAHALSRRGHRVHELPTSSPGGDTGF is encoded by three of the 41-bp tandem nucleotide repeats, and a sequence with 83% identity – MSCPRPFQEGTPGSRAAHVLSRKGPRVHELPTSSPGRDPGF – is encoded by the next three 41-bp tandem nucleotide repeats. Then, deletion of two bases in the 41-bp repeats results in 39-bp nucleotide repeats encoding a 13-aa peptide TSCPRPLQEGTRV. The resultant repeat pattern encoded by pAM1, in terms of number of amino acids in the TR, is $(41)_2 13$, 41, $(13)_4$, $(41)_2$, $(13)_2$ and is followed by a unique 16-aa sequence. Clone pAM1 was subsequently shown by 3′ RACE to be immediately followed by an end codon and a 3′ UTR of 458 bp which contains a polyadenylation signal and a poly A tail [30]. Thus, clone pAM1 encodes the 3′ end of MUC8. Polydisperse bands ranging in size from >9 to 0.5 kb were observed by Northern blot analysis [23].

Antibodies to the TR sequence TSCPRPLQEGTPGS and to the fusion protein encoding the first 325-bp fragment of pAM1 demonstrate localization of MUC8/HTM-1 to both goblet cells and submucosal glands in human tracheal epithelium [30].

3. Regulation of Human Mucin Airway Genes

3.1. Overview

An understanding of the regulation of *MUC* genes expressed in the airways will likely impact on treatment and prevention of chronic obstructive pulmonary diseases. Airway mucins are hypersecreted in response to inflammation, infections and environmental insult. Hypersecretion can persist after removal of chemical or biological insults, resulting in "runny noses" and perhaps ultimately in chronic obstructive pulmonary diseases. Upregulation of all or of specific *MUC* genes must occur in order to maintain hypersecretion of mucins. The ability of various mediators – choliner-

gic agonists, lipid mediators, neuropeptides, adenosine triphosphate ATP, bacterial products and inflammatory cell enzymes – to effect hypersecretion of mucins is reviewed in Chapters 7 and 8. The effects of these mediators on *MUC* gene regulation and expression are now being actively explored. The information generated will enable dissection and correlation of the effects of specific mediators on the mechanisms of *MUC* gene regulation and on secretion of mature, i.e. fully glycosylated, MUC mucins.

3.2. Regulation of MUC Gene Expression

The hypothesis that specific factors released during biological challenges to the airways upregulate specific *MUC* gene expression is currently being investigated in several laboratories. Although bacterial proteinases [50], bacterial endotoxin [51], ATP [52] and human neutrophil elastase [53] are known to result in hypersecretion of mucins, limited information is yet available as to how and whether these factors effect *MUC* gene expression. Further, it is not yet established whether alterations in specific *MUC* expression correlate with altered secretion of specific MUC mucins. Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, and *Pseudomonas aeruginosa* lipopolysaccharide have been shown to upregulate *MUC2* expression in NCI-H292 lung mucoepidermoid carcinoma cells [54, 99]. TNF-α also results in an increased secretion of high molecular weight mucins, although it is not established whether an increased secretion of MUC2 mucins specifically occurs. Recently, transgenic mice that overexpress murine IL-4 have been shown to express increased levels of Muc-5ac, but not Muc-2, mRNA and to induce mucin hypersecretion [100]. Neutrophil elastase, a serine proteinase secreted during chronic neutrophil-dominated inflammation, has very recently been shown to increase *MUC5/5AC* expression in respiratory epithelial cells in a dose and time-dependent manner [55].

3.3. Mucin Gene Regulation and Cell Differentiation

Hyperplasia of goblet cells and of submucosal glands occurs in airway diseases and likely is required to maintain hypersecretion of mucins and mucus. Specific *MUC* genes may provide biomarkers for mucous cell differentiation. The processes that affect cell differentiation and lead to proliferation of goblet or mucosal glandular cells are not well understood. Epidermal growth factor (EGF) has recently been shown to regulate mucus differentiation of rat tracheal epithelial cells in culture. Low levels of EGF result in a two- to threefold decrease in the percentage of mucus cells, a 10-fold decrease in the amount of mucin secreted and an eight-fold reduction in Muc-5 expression [56]. However, in similar human cell cultures, regulation of mucus differentiation may involve a more complex interaction between EGF and retinoic acid. In fact, under certain conditions, EGF

suppresses mucin secretion [57] and *MUC2* and *MUC5* gene expression (P. Nettesheim and K. Guzman, personal communication).

Retinoids are required to maintain differentiation of secretory epithelial cells [58]. Their role in mucous cell hyperplasia and their ability to affect *MUC* gene expression is currently being assessed both in human and rodent model systems. In rat tracheal explants, removal of retinoic acid from the media results in a decrease to non-detectable levels of RMUC 176 expression [59]. For human primary airway epithelial cells plated on collagen gels, conflicting data have been reported. Retinol was reported to downregulate *MUC2* [60], whereas retinoic acid has been reported to upregulate *MUC2* as well as *MUC5* [61].

3.4. Transcriptional Units of Mucin Genes

Elucidation of mucin gene regulation and expression will ultimately require analysis of regulatory components in mucin transcriptional units. *MUC* genes are a recently identified family of genes, and the ORF of only three *MUC* genes have been completely sequenced. Thus, information on *MUC* transcriptional units is limited. Regulatory regions in 5′ upstream sequences have been reported so far only for *MUC1* [62] and murine Muc-1 [63]. The promoter for rat Muc-2 has been identified in a genomic clone [64], but its characterization has not yet been reported.

Promoter analyses have demonstrated the existence of several potential regulatory regions in *MUC1* as detailed in ref. 4. The murine Muc-1 promoter contains cis elements similar to that of *MUC1* [63, 65]. These include perfect homopurine mirror repeats [66], a responsive mucin element that binds a soluble factor capable of stimulating the production of MUC1 by human colon cancer cells [67] and an E-MUC1 domain which may determine epithelial tissue-specific expression [62]. Identification of such epithelial cell-specific cis elements, like that at −84/−72 in the *MUC1* gene [62], will be important in elucidating the basis of the tissue and cell specificity of *MUC* gene expression. Identification of epithelial and mucin-specific transcription factors will facilitate our understanding of the role of mucins in health and disease.

3.5. Cluster of MUC Secretory Genes

The chromosomal localization of *MUC2* [68], *MUC5/5AC* [17, 69], *MUC5B* [69] and *MUC6* [21] onto chromosome 11p15.5 suggests that this region contains a cluster of *MUC* secretory genes. The order has now been established as *MUC6...MUC2...MUC5/5AC...MUC5B*, going from the telomere to the centromere [70]. Further analysis will eventually determine whether the secretory *MUC* genes in this locus share common transcriptional regulatory elements.

4. Expression of *MUC* Genes in the Airways

4.1. Expression of MUC Genes

There appears to be a moderate, but not exclusive, degree to tissue specificity of *MUC* genes in healthy organs (Table 2). To date this has been best investigated for *MUC1* (reviewed in ref. 4), which appears to be an almost universally expressed epithelial mucin. *MUC1* is highly expressed in pancreas and lactating mammary gland, but not well expressed in colon or small intestine. Its expression is also quite strong in lung bronchus and bronchioles, nasal epithelium, excretory ducts of salivary glands, chief cells and epithelial lining of stomach, distal tubules and collecting ducts of kidney, and endometrium and fallopian tubes [71].

MUC2, MUC3, MUC4, MUC5/5AC, MUC5B, MUC6, MUC7 and *MUC8* mRNAs are extensively expressed in epithelial tissues, but with somewhat more limited expression than *MUC1. MUC2* [14] and *MUC3* [15] are well expressed in the intestine, *MUC5/5AC* [19, 33] and *MUC6* [21, 33] in the stomach, *MUC7* in salivary glands [22], and *MUC4* [16], *MUC5/5AC* [17, 18, 33], *MUC5B* [20] and *MUC8* [23] in the larger airways.

4.2. Expression of MUC Genes in the Respiratory Tract

Seven of the nine identified *MUC* genes or MUC gene products are expressed in the upper and/or lower respiratory tract tissues (Table 2), which include nasal and tracheal/bronchial passages, respectively. These tissues are generally considered similar by histological, morphological and ion-conductance criteria.

Quantitative and *in situ* determinations of *MUC* expression in upper respiratory tract tissues and cells have been carried out. A slot blot cRNA assay using non-TR probes quantitatively demonstrated that *MUC5/5AC* is expressed fourfold higher than *MUC2* and 12-fold higher than *MUC1* in nasal polyps [73]. A further study showed that *MUC5/5AC* is expressed 10- to 13-fold higher than *MUC2* or *MUC1* in nasal epithelia cells [74]. These observations are supported by a recent *in situ* hybridization analysis of eight *MUC* genes in nasal tissues (Table 3; ref. 75). The results demonstrate that *MUC3* and *MUC6* are not expressed in upper respiratory tract tissues and that *MUC4, MUC5/5AC* and *MUC5B* are more highly expressed than are *MUC2, MUC1* and *MUC7* [75]. The recently identified *MUC8* gene also appears to be well expressed in the airways on the basis of Northern blot [23] and immunocytochemical data [30], although comparative data are not yet available.

Expression of high levels of *MUC5/5AC* and *MUC8* are supported by the observations that glycosylated mucins with MUC5 and MUC8 backbones

Table 3. Average level of mucin mRNA expression in normal and vasomotor rhinitis turbinates

Gene	Normal E	Normal G	VMR E	VMR G
MUC1	2.4	0.9	0.5	0.0
	$n=19$	$n=19$	$n=4$	$n=4$
MUC2	2.5	0.0	2.5	0.0
	$n=18$	$n=18$	$n=4$	$n=4$
MUC4	3.9	0.4	3.8	0.0
	$n=17$	$n=16$	$n=4$	$n=4$
MUC5/5AC	3.7	1.1	4.0	0.5
	$n=18$	$n=18$	$n=4$	$n=4$
MUC5B	0.1	3.1	0.0	2.8
	$n=17$	$n=19$	$n=4$	$n=4$
MUC7	0.1	2.1	0.0	1.0
	$n=15$	$n=19$	$n=4$	$n=4$

Mucin mRNA expression in epithelium (E) and submucosal glands (G) was graded at five levels from 0 to +4 for each patient and tissue type. Average levels of each *MUC* and tissue type are shown. *MUC3* and *MUC6* showed little or no expression. Normal turbinate samples were obtained from and compared with turbinate samples taken from VMR patients who underwent a partial inferior turbinectomy [75]. n = number of samples.

have been isolated from airway mucus or secretions. The MUC5/TBM mucin was initially isolated from lung mucus of a patient with bronchial asthma [47]. A recent study has shown that mucins with MUC5 backbones are a major fraction of airway mucin in pooled normal airway secretions and in secretions from a patient with status asthmaticus [76]. MUC8/HTM-1 has been biochemically isolated from lung mucus of a patient with cystic fibrosis CF [49].

Whether *MUC3* and *MUC6* are expressed in the respiratory tract is equivocal. *In situ* hybridization studies have yielded conflicting data for *MUC3*. No expression was observed in nasal turbinates [75], or in bronchial epithelium or submucosal glandular cells [72, 77]. However, diffuse staining with a *MUC3* probe was reported in bronchial epithelium and mucosal and serosal glandular cells [78]. Expression of *MUC6* mRNA was not detected by *in situ* hybridization in nasal tissue [75] or large airways [72]. Expression of MUC6 protein was not detected by immunocytochemistry in bronchial epithelium or subglandular mucosal/serosal cells, although it was detected in tracheal epithelium [33].

4.3. Cell-Specific Expression of Airway MUC Genes

Extensive studies to define the localization of eight *MUC* genes in upper and lower respiratory tract epithelium and submucosal glands cells have recently been completed (Table 3; [72, 75]). In nasal surface epithelium, *MUC2* and *MUC5*/5AC mRNAs were expressed in a goblet cell-like pattern in most individuals while *MUC4* and *MUC1* were diffusely expressed along the epithelium. In the submucosal glandular cells, *MUC5B* and *MUC7* were expressed by most individuals; *MUC5*/5AC and *MUC1* were less frequently observed. *MUC2* expression in the glands was not detected. These data are generally, but not completely, consistent with those previously reported for tracheal and bronchial epithelium, as briefly discussed below.

MUC1 presents diffusely in nasal epithelium [75], at low levels in bronchial epithelium [72, 77, 79] and rarely in submucosal glands [72, 77]. *MUC4* has been detected by *in situ* hybridization as a diffuse pattern in nasal [75] and bronchial epithelium [72, 77] and was detected in bronchial epithelium by an MUC4-specific antibody [16]. Low levels of *MUC4* are present in submucosal glands of nasal turbinates [75] and of bronchi [72, 77].

However, differences concerning the cell-specific localization of some *MUC* genes in the upper and lower respiratory tracts have been reported and may reflect differential cell or tissue expression. *MUC2* is expressed in goblet cells in both bronchial [78] and nasal [75] epithelium, but not in the submucosal glands of turbinates [75] or airways [72]. However, *MUC2* expression in both mucous and serous glandular cells in the bronchi has been reported [77]. *MUC5/5AC* gene expression has been observed in goblet cells of nasal turbinates [75] and of bronchial epithelium [72, 77]. Further, MUC5/5AC expression at the protein level is observed in tracheal goblet cells by immunofluorescence with an antibody specific to the octapeptide TR of MUC5/5AC [33]. In addition, expression of *MUC5/5AC* mRNA in submucosal glandular cells has been observed in nasal turbinates [75] and bronchial epithelium [72, 77]. However, expression at the protein level was not observed in bronchial [33] or tracheal [101] submucosal glandular cells. It may be that MUC5 mucins synthesized in bronchial submucosal glands are more extensively glycosylated, thereby masking their TR epitopes. *MUC5B* mRNA is clearly localized to submucosal glands in nasal [75] and bronchial [77] tissues. These data support immunofluorescence studies in which MUC5B is identified in submucosal glandular cells but not in goblet cells [20, 28]. However, *MUC5B* is also expressed by the bronchial epithelium in 6 out of 10 control lung transplant tissues [72]. *MUC7*, which is expressed in most normal turbinate tissues and localized to submucosal glandular cells [75], is not detected in the lower respiratory tract of 20 lung transplant tissues [72]. MUC8 protein has been detected both in tracheal goblet and submucosal glandular cells [30].

5. Animal Mucin Genes in Airway Disease Models

5.1. Overview

The mucus-rich organs of various animals have been evaluated anatomically and histochemically to determine their usefulness as models for studying human airway diseases [80, 81]. These analyses are now being extended to mucin genes, as analogues of *MUC* genes may be more easily studied and manipulated in animals. Some of the mucin genes expressed in canine and rodent airways have now been identified. Canines are good airway models by histological and biochemical criteria, although they are expensive to maintain. The complete cDNA sequence of a canine TBM has been reported [82]. It lacks tandem repeats but, like MUC5B, has non-tandem repeats that are TSP-rich.

Rodents are frequently used as model systems to study biological processes and human disease because of their small size, amenability to manipulation and low cost. The airway surface epithelia of mice, rats and hamsters are similar to those of human airways, although rodents lack the extensive submucosal glands found in human airways [81]. Elucidating the roles of mucins in health and disease with such models will require identification of rodent mucin genes and their human *MUC* gene analogues.

5.2. Mouse Mucin Genes

The murine Muc-1 gene was the first rodent mucin gene identified and characterized [63]. The observed homology between the *MUC1* and Muc-1 cDNAs and promoters serves as a paradigm for rodent homologues of *MUC* genes. Homology between *MUC1* and mouse Muc-1 is 87% in the non-tandem repeat domains [63] and 74% in the promoter regions [65]. However, homology in the TR domains is low (34%), even though the MUC1 TR has 20 aas and the mouse TR varies between 20 and 21 aas. In contrast to the variable number (30–100) of TRs in *MUC1*, the murine Muc-1 gene has 16 TRs. Thus, relative to their human *MUC* analogues, rodent mucins may have (1) a high degree of homology in their non-TR and promoter regions, (2) low homology in their TR domains and (3) decreased transcript size due to a lower, sometimes invariant, number of TRs. However, as demonstrated below, identification of rodent analogues of other *MUC* genes may not be as straightforward as Muc-1 and will require more complete analysis of both human and rodent mucin genes.

At present, the only other mouse Muc homologues reported are the mouse gastric mucin (MGM) candidate clones for Muc-5ac [34]. A 1062-bp sequence from two overlapping clones contains two CR domains with 70% and 77% homology to the two CR domains in the *MUC5AC* cDNA clone JER 47 [18]. By comparison, the sequence similarity to the CR domains in

human *MUC2* and rat Muc-2 is lower – 57% and 54%, respectively. The MGM cDNA clones encode a 16-amino acid TR, rather than the 8-amino acid TR encoded by clones JER 47 and JER 48, and homology of the TR domains is low.

5.3. Rat Mucin Genes

Identification of rat mucin genes is an active area, since rat airways are used as model systems to investigate airway diseases [83, 84]. Based on the partial cDNA clones reported to date, there appear to be at least four rat Muc genes. Determination of full-length cDNA sequences will be required to clearly establish the identity of specific rat mucin genes and their human analogues. Current information on each of the four potential rat mucin genes is summarized briefly below.

Three loci of the rat Muc-2 ORF have been identified independently by three laboratories, as briefly reviewed below in (1), (2) and (3). Identification is based on the high homology of the rat cDNA clones of the CR domains of MUC2. The TR of rat Muc-2 has not yet been clearly identified. The rat Muc-2 transcript is >9 kb [85], apparently 12 kb [86], and is normally well expressed in intestine and colon but not liver, stomach or unstimulated trachea [64, 85]. (1) The 3′ end of the rat Muc-2 gene was initially identified as clone MLP 3500 by polymerase chain reaction (PCR) using primers whose nucleotide sequences were deduced from independently determined peptide fragments of biochemically purified rat intestinal mucin linker glycoprotein [85]. (2) The 705-bp cDNA clone VR-1A, identified by screening a rat jejunal library with antibody to deglycosylated rat mucin, has a CR domain followed by a TSP domain. The CR domain has 54% homology to the CR domain just upstream of the major tandem repeat domain of MUC2 [86]. (3) A third group of cDNA clones was isolated by screening a rat jejunal cDNA library with the human *MUC2* cDNA clone SMUC 313. An ORF of 4546 bp from three overlapping cDNA clones encodes the amino terminus and TSP-rich region of rat Muc-2 and contains irregular repeats presumed to be upstream of the TR domain. The sequences are 80% identical with the 5′ CR domain and 38% homologous with the TSP-rich domain of MUC2 [64].

A second rat mucin gene, encoded by the 390-bp cDNA clone RAM 7s [87], was isolated by screening a cDNA library of hyperplastic rat airways with the human *MUC2* cDNA clone SMUC 41. An ORF of 274 bp is followed by a 113-bp 3′ UTR which includes a polyadenylation signal. The first two-thirds of the cDNA encode seven variable 7–12-amino acid tandem repeats and 5 non-tandem copies of the consensus sequence TTTTIITI. The consensus sequence is 60% homologous to MUC2, although MUC2 has 23 aas per TR. RAM 7s hybridizes to a transcript >9.5 kb and is well expressed in intestine and colon, but not airway, heart, liver,

kidney, brain or testis. Thus it has a similar size and tissue expression to rat Muc-2. However, it encodes the 3′ end of a mucin gene which is different from the 3′ end of rat Muc-2 [85], thus demonstrating that RAM 7s and rat Muc-2 are independent genes.

A third rat mucin gene has been identified from two partial cDNA clones – RMUC 176 [88] and M2-798 [89]. Each clone has TR with the sequence TTTPDV and was identified by screening a rat intestinal cDNA library with an antibody to deglycosylated rat mucin glycoprotein. A 7.5-kb transcript is expressed in rat small intestine and colon, but not in rat lungs, stomach [89], heart, kidney or liver [88].

A fourth rat mucin gene, which is a *MUC5/5AC* analogue, has recently been identified by PCR as a 534-bp amplicon and subcloned [90]. It is 73% identical at the amino acid level to a domain in the carboxyl terminus of MUC5 bounded by two conserved octapeptide sequences.

5.4. Airway Expression of Muc Genes in Rodents

Analysis of mucin gene expression in rat airways is required in order to define model systems for studying specific aspects of airway diseases, including hyperplasia [83, 84]. Neither Muc-2 nor RAM 7s is normally expressed in rat trachea, but expression is seen after exposure to sulfur dioxide and simian virus [64, 87]. The RMUC 176/M2-798 gene was not expressed in rat airways [88, 89]; whether irritants induce upregulation has not been established. RMUC 176 is expressed in rat tracheal cultures only in the presence of retinoic acid [59], as are rat Muc-2 and Muc-5 [60].

Rat Muc-5 is more highly expressed in stomach than in lung or trachea. Further, it is expressed in rat tracheal epithelial cells only when these cells are grown in the presence of retinoic acid [90]. The candidate gene for mouse Muc-5ac is not expressed in some species of mouse or rat lung, although it is highly expressed in rat stomach [34], and its human analogue is highly expressed in both tissues [33]. Completing the ORF and identifying the transcriptional units of Muc genes will facilitate unraveling their complexities of tissue and species expression.

6. MUC Genes and Mucins in Airway Diseases

In airway diseases, infection and inflammation may alter overall or specific expression of *MUC* genes. This hypothesis is supported by the observations that neither rat Muc-2 [64] nor RAM 7s [87] is easily detectable in the airways unless there has been an exposure to a chemical and/or viral irritant. An analogous situation may prevail in human airway epithelial cells; *MUC2*, which is not well expressed in normal bronchial epithelial cells [91, 54], is upregulated by TNF-α [54] and by *Pseudomonas aerugi-*

nosa lipopolysaccharide [99] in H 292 lung mucoepidermoid cells. Analysis of the effects of inflammatory mediators on *MUC* expression will ultimately provide insights into mechanisms that impact on the pathogenesis of mucus overproduction and obstruction in the airways.

6.1. MUC Expression

The question of whether expression levels of specific *MUC* genes are altered in disease is an important one and is being addressed in many laboratories. An extensive study on the expression of eight *MUC* genes in nasal turbinates in patients with vasomotor rhinitis (VMR) has been carried out (Table 3, ref. 75). Essentially no changes in the expression of *MUC2, MUC4* or *MUC5* in nasal epithelium are observed, although there is a decrease in the expression of *MUC1.* Slight but not significant decreases in *MUC1, MUC3, MUC5* and *MUC7* expression occur in submucosal glands [75]. A quantitative comparison of the levels of expression of *MUC5, MUC2* and *MUC1* in nasal epithelial cells from healthy subjects and individuals with rhinitis or CF has also been made. No significant differences in *MUC* transcript levels between the groups was observed [74].

Alterations in tissue expression of *MUC* genes occurs in adenocarcinomas derived from epithelial tissues [92], as some *MUC* genes not normally expressed appear to be upregulated. Whether this simply reflects alteration of *MUC* regulation along with that of other genes in cancer cells, or whether alterations in *MUC* genes are involved in maintaining adenocarcinomas, is not known. Interestingly, the lack of Muc-1 in mammary gland adenocarcinomas in mutant mice (Muc-1–/–) results in highly statistically significant decrease in the growth rate (progression) of tumors [93].

6.2. Mucin Secretion

It is likely that alterations in *MUC* gene expression in airway diseases or by infection or inflammatory mediators will correlate with altered concentrations of mature MUC mucins in airway secretions and mucus. Until recently there have been difficulties even in defining which mature MUC mucins are present in normal and pathological airway secretions. This is due both to difficulties in procuring adequate amounts of secretions from healthy airways and the paucity of molecular tools to identify specific MUCs. Most mucin antibodies recognize carbohydrate or conformational determinants, and lectins have specificity only for oligosaccharide determinants. However, these determinants have not yet been localized to specific MUC mucins in most cases. Antibodies specific to the TR domains of specific MUCs have been raised, but O-glycosylation of mucins frequently renders these epitopes inaccessible. MUC-specific antibodies, raised against specific peptide domains in the CR domains of secretory mucins, are now

becoming available [102, 76] and should prove useful in establishing MUC mucin levels in normal and pathological airway secretions.

Of the seven MUC genes known to be expressed in the upper and lower respiratory tract (Table 2), mature mucins with MUC5/5AC, MUC8 and MUC2 backbones have been identified in or isolated from airway mucus or secretions. TBM/MUC5 was initially isolated as the major mucin fraction from a single patient with bronchial asthma [17, 47]. MUC5 mucins have recently been shown to be a major mucin component both in secretions from an individual with status asthmaticus and in pooled secretions from normal individuals [76] and in some airway epithelial cell secretions [102]. MUC8/HTM-1 was isolated as a major mucin from lung mucus of a CF patient [49]. MUC2 was recently shown, by a MUC2 antibody specific to a sequence in its carboxyl termini CR domain, to be a minor component of secretions from an individual with status asthmaticus [76, 103].

6.3. Altered Glycosylation of MUC Mucins

The glycosylation patterns of MUC protein backbones may be altered in disease, in addition to or instead of alterations in the expression of *MUC* genes and gene products. Changes in terminal glycosylation, i.e. fucosylation, sialylation or sulfation of O-glycans, could alter the biological and physical properties of airway mucus. This is suggested by comparative analyses of mucins isolated from CF and non-CF patients (reviewed in ref. 94), but requires comparison of O-glycans isolated from specific MUC mucins secreted by healthy individuals and patients. It has been proposed that CF mucins may be differently glycosylated as a direct or indirect consequence of mutant CFTR [95], and thus bind differently to bacterial adhesins in CF airway mucus [96, 97]. Evaluation of these hypothesis will require identification and comparison of specific MUC mucins and determination of their O-glycans.

7. Summary

Elucidation of the role of mucins in health and disease requires an understanding at the molecular level of mucin genes and their gene products. With the identification of *MUC1* in 1987 [13] and the identification of an additional eight *MUC* genes by 1995, the tools to carry out this formidable task are becoming available. Knowledge of the regulation and expression of *MUC* genes and of the structure and glycosylation of their gene products will require elucidation of cis elements and trans-activating factors that regulate *MUC* gene expression. Delineation of the structural features which define the physical and biological functions of mature glycosylated

mucins with respect to their MUC protein backbones and their O-glycans is also required. The physical properties of mucins may be solely due to structural features in their MUC protein backbone, especially in their CR domains, and/or to charge and hydrophobic properties contributed by terminal glycosylators of O-glycans, i.e. sialic acid, sulfate, fucose. The biological properties of mucins likely correlate to MUC glycosylation status; the effects of MUC primary structure on O-glycosylation are still unknown.

Acknowledgments

We thank Drs. Judith Voynow, John Berger and Franco Piazza for critical reading of the manuscript, Drs. K. Guzman, P. Nettesheim and G. Sachdev for sharing their manuscripts in press, Li Huang for graphing Figure 1, and Laura Chapman for assistance with tables and figures. This review was supported by NIH HL33152 to MCR, CA64389 and DK49184 to SJG.

References

1. Rose MC (1992) Mucins: Structure, function and role in pulmonary diseases. *Am J Physiol* 263: L413–L429.
2. Gum Jr (1992) Mucin genes and the proteins they encode: Structure, diversity and regulation. *Am J Respir Cell Mol Biol* 7: 557–564.
3. Kim YS, Gum JR Jr (1995) Diversity of mucin genes, structure, function and expression. *Gastroenterology* 109: 999–1013.
4. Gendler SJ, Spicer AP (1995) Epithelial mucin genes. *Annu Rev Physiol* 57: 607–634.
5. Carlstedt I, Sheehan JK, Corfield AP, Gallagher JT (1985) Mucous glycoproteins: A gel of a problem. *Essays in Biochem* 20: 40–76.
6. Neutra MR, Forstner JF (1987) Gastrointestinal mucus: Synthesis, secretion and function. In: Johnson R ed. *Proceedings of the gastrointestinal tract.* New York: Raven Press, 975–1009.
7. Strous GJ, Dekker J (1992) Mucin-type glycoproteins. *Crit Rev Biochem Mol Biol* 27: 57–92.
8. Rose MC (1994) Mucin glycoproteins are the major macromolecular component of airway secretions. *Am J Physiol* 26: L109–L111.
9. Van Halbeek H, Strang A-M, Lhermitte M, Rahmoune H, Lamblin G, Roussel P (1994) Structures of monosialyl oligosaccharides isolated from the respiratory mucins of a non-secretor (O, Le^{a+b-}) patient suffering from chronic bronchitis: Characterization of a novel type of mucin carbohydrate core structure. *Glycobiology* 4:203–219.
10. Hounsell EF (1994) Physicochemical analyses of oligosaccharide determinants of glycoproteins. *Adv Carbo Chem Biochem* 50: 311–350.
11. Schachter H, Williams D (1982) Biosynthesis of mucus glycoproteins. In: Chantler EN, Elden JB, Elstein M eds. Adv Exp Med Biol, vol. 144. New York: Plenum Press, 3–28.
12. Hull SR, Bright A, Carraway KL, Abe M, Hayes DF, Kufe DW (1989) Oligosaccharide differences in the DF3 sialomucin antigen from normal human milk and the BT-20 human breast carcinoma cell line. *Cancer Comm* 1: 261–267.
13. Gendler SJ, Burchell JM, Duhig T, Lamport D, White R, Parker M, Taylor-Papadimitriou J (1987) Cloning of partial cDNA encoding differentiation and tumor-associated mucin glycoproteins expressed by human mammary epithelium. *Proc Natl Acad Sci USA* 84: 6060–6064.
14. Gum JR, Byrd JC, Hicks JW, Toribara NW, Lamport DTA, Kim YS (1989) Molecular cloning of human intestinal mucin cDNAs. *J Biol Chem* 264: 6480–6487.

15. Gum JR, Hicks JW, Swallow DM, Lagace RL, Byrd JC, Lamport DTA, Siddiki B, Kim YS (1990) Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. *Biochem Biophys Res Comm* 171: 407–415.
16. Porchet N, Nguyen VC, Dufosse J, Audie JP, Guyonnet-Duperat V, Gross MS, Denis C, Degand P, Bernheim A, Aubert JP (1991) Molecular cloning and chromosomal localization of a novel human tracheobronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. *Biochem Biophys Res Comm* 175: 414–422.
17. Meerzaman D, Charles P, Daskal E, Polymeropoulos MH, Martin BM, Rose MC (1994) Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (MUC5). *J Biol Chem* 269: 12932–12939.
18. Guyonnet Duperat V, Audie J-P, Debailleul V, Laine A, Buisine M-P, Galiegue-Zouitina S, Pigny P, Degand P, Aubert J-P, Porchet N (1995) Characterization of the human mucin gene MUC5AC: A consensus cysteine-rich domain for 11p15 mucin genes? *Biochem J* 305: 211–219.
19. Klomp LWJ, Van Rens L, Strous GJ (1995) Cloning and analysis of human gastric mucin cDNA reveals two types of conserved cysteine-rich domains. *Biochem J* 308: 831–838.
20. Dufosse J, Porchet N, Audie J-P, Guyonnet Duperat V, Laine A, Van-Seuningen I, Marrakchi S, Degand P, Aubert J-P (1993) Degenerate 87-base-pair tandem repeats create hydrophilic/hydrophobic alternating domains in human mucin peptides mapped to 11p15. *Biochem J* 293: 329–337.
21. Toribara NW, Roberton AM, Ho SB, Kuo W-L, Gum E, Hicks JW, Gum JR Jr, Byrd JC, Siddiki B, Kim YS (1993) Human gastric mucin: Identification of a unique species by expression cloning. *J Biol Chem* 266: 5879–5885.
22. Bobek LA, Tsai H, Biesbrock AR, Levine MJ (1993) Molecular cloning, sequence and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J Biol Chem* 268: 20563–20569.
23. Shankar V, Gilmore MS, Elkins RC, Sachdev GP (1994) A novel human airway mucin cDNA encodes a protein with unique tandem-repeat organization. *Biochem J* 300: 295–298.
24. Gum JR, Hicks JW, Toribara NW, Siddiki B, Kim YS (1994) Molecular cloning of human intestinal mucin (MUC2) cDNA. *J Biol Chem* 269: 2440–2446.
25. Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani E, Wilson D (1990) Molecular cloning and expression of the human tumour-associated polymorphic epithelial mucin, PEM. *J Biol Chem* 265: 15286–15293.
26. Lan MS, Batra SK, Qi W, Metzgar RS, Hollingsworth MA (1990) Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J Biol Chem* 265: 15294–15299.
27. Xu G, Huan L, Khatri I, Sajjan US, McCool D, Wang D, Jones C, Forstner G, Forstner J (1992) Human intestinal mucin-like protein (MLP) is homologous with rat MLP in the C-terminal region, and is encoded by a gene on chromosome 11p15.5. *Biochem Biophys Res Comm* 183: 821–828.
28. Crepin M, Porchet N, Aubert JP, Degand P (1990) Diversity of the peptide moiety of human airway mucins. *Biorheology* 27: 471–484.
29. Aubert JP, Porchet N, Crepin M, Duterque-Coquillaud M, Vergnes G, Mazzuca M, Debuire B, Pettiprez D, Degand P (1991) Evidence for different human tracheobronchial mucin peptides deduced from nucleotide cDNA sequences. *Am J Respir Cell Mol Biol* 5: 175–185.
30. Shankar V, Pichan P, Eddy RL Jr, Tonk V, Nowak N, Sait SNJ, Shows TB, Schultz RE, Gotway G, Elkins RC et al. (1997) Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxyl terminus. *Am J Respir Cell Mol Biol.* 16: 232–241.
31. Pemberton L, Taylor-Papadimitriou J, Gendler SJ (1992) Antibodies to the cytoplasmic domain of MUC1 mucin show conservation throughout mammals. *Biochem Biophys Res Comm* 185: 167–175.
32. Yan P-S, Ho SB, Itskowitz SH, Byrd JC, Siddiqui B, Kim YS (1990) Expression of native and deglycosylated colon cancer mucin antigens in normal and malignant epithelial tissues. *Lab Invest* 62: 698–706.
33. Ho SB, Roberton AM, Shekels LL, Lyftogt CT, Niehans GA, Toribara NW (1995) Expression cloning of gastric mucin complementary DNA and localization of mucin gene expression. *Gastroenterology* 109: 735–747.

34. Shekels LL, Lyftogt C, Kieliszewski M, Filie JD, Kozak CA, Ho SB (1995) Mouse gastric mucin: Cloning and chromosomal localization. *Biochem J* 311: 775–785.
35. Troxler RF, Offner GD, Zhang F, Iontcheva I, Oppenheim FG (1995) Molecular cloning of a novel high molecular weight mucin (MG1) from human sublingual gland. *Biochem Biophys Res Comm* 217: 1112–1119.
36. Sadler JE, Shelton-Inloes BB, Sorace, JM, Harlan JM, Titani K, Davie EW (1985) Cloning and characterization of two cDNAs coding for human von Willebrand factor. *Proc Natl Acad Sci USA* 82: 6394–6498.
37. Ruggeri ZM, Ware J (1993) von Willebrand factor. *FASEB J* 7: 308–316.
38. Voorberg J, Fontijn R, Calafat J, Janssen H, van Mourik JA, Pannekoek H (1991) Assembly and routing of von Willebrand factor variants: The requirements for disulfide-linked dimerization reside within the carboxy-terminal 151 amino acids. *J Cell Biol* 113: 195–205.
39. Desseyn JL, Guyonnet-Duperat V, Porchet N, Aubert JP, Laine A (1997) Human Mucin Gene *MUC*5B, the 10.7-kb large central exon, encodes various alternate subdomains resulting in a superrepeat. *J Biol Chem* 272: 3168–3178.
40. Nishimori I, Johnson NR, Sanderson SD, Perini F, Mountjoy KP, Cerney R, Gross ML, Finn O, Hollingsworth MA (1994) The influence of acceptor substrate primary amino acid sequence on the activity of human UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase: Studies with the MUC1 tandem repeat. *J Biol Chem* 269: 16123–16130.
41. Nehrek K, Hagen FK, Tabak LA (1996) Charge distribution of flanking amino acids influences O-glycan acquisition in vivo. *J Biol Chem* 271: 7061–7065.
42. Bennett EP, Hassan H, Clausen H (1996) cDNA cloning and expression of a novel human UDP-*N*-acetyl-alpha-D-galactosamine. *J Biol Chem* 271: 17006–17012.
43. Hilkens J, Buijs F (1988) Biosynthesis of MAM-6, and epithelial sialomucin. *J Biol Chem* 263: 4215–4222.
44. Linsley PS, Kallestad JC, Horn D (1988) Biosynthesis of high molecular weight breast carcinoma associated mucin glycoproteins. *J Biol Chem* 263: 8390–8397.
45. Ohara S, Byrd JC, Gum JR Jr, Kim YS (1994) Biosynthesis of two distinct types of mucin in HM3 human colon cancer cells. *Biochem J* 297: 509–516.
46. McCool DJ, Forstner JF, Forstner GG (1994) Synthesis and secretion of mucin by the human colonic tumour cell line LS 180. *Biochem J* 302: 111–118.
47. Rose MC, Kaufmann B, Martin BM (1989) Proteolytic fragmentation and peptide mapping of human carboxyamidomethylated tracheobronchial mucin. *J Biol Chem* 264: 8193–8199.
48. Lesuffleur T, Roche F, Hill AS, Lacasa M, Fox M, Swallow DM, Zweibaum A, Real FX (1995) Characterization of a mucin cDNA clone isolated from HT-29 mucus-secreting cells. *J Biol Chem* 270: 13665–13673.
49. Shankar V, Naziruddin B, Reyes de la Rocha S, Sachdev GP (1990) Evidence of hydrophobic domains in human respiratory mucins: Effects of sodium chloride on hydrophobic binding properties. *Biochemistry* 29: 5856–5864.
50. Klinger JD, Tandler B, Liedtke CM, Boat TF (1984) Proteinases of *Pseudomonas aeruginosa* evoke mucin release by tracheal epithelium. *J Clin Invest* 74: 1669–1678.
51. Steiger D, Hotchkiss J, Bajaj L, Harkema J, Basbaum C (1995) Concurrent increases in the storage and release of mucin-like molecules by rat airway epithelial cells in response to bacterial endotoxin. *Am J Respir Cell Mol Biol* 12: 307–314.
52. Kim KC, Park HR, Shin CY, Akiyama TA, Ko KH (1996) Nucleotide-induced mucin release from primary hamster tracheal surface epithelial cells involves the P-2U purinoceptor. *Eur Respir J* 9: 542–548.
53. Kim KC, Wasano K, Niles RM, Schuster JE, Stone PJ, Brody JS (1987) Human neutrophil elastase releases cell surface mucins from primary cultures of hamster tracheal epithelial cells. *Proc Natl Acad Sci USA* 84: 9304–9308.
54. Levine SJ, Larivee P, Logun C, Angus CW, Ognibene FP, Shelhamer JH (1995) Tumor necrosis factor-alpha induces mucin hypersecretion and MUC-2 gene expression by human airway epithelial cells. *Am J Respir Cell Mol Biol* 12: 196–204.
55. Young LR, Wang Y, Voynow JA (1996) Neutrophil elastase up-regulates MUC5AC gene expression in respiratory epithelial cells. *Pediatric Pulmonology* S 13: 288.
56. Guzman K, Randell SH, Nettesheim I (1995) Epidermal growth factor regulates expression of the mucous phenotype of rat tracheal epithelial cells. *Biochem Biophys Res Comm* 217: 412–418.

57. Gray TE, Guzman K, Davis CW, Abdullah LH, Nettesheim P (1996) Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am J Respir Cell Mol Biol* 14: 104–112.
58. De Luca LM (1991) Retinoids and their receptors in differentiation, embryogenesis and neoplasia. *FASEB* 5: 2924–2933.
59. Manna B, Lund M, Ashabush P, Kaufman B, Bhattacharyya SN (1994) Effect of retinoic acid on mucin gene expression in rat airways *in vitro*. *Biochem J* 297: 309–313.
60. An G, Lou G, Wu R (1994) Expression of MUC2 gene is down-regulated by vitamin A at the transcriptional level *in vitro* in tracheobronchial epithelial cells. *Am J Respir Cell Mol Biol* 10: 546–551.
61. Guzman K, Gray TE, Yoon J-H, Nettesheim P (1996) Quantitation of mucin RNA by PCR reveals induction of both MUC2 and MUC5 mRNA levels by retinoids. *Am J Physiol.* 271: L1023–1028.
62. Kovarik A, Peat N, Wilson D, Gendler SJ, Taylor-Papadimitriou J (1993) Analysis of the tissue-specific promoter of the MUC1 gene. *J Biol Chem* 268: 9917–9926.
63. Spicer AP, Parry G, Patton S, Gendler SJ (1991) Molecular cloning and analysis of the mouse homologue of the tumor-associated mucin, MUC1, reveals conservation of potential O-glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. *J Biol Chem* 266: 15099–15109.
64. Ohomori H, Dohrman AF, Gallup M, Tsuda T, Kai H, Gum JR Jr, Kim YS, Basbaum CB (1994) Molecular cloning of the amino-terminal region of a rat MUC 2 mucin gene homologue. *J Biol Chem* 269: 17833–17840.
65. Vos HL, de Vries Y, Hilkens J (1991) The mouse episialin (MUC1) gene and its promoter: rapid evolution of the repetitive domain in the protein. *Biochem Biophys Res Comm* 181: 121–130.
66. Hollingsworth MA, Closken C, Harris A, McDonald CD, Pahwa GS, Maher JI (1994) A nuclear factor that binds purine-rich, single-stranded oligonucleotides derived from S1-sensitive elements upstream of the CFTR gene and the MUC1 gene. *Nucleic Acids Res* 22: 1138–1146.
67. Irimura T, McIssac AM, Carlson DA, Yagita M, Grimm EA (1990) Soluble factor in normal tissues that stimulates high-molecular weight sialoglycoprotein production by human colon carcinoma cells. *Cancer Res* 50: 3331–3338.
68. Griffiths B, Matthews DJ, West L, Attwood J, Povey S, Swallow DM, Gum JR, Kim YS (1990) Assignment of the polymorphic intestinal mucin gene (MUC2) to chromosome 11p15. *Ann Hum Genet* 54: 277–285.
69. Nguyen VC, Aubert JP, Gross MS, Porchet N, Degand P, Frezal J (1990) Assignment of human tracheobronchial mucin gene(s) to 11p15 and a tracheobronchial mucin-related sequence to chromosome 13. *Hum Genet* 86: 167–172.
70. Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS, Galiegue S, D'Hooge C, Laine A, Van Seuningen I, Gum JR, Kim YS et al. (1996) Human mucin genes assigned to 11p15.5: identification and organization of a cluster of genes. *Genomics* 38: 340–352.
71. Zotter S, Hageman PC, Lossnitzer A, Mooi WJ, Hilgers J (1988) Tissue and tumor distribution of human polymorphic epithelial mucin. *Cancer Rev* 11–12: 55–101.
72. Gendler SJ, Madsen C, Yankaskas JR, Mucin mRNA expression in cystic fibrosis and normal bronchial epithelium. Manuscript submitted.
73. Voynow JA, Rose MC (1994) Quantitation of mucin mRNA in respiratory and intestinal epithelial cells. *Am J Respir Cell Mol Biol* 11: 742–750.
74. Voynow JA, Selby D, Rose MC. Comparison of mucin gene expression in CF and control nasal epithelial cells. Manuscript submitted.
75. Aust MR, Madsen CS, Jennings A, Kasperbauer JL, Gendler SJ (1997) Mucin mRNA expression in normal and vasomotor inferior turbinates. *Am J Rhinology*. In press.
76. Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK (1996) Respiratory mucins: Identification of core proteins and glycoforms. *Biochem J* 316: 967–975.
77. Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP (1993) Expression of human mucin genes in respiratory, digestive and reproductive tracts ascertained by in situ hybridization. *J Histochem Cytochem* 41: 1479–1485.
78. Dohrman A, Tsuda T, Escudier E, Cardone M, Jany B, Gum J, Kim Y, Basbaum C (1994) Distribution of lysozyme and mucin (MUC2 and MUC3) mRNA in human bronchus. *Exp Lung Res* 20: 367–380.

79. Chambers JA, Hollingsworth MA, Trezise AEO, Harris A (1994) Developmental expression of mucin genes MUC1 and MUC2. *J Cell Science* 107: 413–424.
80. Plopper CG, George JS, Mariassy A, Nishio S, Heidsiek J, Weir A, Tyler N, Wilson D, Cranz D, Hyde D (1989) Species differences in airway cell distribution and morphology. In: *Extrapolation of Dosimetric Relationships of Inhaled Particles and Gases.* Academic Press, 19–33.
81. Plopper CG, Weir A, George JS, Tyler N, Mariassy A, Wilson D, Nishio S, Cranz D, Heidsiek J, Hyde D (1988) Cell populations of the respiratory system: Interspecies diversity in composition, distribution and morphology. In: Dungworth D, Kimmerle G, Lewkoski J, McClellan R, Stober W eds. *Inhalation toxicology.* New York: Springer-Verlag, 25–40.
82. Verma M, Davidson EA (1993) Molecular cloning and sequencing of a canine tracheobronchial mucin cDNA containing a cysteine-rich domain. *Proc Natl Acad Sci USA* 90: 7144–7148.
83. Lamb D, Reid L (1968) Mitotic rates, goblet cell increase and histochemical changes in mucus in rat bronchial epithelium during exposure to sulphur dioxide. *J Pathol Bacteriol* 96: 97–111.
84. Basbaum C, Gallup M, Gum J, Kim Y, Jany B (1990) Modification of mucin gene expression in the airways of rats exposed to sulfur dioxide. *Biorheology* 27: 485–489.
85. Xu G, Huan L-J, Khatri IA, Wang D, Bennick A, Fahim REF, Forstner GG, Forstner JF (1992) cDNA for the carboxyl-terminal region of a rat intestinal mucin-like peptide. *J Biol Chem* 267: 5401–5407.
86. Hansson GC, Baeckstrom D, Carlstedt I, Klinga-Levan K (1994) Molecular cloning of a cDNA coding for a region of an apoprotein from the "insoluble" mucin complex of rat small intestine. *Biochem Biophys Res Comm* 198: 181–190.
87. Tsuda T, Gallup M, Jany B, Gum J, Kim Y, Basbaum C (1993) Characterization of a rat airway cDNA encoding a mucin-like protein. *Biochem Biophys Res Comm* 195: 363–373.
88. Gum JR Jr, Hicks JW, Lagace RE, Byrd JC, Toribara NW, Siddiki B, Fearney FJ, Lamport DTA, Kim YS (1991) Molecular cloning of rat intestinal mucin: Lack of conservation between mammalian species. *J Biol Chem* 266: 22733–22738.
89. Khatri IA, Forstner GG, Forstner JF (1993) Suggestive evidence for two different mucin genes in rat intestine. *Biochem J* 294: 391–399.
90. Guzman K, Bader T, Nettesheim P (1996) Regulation of MUC5 and MUC1 gene expression: Correlation with airway mucous differentiation. *Am J Physiol* 270: L846–L853.
91. Wu R, Plopper CG, Cheng PW (1991) Mucin-like glycoprotein secreted by cultured hamster tracheal epithelial cells. *Biochem J* 277: 713–718.
92. Ho SB, Shekels LL, Toribara NW, Kim YS, Lyftogt C, Cherwitz DL, Niehans GA (1995) Mucin gene expression in normal, preneoplastic and neoplastic human gastric epithelium. *Cancer Res* 55: 2681–2690.
93. Spicer AP, Rowse GJ, Lidner TK, Gendler SJ (1995) Delayed mammary tumor progression in MUC-1 null mice. *J Biol Chem* 270: 30093–30101.
94. Rose MC (1988) Epithelial mucous glycoproteins and cystic fibrosis. *Horm Metabol Res* 20: 601–608.
95. Barasch J, Kiss B, Prince A, Saiman L, Gruenert D, Al-Awqati Q (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature* 352: 70–73.
96. Lamblin G, Roussel P (1993) Airway mucins and their role in defence against microorganisms. *Resp Med* 87: 421–426.
97. Devarj N, Sheykhnazari M, Warren WS, Bhavanandan VP (1994) Differential binding of *Pseudomonas aeruginosa* to normal and cystic fibrosis tracheobronchial mucins. *Glycobiology* 4: 307–315.
98. Nielsin PA, Bennett EP, Wandall HH, Therkildsen MH, Hannibal J, Clausen H (1997) Identification of a major human high molecular weight salivary mucin (MG1) as tracheobronchial mucin MUC5B. *Glycobiology* 7: 413–419.
99. Li J-D, Dohrman AF, Gallup M, Miyata S, Gum JR, Kim YS, Nadel JA, Prince A, Basbaum CR (1997) Transcriptional activation of mucin by *Pseudomonas aeruginosa* lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. *Proc Natl Acad Sci USA.* 94: 967–972.

100. Temann U-A, Prasad B, Gallup MW, Basbaum C, Ho SB, Flavell RA, Rankin JA (1997) A novel role for murine IL-4 in vivo: induction of Muc-5ac gene expression and mucin hypersecretion. *Am J Respir Cell Mol Biol* 156: 471–478.
101. Hovenberg HW, Davies JR, Carlstedt I (1996) Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. *Biochem J* 318: 319–324.
102. Rose MC, Peters KW, Harris-Evans C, Gelfand HJ (1996) Isolation of Glycosylated mucins with the MUC5 protein backbone from airway cell secretions. *Ped Res* 39: 390A.
103. Hovenberg HW, Davies JR, Herrmann A, Linden C-J, Carlstedt I (1996) MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. *Glycoconjugate J* 13: 1–9.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 4
The Microanatomy of Airway Mucus Secretion

Terence M. Newman[1,*] and Duncan F. Rogers[2]

[1] *The Secretory Mechanisms Group, Department of Physiology, University College London, UK*
[2] *Thoracic Medicine, National Heart and Lung Institute (Imperial College), London, UK*

1. Introduction

Despite the importance of mucus secretion in airway physiology and pathophysiology, our understanding of the functional microanatomy of mucus release in mammalian airways is rudimentary. It is not clear how many different types of secretory granule may be present within the different mucus-producing cells (the goblet cells of the surface epithelium and the mucous and serous cells of the submucosal glands), or whether different pathways for granule release exist. There is no clear understanding of the sequence of intracellular events leading to basal secretion of a mucin granule, of the intracellular mechanisms involved or of the effect of secretagogues upon degranulation. The culmination of these events is generally considered to be fusion of the cytoplasmic mucin granule membrane with the plasma membrane, the process of exocytosis, with complete incorporation of the granule membrane into the apical plasma membrane. However, ultrastructural support for this proposal has been surprisingly sparse, and it has been particularly difficult to find changes that can be quantitatively

* Author for correspondence: The Secretory Mechanisms Group, Department of Physiology, University College London WC1E 6JJ, UK.

correlated with secretagogue action. The reasons for this are most probably associated with the investigative methods. In order to understand the functional morphology of mucin secretion, it is vital to appreciate what occurs during specimen preparation.

In this chapter, we consider the methodology of the different approaches that have been used to investigate the microanatomy of the release process, especially as they relate to airway secretion. We then consider the exocytotic process in one mucin-secreting airway cell type (the guinea pig tracheal epithelial secretory cell) that we have investigated in some detail. Because it must not be assumed *a priori* that exocytosis is the only, or even the major, method of release in airway secretory cells, we discuss modes of release in relation to what is known about constitutive and regulated secretion in other systems. Although much can be learnt by this approach, in particular by comparison with other mucin-containing cells such as the crypt and villus intestinal goblet cells, important differences exist. Therefore, we conclude the chapter with a model for mucin release in the airways. Although not definitive, we hope that it will be useful in stimulating discussion and illustrate the important contributions still to be made before we gain a full understanding of the microanatomy of secretion in airway mucin-containing cells.

2. Methods for Investigating the Morphological Basis of Release Processes

2.1. Investigations in Living Preparations by Light Microscopy

Information concerning the microanatomy of secretory events in living cells is limited by the resolution possible with light microscopy. Although it is possible using only conventional light microscopy to see evidence of degranulation in certain secretory cells, for example mast cells which have large granules, this does not provide information about the microanatomy of the secretory event. The development of video-enhanced microscopy and confocal scanning light microscopy, which maximize the information obtainable with light microscopy, has to some extent overcome this problem. Video-enhanced microscopy uses image intensification and noise subtraction systems to process an electronic signal produced from an image obtained using predominantly differential-interference-contrast (DIC) microscopy. In this manner, even objects as small as microtubules, with diameters of less than 0.025 μm (smaller than the wavelength of light), can be imaged. As microtubules are implicated in some forms of mucin release [1] this has great potential for analysis of airway secretion. However, because of diffraction effects, these small structures appear much wider (approximately 0.2 μm). Consequently, the detail of these structures,

and hence the initial stages of release from the cell (whether by exocytosis or other mechanisms), is lost. This does not, however, detract from investigation of the dynamics of the process itself.

Using video-enhanced microscopy, studies of release of mucins, glycoproteins and lipids from untreated airway goblet cells have investigated the action of putative secretagogues [2–5]. In this way the stimulatory effect of extracellular triphosphate nucleotides on the rate of granule release from goblet cells in both normal and cystic fibrosis (CF) airway epithelial explants has been demonstrated in single cells and correlated with concurrent enzyme-linked immunosorbent assay (ELISA) measurements of mucin releases [3]. The dynamics of secretion have been investigated: release takes place over tens of milliseconds, following a postulated delay of tens of seconds [3, 4]. Volume increases in the secreted product of several hundredfold take place within 20–30 ms [5]. This process can also be monitored in suitable preparations with a video camera attached to a standard microscope equipped with phase contrast (Figure 1). Using this type of set-up, discrete "packages" of mucus can be seen "erupting" from isolated guinea pig tracheal cells. The packages remain in close proximity to the cell and, after attaining a maximal expansion, dissipate slowly into the surrounding medium. Packages are released sequentially. In general, packages attain maximal expansion before another package is released. Rates of release could be altered by drug application to the petri dish. However, although a useful secretion assay, this approach still leaves unanswered basic microanatomical questions, such as whether release is from one or a number of granules, and whether there was membrane fusion before mucus release.

The effect of polyions, such as soluble proteins, on the swelling of mucin granules has also been monitored by video microscopy [6]. These experiments evaluated the effect of albumin on swelling kinetics of mucin granules after release from respiratory goblet cells grown in culture.

Other secretory events that have been analysed in some detail include release from salivary glands [7] using a DIC microscope and charge coupled device (CCD) camera in conjunction with a high-speed image 0processor. Changes in the number and distribution of secretory granules during secretion were measured and correlated with the appearance in the apical plasma membrane of omega-shaped invaginations imaged using confocal microscopy. The fluid-phase tracer Lucifer Yellow was used to give evidence for exocytotic release. Differences were found between this approach and results from previous electron-microscopical investigations, particularly with regard to the extent of enlargement of the plasma membrane found after granule fusion [7], which illustrates the contribution that modern light-microscopical techniques can make to investigation of the functional morphology of release. Video-enhanced microscopy has extended application of the technique to investigation of the location of proteins suggested to be involved in the process of secretion. For example, the role of synapsin 1 in regulating the availability of synaptic vesicles for neuro-

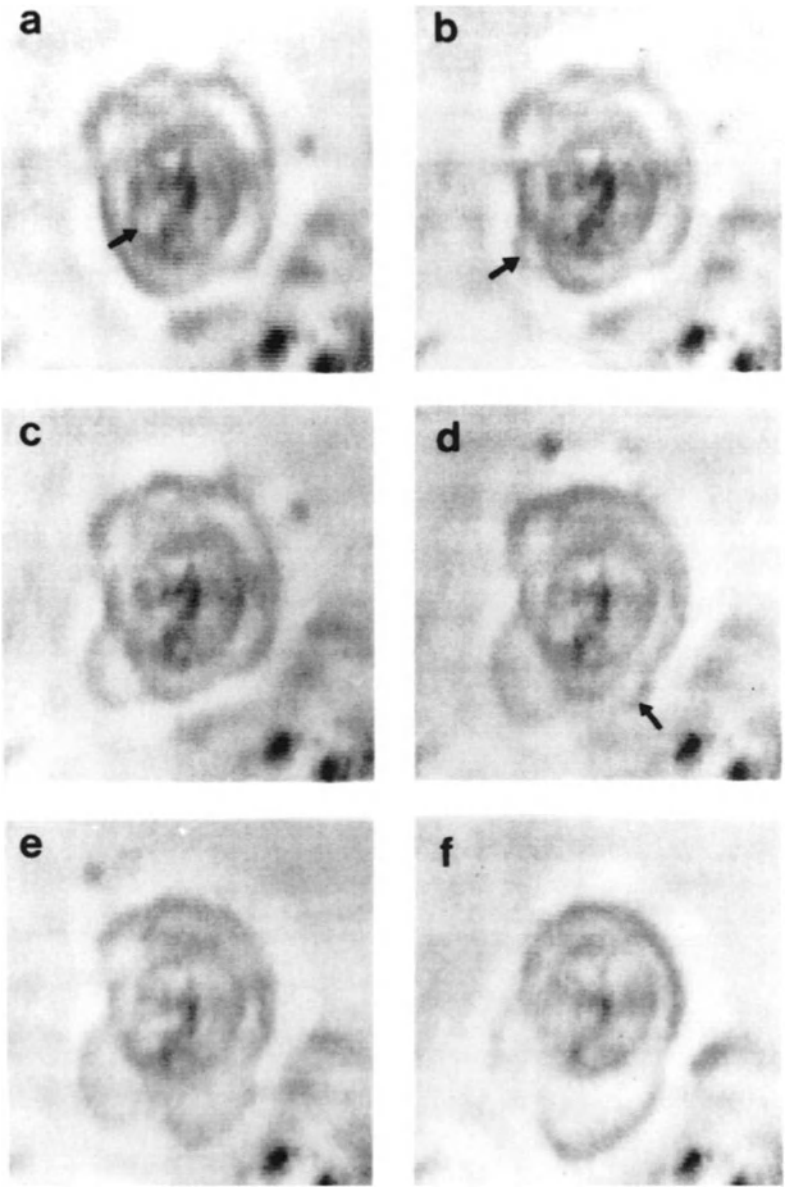


Figure 1. Mucus exocytosis sequence *in vitro* by an isolated cultured guinea pig tracheal epithelial secretory cell. (a) Secretory cell (arrow) surrounded by a "halo" of secretion. (b–c) A secretory event (arrow), considered to be mucus release, is seen as a discrete rapidly-developing volumetric expansion from the surface of the cell. (d–e) A second secretory event is initiated (arrow) as the previous one reaches maximal size. (f) Second secretory event reaches maximal size as initial expansion begins to disperse in culture medium. Whole sequence takes 25 s. Original videorecordings at ×600 magnification.

transmitter release has been studied [8]. Using this approach, dephosphorylated synapsin 1 was found to anchor vesicles to the actin cytoskeleton at nerve terminals prior to exocytosis. This observation is of interest to all studies of secretion, as well as illustrating the efficacy of video-enhanced microscopy.

Interference-reflection microscopy [9] is another light-microscopical technique that has yielded information about secretory activities in living cells, in particular the contractile vacuoles of the *Dictyostelium* amoebae [10]. This approach relies upon an interference effect due to the reflection from an organelle lying close to the surface of a cell, less than a wavelength of light away. Although the technique is necessarily limited by the epidermal location of many mucin-containing cells, there is potential for investigation of secretion in living airway cells.

2.2. Release Investigated in Chemically Fixed Cells

2.2.1. The use of aldehyde primary fixation: The majority of morphological studies of secretion have used chemical fixatives for light microscopy and, in conjunction with ultrathin sectioning, for transmission electron microscopy. These techniques have contributed to our understanding of exocytosis with transmission electron microscopy, in particular shaping the way the process has been modeled. However, because of the use of chemical fixatives many models, especially of the membrane fusion event itself, have proven erroneous or at best misleading as the introduction of physical, ultrarapid freezing techniques has shown (see Section 2.3 below). In comparison with studies of mucin release in fixed intestinal goblet cells, there have been far fewer investigations of airway mucin-containing cells. Most studies of mucin-containing cells have used a standard method of chemical fixation, namely aldehyde primary fixation followed by osmium tetroxide post-fixation [see, for example, refs. 11, 12]. From these and similar studies it has been possible to characterise the microanatomy of the principle mucin-containing cell types in the airways (Figure 2), namely the goblet cells of the epithelium and the mucus cells of the submucosal gland cells, as they appear in chemically fixed sections. They are characterised by basally located nuclei with a large Golgi apparatus and abundant rough endoplasmic reticulum. Apically the cell is larger, often protruding into the lumen, and filled with large numbers of granules varying in diameter between 200 and 1800 nm. Granules are reported to coalesce to form a sincitium, although this along with the swollen appearance of the apex of the cell may be an artifact of fixation (see Section 2.3). Granules are usually clear, although variation does exist in different animals. For example, the guinea pig has a granule structure intermediate between mucin granules and the electron-dense core granules of serous cells. Similarly histochemical staining of fixed sections has shown that even within the same

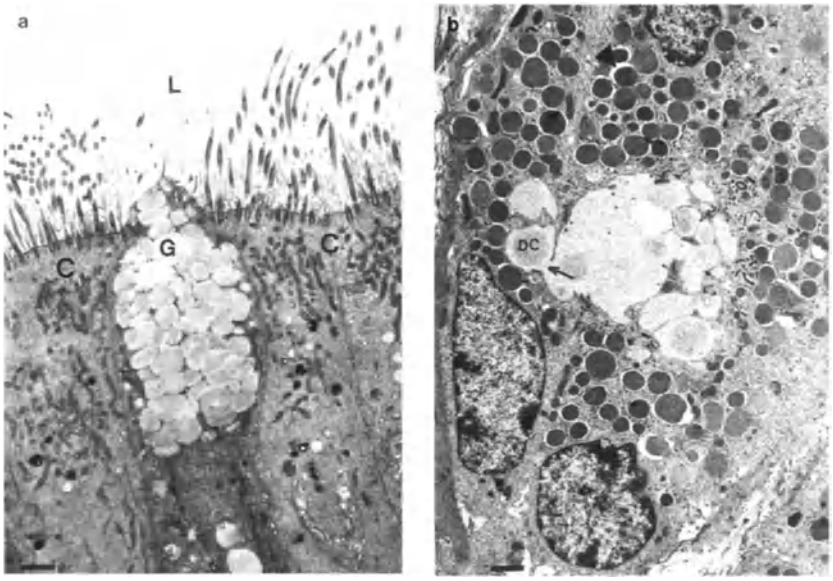


Figure 2. Examples of airway mucin-containing cells prepared by conventional fixation and ultrathin sectioning. (a) Human bronchial goblet cell with clear granules (G), many being fused together, present in the swollen apical portion of the cell. Exocytosis is difficult to visualise. L = lumen; C = ciliated cells. (b) Sheep submucosal gland cells with exocytotic site (arrowed). DC = dense core, arrowhead = "mucin-like halo". Bars for (a) and (b) = 1 μm.

cell, granule contents may have different pHs, reflecting variation in sulphate or sialic acid residues. The presence of this spectrum of structure and the ability to alter it by experimental intervention has suggested that these epithelial cells may be capable of differentiating (for details see Chapter 10 of this volume).

The relative contribution of the two main cell types to total mucus production varies in different animals, and it is difficult to assess this ultrastructurally. If release is by exocytosis, membrane fusions should be evident by electron microscopy (something that is beyond the resolution of video microscopy). However, it has been difficult to find such sites, probably because of technical limitations. Although the mechanisms behind granule release and mucin decondensation have not been fully characterised, it would appear that the process is not particularly amenable to fixation by aldehyde primary fixatives, for example glutaraldehyde. Such fixatives require minutes for their actions and so are not ideal for capturing very rapid events. Also, because aldehydes are primarily protein fixatives, they are not suited for the arrest of the mucin core of the granule, which has been shown by light microscopy to undergo a rapid expansion (see Figure 1). In addition, with the exception of phospholipids containing a free amino group (for example phosphatidylserine and phosphatidylethanolamine),

glutaraldehyde does not cross-link lipids and is therefore unlikely to immobilise any labile lipid intermediaries that may be present during the early stages of membrane fusion.

Nevertheless, despite the problems outlined above, exocytosis has been illustrated in many secretory cells using the standard method of chemical fixation. However, it can be seen to be very dependent on the method of preparation. Early attempts at finding electron-microscopical evidence for exocytosis were fraught with difficulty when using tissue that was perfused, stimulated and fixed *in situ* (see ref. [13] for review). It was not until the hitherto "inferior" approach of postmortem immersion-fixation of hemisected adrenal glands was tried that images of exocytosis were obtained. The conclusion from these observations, that slow penetration of the fixative by immersion slows exocytosis and allows accumulation of exocytotic figures, may not be a sufficient explanation of the phenomenon [13].

Examples of mucin exocytosis have been reported, for example in rabbit gallbladder treated with free fatty acids [14] and in sheep submucosal gland cells [15] (Figure 2b). However, it appears to be harder to get clear examples of exocytosis from the more accessible goblet cells of the trachea using conventional fixation. Perhaps, as with the study of chromaffin cells above, there is a slowing effect on exocytosis caused by the relative inaccessibility of the gland and the presence of mucus in the gland duct. For many cell types, where release is very rapid, for example neuronal cells, other approaches have been employed (see below). Also, even in the chromaffin cell, for which many examples of exocytosis have been obtained, a recent morphometric study has suggested that although exocytosis can occur, analysis of granule numbers and movement does not support the conclusion that this is how most secreted product is released [16]. Although these observations are contrary to current wisdom, they indicate an alternative interpretation of secretory event data.

2.2.2. Tannic acid arrest procedures: Another approach that uses chemical fixatives to investigate secretion, although in a different manner to conventional fixation, is the technique of tannic acid arrest. Tannic acid, when applied in physiological saline to secretory cells (including neuronal tissue) prior to a subsequent fixation step, arrests exocytosis of granule contents, which causes an accumulation of fusion sites [17]. This is a property shared with other polymers, for example dextran, which suggests that there may be a class of cell surface-acting agents with similar properties [18]. Investigations in neural, neuroendocrine and endocrine systems have shown the efficacy of tannic acid arrest for quantifying secretory events [19–21]. In the case of atrial natriuretic peptide secretion, linear release of the peptide was found over a 30-min tannic acid perfusion period [22]. Cells can be stimulated prior to tannic acid application or in the presence of tanic acid. Either way, an increase in fusion sites can be found. This approach provides support for the overall idea that during secretion in the majority of cell

types, granules are lost by exocytosis. A significant increase in arrested-fusion sites can be found in cells even where there is no discernible decrease in cytoplasmic granules.

Tannic acid is in fact a collective name given to a variety of polymers of plant origin that have protein-, lipid- and carbohydrate-fixing actions [23–25]. How these actions are linked to its ability to arrest both exocytotic and endocytotic processes is not clear. The rationale behind the technique, namely the ability to increase the number of fusion figures by allowing continued secretion to occur, is dependent upon the arrest agent not crossing the plasma membrane to fix the interior of the cell, as does glutaraldehyde. However, the technique can even be used to arrest and accumulate exocytotic sites in permeabilised cells [20]. If tannic acid has a lipid-fixing action, then it would appear that the fusion process can persist even when the outer leaflet of the lipid bilayer, at least, has been affected. Similarly, if tannic acid's protein-fixing ability is important, then exocytosis cannot necessitate the reorganization of "fixable" intercalated membrane proteins. Support for the idea that it is the carbohydrate-fixing ability of tannic acid that is vital for the arrest of fusion comes from cell systems such as atrial cells, where an intact glycocalyx is found over the forming channel at the fusion site of the secretory granules. Retention of the glycocalyx appears to be important because dextran, another long-chain polymer, also arrests exocytosis in atrial cells [18] even though it does not have a fixative action. Dextran also produces a similar intact glycocalyx over the forming exocytotic channel. Retention of the glycocalyx over the fusion pore is not obligatory to arrest, as is seen at sites of fusion in the eosinophil [26] and cultured chromaffin cells [20].

We have recently used tannic acid arrest in a study of guinea pig airway secretory cells [27]. These cells are useful for secretory arrest studies because they contain a homogenous population of granules with a dense core surrounded by a clear mucin component (Figure 3). Using tannic acid arrest, exocytotic release can be clearly demonstrated (Figure 3). Retention of the dense core enables sites of exocytosis to be easily recognised and distinguished from other membrane perturbations. We found that the number of fusion sites increases upon stimulation, which provides support for the occurrence of exocytosis in these cells and its involvement in both basal and stimulated secretion. The use of tannic acid perfusion combined with freeze-fracture replication provides an unparalleled appreciation of the membrane systems involved during the exocytotic process (Figure 4).

2.3. Exocytosis Investigated in Ultrarapidly Frozen Cells

The use of ultrarapid freezing was promoted by the work of Heuser, Reese and colleagues [28, 29], who illustrated the efficacy of the technique for investigating synaptic vesicle fusion, particularly when used in conjunction

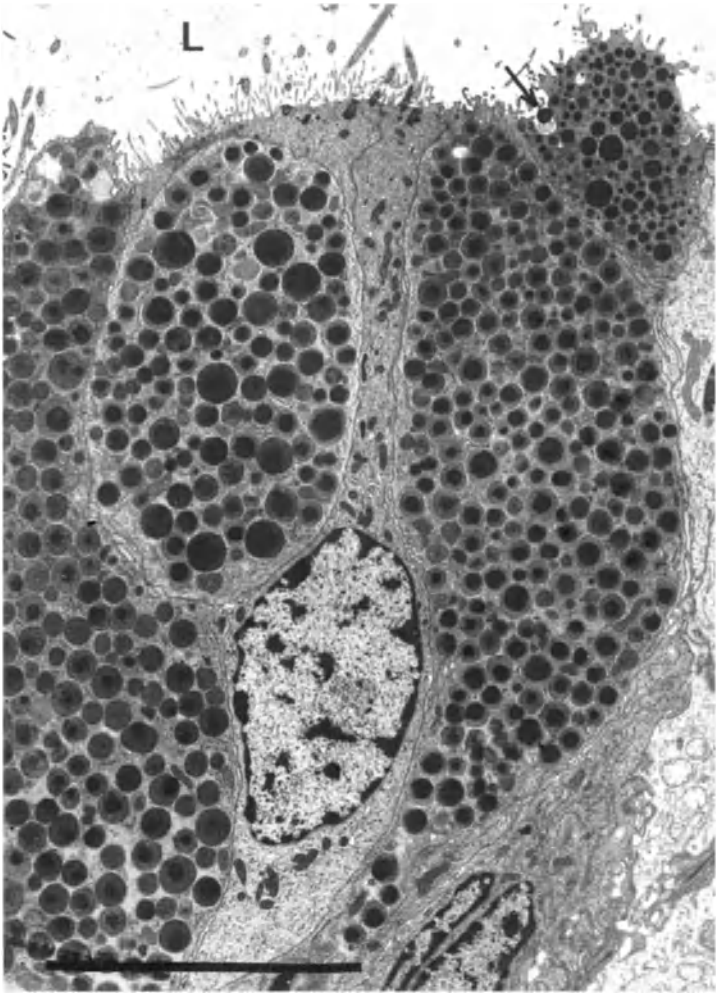


Figure 3. Guinea pig tracheal mucin-containing cells prepared after tannic acid arrest. Secretory cells have predominantly one type of granule, sometimes fused in a mass. Each granule contains a dense core similar to that found in serous cells with a lighter "mucin-like" halo. The cell is swollen apically. Sites of exocytosis are common (e.g. arrowed), frequently with the dense core retained at the fusion site. L = lumen. Bar = 10 µm.

with freeze-fracture replication. Neurotransmitter release is very rapid and not easily investigated by chemical fixatives, because in addition to the problem of capturing the sites of exocytosis, the fixative can also cause release of neurotransmitter.

Ultrarapid freezing can now be achieved by a number of different methods. These are "plunge" (or immersion) freezing, jet freezing, high-

Figure 4. Apical portion of a guinea pig tracheal epithelial mucin-containing cell prepared after tannic acid arrest and freeze-fracture replication. The complexity of membrane systems involved in secretion is evident. A nerve (N) with synaptic vesicles is present, running in close proximity to this portion of the cell. L = lumen. Bar = 1 μm.

pressure freezing and metal mirror ("slam") freezing. Although there are variations in use, the objective remains essentially the same in each case: to remove heat as rapidly as possible from the specimen to immobilise cellular constituents at their sites of action. The main method of investigation for mucin-containing cells has been slam freezing. The approach utilises the rapid transfer of heat attainable across a solid/solid interface. The specimen, for example a segment of trachea split and pinned on the

basal surface, is brought rapidly into contact with a helium- or nitrogen-cooled copper or silver block. In untreated specimens this produces a zone of "good" freezing where ice crystals are small enough not to affect cell structure, as visualised by electron microscopy. This zone will not be consistent throughout the specimen because of its variable surface topography. The depth of freezing can also be affected by the amount of saline left around the specimen, which needs to be controlled by careful blotting. Slamming provides the best method for investigating secretion from epithelial mucin-secreting cells, as these are located at the airway surface. The length of some cells means that although freezing at the surface may be excellent, there may be some ice crystal damage at the apical portion of the cell.

If ultrarapid freezing at rates $>10^5$ °C s^{-1} is used for primary fixation, after subsequent processing for ultrathin section electron microscopy (freeze substitution), the shape of "goblet" cells of the trachea is different to that produced by chemical fixation. The cells have a more linear and conventional columnar appearance (Figure 5) which is similar to that described in other mucin-containing cells [30, 31]. The commonly accepted chalicelike shape of airway goblet cells (Figures 2 and 3) is therefore probably erroneous and caused by an artifact of chemical fixation. The mechanism underlying production of the artefactual appearance is unknown but probably involves swelling of the intensely hydrophilic secretory granules that occurs too rapidly to be halted by glutaraldehyde which, in any event, is not a good fixative for mucin granule core contents. Although freeze substitution also involves a chemical fixation step, the latter takes place at low temperature with cellular constituents still frozen *in situ*. Fixation of the mucins by osmium tetroxide takes place before expansion can occur. This concept is important for an understanding of mucin release, because granule swelling during aldehyde chemical fixation may also be implicated in other observations, for example apparent granule–granule fusion. Granule swelling may also affect other properties such as fusion with the plasma membrane. Although aiding our understanding of the normal microanatomy of the goblet cell, ultrarapid freezing has not been applied in a systematic or quantitative study of stimulated and secreting cells. However, ultrarapid freezing has been used in conjunction with X-ray microanalysis to examine the ion content of mucin granules in the respiratory epithelium, which is another aspect vital to an understanding of secretion [32].

3. Mucin Granule Exocytosis

Studies of mucin secretion from intestinal goblet cells may aid our understanding of how mucin granule exocytosis is regulated in the airways. However, as with studies of airway secretion, images of intestinal cells illustrating full fusion between secretory granules and the plasma membrane

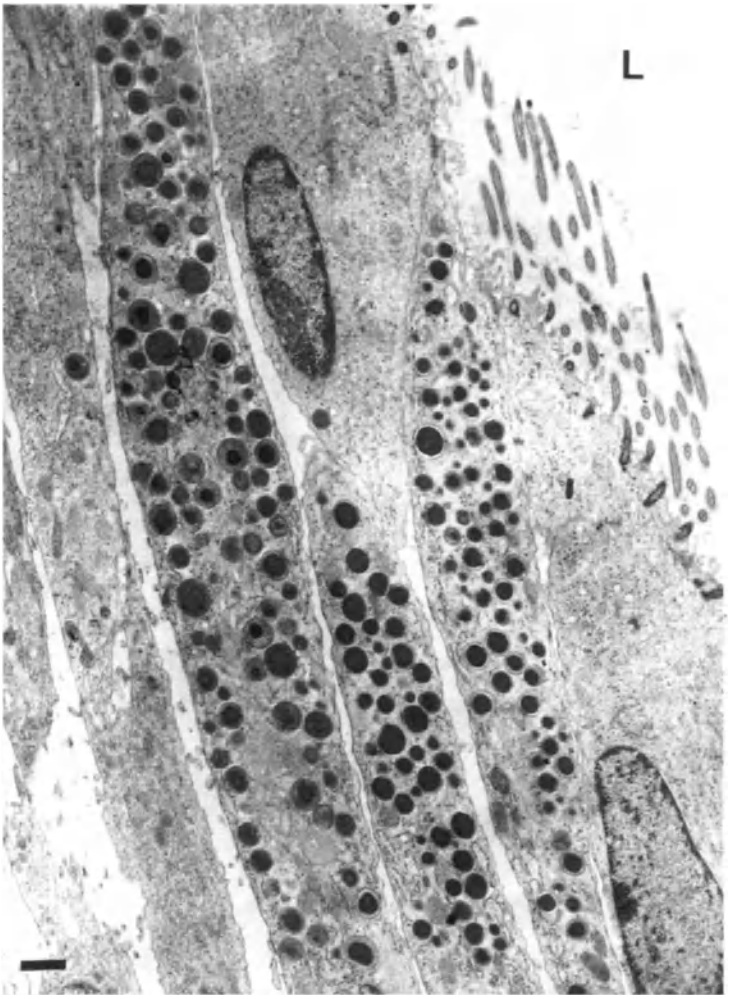


Figure 5. Guinea pig tracheal mucin-containing cells prepared by ultrarapid freezing and freeze substitution. The cells have a much more columnar shape than those prepared with conventional chemical fixation (see Figure 1). In comparison with conventional fixation, the "mucin" layers around the dense cores are less swollen. Granules remain distinct and do not coalesce. L = lumen. Bar = 1 μm.

appear difficult to obtain. Results using the tannic acid arrest procedure offer the best examples of exocytotic release from airway mucin-containing cells. Three types of fusion have been reported in these cells [27]. The first and most common is simple fusion, which involves a single granule fusing with the plasma membrane with full incorporation of the granule membrane into the plasma membrane (Figure 6). Second, more complicat-

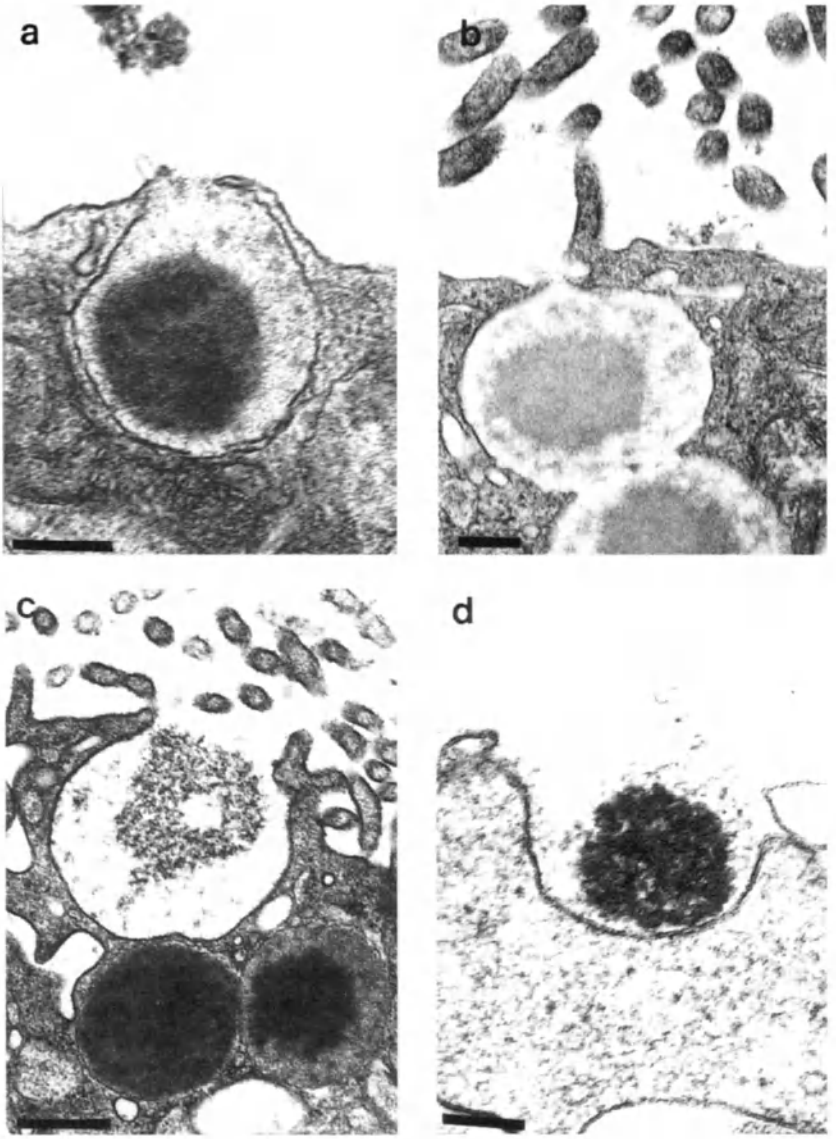


Figure 6. Hypothetical fusion sequence assembled from tannic acid arrested mucin-containing granules of guinea pig tracheal epithelial secretory cells. (a) initial stages of pore formation; (b) beginnings of mucus release; (c) pore widening and greater release of electron-lucent material; (d) later stages of release with pore greatly expanded and retention of electron-dense material at the exocytotic site. In each panel, lumen is at top of micrograph. Bar = 0.1 μm.

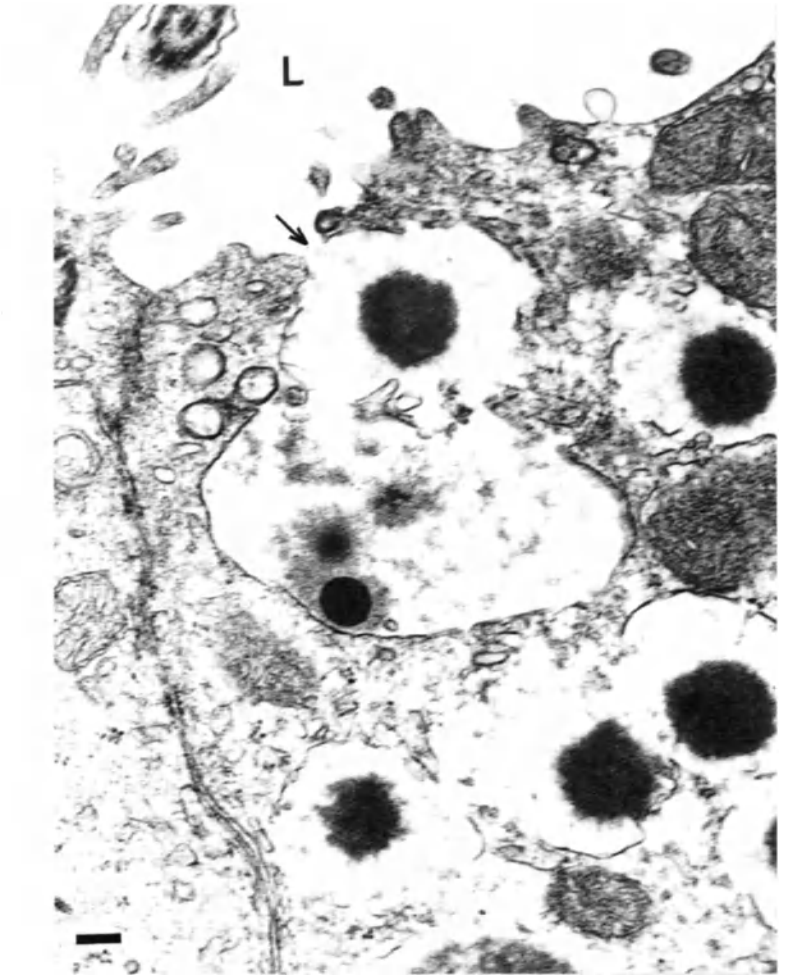


Figure 7. Compound exocytosis in a tannic acid-arrested guinea pig tracheal epithelial secretory cell, illustrating the dense cores present in the cell cavity. Arrow = fusion site; L = lumen. Bar = 0.1 μm.

ed fusion sites are found where several granules, connected together intracellularly, fuse with the plasma membrane (Figure 7). The latter may be referred to as compound exocytoses whereby a cavity appears to become formed within the cell which, in ultrathin section, can be seen to be in continuity with the airway lumen. In some cells the intracellular cavity can occupy most of the apical portion of the cell. The cavity will often still contain both dispersing dense cores and more diffuse secretory material. Com-

pound exocytoses are found in both stimulated and unstimulated preparations. They may be the result of an accumulation of individual granules fusing sequentially with granules already continuous with the plasma membrane. Each intergranule fusion is, therefore, equivalent to a simple fusion. If this really occurs, and it is as yet only a supposition, then the action of tannic acid, which will act upon all aspects of membrane function, may be abetting the process by slowing membrane retrieval. Alternatively, there may a prefused mass of granules already in the cell which only needs one apical granule to fuse with the plasma membrane to cause release of a large mass of mucus. A similar process is suggested for the accelerated secretion of intestinal goblet cells. Compound exocytoses are known to occur in other secretory systems, for example eosinophils, where they are thought to allow the co-ordinated release of large amounts of enzymatic secretory product in order to attack bacteria. Results from tannic acid-treated eosinophils also illustrate the compound nature of many of the exocytoses [26]. In these cells compound exocytoses have been studied electrophysiologically, showing that tannic acid *per se* is not causing the compound fusions found ultrastructurally. Also, in tannic acid-treated atrial cells, compound fusion sites are never found [18]. Apart from being a method of directing large amounts of secretory product, compound exocytoses would, especially in the case of airway secretory cells, seem to be an inevitable consequence of the limited area of plasma membrane available for fusion sites. Large amounts of membrane are added to the apical plasma membrane by fusing secretory granules. It is unlikely that any membrane retrieval mechanism could recycle granule membrane fast enough to allow continued fusion without the facility to utilise compound exocytoses. Whether in the airway cells this compound exocytosis is of prefused or sequentially added granules is difficult to ascertain. Many cells show prefused granules in the cytoplasm without obvious connections to the exterior, even where this can be traced through serial sections. It is possible that this apparent fusion is due to granule ruptures caused by swelling of the granule contents during fixation. However, compound exocytoses have also been found in ultrarapidly frozen tissue, which suggests that they are not just an effect of swelling.

In addition to the simple and compound exocytotic mechanisms discussed above, we have noted another possible secretory mechanism in the guinea pig tracheal secretory cells which involves loss of a large portion of the cytoplasm [27]. This "apocrine-like" secretion could represent cases where the prefused granule mass is of such a size that fusion has resulted in the breakdown and loss of integrity of the cell itself. Given the nature of the secretory event, it is not possible to conclude that the apocrine-like release is by exocytosis.

4. Pathways of Mucin Secretion

4.1. Constitutive and Regulated Secretion

Two different secretory pathways are usually considered to operate in secretory cells, namely constitutive (non-regulated) secretion and regulated secretion. The latter pathway is typified by the production of storage granules which are released in response to an external stimulus, for example a hormone or stretch. The constitutive pathway is characterised by small intracellular vesicles containing secretory product which is unpackaged and released without the need for a regulating stimulus, at approximately the rate of protein production. In studies of secretion, including mucin secretion, the term "basal" secretion is often used to describe what is considered to represent normal release from the cell without any intervention, either experimentally or from endogenous factors. However, in any experimental system it is difficult to ensure that all causes of stimulation have been eliminated, and so it is not possible to equate basal release with constitutive release. The simple fusion sites described above, with fusion of individual large dense-core granules with the plasma membrane, could represent sites of basal secretion. However, simple fusions have been found in both stimulated and unstimulated preparations. Thus, if this is basal secretion, it is not likely to be constitutive. The granules themselves are also not typical of constitutive granules, being much larger. In fact, because mucin molecules undergo such an involved condensation process during packaging, it is difficult to imagine mucin being released from a small constitutive type of vesicle. Examination of the *trans*-Golgi network (TGN) (Figure 8), that area of the guinea pig mucin-containing cell where the secretory granules are packaged and bud off, illustrates the complexity of this process. Large stacks of TGN membrane reflect the secretory prowess of this cell type. After chemical fixation and freeze-fracture replication, the budding granules do appear to be of a variety of sizes. In other secretory cells it has often been supposed that there exist both immature and mature granules and that large granules are formed by fusion of the smaller immature granules. In mucin-containing cells it is difficult to conclude that this happens. It would be difficult to separate such fusions from artefactual swelling induced by chemical fixation. Ultrarapidly frozen mucin-containing cells of the quail oviduct illustrated that as they bud off the Golgi the mucin granules appear to be large and comparatively electron-lucent and appear to condense to form the smaller electron-dense mature granules [30]. Examination of ultrapidly frozen guinea pig tracheal secretory cells has shown a similar mixture of large electron-lucent and smaller dense cores (Figure 9). Again some of the apparent "disorder" of the granules in conventionally prepared material appears to be linked with the use of chemical fixatives. However, even in ultrarapidly frozen material no separate class of constitutive granules has been found.

Figure 8. Golgi region of a guinea pig tracheal epithelial mucin-containing cell prepared by chemical fixation and freeze-fracture replication. Small, newly-formed mucus granules (arrowed) bud from the membrane "stacks" of the *trans*-Golgi network (G). Bar = 1 µm.

Investigation of whether the rate of baseline secretion could be altered, in particular reduced, would aid understanding of whether it equates with constitutive secretion. Recently, nitric oxide (NO), both exogenously applied or endogenously produced, has been shown to reduce baseline secretion from ferret trachea [33], a preparation in which the principal source of mucus is the submucosal glands. This has interesting ramifications for understanding the microanatomy of secretion, because NO has been seen by flow cytometry to affect the cell cycle by interfering with actin polymerisation [34], in a similar fashion to cytochalasin. Experimental depolymerisa-

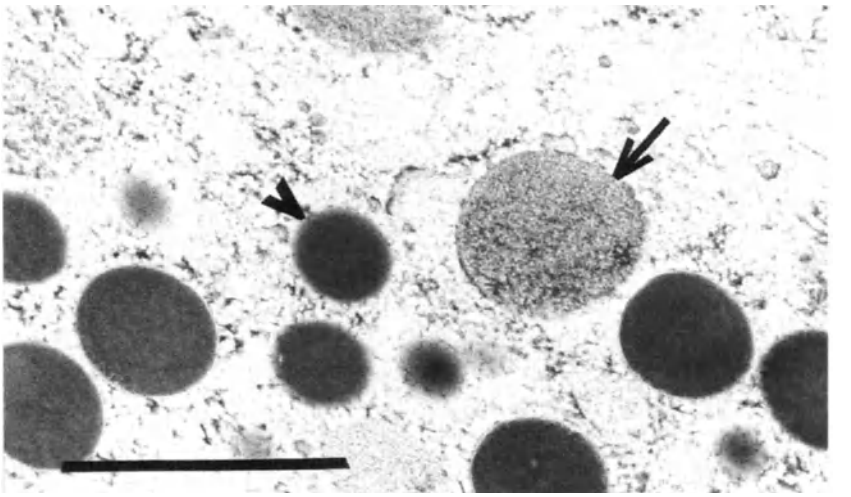


Figure 9. Immature (arrowed) and mature (arrowhead) secretory granules in the Golgi region of a guinea pig tracheal epithelial mucin-containing cell prepared by ultrarapid freezing and freeze substitution. Bar = 1 μm.

tion of actin has also been found to increase the rate of basal release of mucin from intestinal goblet cells without causing accelerated (regulated) secretion [35]. The state of actin may, therefore, be important in baseline release from airway mucin-containing cells. The mechanism(s) for this action are unexplored but may be the result of adenosine 5′-diphosphate (ADP) ribosylation, which involves the transfer of the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to acceptors such as arginine, other guanidino compounds and proteins. There is some support for this suggestion, because NO stimulates ADP ribosylation of actin in human neutrophils [36]. NO may help regulate the state of actin at the inner leaflet of the plasma membrane, which is a site of obvious importance for affecting secretory granule release. ADP ribosylation modifies F-actin [37] by acting as a capping protein, binding at the barbed end of the filament to prevent further growth [38]. Clostridial toxins, which also ADP-ribosylate proteins, inhibit carbachol-stimulated histamine release from mast cells [39]. Consequently, there appears to be a mechanism whereby NO can depress basal release of mucin by acting directly at the level of the goblet cell. If it is simply accessibility to the plasma membrane, via a subplasmalemmal actin network, that is enough to control release, then at least some of these granules may be fusing without a further signal. This would represent constitutive secretion.

It has been proposed from studies of intestinal goblet cells [35] that there are two organisationally distinct secretory pathways for mucin release. One, termed accelerated secretion, involves the loss of large numbers of

centrally located granules in response to a secretagogue. The second pathway is termed basal or baseline secretion and involves the release, on peripherally located microtubular tracks, of recently produced individual granules that fuse with the plasma membrane in a constitutive fashion. Again, although no different classes of mucin granules are distinguished morphologically, the assumption is that constitutive and regulated secretion, although being through different routes, involves essentially similar granules. It is possible that different classes of mucin-containing cells could also secrete differently in response to a secretagogue. A study of intestinal secretion showed that, after a 5-min application of acetylcholine, crypt goblet cells were depleted of mucin secretory granules. The apical membranes had the deep cavitation considered indicative of compound exocytotic activity. Although villus goblet cells did not show similar signs of recent compound exocytosis, morphometric analysis revealed a significant, although not so large, decrease in the cell volume occupied by granules. The possibility here was that it was secretion via simple and not compound release.

4.2. A Model for Airway Mucin Secretion

Recent work on tannic acid arrest of mucin granule exocytosis has suggested that release from guinea pig tracheal airway cells has features of both regulated and constitutive secretion [27]. In this model (Figure 10), large mucin granules produced and packaged in the regulated pathway would initially have the capacity to be released either constitutively or by regulated secretion. After budding from the Golgi, these granules are either transported to the surface or they enter the "storage pool" of granules located centrally in the cell. At the surface, baseline release is regulated by actin-controlled accessibility to the membrane, which is itself controlled by NO. However, the actual fusion event with the plasma membrane does not in itself require a regulatory signal. Upon entering the storage pool, some granules lose the ability to be released constitutively (mature) by masking or removal of their fusion machinery. In our model stimulation will initially cause granule–granule fusion, which does not occur in unstimulated cells except as an artefact of chemical fixation, followed by fusion with the plasma membrane. Although it is not possible to say with certainty that granules are not added sequentially to a granule already fused with the plasma membrane, this seems unlikely from the ultrastructure of the sites. If this were the case, given the speed of expansion of mucin found experimentally, it would be expected that at a compound site (see Figure 6b) there would be a progressive loss of material, with the granule next to the plasma membrane disappearing first. This does not seem to be the case. There are similarities here to the compound release seen in eosinophils, where electrophysiological capacitance measurements have shown that many pre-

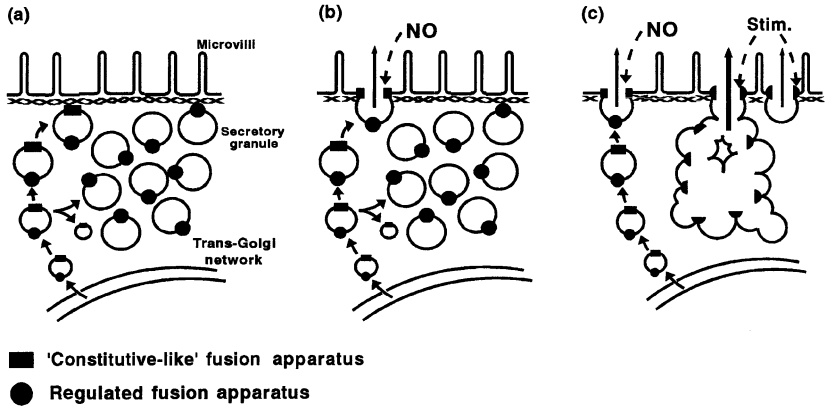


Figure 10. Model for secretion of mucin-containing granules of the guinea pig respiratory epithelium. (a) Granules are produced from the *trans*-Golgi network on the regulated pathway. (b) Some are transported to the apical plasma membrane where they are released constitutively; i.e. an external stimulus is not necessary to bring about fusion of granule and plasma membrane. This baseline secretion is still governed by accessibility to the membrane through the actin cytoskeleton (X), which can be altered by nitric oxide (NO). (c) In response to a secretory stimulus (Stim., e.g. acetylcholine), granules which have entered the storage pool, with loss of their ability to be released constitutively, fuse directly with the plasma membrane, or with each other and then with the plasma membrane, to produce compound exocytotic release.

fused granules can be added to the plasma membrane. These eosinophil granules appear, therefore, to be able to fuse both with the plasma membrane and with each other to produce a highly focal release. Extended secretagogue exposure could also lead eventually to the apocrine-like secretions which were found in a number of goblet cells, particularly after acetylcholine stimulation.

In recent studies of atrial natriuretic peptide release, the concept has been extended to suggest that recently produced secretory granules may retain the ability to be released in either a constitutive or regulated fashion, with removal or masking of the machinery for constitutive fusion occurring during maturation. The rate of this maturation process would depend upon the secretory exigency of the cell. We propose that a similar process could take place in airway goblet cells.

The above hypothesis can be tested in evaluation of other putative secretagogues which have been shown to affect mucus secretion. The retention of secretory cores at the site of fusion in tannic acid-treated tracheae offers an opportunity for investigating and quantifying mucus release directly at the cellular level, in particular the role of the cytoskeleton in regulating secretion [40]. When use in parallel with ultrarapid freezing, a better understanding of the microanatomy of mucin-secreting cells of the respiratory epithelium should be obtained.

5. Summary

The microanatomy of mucin-containing secretory cells of mammalian airways is far from understood. Studies using chemical fixatives have produced results that have led to assumptions being made concerning secretory cell ultrastructure. In particular, that the cell is swollen apically to give a goblet shape and that the large electron-lucent secretory granules are confluent prior to stimulation. Studies using ultrarapid freezing suggest that this is not the case, at least for those cells so far investigated. Other ultrastructural methods, such as tannic acid arrest procedures, which allow secretion to be quantified at the cellular level can contribute to a better understanding of secretion from these important components of the respiratory system.

Acknowledgement

The authors thank the Clinical Research Committee of the Royal Brompton National Heart and Lung Hospitals and the Wellcome Trust for financial support. We also thank Dr. J.N. Skepper, Cambridge University, for assistance with the freeze-substitution studies, A. Griffin and Dr. R. Scott, University of Aberdeen, for collaboration on the submucosal gland studies, and P. O'Gara, NHLI, for his expertise in converting video to computer images for Figure 1. Figure 2 was kindly provided by A. Dewar, Brompton Hospital.

References

1. Specian RD, Neutra MR (1980) Mechanism of rapid mucous secretion in goblet cells stimulated by acetylcholine. *J Cell Biol* 85: 626–640.
2. Davis CW, Dowell ML, Lethem M, van Scott M (1992) Goblet cell degranulation in isolated canine tracheal epithelium: Response to exogenous ATP, ADP and adenosine. *Am J Physiol* 262: C1313–C1323.
3. Lethem MI, Dowell ML, Van-Scott M, Yankaskas JR, Egan T, Boucher RC, Davis CW (1993) Nucleotide regulation of goblet cells in human airway epithelial explants: normal exocytosis in cystic fibrosis. *Am J Respir Cell Mol Biol* 9: 315–322.
4. Kamijo AS, Terakawa S, Hisamatsu K (1993) Neurotransmitter-induced exocytosis in goblet and acinar cells of rat nasal mucosa studied by video microscopy. *Am J Physiol* 265: L200–L209.
5. Verdugo P (1984) Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: a new model. In: *Mucus and mucosa,* Ciba Foundation Symposium 109 Pitman: London, 212–225.
6. Aitken ML, Verdugo P (1989) Donnan mechanism of mucin release and conditioning in goblet cells: The role of polyions. *Symp Soc Exp Biol* 43: 73–80.
7. Segawa A, Terakawa S, Yamahina S, Hopkins CR (1991) Exocytosis in living salivary glands: direct visualisation by video-enhanced microscopy and confocal laser microscopy. *Eur J Cell Biol* 54: 322–330.
8. Ceccaldi PE, Grohavaz F, Benfenati F, Chieregatti E, Greengard P, Valtorta F (1995) Dephosphorylated synapsin 1 anchors synaptic vesicles to actin cytoskeleton: An analysis by videomicroscopy. *J Cell Biol* 128: 905–912.
9. Curtis ASG (1964) The mechanism of adhesion of cells to glass: A study by interference reflection microscopy. *J Cell Biol* 20: 199–215.
10. Heuser J, Zhu Q, Clarke M (1993) Proton pumps populate the contractile vacuoles of *Dictyostelium* amoebae. *J Cell Biol* 121: 1311–1327.

11. Jeffery PK (1978) Structure and function of mucus-secreting cells of cat and goose airway epithelium. In: *Respiratory Tract Mucus*, Porter R (ed), *Ciba Foundation Symposium* 54: 5–24.
12. Jeffery PK (1983) Morphological features of airway surface epithelial cells and glands. *Am Rev Respir Dis* 128: S14–S20.
13. Douglas WW (1993) Calcium, stimulus-secretion coupling and exocytosis – glancing back 30 years; and recent lessons on *spontaneous* secretion from melanotrophs revealing calcium-driven autonomous secretion, independent of action potential discharge and its *un*coupling by the secretacurbins; dopamine, GABA and neuropeptide Y. *Biomed Res* 2: 9–19.
14. Mingrone G, Greco AV, Arcieri Mastromattei E (1990) Free fatty acids stimulate mucin hypersecretion by rabbit gall-bladder epithelium *in vitro*. *Clin Sci* 78: 175–180.
15. Griffin A, Newman TM, Scott R (1996) Electrophysiological and ultrastructural events evoked by methacholine and intracellular photolysis of caged compounds in cultured ovine trachea submucosal gland cells. *Exp Physiol* 81: 27–43.
16. Fox GQ (1996) A morphometric analysis of exocytosis in KCl-stimulated bovine chromaffin cells. *Cell Tiss Res* 284: 303–316.
17. Buma P, Roubos EW, Buijs RM (1984) Ultrastructural demonstration of exocytosis of neural, neuroendocrine and endocrine secretions with an *in vitro* tannic acid (TARI) method. *Histochemistry* 74: 247–256.
18. Newman TM, Severs NJ, Skepper JN (1991) The pathway of atrial natriuretic peptide release – from cell to plasma. *Cardioscience* 2: 263–272.
19. Newman TM, Severs NJ (1990) Arrested exocytosis of synaptic vesicles at a glutamatergic synpase. *Cell Biol Int Reps* 14: 849–863.
20. Brooks JC, Carmichael SW (1988) Ultrastructural demonstration of exocytosis in intact and saponin-permeabilized cultured bovine chromaffin cells. *Am J Anat* 178: 85–89.
21. Newman TM, Severs NJ (1990) Arrested exocytosis of atrial secretory granules. *J Mol Cell Cardiol* 22: 771–786.
22. Newman TM, Sever NJ (1993) Stretch and anaesthetic dependency of atrial natriuretic peptide release demonstrated by an ultrastructural assay. *J Cell Physiol* 155: 240–247.
23. Meek KM (1981) The use of glutaraldehyde and tannic acid to preserve reconstituted collagen for electron microscopy. *Histochemistry* 73: 115–120.
24. Kalina M, Pease DC (1977) The preservation of ultrastructure in saturated phosphatidyl cholines by tannic acid in model systems and type two pneumocytes. *J Cell Biol* 74: 726–741.
25. Singley CT, Solursch M (1980) The use of tannic acid for the ultrastructural visualization of hyaluronic acid. *Histochemistry* 65: 93–98.
26. Newman TM, Tian M, Gomperts BD (1996) Ultrastructural characterisation of tannic acid arrested degrenulation of permeabilised guinea pig eosinophils stimulated with GTP-γ-S. *Eur J Cell Biol* 70: 209–220.
27. Newman TM, Robichaud A, Rogers DF (1996) Microanatomy of secretory granule release from guinea pig tracheal goblet cells. *Am J Respir Cell Mol Biol* 15: 529–539.
28. Heuser JE,Reese TS, Landis DM (1976) Preservation of synaptic structure by rapid freezing. *Cold Sprig Harb Symp Quant Biol* 40: 17–24.
29. Chandler DE, Heuser JE (1980) Arrest of membrane fusion events in mast cells by quick-freezing. *J Cell Biol* 86: 666–674.
30. Sandoz D, Nicolas G, Laine MC (1985) Two mucous cell types revisited after quick-freezing and cryosubstitution. *Biol Cell* 54: 79–88.
31. Puchelle E, Beorchia A, Menager M, Zahm JM, Ploton D (1991) Three-dimensional imaging of the mucus secretory process in the cryofixed respiratory epithelium. *Biol Cell* 72: 159–166.
32. Wagner D, Puchelle E, Hinnrasky J, Girard P, Balossier G (1994) Quantitative X-ray microanalysis of P, Ca and S in the mucus secretory granules of the cryofixed frog palate epithelium. *Microsc Res Tech* 28: 141–148.
33. Ramnarine SI, Haddad EB, Khawaja AM, Mak JCW, Rogers DF (1996) On muscarinic control of neurogenic mucus secretion in ferret trachea. *J Physiol* 494: 577–586.
34. Takagi K, Isobe Y, Yasukawa K, Okouchi E, Suketa Y (1994) Nitric oxide blocks the cell cycle of mouse macrophage-like cells in the early G2+M phase. *FEBS Lett* 340: 159–162.
35. Oliver MG, Specian RD (1991) Cytoskeleton of intestinal goblet cells: Role of microtubules in baseline secretion. *Am J Physiol* 260: G850–G857.

36. Clancy R, Leszczynska J, Amin A, Levartovsky D, Abramson SB (1995) Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. *J Leukoc Biol* 58: 196–202.
37. Terashima M, Mishima K, Yamada K, Tsuchiya M, Wakutani T, Shimoyama M (1992) ADP-ribosylation of actins by arginine-specific ADP-ribosyltransferase purified from chicken heterophils. *Eur J Biochem* 204: 305–311.
38. Wegener A, Aktories K (1988) ADP-ribosylated actin caps the barbed ends of actin filaments. *J Biol Chem* 263: 13739–13742.
39. Bottinger H, Reuner KH, Aktories K (1987) Inhibition of histamine release from rat mast cells by botulinum C2 toxin. *Int Arch Allergy Appl Immunol* 84: 380–384.
40. Cheek T, Burgoyne RD (1991) Cytoskeleton in secretion and neurotransmitter release. In: *The neuroronal cytoskeleton,* Wiley-Liss, 309–325.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 5
Mechanisms Controlling Airway Ciliary Activity

Michael J. Sanderson

Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts, USA

1. Introduction

The principal activity of respiratory tract cilia, in conjunction with mucus secretion, is the expulsion of contaminating material from the airways. Without mucociliary clearance, the airways would remain exposed to infectious agents or reactive substances – a situation that often results in chronic obstructive lung disease. The conditions that predispose mucociliary clearance to failure are not well established, but if therapies are to be designed to counter obstructive lung disease, a better understanding of mucociliary activity is required. In comparison with other cilia, the mechanisms that drive airway ciliary activity have not been extensively investigated a consequence of poor accessibility to airway tissue and the incompatibility of mucosal tissue with microscopy. From an experimental viewpoint, flagella have the advantage that they are readily obtained and easily

observed with microscopy. Consequently, our understanding of ciliary activity relies on a significant amount of information derived from studies of flagella. With the exception of length, the structure of flagella is almost indistinguishable from cilia. In addition, airway cilia display a sliding-microtubule mechanism of motility and dynein structure that are similar to flagella or cilia of other eukaryotes. In this chapter, the structure of the mucociliary interface, the mechanics and coordination of ciliary activity, and the intra- and intercellular mechanisms that regulate airway ciliary activity will be briefly reviewed. Limited space necessitates that reference to many original studies is directed through reviews and recent reports [1–7].

2. Mucociliary Interface

The mucociliary interface consists of three basic components: the cilia, a thin layer of periciliary fluid and an overlying layer of mucus (Figure 1) [1, 2, 8, 9]. The periciliary fluid is a watery, ionic solution and is generated by trans-epithelial ion transport, although composition and net transport varies with airway location [10]. The major functions of the periciliary layer are to provide (1) an environment within which the cilia beat, (2) the correct spatial organization of the interface by supporting the mucus at the ciliary tips and, in the upper airways, (3) a reservoir of fluid for humidification of inhaled air. The mucus is a viscous secretion, released into the airway lumen from either goblet cells or mucus glands, and is composed mainly of glycoproteins. The reader is referred to Chapters 1 and 2 of this volume for information on the structure and composition of mucus.

3. Mechanisms of Mucus Transport

Particles deposited on the epithelial surface become trapped within mucus, and these mucus-particle aggregates are moved by ciliary activity. It is important to emphasize that mucus is essential for particle transport; particles resting on a ciliated epithelium devoid of mucus do not move even though the cilia are active [1]. In the lower airways, mucus exists in discrete plaques which coalesce into larger sheets, due to the convergence of the surface area of the airways, as they are transported towards the larynx. To compensate for airway convergence, mucus transport rates increase towards the trachea; this mucus transport velocity gradient correlates with a gradient of increased ciliary activity and ciliary length [1, 2, 8, 9].

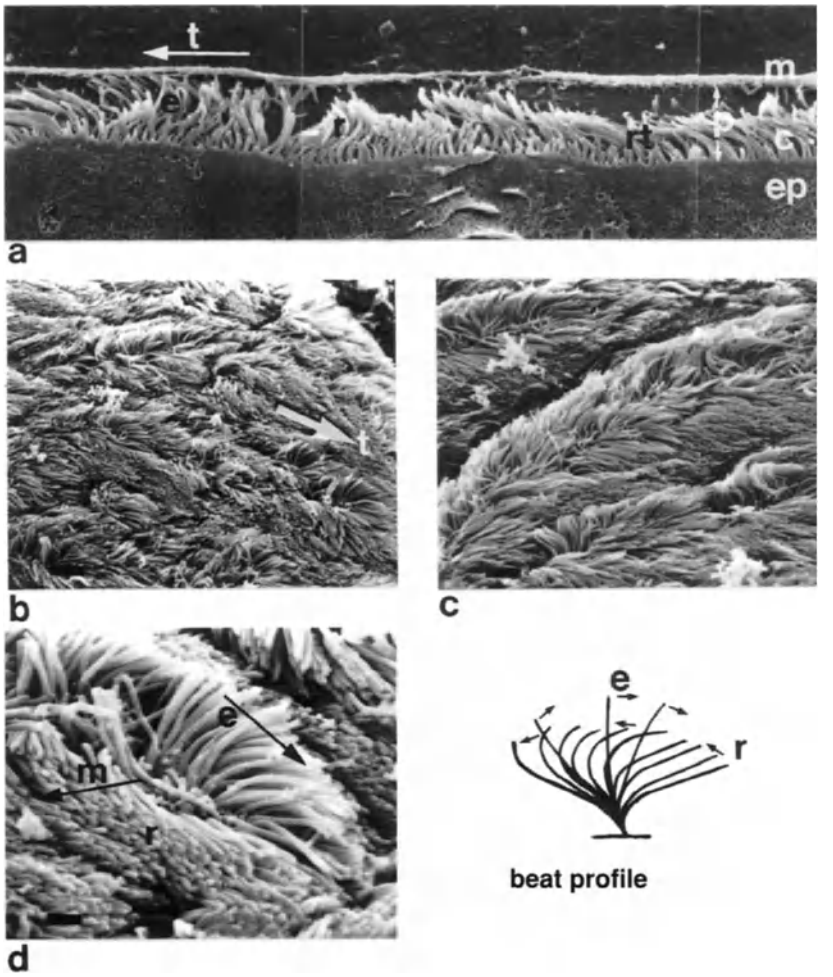


Figure 1. The beat and metachronal organization of airway cilia. (a) A profile scanning electron micrograph (SEM) (×2500) of the mucociliary interface: mucus (m) forms a layer over the cilia (c) that arise from epithelial cells (ep) and are active in the periciliary layer (p). The effective stroke (e) is directed towards the left and contacts the mucus. Cilia performing recovery (r) strokes move towards the right underneath the mucus. Cilia lie in the direction of mucus transport (t) when at rest (rt). (b, c) Low power SEMs (b, ×1000 and c, ×1500) of the surface of the airway ciliated epithelium showing the multiple sites of discontinuous metachrony associated with ciliary activity. The majority of the cilia are at rest at the instant of fixation and lie in the direction of mucus transport (t). (d) A higher-power SEM (×4000) of a single site of metachronal activity: cilia performing recovery strokes (r) move backwards and to the right relative to the forward direction of the effective stroke (e), and generate a metachronal sequence that appears to move in the direction of the arrow m. (beat profile) The profile of the beat cycle of a single cilium. Arrows indicate direction of movement. All images show rabbit tracheal tissue.

3.1. Ciliary Beat Pattern

The beat pattern of respiratory tract cilia is similar to other cilia, and each beat cycle begins with a recovery or reverse stroke in preparation to perform an effective (forward) stroke [8, 9]. In contrast to water-transporting cilia, airway cilia also display a brief rest phase in which the cilia lie, in a slightly curved configuration, at the end of the effective stroke, with their tips pointing in the direction of mucus transport. This rest state may have an advantage for mucus transport by creating a surface that would tend to oppose the retrograde motion of mucus. Each beat cycle begins from the rest position. During the recovery stroke the cilium moves backwards and slightly to the right (relative to the forward direction of the effective stroke) by propagating a bend from the base to tip to produce an "unrolling" type of motion. At the end of the recovery phase, the cilium continues into the effective stroke, during which the cilium maximizes its height and moves forward in an almost planar arc, bending mainly at the base. The cilium finishes the cycle upon reaching the rest position. During the effective stroke, the ciliary tip contacts the lower surface of the mucus to propel the mucus forward. At the end of the effective stroke, the cilium disengages from the mucus and the unrolling action of the recovery stroke takes place within the periciliary layer to avoid retrograde mucus transport (Figure 1).

3.2. Ciliary Metachronism

Each ciliated cell bears approximately 200 cilia that are densely packed on its apical surface in a loose hexagonal pattern [11]. Individual cilia cannot move without influencing neighboring cilia, and in order to perform an unhindered beat cycle, the cilia move in a coordinated or metachronal manner with respect to neighboring cilia. Along a metachronal sequence, each cilium is slightly out of phase with the previous cilium. The distance over which all phases of one beat cycle are represented is equal to a single metachronal wavelength. Almost any line of observation of a field of beating cilia will contain cilia at different phases of the beat cycle, but the true metachronal wavelength will be in the direction of the shortest distance [12–14]. Another defining characteristic of metachrony is that the cilia in a line perpendicular to the plane of metachrony all beat in synchrony, i.e. the cilia along the plane of synchrony are at the same phase of the beat cycle [12–14]. In view of this two-dimensional pattern of activity, it should be appreciated that the application of the term "beating synchronously" to fields of active cilia is inaccurate. The cilia within the field do not all beat in phase. However, the phase relationship of their beat patterns is synchronized. The metachronal activity of airway cilia can be classified as anti-laeoplectic: the effective stroke is directed backwards and towards the left if the observer looks in the direction towards which the

metachronal wave travels [8, 15, 16]. When observing the metachronal activity relative to the direction of the effective stoke, the metachronal wave appears to move back and to the right.

The mechanism coordinating metachronism relies solely on the physical interactions occurring between the cilia through the surrounding periciliary fluid – a process known as hydrodynamic coupling. Intercellular signals do not determine metachronal sequences, even though epithelial cells are coupled by gap junctions. It is common to observe within the boundaries of a single cell, especially in water-transporting cilia where metachrony is highly organized, multiple lines of both metachrony and synchrony [13].

Metachronal activity provides two important functions to cilia. First, metachrony allows every cilium to move through its beat envelope without interference from other cilia. Second, metachrony establishes cooperative ciliary activity that is required to overcome the elastic or viscous forces associated with transportation of mucus or fluid loads. At the level of the cilium, inertial forces are negligible, and fluid motion is primarily influenced by viscosity [1, 2]. As a cilium moves forward, it carries with it a volume of fluid that adheres to the cilium due to viscosity. As the cilium moves backwards, it reduces its profile and speed so that the volume of fluid moved in the opposite direction is reduced. Once the cilium changes direction, the transported fluid will come to a standstill because of the relatively high viscous and low inertial forces. As a result, a single cilium is not able to generate a continuous fluid flow or swimming motion. However, the organization of cilia into repetitive metachronal sequences enables multiple volumes of fluid to be moved in a continuous sequence to generate flow [1, 2, 12, 13].

The metachrony associated with airway cilia is not as well organized as that of water-transporting cilia, and this reflects the specialized requirements of transporting mucus, a viscoelastic substance. Each ciliary beat cycle begins from the rest phase. As the cilium moves backwards through the recovery stroke, it activates, via hydrodynamic coupling, other cilia in the direction of the movement. These cilia, in turn, activate more cilia. As will be discussed, this sequential activation of motility may result from distorting the axoneme of each cilium so that the probability of dynein cross-bridges being formed is increased, allowing bend formation to be initiated. As the cilium progresses into the effective stroke, the ciliary tip contacts the mucus (Figure 1). "Claw-like" projections, found on the tips of mucus-transporting cilia (Figure 2, see below), may enhance the "grip" on the mucus [17, 18]. The mucus-cilia interaction rapidly reduces the velocity of ciliary movement, an event that reduces hydrodynamic coupling between cilia. If metachronal sequences of airway cilia relied only on the hydrodynamic coupling of cilia in the effective phase, the metachronal activity would rapidly become disorganized, and few cilia would be recruited into mucus transport. However, by relying on the recovery phase to stimulate and coordinate ciliary movements, more cilia can be recruited to

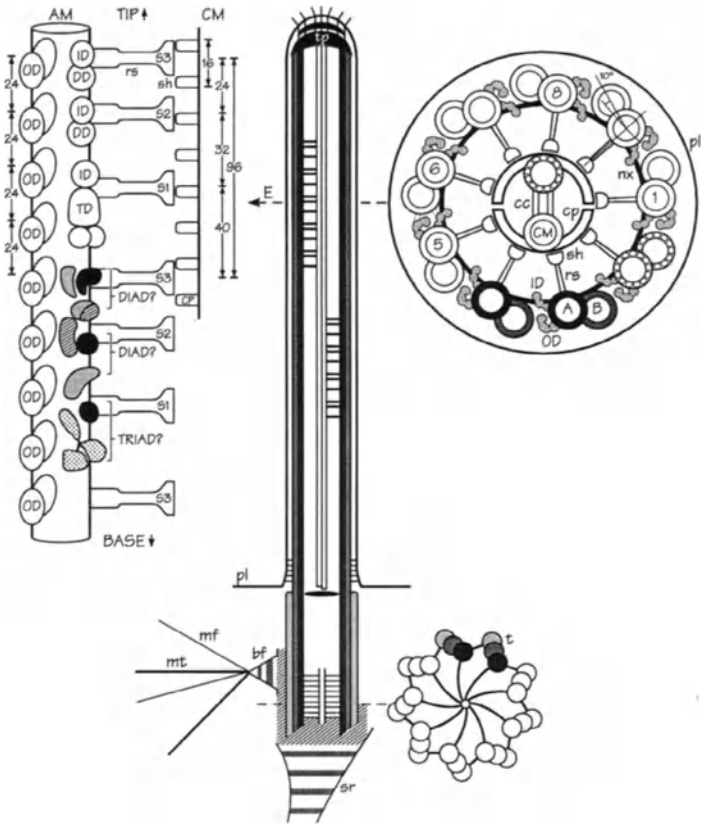


Figure 2. The generalized ultrastructure of airway cilia. The ciliary axoneme is composed of nine outer doublets, comprising an A and B subfiber, and two central microtubules (CM). The axoneme is surrounded by the plasmalemma (pl) and extends into the cell to form the basal body. The addition of a third microtubule forms a triplet (t). Attached to the basal body is a basal foot (bf) that points in the direction of the effective stroke (E) and interconnects with cytoskeletal microtubules (mt) and microfilaments (mf), and a striated rootlet (sr). These structures provide stability to the cilium. At the ciliary tip, the microtubule doublets terminate in an electron dense tip plate (tp). Filaments extending from this plate form the ciliary crown. From each microtubule doublet, inner (ID) and outer dynein (OD) arms project from doublet "n" towards doublet "n+1". A nexin (nx) link joins adjacent doublets. Radial spokes (rs) project from each A subfiber and associates, via the spoke head (sh), with the projections (CP) of the central microtubules. The cross-sections of the cilium are viewed from base to tip. The detail of the proposed organization of the dynein arms is shown on the left, as viewed from the B subfiber of doublet "n+1" towards the A subfiber of doublet "n". An outer dynein (OD) is located every 24 nm. The inner dynein (ID) arm has a variety of appearances. From freeze-etch images (upper half of left figure) a triad structure is associated with radial spoke S1, while a diad structure is associated with spokes S2 and S3 [24]. By subtractive image analysis (lower half of left figure), 10 structures have been identified associated with spokes S1 and S2 [25] (the relationship of structures to S3 are suggested, as S3 spoke is not present in *Chlamydomonas*). The shading represent structures that appear to be genetically related. Dotted: inner dynein I1; cross-hatch shading: components of the DRC; black: components of I2; grey: components not fully identified. A relationship between the structures identified by freeze-etch and subtractive analysis is proposed by the brackets and question marks.

mucus transport irrespective of the disruptive events that occur during the effective stroke [8]. The cilia return to the rest position after the mucus has been moved forward and the cilia can disengage from the mucus. A consequence of these mucociliary interactions is that the duration of the effective stroke is extended. As a result, the number of times (i.e. beat frequency) that the cilia can contribute to mucus transport is reduced, a situation that may reduced mucus clearance. Increases in airway ciliary beat frequency can be achieved by differentially shortening the duration of the rest phase, and this mechanism may compensate for the slowing of the effective stroke. Airway cilia may also respond to the increasing resistance forces by increasing the power production of the axoneme [19]. These auto-regulatory mechanisms for beat frequency and power production would both help to maintain a constant mucus transport velocity. During mucus contact, the cilia do not appear to penetrate deep into the mucus (Figure 1). This might be expected in order to prevent the cilia from just passing through the mucus. The consequence of restricted penetration appears to be that the cilia lift the mucus a small amount as the cilium reaches its full height during the effective stroke (Figure 1).

The metachronal sequences initiated by the recovery strokes do not travel extensive distances. Epithelial discontinuity, such as ridges or cell boundaries, may account for wave termination, but waves often terminate in areas devoid of an obvious physical barrier. If the metachronal propagation relies on the stimulation of bend initiation, cilia that could be in an unresponsive state, for example during the early stages of the rest phase, would terminate the metachronal wave. This restriction of metachronal wave propagation creates a multitude of small, localized areas of ciliary activity (Figure 1). The ciliary activity within each area is coordinated, but the activity between areas is not coordinated. This configuration enhances mucus transport by compensating for the elastic recoil of mucus. Random groups of cilia hold and move the meshwork of mucus forward, while other groups are disengaging from the mucus. If all the cilia acted together, the mucus would be extended forward, only to recoil when the cilia disengaged at the end of the effective stroke [1, 2, 8, 9]. The length of the cilium is also adaptive to mucus transport as a short structure can develop and maintain stiffness more easily. A short cilium will influence the displacement of the periciliary layer in both the effective and recovery strokes, but because the fluid only reaches to the tip of the cilia, the net fluid transport is greatly reduced. In summary, mucus transport at an air interface has been optimized by modification of ciliary size and structure and by the development of discontinuous metachronal activity.

4. Ciliary Ultrastructure

The ultrastructure of cilia and flagella is extremely similar in organisms ranging from protozoa to mammals, and airway cilia conform to this com-

mon architectural design [7, 11, 20, 21, 22]. Airway cilia are about 0.25 μm in diameter and range from 4 to 6 μm in length depending on location [23]. Each cilium is surrounded by an extension of the cell membrane and contains an axoneme – a structure consisting of two central microtubules surrounded by nine microtubule doublets (Figure 2). Each microtubule doublet consists of a whole microtubule (A subfiber) and a partial microtubule (B subfiber). At the base of the cilium, the central pair of microtubules terminate, while the outer doublets continue into the cytoplasm to become microtubule triplets and form the basal body of the cilium. A basal foot, projecting in the direction of mucus transport, along with a striated ciliary rootlet and interconnections with the apical cytoskeleton provides stability to the basal body.

The circumferential doublet microtubules support a variety of projections, the most important of which are the dyneins arms: a series of complex macromolecules that project from the A subfiber of each microtubule doublet towards the B subfiber of the adjacent microtubule. Outer dynein arms occur every 24 nm along the outer edge of the A microtubule of the doublet. The inner dynein arms are more varied in appearance, but these occur as a series that repeats every 96 nm [24, 25]. Dynein arms utilize adenosine 5′-triphosphate (ATP) and undergo a mechanochemical cycle that is responsible for the generation of ciliary motility. In addition, a series of nexin links extend between the doublets and are thought to provide an elastic resistance to shape changes. A series of curved projections arises every 16 nm from the microtubules of the central pair to form a "central sheath" (Figure 2). Structures called radial spokes extend from the A subfiber of each outer doublet towards the central sheath. The longitudinal organization of the radial spokes matches the spatial organization of the inner dynein arm complexes and repeats every 96 nm. In metazoan cilia, these radial spokes are often organized in groups of three, whereas in *Chlamydomonas* flagella the spokes are organized in pairs. The spacing between radial spokes S1 and S2 is about 32 nm and between S2 and S3 is 24 nm; 40 nm separate the repeat groupings (S3-S1) [24]. The radial spokes appear to be important in regulating dynein activity and determining the pattern of the beat cycle.

An unusual feature of airway cilia is the presence of a ciliary crown (Figure 2). In water-propelling cilia, the outer microtubules terminate near the tip of the cilium [26]. However, in mucus-transporting cilia, the tips of the microtubules are connected together and terminate in an electron-dense end plate. As will be discussed, this configuration has interesting implications for the behavior of the axoneme during bending. From the end plate, a number of hair-like structures, arranged in a regular pattern, arise and project through the membrane at the ciliary tip for about 30 nm to form the "ciliary crown" [17, 18]. As mentioned, this structure may increase the traction between cilia and mucus.

5. Mechanism and Regulation of Ciliary Activity

Cilia and flagella are utilized by a diversity of organisms, and these organelles can display a range of beat patterns, each specialized for activities such as swimming, feeding and mucus transport. In addition, the pattern of ciliary activity frequently must be changed to accommodate changes in the prevailing conditions. This process is illustrated by the avoidance reaction of the protozoan *Paramecium*: to navigate around obstacles, the cilia can change beat frequency and beat direction [27]. Similarly, the swimming direction of *Chlamydomonas* is reversed by changing the beat pattern of its flagella from one typical of cilia to a waveform typical of flagella [28]. These behaviors of ciliary activity clearly demonstrate that the axoneme contains mechanisms to generate cyclic forward and reverse stokes as well as changes in direction, waveform and frequency.

To help understand the mechanisms that control or regulate ciliary activity, these behavior patterns can be separated into two groups – a steady-state behavior and a response-state behavior [29]. During steady-state behavior, the structural configuration of the axoneme produces the normal repetitive beat pattern consisting of a forward and reverse stroke. This beat pattern may be symmetrical or asymmetrical. As will be discussed, the control of ciliary beating is determined by the intrinsic patterns and activity of dynein–microtubule sliding. During the response state, this intrinsic configuration and activity of the axoneme is transiently modified to alter beat pattern or frequency. Control of the response state is mediated by cellular factors, such as cyclic adenosine monophosphate (cAMP) and Ca^{2+}, that arise from signal transduction at the cell membrane.

6. Mechanisms of Ciliary Movement (Steady-State Behavior)

6.1. Microtubule Sliding-Based Motility

The hypothesis that microtubule sliding accounted for ciliary motion was initially supported by an ultrastructural analysis of the relative microtubule positions at the ciliary tip throughout the beat cycle [2, 26]. The differential termination of the microtubules at the tip of water-transporting cilia allows each doublet to be identified, and it was possible to show that doublets positioned on the inside curvature of ciliary bends slide towards the ciliary tip relative to those doublets positioned towards the outside curvature of the bend. These observations were followed by a physiological demonstration of sliding between microtubules in sperm flagella axonemes [30]. In the presence of ATP, demembranated, isolated flagella resumed a beat pattern almost identical to that of live sperm. After treatment with proteolytic enzymes, to cleave interdoublet links, the addition of ATP led to an axonemal disintegration of these flagellar "models"; the component

microtubule doublets slide out of the axoneme in a telescopic manner. Mammalian and newt lung cilia display similar properties of ATP reactivation [17, 31, 32] and microtubule sliding [33]. Confirmation that microtubule sliding actually occurs in intact axonemes has been provided by the generation of bends by local application of ATP [34] and the oscillatory motion of beads attached to adjacent microtubules of beating flagella [35]. An interesting "twist" to the mechanism of microtubule sliding is found in airway cilia and appears to result from the axonemal capping structure. Unlike other cilia, the tips of the microtubules in airway ciliary axonemes are embedded in the capping structure and are not completely free to slide. In order to accommodate the microtubule sliding, the axoneme twists as well as bends [17]. This twisting may contribute to the generation of the beat envelope or it may provide some recoil to enhance the power of the effective stroke.

6.2. Molecular Structure of Dynein

The dynein arms are the molecular structures responsible for driving microtubule sliding and have been extensively investigated in order to understand their structure and function [4, 7, 36]. The identification of dynein as the motor molecule was initially made by the discovery that proteins with ATPase activity could be selectively extracted from the axoneme by high ionic strength solutions. Electron microscopy of salt-extracted axonemes confirmed that the projections of the microtubule doublets had been removed. The loss of the ability of salt-extracted axonemes to be reactivated with ATP also correlated with the loss of the dynein arms [7]. The role of dynein arms in ciliary motility has also been emphasized by the human disease "immotile-cilia syndrome". In this condition, patients commonly suffer from bronchitis, rhinitis and otitis media, and male patients are sterile. These symptoms arise from the inability of their cilia to move due to the absence of dynein arms [20].

Since the initial studies, refinements in the ionic extraction procedures and ultrastructural studies have been made, and it is now well established that the ciliary dyneins can be classified into two major groups: the *outer* and *inner* dynein arms. The names reflect their ultrastructural location, but the outer and inner dynein arms also appear to have different roles in the generation and control of beating [5, 27–40].

6.3. Outer Dynein Arms

The outer dynein arms are complex macromolecules spaced at 24 nm intervals along the outer edge of the A subfiber of the microtubule doublets (Figure 2). The outer dynein arm has been extensively studied and, in most

species, the structure of all outer dynein arms appears to be similar along the length of the axoneme. However, for each species, the polypeptide chains that construct the outer dynein arm vary both in number and molecular weight [4]. The outer dynein arms of mammalian or vertebrate lung cilia, like other metazoan outer dynein arms, contain two heavy chains (α and β, MW > 300 Da) and a variable number of intermediate chains. Protozoan dyneins usually contain three heavy chains. Porcine lung cilia were found to have two intermediate chains [32], while newt lung cilia appear to have eight intermediate chains (MWs 56–175 Da) [41]. The ultrastructure of the extracted outer dyneins arms of both pig and newt cilia consisted of two connected globular "heads", with each head containing one heavy chain [41, 42]. This dynein structure of lung cilia is consistent with the structure of outer dynein arms isolated from other vertebrate sources [4].

6.4. Inner Dynein Arms

Little information regarding the structure of the inner dynein arm of lung cilia is available, a situation that results from the difficulty of isolation and the apparent greater variability of this structure [37]. Current information regarding the inner dynein arm has been obtained mainly from studies of flagella of *Chlamydomonas* mutants. In contrast to the homogenous form of the outer dynein arm containing two or three heavy chains, the inner dynein arms appear to exist as multiple isoforms constructed from eight different heavy chains. Ultrastructural studies of *Tetrahymena* and *Boroë* cilia have suggested that the inner arms are organized into groupings consisting of one triad structure and two diad structures that were associated with and matched the radial spoke alignment pattern (in pairs or triplets depending on the organism) (Figure 2) [24]. Other studies classified the inner dynein arms of *Chlamydomonas* into three basic types – I1, I2 and I3 [40, 43]. These three isoforms are constructed from different sets of heavy, intermediate and light chains and are also grouped together to form a repeat sequence every 96 nm along the axoneme (Figure 2). Isoform I1 is a "two-headed" form of dynein residing at one end of the grouping containing heavy chains 1α and 1β [43, 44]. However, the characterization of the dynein isoforms I2 and I3 has become less certain, and it now appears that the composition of the I2 and I3 dyneins varies with respect to position along the axoneme and that these are constructed from a subset of five different heavy chains [44]. Image analysis of the axonemes of *Chlamydomonas* mutants has identified a number of related structures that are believed to represent the organization of the inner dynein arms [25]. A comparison of the models derived by ultrastructural analysis and subtractive image analysis is provided in Figure 2.

The selective salt extraction of the outer dynein arms was found to reduce beat frequency of other flagella or cilia by about 50% [38, 45]. This

initially led to the suggestion that, although the dynein arms were biochemically different, their contribution to the beat cycle was similar. However, it is now hypothesized that the function of the outer dynein arm is to contribute power to the ciliary beat, while the function of the inner dynein arm is to control the ciliary beat envelope [38]. This latter function may reflect the greater complexity of the inner dynein arms.

6.5. Dynein Regulatory Complex

In one group of motility mutants of *Chlamydomonas*, the loss of radial spoke components results in paralyzed flagella. Surprisingly, motility can be restored to these flagella by the presence of a second or suppressor mutation [46]. A variety of *Chlamydomonas* suppressor mutations have been isolated and analyzed. These mutations do not restore the original radial spoke structures to generate motility, but each results in the loss or reduction of another specific group of axonemal proteins. This group of proteins has been called the dynein regulatory complex (DRC) and consists of seven proteins located within each 96 nm inner arm complex at a site located between the outer and inner dynein arms near the junction of the second radial spoke (Figure 2) [40, 47, 48]. The restoration of motility following the loss of a component of the DRC suggests that an intact DRC, in the absence of a radial spoke, inhibits dynein activity. From this it follows that the regulation of dynein activity may be mediated through the interaction of radial spokes with the DRC [49]. A close association of the DRC with some isoforms of the inner dynein arms is predicted by the correlation of a deficiency of inner-arm heavy chains with suppressor mutations [40]. The DRC may also vary along the length of the axoneme, and this has implications for the control of ciliary waveform.

6.6. Mechanochemical Cycle of Dynein

The general form of the mechanochemical cycle of dynein has been extensively described, but it will be briefly outlined here because the polarity of the cycle has important implications for understanding the generation and control of the ciliary beat cycle [3, 50]. The dynein arms of subfiber A of one doublet project towards the B subfiber of the adjacent doublet. To clarify this orientation, the following labelling convention has been adopted: dynein arms project from doublet "n" towards doublet "n+1", e.g. dynein arms of doublet 3 point towards doublet 4. By definition, doublet 1 lies on the axis that symmetrically bisects the axoneme and passes between the central pair and doublets 5 and 6 (Figure 2).

A characteristic of dynein that makes the mechanochemical cycle possible is that it binds to the B microtubules with a high affinity, but this

dynein–microtubule bond is broken by the binding of ATP with the dynein. In the absence of ATP, cilia develop a rigor state where rows of dynein cross-bridges form between the microtubules. If we enter the mechanochemical cycle at this point, the dynein arms are attached approximately perpendicular to the B subfiber of the microtubule. In the living cell, the abundance of ATP ensures the rapid association of ATP with dynein. Following the binding of ATP, the dynein–microtubule bond is broken, and the dynein arm undergoes a conformational change to reorientate the dynein arm to point obliquely towards the base of cilium. The inherent ATPase activity of the dynein arm hydrolyzes the ATP to adenosine diphosphate (ADP), an event that allows the rebinding of the dynein to the B subfiber at a new site lower on the adjacent microtubule. This microtubule binding, in turn, causes a conformational change within the dynein arm that reorients the dynein arm back towards a perpendicular position. The result of this reorientation is that the n+1 microtubule is moved towards the ciliary tip. During the conformational change of the dynein, ADP is released from the dynein and, at the end of this phase, ATP can once again bind to the dynein, to break the dynein–microtubule cross-bridge and reinitiate the mechanochemical cycle [2–4, 50].

6.7. Conversion of Microtubule Sliding into Ciliary Bending

The early demonstration that proteolytic enzyme treatment of axonemes changed the behavior of ATP-activated axonemal models from beating to sliding strongly supported the idea that bending results from restricted sliding. There are a considerable number of transverse structures within the axoneme, including the radial spokes and nexin links, that may serve to restrict sliding. However, it has not been possible to assign this function to any individual element. Surprisingly, protein analysis of the flagella of mutant strains of *Chlamydomonas* revealed that normal beat patterns can occur in the absence of some of the key structures. Furthermore, the mechanism that converts sliding into bending does not appear to be static; propagating bends of flagella or cilia contain multiple regions of sliding and restraint, and because cilia beat at frequencies of 10–30 Hz, the propagating process that converts sliding into bending must be dynamic.

6.8. Generation of Bidirectional Ciliary Motility

The problem of generating bidirectional movement from a unidirectional siding filament mechanism has been solved for skeletal muscle by organizing muscles in antagonistic pairs. The alternative contraction of each muscle with the concomitant relaxation of the partner muscle generates movement in opposite directions. By contrast, the axoneme of an indi-

vidual cilium generates both forward and reverse strokes. This ability results from the polarized arrangement of the dynein arms around the axoneme.

In the extended form, the dynein arms are angled so as to project from doublet n towards doublet n+1 and the base of the cilium. Because of the circular arrangement of the doublets, dynein arms on opposite sides of the axoneme project towards opposite sides of the cilium, i.e. dynein arms of doublets 2–4 project downwards and towards doublets 5–6, dynein arms of doublets 7–9 project downwards and in the opposite direction towards doublet 1 (Figure 2). Active microtubule sliding, driven by ATP-dependent dynein activity, can only move microtubules n+1 in a tipward direction relative to doublet n [51]. Ultrastructural analysis demonstrated that microtubules on the inside curvature slide tipwards, but the relative sliding of only half of these doublets conforms to that predicted for active sliding [26]. Consequently, to obtain bidirectional bending, dynein activity must alternate from one side of the axoneme to the other [52]. If the dynein arms on each side of the axoneme were active simultaneously, their efforts would be counteractive. Active microtubule sliding on one side of the axoneme (driven by dynein activity on doublets 1 through 4) will bend the axoneme in the direction of the effective stroke (towards doublets 5 and 6). In order to accommodate the bend forming in the cilium, passive sliding of microtubules with non-active dynein must occur. Active sliding on the opposite side of the axoneme (driven by dynein activity on doublets 6 through 9) will bend the axoneme in the direction of doublet 1. This concept of alternating dynein activity from side to side of the axoneme has led to the "switch point hypothesis" of activity [2, 52] because there appear to be two distinct "switch points" that control the generation of the effective and recovery strokes. By preventing the progression of sliding through these transition points with either Ca^{2+} or vanadate, the cilia are immobilized at the end of the recovery stroke or at the end of the effective stroke, respectively. Similar arrest positions have been identified for flagella [53]. Immobilization of the cilia occurs only when the cilium reaches the appropriate position in the beat cycle and not at the instant when exposed to the inhibitor. The replacement of one inhibitor with the other will also allow the cilia to change position without resuming the beat cycle. Thus, active sliding can occur in the presence of the inhibitors, but only the transition from one phase of the beat cycle to the next is inhibited [2, 52]. In terms of steady-state behavior of cilia, it would be predicted that these transition sites retain a stable configuration (i.e. "on") and that only during control responses are these sites altered. The idea that dynein activity occurs on one side of the axoneme is also supported by observation that *in situ* axonemes, treated with enzymes to partially destroy interdoublet connections, will split or fragment into groups of doublets rather than fraying into individual doublets [54]. The axonemal split appears to occur along microtubule doublets that have inactive dynein arms.

In contrast to the simplified pattern of cilia movement envisaged by the switch hypothesis, the beat pattern of cilia or flagella often consists of a complex three-dimensional temporal-spatial waveform. This is emphasized by flagella where multiple bends of opposite curvature exist. Therefore, it is an oversimplification to predict that the beat envelope is generated solely by alternating dynein activity from one side of the axoneme to the other. A modified mechanism is required that simultaneously controls dynein activity both around the axoneme as well as along the axoneme. Currently, the mechanisms that may mediate this control are poorly understood, but a number of models have been proposed [3, 5, 6, 55].

The discovery that the central pair of microtubules can rotate in certain organisms [3] has led to the hypothesis that central pair rotation may regulate, via the radial spokes, the dynein arms and hence the zones of active sliding. The association of the dynein regulatory complex with the radial spoke lends support to this idea. However, the finding that mutant strains of *Chlamydomonas* that lack a central pair can display a normal beat form suggests this may not be a universal mechanism.

After the establishment of microtubule sliding as the mechanism for ciliary motility, Brokaw [3, 5] proposed a model of axonemal movement in which the shearing force per unit length (active microtubule sliding) was determined by the curvature of the flagella. Because the curvature itself is generated by the shear (sliding) force, it can act as a feedback mechanism to regulate the amount of sliding. The prediction of this model was that bends would self-propagate to the end of the flagella [3, 5], a behavior matched by experimental observation of flagella motility. Later models of flagellar activity were modified to incorporate the specific mechanisms of bend initiation and shear generation by dynein arms [3, 5]. The cross-bridge cycling of the dynein arms was again controlled by the curvature of the flagella, and these models produce good simulations of flagellar movement. These ideas have been extended in a "geometric clutch" hypothesis of ciliary motility to explain curvature control of sequential dynein activity [55]. This hypothesis suggests that curvature-control of dynein activity results from geometrical changes in the axoneme. In addition to longitudinal forces, dynein–microtubule interactions would also generate transverse forces that alter interdoublet spacing. A decrease in doublet spacing would increase the probability that dynein arms bind to adjacent microtubules which in turn would initiate a cascade of dynein attachments. The probability of dynein cycling is decreased by increasing the doublet spacing – a change induced by the forces developed within the axoneme as it bends in response to microtubule sliding. Computer simulations of this mechanism reproduce asymmetrical ciliary behavior if the initial association constant of dynein arms on one side of the axoneme is higher than on the opposite side [55].

The reproduction of beat cycles by computer simulations is gratifying, but models of flagellar activity must reflect all aspects of behavior. Thus,

current models should be considered with the caveat that they do not account for some important experimental observations [5]. Of particular importance is the different contribution of the inner and outer dyneins to the beat cycle. The removal [37, 45] or lack of the outer dyneins [38] reduces beat frequency but does not dramatically alter the beat waveform. Originally, these results were interpreted as each arm contributing equally to sliding; the control of sliding being mediated by other structures. However, inner-arm mutants display changes in bend angle of the flagellar waveform but not changes in the beat frequency [38]. These results suggest that the inner dynein arms are fully capable of overcoming the resistance forces within the axoneme to generate a normal waveform. Inner and outer dynein arms of lung cilia also impart different behaviors [37]. Brokaw [5] has reasoned that, because of the differences in structure and function of the inner and outer dyneins [37, 39], hypotheses that assume homogeneity of inner and outer dyneins are obsolete. As an alternative, Brokaw [5] hypothesizes that the data indicate that inner dynein arms determine the parameters of bend propagation and that inner arms may also be responsible for control of bend initiation. Bend formation can occur anywhere along the axoneme [34], but the characteristics of the bends generated distally are different from those generated at the base – the normal site for bend initiation. These observations reflect the findings that the inner dyneins also appear to vary in composition along the length of the axoneme [40]. The idea that different inner dyneins influence bend initiation and bend propagation appears to be compatible with the switch hypothesis. If bend initiation is sensitive to inhibitors (Ca^{2+} and vanadate), ciliary arrest would occur at the end of either the effective or recovery stroke, because a new bend in the opposite direction cannot be initiated. However, once initiated, a bend propagates normally to the end of the cilium – a prediction compatible with the observations that cilia arrest at the end of each phase of the beat cycle, and a shift in arrest position can occur after changing inhibitors [52]. The requirement for the outer dynein arms appears to be the addition of power to overcome the external load or viscous forces [4]. This function appears to be reflected by the homogeneous structure of the outer dynein.

In summary, the dynein arms drive axonemal sliding and therefore are obvious sites for the regulation of beat parameters. In view of the different functions assigned to inner and outer dynein arms, it may speculated that changes in waveform would be mediated by alterations of inner dynein arm activity. Changes in inner-arm activity may also influence the rate of bend initiation, while changes in outer-arm activity may increase bend propagation rates, both of which would contribute to changes in beat frequency. By resting at the end of the effective stroke, airway cilia display behavior that matches the switch-point hypothesis. Because the development of metachrony appears to rely on the initiation of the recovery stroke, it may be speculated that bend initiation is a primary control site for airway ciliary activity, especially since airway cilia do not show arrest in response to Ca^{2+}.

7. Regulation of Ciliary Activity (Response-State Behavior)

A wide variety of compounds or conditions lead to changes in airway ciliary activity, but little is understood regarding the intracellular mechanisms that mediate these changes. The identity of these compounds or conditions is important for characterizing the pharmacological response of airway ciliated cells, but it is the second messengers produced by these stimuli that are the key to understanding the mechanisms by which axoneme modifications induce changes in beat frequency and pattern. This review will focus on the fewer, but common, secondary intracellular events rather than cataloging the extensive primary extracellular stimuli.

7.1. Source of Control Signals

The origin of control signals can be of two general types – direct and indirect. Direct control signals originate within each cell (intracellular signaling) and are initiated by ligand-receptor interactions at the cell membrane. By contrast, control signals may arise from adjacent cells; the regulatory messenger reaching the target cells via gap junctions or transmembrane diffusion, i.e. intercellular signaling [56]. Gap junction signaling is important to multicellular systems, as it represents one way in which cooperative cellular activity may be achieved. Messengers mediating intercellular communication can be inositol trisphosphate (IP_3), cAMP, Ca^{2+} or nitric oxide (NO), formed or released by receptor–ligand interactions in distal cells.

7.2. Regulation of Ciliary Activity by Phosphorylation

The beat frequency of airway cilia is increased by a wide variety of β-adrenergic drugs [1, 2, 57], agonists that generally act via G proteins to elevate $[cAMP]_i$ and stimulate cAMP-dependent phosphorylation. The role for cAMP as a regulator of mammalian ciliary activity is supported by the findings that cAMP analogs, or treatments designed to increase $[cAMP]_i$, elevated the beat frequency of intact airway cilia [58, 59]. Conversely, extracellular adenosine decreased $[cAMP]_i$ and ciliary activity [60]. The possibility that cAMP was altering activity via phosphorylation was indicated by the inhibition of cAMP action by a protein kinase inhibitor [58]. A similar sensitivity to cAMP has been found associated with activation of flagellar activity [53], the control of cilia of *Paramecium* [27] and of gill cilia of mussels [61]. Although an increase in beat frequency induced by β-adrenergic agents was achieved by reducing all phases of the beat cycle [62], the mechanism by which cAMP-dependent phosphorylation may mediate these events is not well understood. Salathe et al. [63] have established that cAMP induces specific phosphorylation of a 26-kD

protein from ovine axonemes. This phosphorylation occurred in isolated axonemes, suggesting that the kinase is part of the axoneme. A similar conclusion is drawn from the cAMP-sensitivity of demembranated cilia of *Paramecium* [27]. By contrast, demembranated rabbit airway cilia were insensitive to cAMP [62].

The relationship of the phosphorylated 26-kD protein to ciliary activity is unknown [63]. However, cAMP-dependent phosphorylation of proteins of a similar molecular weight also occurs in axonemes of mussels (20, 21 and 27 kD, ref. [6]) and *Paramecium* (29 kD, ref. [64]). The 20-, 21-, 27- and 29-kD proteins are considered to be light chains specific to the outer dynein – these proteins were copurified with the outer dynein arms. The phosphorylation of the proteins from mussel and ovine axonemes was independent of Ca^{2+}, whereas the phosphorylation of the 29-kD protein of *Paramecium* was prevented by Ca^{2+} [64]. In support of the enticing hypothesis that light-chain phosphorylation regulates axonemal function, Hamasaki *et al.* [64] have demonstrated that phosphorylated 29-kD protein (p29) increased the translocation velocity of microtubules with isolated 22S dynein. It is proposed that an increase of microtubule sliding rates, achieved by a p29-dependent increase in the numbers of dynein arms contributing to sliding, will translate into increased frequency [65]. Although a relationship between sliding velocity and beat frequency is consistent with earlier hypotheses of wave propagation and outer dynein arm function, the effect of cAMP on the activity on *Paramecium* cilia is not as well defined [27]. Increases in cAMP do increase the swimming velocity of whole demembranated *Paramecia* [64]; however, this increase in velocity appears to be achieved by a change in the beat orientation rather than by an increase in beat frequency [27]. As a result, the regulatory function of p29 or other proteins may also affect beat orientation.

The speed of microtubule sliding may also be regulated by inner dynein arm activity [66]. In contrast to *Paramecium*, these experiments, with radial spoke-deficient *Chlamydomonas* mutants, demonstrated that a dephosphorylated state, achieved by inhibition of axonemal cAMP-dependent protein kinases, was associated with increased microtubule sliding velocity. This increase in microtubule sliding was prevented by maintaining a phosphorylated state with phosphatase inhibitors. These experiments were performed on isolated axonemes in the absence of radial spokes, and Habermacher and Sale [49] suggest that a kinase and phosphatase as well as a regulatory substrate are part of the dynein regulatory complex. Preliminary studies indicate that this phosphorylation-dependent regulation of microtubule-sliding velocity is mediated via the I1 inner dynein arm [49]. It is not clear how this regulation is modified by the presence of the radial spoke, but it is suggested that the radial spoke overrides the phosphorylated state to relieve inhibition of sliding.

Protein phosphorylation is also induced by non-cAMP kinases such as protein kinase C, G or calcium/calmodulin-dependent kinase. A variety of

compounds (neuropeptides and inflammatory mediators) have been found to stimulate membrane-bound guanylyl cyclase activity to elevate cyclic guanosine 5′-phosphate (cGMP), and this suggests that cGMP-dependent phosphorylation plays a role in beat frequency regulation [67, 68]. Little is known regarding the mechanisms by which cGMP controls cilia, but cGMP has been shown to influence flagellar activity [53] and the orientation of the beat pattern of demembranated *Paramecium* cilia in a manner distinct from that induced by cAMP [27]. In airway cells, the results regarding the effects of cGMP are conflicting in that in human cells, increases in cGMP stimulated by C-type natriuretic factor increased ciliary beat frequency [68], while in rabbit epithelial cells, increases in cGMP stimulated by atrial natriuretic factor induced decreases in beat frequency [67]. An alternative method of increasing cGMP is by the activation of soluble guanylyl cyclase with nitric oxide (NO). Surprisingly, the generation of NO failed to increase beat frequency in human epithelial cells [68]. NO can be produced in response to β-adrenergic stimulation, and this enhances ciliary activity in bovine or rabbit epithelial cells, but this effect of NO occurs only in conjunction with β-adrenergic stimulation [69, 70]. Similar results were obtained with substance P and human cells [71]. In view of the finding that cGMP could decrease beat frequency, Tamaoki *et al.* [67] suggested that NO may catalyze the production of ADP-ribose, an intracellular messenger that can release Ca^{2+} from internal stores via a ryanodine receptor to elevate $[Ca^{2+}]_i$. However, cultured rabbit epithelial cells seem to lack ryanodine receptors [72], and human cilia do not respond to NO generators [68]. Collectively, these results suggest that NO itself does not directly influence ciliary beat frequency.

The activation of protein kinase C [73] was found to reduce ciliary beat frequency and phosphorylate a membrane-associated protein (p37). The mechanism of control is unknown but would appear to be indirect, as the protein is not associated with the axoneme.

7.3. Regulation of Ciliary Activity by Calcium

Increases in $[Ca^{2+}]_i$ stimulate an increase in airway ciliary beat frequency without an alteration in beat pattern [57, 62, 74–78]. By contrast, in protozoa such as *Paramecium* [27] and *Chlamydomonas* [28], elevated $[Ca^{2+}]_i$ induced a reverse or change in beat pattern, and in gill cilia of mussels [52] elevated $[Ca^{2+}]_i$ induced ciliary arrest. In most of these organisms, this Ca^{2+} sensitivity was retained by demembranated axonemes, a result that suggests the Ca^{2+} sensor is a component of the axoneme. Reactivated ciliary models of airway cilia showed either moderate [74, 79] or no [62, 80] sensitivity to Ca^{2+}. If the Ca^{2+} sensor in airway cells is part of the axoneme, it may be easily lost by detergent extraction. Calmodulin, a ubiquitous Ca^{2+} regulatory protein, enhanced the Ca^{2+} sensitivity of model

airway cilia [74, 79], and inhibitors of calmodulin activity (i.e. W-7 or trifluoroperazine) slowed the beat frequency in intact cilia [74, 76, 79]. A similar suggestion that calmodulin can affect outer-arm activity has been made [80]. Calmodulin has been found to be localized in a variety of cilia [52]. In view of the current opinion that inner dynein arm regulates beat form and outer dynein arm regulates beat frequency, these data lead to the suggestion that increases in beat frequency induced by Ca^{2+} in airway cilia may be mediated through changes in outer dynein arm activity. At present, the site of Ca^{2+} action is not clear. Phosphorylation appears to provide a mechanism to control either inner- or outer-arm activity; thus, calcium/calmodulin kinase may mediate Ca^{2+} effects. The site of phosphorylation may be similar to those influenced by cAMP, because no differences in the changes in the timing of the beat cycle could be detected when beat frequency was elevated by either β-adrenergic agonists or Ca^{2+} [62].

7.4. Regulation by Intercellular Signaling via Gap Junctions

Mucociliary transport requires contact between the ciliary tips and the underside of the mucus layer. In view of this physical mucus–ciliary interaction, mechanical stimuli may play a role in the regulation of mucociliary transport. Mechanical stimulation of ciliated airway epithelial cells in culture, by the brief distortion of the apical membrane with a microprobe, induced a transient increase in ciliary beat frequency of the stimulated cell [56]. The beat frequency was restored to the original frequency after about 40–60 s. It is perhaps of greater interest, that in response to the same mechanical stimulus, the beat frequency of cilia of immediately adjacent, as well as more distal, ciliated cells also transiently increased. This increase in frequency spread through the surrounding cells in a wavelike manner, with each cell responding in turn after a delay of about 0.5–1 s between cells. This ciliary response to a single stimulus was displayed by 5–7 cells in all directions. A similar response could be initiated by mechanically stimulating a non-ciliated cell, a result that suggests the transduction mechanism does not require cilia. An implication of these results is that airway cilia have, in addition to pharmacological control of beat frequency, a mechanochemical control of beat frequency. The ability of airway cilia to autoregulate their beat frequency under increasing load conditions is compatible with this idea [19], but autoregulation can also occur in the absence of a cell membrane.

The cellular mechanism underlying these multicellular changes in beat frequency is the intercellular propagation of Ca^{2+} waves. Digital video microscopy of the Ca^{2+}-specific reporter dye fura-2 revealed that mechanical stimulation elevates $[Ca^{2+}]_i$ not only in the stimulated cell but also in surrounding adjacent cells (Figure 3). A wave of increasing Ca^{2+} spreads concentrically from the point of contact to the periphery of the cell. After a

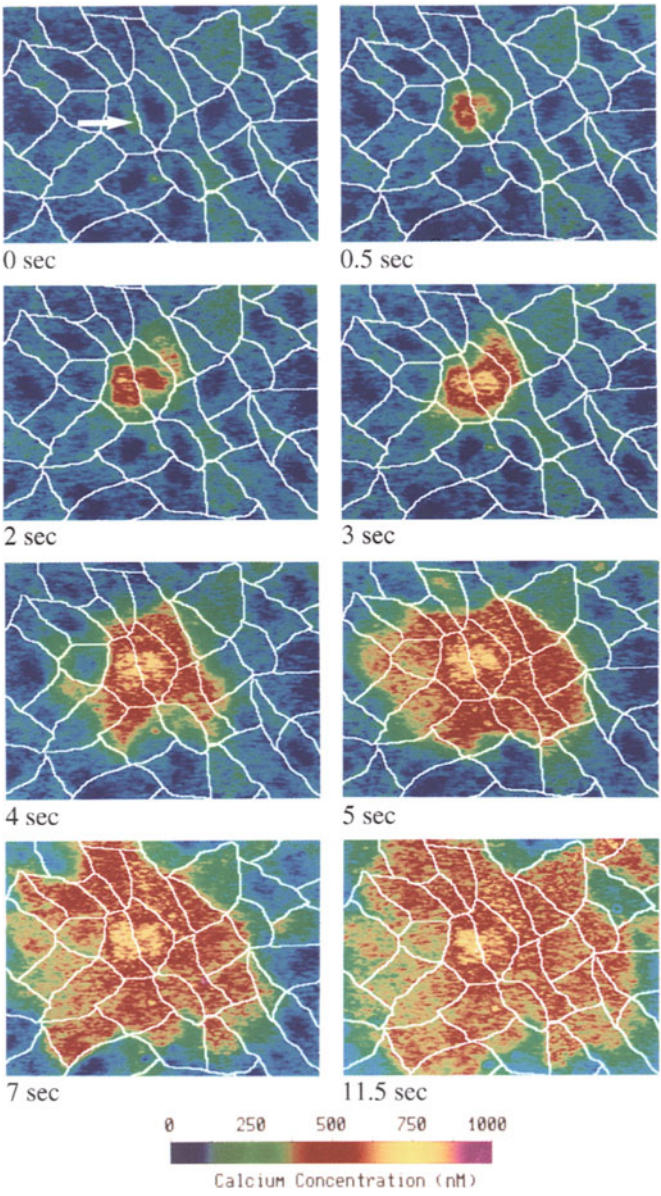


Figure 3. The propagation of an intercellular Ca^{2+} wave through airway epithelial cells. The boundaries of individual cells are outlined in white. A single cell is stimulated at time 0 s (arrow). An increase in $[Ca^{2+}]_i$, as indicated by the scale bar, occurs in the stimulated cell and is communicated to adjacent cells. At each cell border the wave propagation is interrupted by a short delay. After 11.5 s a field of cells have participated in the propagation of a Ca^{2+} wave. Vertical distance = 140 µm; horizontal distance = 240 µm.

short delay of about 0.5–1.0 s, Ca^{2+} waves were initiated in immediately adjacent cells from points of contact with the stimulated cell. This process was repeated in more distal cells to form an intercellular Ca^{2+} wave that propagates through the culture in a cell-by-cell manner. The extent of travel by the Ca^{2+} wave was similar to that of the ciliary response. The increase in $[Ca^{2+}]_i$ precedes the increase in beat frequency. Non-ciliated cells also demonstrated increases in $[Ca^{2+}]_i$ with similar kinetics [56].

Intercellular Ca^{2+} wave propagation does not appear to occur by neural activity, membrane depolarization, mechanical shock waves or hydrodynamic disturbances due to ciliary interactions. Propagation of waves of Ca^{2+} and increases in beat frequency occur in tissue-cultured cells devoid of innervation, under conditions of high extracellular K^+ designed to cause membrane depolarization, at velocities much slower than those associated with electrical events or mechanical vibrations and through cells that are non-ciliated.

The current hypothesis for the propagation of an epithelial intercellular Ca^{2+} wave is that the second messenger IP_3 diffuses through gap junctions to adjacent cells. It is proposed that IP_3 is produced in the stimulated cell by mechanical stimulation. The exact mechanism by which this occurs is unknown, but stretching airway epithelial cells increases the $[IP_3]_i$ [81]. A β-phospholipase C (PLC) inhibitor (U73122) also prevents Ca^{2+} wave initiation, and this suggests that mechanical stimulation might directly activate PLC or a regulatory G protein [72]. The IP_3 diffuses across the stimulated cell from the point of stimulation and releases Ca^{2+} from intracellular stores via the IP_3 receptor. A Ca^{2+} feedback mechanism amplifies the Ca^{2+} release process through the IP_3 receptor [82] to form an intracellular Ca^{2+} wave. Airway epithelial cells are electrically coupled by gap junctions [56, 83], and these allow IP_3 to diffuse to adjacent cells to initiate a similar cascade of Ca^{2+} release that produces an intercellular Ca^{2+} wave. A regenerative production of IP_3 is not required for the propagation of a Ca^{2+} wave through multiple cells [84]. While gap junctions may be permeable to Ca^{2+}, Ca^{2+} does not appear to be required for the initiation or propagation of intercellular Ca^{2+} waves. This conclusion is supported by the evidence that elevations in $[Ca^{2+}]_i$ associated with Ca^{2+} oscillations or following experiments in which the IP_3 receptor or PLC was inhibited did not propagate to adjacent cells. Ca^{2+} waves could also be initiated without increases in $[Ca^{2+}]_i$ in the stimulated cell [56].

The ability of signaling molecules to move from one cell to the next provides the multicellular system with a mechanism to generate cooperative cellular activity. This would be an advantage to a ciliated epithelium, as the communication of a Ca^{2+} signal (via IP_3) would enhance beat frequency of many cells – a response that would seem beneficial to the process of metachrony. It should be pointed out that the Ca^{2+} waves are not responsible for metachronal activity. As mentioned earlier, metachrony is governed by hydrodynamic interactions. At present, it is unknown if such waves exist

in the airway epithelium *in situ*, but intercellular Ca^{2+} waves have been observed in slice preparations of liver and brain. In addition to IP_3, cAMP can permeate gap junctions, and this may also serve as an intercellular messenger to regulate ciliary function.

8. Conclusions

Airway cilia are complex biochemical engines that drive mucus transport. Airway cilia have a general ultrastructure and principle of operation that is similar to those of other cilia and flagella. The structural characteristics that specialize airway cilia for mucus transport include length, a ciliary crown and a twisting axoneme. The specialized behavioral characteristics of airway cilia are a discontinuous metachronal activity and a beat cycle with a rest phase. The distinguishing regulatory mechanisms of airway ciliated cells includes an ability to autoregulate power output, mechanosensitivity and intercellular signaling, the restriction of Ca^{2+} sensitivity to changes in beat frequency and sensitivity to a wide range of neurohormonal agonists. However, the details of the molecular mechanisms that convert microtubule sliding into bending and control ciliary activity remain poorly defined for all types of cilia, and these will form research challenges for the future.

Acknowledgements

The editors would like to thank Prof. M.A. Sleigh for reviewing this chapter. This work was supported by NIH grant HL49288.

References

1. Sleigh MA, Blake JR, Liron N (1988) The propulsion of mucus by cilia. *Am Rev Resp Dis* 137: 726–741.
2. Satir P, Sleigh MA (1990) The physiology of cilia and mucociliary interactions. *Annu Rev Physiol* 52: 137–155.
3. Omoto CK (1991) Mechanochemical coupling in cilia. *Int Rev Cytol* 131: 255–292.
4. Mitchell DR (1994) Cell and molecular biology of flagellar dyneins. *Int Rev Cytol* 155: 141–180.
5. Brokaw CJ (1994) Control of flagellar bending: A new agenda based on dynein diversity. *Cell Motil Cytoskeleton* 28: 199–204.
6. Cosson J (1996) A moving image of flagella: News and views on the mechanisms involved in axonemal beating. *Cell Biol International* 20: 83–94.
7. Gibbons IR (1981) Cilia and flagella of eukaryotes. *J Cell Biol* 91: 107s–124s.
8. Sanderson MJ, Sleigh MA (1981) Ciliary activity of cultured rabbit tracheal epithelium: Beat pattern and metachrony. *J Cell Sci* 47: 331–347.
9. Sanderson MJ, Sleigh MA (1982) The function of respiratory tract cilia. In: Bonsignore G and Cumming G, ed. *The lung in its environment.* New York. Plenum 81–120.
10. Liedtke CM (1992) Electrolyte transport in the epithelium of pulmonary segments of normal and cystosis lung. *FASEB J* 6: 3076–3084.
11. Rhodin JAG (1966) Ultrastructure and function of the human tracheal mucosa. *Am Rev Resp Dis* 93: 1–15.

12. Machemer H (1974) Ciliary activity and metachronism in protozoa. In: Sleigh MA ed. *Cilia and flagella.* London. Academic Press, 199–286.
13. Sleigh MA (1974) Metachronism of cilia of metazoa. In: Sleigh MA ed. *Cilia and flagella.* London. Academic Press, 287–304.
14. Gheber L, Priel Z (1990) On metachronism in ciliary systems: A model describing the dependence of the metachronal wave properties on the intrinsic ciliary parameters. *Cell Motil Cytoskeleton* 16: 167–181.
15. Marino MR, Aiello E (1982) Cinemicrographic analysis of beat dynamics of human respiratory cilia. *Cell Motil* Suppl 1: 35–39.
16. Wong LB, Miller IF, Yeates DB (1993) Nature of the mammalian ciliary metachronal wave. *J Appl Physiol* 75: 458–467.
17. Dentler WL, LeCluyse EL (1982) Microtubule capping structures at the tips of tracheal cilia: Evidence for their firm attachment during ciliary bend information and the restriction of microtubule sliding. *Cell Motility* 2: 549–572.
18. Foliguet B, Puchelle E (1986) Apical structure of human respiratory cilia. *Bull Eur Physiopathol Respir* 22: 43–47.
19. Johnson NT, Villalon M, Royce FH, Hard R, Verdugo P (1991) Autoregulation of beat frequency in respiratory ciliated cells. *Am Rev Respir Dis* 144: 1091–1094.
20. Afzelius BA (1995) Role of cilia in human health. *Cell Motil Cytoskeleton* 32: 95–97.
21. Hard R, Rieder CL (1983) Mucociliary transport in newt lungs: The ultrastructure of the ciliary apparatus in isolated epithelial sheets and functional triton-extracted models. *Tissue Cell* 15: 227–243.
22. Sanderson MJ, Dirksen ER, Satir P (1990) Electron microscopy of respiratory tract cilia. In: Schraufnagel DE ed. *Lung biology in health and disease,* vol. 48, *Electron Microscopy of the Lung.* New York. Marcel Dekker, 47–69.
23. Serafini SM, Michaelson ED (1977) Length and distribution of cilia in human and canine airways. *Bull Europ Physiopath Resp* 13: 551–559.
24. Goodenough UW, Heuser JE (1989) Structure of the soluble and *in situ* ciliary dyneins visualized by quick-freeze deep-etch microscopy. In: Warner FD, Satir P, Gibbons IR eds. *Cell movement,* vol. 1. New York. Alan Liss, 121–140.
25. Mastronarde DN, O'Toole ET, McDonald KL, McIntosh JR, Porter ME (1992) Arrangement of inner dyneins arms in wild-type and mutant flagella of *Chlamydomonas. J Cell Biol* 118: 1145–1162.
26. Satir P (1965) Studies on cilia II: Examination of the distal region of the ciliary shaft and the role of the filaments in motility. *J Cell Biol* 26: 805–834.
27. Pech LL (1995) Regulation of ciliary motility in *Paramecium* by cAMP and cGMP. *Comp Biochem Physiol* 111A: 31–37.
28. Whitman GB (1993) *Chlamydomonas* phototaxis. *Trends Cell Biol* 3: 403–408.
29. Sanderson MJ (1984) Cilia. In: Bereiter-Hahn J, Matoltsy AG, Richards KS eds. *Biology of the integument,* vol. 1, *Invertebrates.* Berlin. Springer-Verlag, 17–42.
30. Summers KE, Gibbons IR (1971) Adenosine-triphosphate induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. *Proc Natl Acad Sci USA* 68: 3092–3096.
31. Weaver A, Hard R (1985) Isolation of newt lung ciliated cell models.: Characterization of motility and coordination thresholds. *Cell Motility* 5: 355–375.
32. Hastie AT, Dicker DT, Hingley ST, Kueppers F, Higgins ML, Weinbaum G (1986) Isolation of cilia from porcine tracheal epithelium and extraction of dynein arms. *Cell Motil Cytoskeleton* 6: 25–34.
33. Dirksen ER, Zeira M (1981) Microtubule sliding in cilia of the rabbit trachea and oviduct. *Cell Motility* 1: 247–260.
34. Shingyoji C, Takahashi K (1995) Cyclical bending movements induced by successive iontophoretic application of ATP to an elastase-treated flagellar axoneme. *J Cell Sci* 108: 1359–1369.
35. Brokaw CJ (1991) Microtubule sliding in swimming sperm flagella: Direct and indirect measurements on sea urchin and tunicate spermatozoa. *J Cell Biol* 114: 1201–1215.
36. Gibbons IR (1995) Dynein family of motor proteins: Present status and future questions. *Cell Motil Cytoskeleton* 32: 136–144.
37. Hard R, Blaustein K, Scarcello L (1992) Reactivation of outer-arm-depleted lung axonemes: Evidence for functional differences between inner and outer dynein arms *in situ. Cell Motil Cytoskeleton* 21: 199–209.

38. Brokaw CJ, Kamiya R (1987) Bending patterns of *Chlamydomonas* flagella. IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. *Cell Motil Cytoskeleton* 8: 68–75.
39. Kamiya R (1995) Exploring the function of inner and outer dynein arms with *Chlamydomonas* mutants. *Cell Motil Cytoskeleton* 32: 98–102.
40. Piperno G (1995) Regulation of dynein activity within *Chlamydomonas* flagella. *Cell Motil Cytoskeleton* 32: 103–105.
41. Rupp G, Hard R (1995) Outer arm dynein from newt lung respiratory cilia: Purification and polypeptide composition. *Cell Motil Cytoskeleton* 31: 22–33.
42. Hastie AT, Marchese-Ragona SP, Johnson KA, Wall JS (1988) Structure and mass of mammalian respiratory ciliary outer arm 19S dynein. *Cell Motil Cytoskeleton* 11: 157–166.
43. Piperno G, Ramanis Z, Smith EF, Sale WS (1990) Three distinct inner dynein arms in *Chlamydomonas* flagella: molecular composition and location in the axoneme. *J Cell Biol* 110: 379–389.
44. Piperno G, Ramanis Z (1991) The proximal portion of *Chlamydomonas* flagella contains a distinct set of inner dynein arms. *J Cell Biol* 112: 701–709.
45. Gibbons BH, Gibbons IR (1973) The effect of partial extraction of dynein arms on the movement of reactivated sea urchin sperm. *J Cell Sci* 13: 337–357.
46. Haung B, Ramanis Z, Luck DJ (1982) Suppressor mutations in *Chlamydomonas* reveal a regulatory mechanism for flagellar function. *Cell* 28: 115–124.
47. Gardner LC, O'Toole E, Perrone CA, Giddings T, Porter ME (1994) Components of a "dynein regulatory complex" are located at the junction between radial spokes and the dynein arms in *Chlamydomonas* flagella. *J Cell Biol* 127: 1311–1325.
48. LeDizet M, Piperno G (1995) The light chain p28 associates with a subset of inner dynein arm heavy chains in *Chlamydomonas* axonemes. *Mol Biol Cell* 6: 697–711.
49. Habermacher G, Sale WS (1995) Regulation of dynein-driven microtubule sliding by an axonemal kinase and phosphatase in *Chlamydomonas* flagella. *Cell Motil Cytoskeleton* 32: 106–109.
50. Brokaw CJ, Johnson KA (1989) Dynein-induced microtubule sliding and force generation. In: Warner FD, Satir P, Gibbons IR eds. *Cell movement,* vol 1. New York. Alan Liss, 191–198.
51. Sale WS, Satir P (1977) Direction of active sliding of microtubules in *Tetrahymena* cilia. *Proc Natl Acad Sci USA* 74: 2045–2049.
52. Satir P (1985) Switching mechanisms in the control of ciliary motility. *Mod Cell Biol* 4: 1–46.
53. Walczak CE, Nelson DL (1994) Regulation of dynein-driven motility in cilia and flagella. *Cell Motil Cytoskeleton* 27: 101–107.
54. Holwill MEJ, Satir P (1994) Physical model of axonemal splitting. *Cell Motil Cytoskeleton* 27: 287–298.
55. Lindemann CB, Kanous KS (1995) "Geometric clutch" hypothesis of axonemal function: Key issues and testable predictions. *Cell Motil Cytoskeleton* 31: 1–8.
56. Sanderson MJ, Charles AC, Boitano S, Dirksen ER (1994) Mechanisms and function of intercellular calcium signaling. *Mol Cellul Endocrinol* 98: 173–187.
57. Sanderson MJ, Dirksen ER (1989) Mechanosensitive and beta-adrenergic control of the ciliary beat frequency on mammalian respiratory tract cells in culture. *Am Rev Respir Dis* 139: 432–440.
58. Di Benedetto G, Manara-Shediac FS, Mehta A (1991) Effect of cyclic AMP on ciliary activity of human respiratory epithelium. *Eur Respir J* 4: 789–795.
59. Tamaoki J, Kondo M, Takizawa T (1989) Effect of cAMP on ciliary function in rabbit tracheal epithelial cells. *J Appl Physiol* 66: 1035–1039.
60. Tamaoki J, Kondo M, Takizawa T (1989) Adenosine-mediated cyclic AMP-dependent inhibition of ciliary activity in rabbit tracheal epithelium. *Am Rev Resp Dis* 139: 441–445.
61. Stephens RE, Prior G (1992) Dynein from serotonin-activated cilia and flagella: extraction characteristics and distinct sites for cAMP-dependent protein phosphorylation. *J Cell Sci* 103: 999–1012.
62. Lansley AB, Sanderson MJ, Dirksen ER (1992) Control of the beat cycle of respiratory tract cilia by Ca^{2+} and cAMP. *Am J Physiol* 263 (Lung Cell Mol Physiol 7): L232–242.

63. Salathe M, Pratt MM, Wanner A (1993) Cyclic AMP-dependent phosphorylation of a 26-kD axonemal protein in ovine cilia isolated from small tissue pieces. *Am J Respir Cell Mol Biol* 9: 306–314.
64. Hamasaki T, Barkalow K, Satir P (1995) Regulation of ciliary beat frequency by a dynein light chain. *Cell Motil Cytoskeleton* 32: 121–124.
65. Hamasaki T, Holwill MEJ, Barkalow K, Satir P (1995) Mechanochemical aspects of axonemal dynein activity studied by *in vitro* microtubule translocation. *Biophys J* 69: 2569–2579.
66. Smith EF, Sale WS (1992) Regulation of dynein-driven microtubule sliding by the radial spokes in flagella. *Science* 257: 1557–1559.
67. Tamaoki J, Chiyotani A, Kondo M, Konno K (1995) Role of NO generation in β-adrenoceptor-mediated stimulation of rabbit airway ciliary motility. *Am J Physiol* 268 (Cell Physiol 37): C1342–1347.
68. Geary CA, Davis CW, Paradiso AM, Boucher RC (1995) Role of CNP in human airways: cGMP-mediated stimulation of ciliary beat frequency. *Am J Physiol* 268 (Lung Cell Mol Physiol 12): L1021–1028.
69. Jain B, Rubinstein I, Robbins RA, Leise KL, Sisson JH (1993) Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem Biophys Res Comm* 191: 83–88.
70. Tamaoki J, Kobayashi K, Sakai N, Kanemura T, Horii S, Isono K, Takeuchi S, Chiyotani A, Yamawaki I, Takizawa T (1991) Atrial natriuretic factor inhibits ciliary motility in cultured rabbit tracheal epithelium. *Am J Physiol* 260 (Cell Physiol 29): C201–205.
71. Schlosser RJ, Czaja JM, Yang B, McCaffrey TV (1995) Signal transduction mechanisms in substance P-mediated ciliostimulation. *Otolaryngol Head Neck Surg* 113: 582–588.
72. Hansen M, Boitano S, Dirksen ER, Sanderson MJ (1995) A role for phospholipase C but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. *J Cell Sci* 108: 2583–2590.
73. Salathe M, Pratt MM, Wanner A (1993) Protein kinase C-dependent phosphorylation of a ciliary membrane protein and inhibition of ciliary beating. *J Cell Sci* 106: 1211–1220.
74. Kakuta Y, Kanno T, Sasaki H, Takishima T (1985) Effect of Ca^{2+} on the ciliary beat frequency of skinned dog tracheal epithelium. *Respir Physiol* 60: 9–19.
75. Girard PR, Kennedy JR (1986) Calcium regulation of ciliary activity in rabbit tracheal epithelial explants and outgrowth. *Eur J Cell Biol* 40: 203–209.
76. Di Benedetto G, Magnus CJ, Gray PTA, Mehta A (1991) Calcium regulation of ciliary beat frequency in human respiratory epithelium *in vitro*. *J Physiol* 439: 103–113.
77. Korngreen A, Priel Z (1994) Simultaneous measurement of ciliary beating and intracellular calcium. *Biophys J* 67: 377–380.
78. Salathe M, Bookman RJ (1995) Coupling of $[Ca^{2+}]_i$ and ciliary beating in cultured tracheal epithelial cells. *J Cell Sci* 108: 431–440.
79. Verdugo P, Raess BV, Villalon M (1983) The role of calmodulin in the regulation of ciliary movement in mammalian epithelial cilia. *J Submicrosc Cytol* 15: 95–96.
80. Hard R, Cypher C (1992) Reactivation of newt lung cilia: Evidence for a possible temperature- and MgATP-induced activation mechanism. *Cell Motil Cytoskeleton* 21: 187–198.
81. Felix JA, Woodruff ML, Dirksen ER (1996) Stretch increases inositol 1,4,5-triphosphate concentration in airway epithelial cells. *Am J Respir Cell Mol Biol* 14: 296–301.
82. Berridge MJ (1993) Inositol trisphosphate and calcium signalling. *Nature* 361: 315–325.
83. Carson JL, Willumsen NJ, Gambling TM, Hu SS, Collier AM (1989) Dynamics of intercellular communication and differentiation in a rapidly developing mammalian airway epithelium. *Am J Respir Cell Mol Biol* 1: 385–390.
84. Sneyd J, Wetton BTR, Charles AC, Sanderson MJ (1995) Intercellular calcium waves mediated by diffusion of inositol trisphosphate: A two-dimensional model. *Am J Physiol* 268 (Cell Physiol 37): C1537–1545.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 6
Rheological Properties and Hydration of Airway Mucus

Gary J. Phillips*, Stuart L. James and Michael I. Lethem

Department of Pharmacy, University of Brighton, Brighton, UK

1. Introduction

The protective functions of mucus may chiefly be defined by its barrier and transport properties. These properties are largely, but not exclusively, determined by the physical characteristics of the mucus which, in turn, may be most usefully considered as its characteristic behaviour under physical stress, that is, its rheological properties [1]. Abnormalities in the rheological properties of respiratory mucus are reliable indicators of suboptimal transport properties. Increases in viscoelastic parameters, common in cystic fibrosis, for example, lead to poor mucociliary clearance, whilst it has been suggested that a reduction in viscoelastic parameters, a situation possibly found in fucosidosis, will also result in impaired clearance [2].

* Author for correspondence.

The rheological properties of mucus are determined primarily by high relative molecular mass, O-glycosidically linked glycoproteins, the mucins. However, substantial modification of these properties occurs as a result of the presence, and variations in the amounts of, other mucus constituents. Of fundamental importance is the state of hydration of the mucus, with comparatively small changes in the water content of a mucus gel resulting in large changes in its rheological properties [3]. Indeed, suboptimal hydration may be of major clinical importance in many obstructive airway diseases, e.g. cystic fibrosis (CF). Other mucus constituents which have been shown to modify the rheological properties of mucus are high molecular weight species, such as nucleic acids [4] and polysaccharides [5], and ions, in particular metal ions [6]. In a therapeutic context, drugs may also affect mucus rheology, either intentionally, with the use of mucolytics such as *N*-acetylcysteine, or unintentionally as with the use of tetracycline, a mucospissic antibiotic [7].

This chapter sets out to discuss the role of hydration processes in mucus rheology, describe briefly the basic rheological principles, comment on measurement methodologies and place these principles within the context of airway diseases.

2. Mucus Exocytosis and Hydration

In a normal healthy lung the mucociliary escalator provides an efficient system for the removal of particulate matter from the upper airway. It is generally accepted that tracheobronchial secretions are present in two layers, i.e. a biphasic system in which the cilia, present on the apical surface of ciliated epithelial cells, are bathed in periciliary liquid (sol or airway surface liquid) and covered by a discontinuous mucus (gel) layer which interacts with the tips of the cilia. However, it is perhaps more correct to envisage a situation in which the layers are less distinct, with the periciliary layer merging into the gel layer as the mucin granules are released, hydrate and anneal with the gel layer and are transported up the airway via the mucociliary escalator. Whatever the true situation regarding the distinction between the two phases, it is clear that the efficiency of the system is dependent upon the ability of the cilia to beat at the appropriate frequency (15–21 Hz) [8] and the presence of mucus with physical properties falling between critical limits.

From a clinical viewpoint, the failure of this system is manifested most dramatically in CF. The majority of the morbidity and mortality resulting from this inherited disease is associated with repeated respiratory infections which recur due to the failure of sufferers to clear excessively thick an sticky mucus from their lungs. It appears that poor clearance of mucus secretions from cystic fibrotic lungs is not associated with a defect in cilia *per se* [9]. It has been shown, however, that CF tracheobronchial and

cervical secretions have a reduced water content when compared with controls [10]. This is a key finding, since small variations in the water content of mucus can produce significant changes in its rheological properties [3, 11]. It is clear, therefore, that the exocytotic and post-secretion hydration of mucus is a potentially effective mechanism by which mucus rheology, and thus transportability, may be controlled physiologically [12].

In CF, the under-hydration of mucus secretions is widely assumed to be due to a paucity of water into which the mucin polymer can swell. This lack of liquid is suggested to be a consequence of abnormal epithelial Cl^- secretion and increased reabsorption of Na^+ and water across the airway epithelium as a result of defective functioning of the CF gene product, cystic fibrosis transmembrane conductance regulator (CFTR). This assumption, however, does not take into account the suggestion that the swelling process is governed by a Donnan effect which is dependent upon polymer charge, ionic and polyionic milieu, and pH of the airway surface liquid into which the mucin is released [12]. A full appreciation of the process or set of processes responsible for the hydration of mucus may, therefore, lead to a greater appreciation of the causes of increased mucus viscoelasticity in pathological conditions such as CF.

2.1. Formation of the Mucus Gel

Fundamental to the understanding of the process of mucin exocytosis and hydration is an appreciation of mucus as an entangled network of polymers which may be packed in a condensed state into secretory vesicles [13–16]. Upon release, the mucin polymers undergo post-exocytotic hydration and anneal to form a coherent mucus gel as the glycoprotein polymers, free of cross-links, migrate from one entanglement to another [15, 17, 18]. There are, therefore, a number of complex biophysical processes associated with the formation of an optimally transportable mucus gel. These include the mechanism of condensation of the mucins within the secretory granule, the means by which they are released into airway lumen and how they hydrate to produce a coherent viscoelastic gel with appropriate rheological properties.

In the process of mucogenesis the protein backbone of mucus glycoproteins is translated from specific *MUC* genes (see Chapter 3 of this volume for full review) and glycosylated as it passes through the endoplasmic reticulum and Golgi apparatus of epithelial goblet cells and submucosal mucus cells. The addition of sialic acid residues and sulphated sugars to the terminals of many of the oligosaccharide side chains results in the production of highly negatively charged mucin polymers which are stored in a condensed state within membrane-bound vesicles. It is the polyanionic nature of the mucins that drives and regulates their release and hydration [12, 15–17].

2.2. *"Jack-in-the-Box" Mechanism of Mucin Exocytosis*

Following the excitation of a membrane receptor via an agonist, for example exogenous ATP, the process of stimulus–secretion coupling results in the juxtaposition of granule membrane and apical membrane, which undergo a series of fission and fusion processes culminating in the formation of a pore through which the mucin is released into the airway. This exocytosis of mucin from the apical regions of goblet cells has been said to follow a "jack-in-the-box" mechanism with the post-exocytotic hydration and expansion being driven by a Donnan potential [15, 16, 19].

The "jack-in-the-box" mechanism of exocytosis (Figure 1) is an all-or-nothing concept based on evidence including that from X-ray microanalytical studies which have demonstrated the presence of large amounts of calcium in a number of secretory granules, including mucin granules [19, 20]. This intragranular calcium is believed to shield the anionic residues on the mucin molecules resulting in a loss of charge repulsion and consequent condensation of the molecules within the granule. Following the docking of the granule with the apical membrane, exocytosis is initiated by the formation of a pore, the nature of which remains elusive [21, 22]. It is proposed that, following its formation, the pore exhibits a state of high conductance resulting in the free flow of ions between the granule and the extracellular fluid [18]. The calcium ions, which up to this point had shielded the anionic mucins, thereby maintaining their condensed state in the granule, are exchanged for Na^+ from the extracellular space, Na^+ having the ability to reduce calcium binding to mucus [23, 24]. This exchange, with a consequent loss of charge shielding, results in charge repulsion between the polyanionic mucin polymers and rapid expansion of the mucin polymer network. This process has been verified by the demonstration that calcium release precedes the bursting of intact giant mucin granules isolated from the slug *Ariolimax columbianus* [15].

2.3. *Phase Transition*

Although the shielding of the polyanionic mucin molecules by Ca^{2+} is essential for their condensation, it has been suggested that the condensation and expansion of granule contents can only be fully explained in terms of a phase transition [19]. This phase transition appears to be similar to that previously observed in artificial polyanionic gels [25] in that mucin, like other hydrogels, exists in one of two phases, either condensed or expanded/hydrated. Evidence to support this hypothesis was provided by Verdugo when it was demonstrated by video microscopy that slug giant mucin granules could reversibly expand up to 600 times their original volume in solutions of varying water:acetone ratios [19]. The volume changes of the mucus were shown to be discontinuous, remaining stable

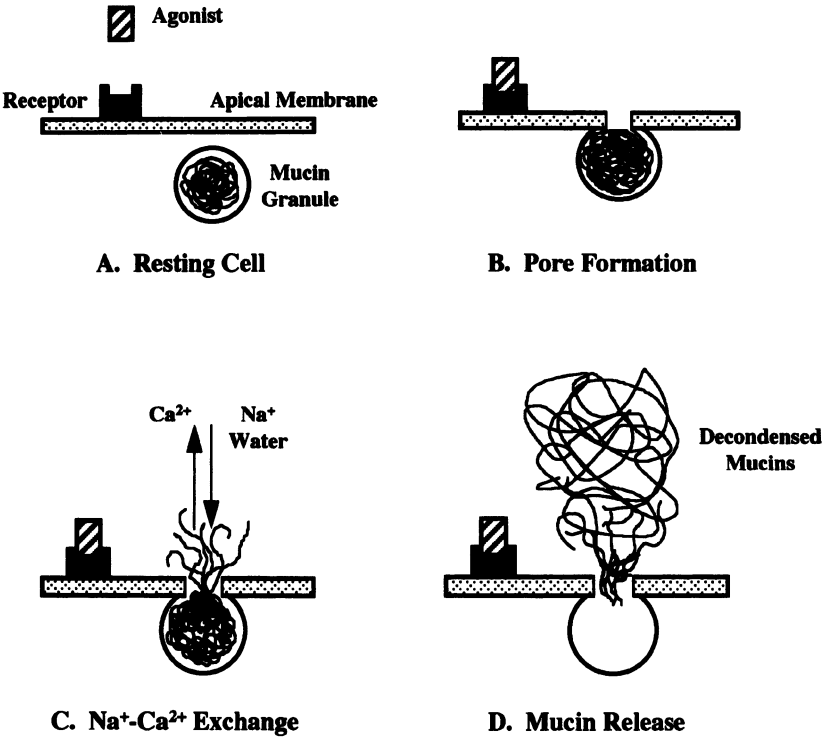


Figure 1. Proposed mechanism of mucin exocytosis and hydration. (A) Interaction between an agonist and its membrane receptor initiates the process of stimulus–secretion coupling. (B) Stimulus–secretion coupling results in the granule docking with the apical membrane of the cell, and membrane fusion events result in the formation of a pore. (C) A switch in the pore to a state of high ionic conductance allows the release of shielding Ca^{2+} from the granule and an influx of Na^{+} and water. (D) This process triggers a phase transition resulting in rapid swelling and subsequent release of mucin from the granule. The mucin hydrates via a Donnan mechanism to produce droplets which anneal to form a mucus gel.

over a wide range of solvent temperatures, pH and ionic compositions. However, at a critical point (a feature typical of phase transitions), dependent upon solvent temperature and Ca^{2+} concentration, the mucin displayed a transition from a condensed to a hydrated state. Upon further change in solvent characteristics, the process could be reversed and the gel returned to a condensed state. Such discontinuity is characteristic of phase transitions in artificial polymer gels [25]. In an *in vivo* situation the formation of a pore would allow a shift in the equilibrium with Na^{+}–Ca^{2+} cation exchange allowing the condensed mucin to undergo transition to the hydrated phase [26]. The "explosive" swelling of the gel, driven by a Donnan potential, results in the release of the polymer network in to the lung lumen and ultimately the production of a viscoelastic mucus gel. The critical point in the

phase transition process will be dependent on the charge density of the polymer together with the dielectric constant of the non-solvent species and the amount of charge shielding of the polyanionic mucins. In subsequent studies, granules from mammalian mast cells and respiratory goblet cells have both been demonstrated to undergo phase transitions [26, 27]. This stage of swelling is very rapid, with the post exocytotic expansion of the mucin networks reaching several hundredfold within 20–30 ms [19].

2.4. Post-Exocytotic Hydration and the Donnan Potential

As the continuum between the granule and extracellular space is formed and the mucin is released, it is proposed to undergo post-exocytotic hydration driven by a Donnan potential [12]. A Gibbs–Donnan effect, shown theoretically by Gibbs and experimentally by Donnan [28], is dependent upon the presence within a system of a non-diffusible polyion, such as a mucin molecule, on one side of a semipermeable membrane which effects the distribution of diffusible ions and consequently the movement of water (e.g. into the mucus network). The driving force for swelling does not rely upon the relatively limited osmotic effect of the mucin polymers; rather, it is derived from the polyanionic nature of the mucin molecules, the entanglements of which act as a semipermeable membrane, and on the asymmetric distribution of diffusible ions [12, 29]. This hypothesis arose from experiments which demonstrated a reduction in the *in vitro* hydration of bovine cervical mucus with fluids of increasing ionic concentration and pH [12]. Initially, some doubt surrounded the validity of this hypothesis, since these workers demonstrated an increased hydration at low pH, contrary to the effect expected from a polyanionic polymer. This discrepancy was presumably due to the presence in the mucus of contaminant proteins with varying isoelectric points resulting in a higher net charge at low pH. More recently, using partially purified porcine gastric mucus gels, we have demonstrated clearly that hydration can be increased dramatically by increasing pH [29]. This supports the proposal that pH affects the movement of water by altering the electrostatic charge on the polymer in a manner dependent upon the ionisation constant of the dissociable groups present, which in the case of mucins are predominantly the terminal sialic acid residues [12]. The notion that polymer charge plays a central role in post-exocytotic swelling has been further reinforced by the demonstration that the hydration of mucins released from rabbit tracheal epithelial cells under culture conditions follows first-order kinetics [17]. This finding has been used to estimate the diffusivity of freshly secreted mucins released from the secretory granule and has indicated that, when compared with reptational diffusion of hydrated gels using dynamic laser scattering, diffusivities are too great to be driven by simple concentration gradients [13, 14, 17].

It is clear, therefore, that through a Donnan process, the extent of hydration could be determined by the fixed charge on the polymer and the concentration of freely diffusible ions in the hydrating liquid, since either an increase in the ionic strength, or a decrease in polyanionicity would result in reduced swelling. This scenario has important implications for CF, as it provides an alternative explanation for mucus under-hydration in a situation where mucin charge and ionic milieu are known to be altered [10, 30, 31].

2.5. CF Mucus and the Donnan Potential

The study of mucus hydration as a Donnan process has, in fact, shown that the provision of excess water may not be the panacea for CF. Even in the presence of excess liquid, the hydration of bovine cervical, porcine gastric and human respiratory mucus gels can be limited by the presence of ions in the mucin and swelling medium at, or below, the concentrations reported in the luminal environment [12, 29]. It is reasonable to assume, therefore, that the rheological properties of the mucus may be adjusted via control of luminal ion concentration, of which calcium and potassium may be of particular importance in a CF lung where Na^+ and Cl^- levels are reduced. Verdugo and co-workers have demonstrated that increases in extracellular calcium concentrations, equivalent to levels found in the CF lung, can produce a significant reduction in the diffusivity of mucins released from cultured rabbit tracheal epithelial cells [16]. As the release of shielding ions from the granule is driven by a diffusion gradient between the granule and extracellular space, if the concentration of Ca^{2+} in the hydrating liquid is increased, the release of Ca^{2+} from the charged sites on the mucin polymers should decrease, resulting in a slowing in the rate of swelling [16]. Further to this we have demonstrated that, as predicted by Donnan theory, diffusible ion identity is an important factor in the determination of swelling rate, with the swelling process being reduced by a greater extent in the presence of divalent cations and their co-ions than monovalent ions, this effect being a function of particle number [29]. It is important to consider, however, that the extent of mucus hydration via a Donnan effect will depend on the combined ionic concentration in both the gel matrix and hydrating liquid throughout and immediately after phase transition of the mucin. It is difficult to predict such effects, as previous studies have only investigated the effect of individual ions in isolation, and the ionic status of the gel and the hydrating liquid at the point of release are not well characterised.

The realisation that free swelling can be limited by an increase in the ionic concentration of gel and swelling liquid also has important implications for contemporary CF therapies. Over the last few years, the sodium channel blocker amiloride has been used in cystic fibrosis patients in an

attempt to halt the reabsorption of Na^+ across the epithelial cells and thus the absorption of water [32, 33]. However, even if the water content is increased in this way, the increased Na^+ content, together with Ca^{2+} and K^+, both of which may be higher in CF secretions, may limit the free hydration due to charge effects. Therefore, simply increasing the amount of liquid present may not be sufficient to increase hydration enough to modify the rheological properties of the mucus. Recently, this hypothesis has been supported by studies which have failed to show any beneficial effect of nebulised amiloride in CF, either in terms of clinical status or sputum rheology [34, 35].

In addition to manipulation of luminal ionic composition, it is possible to envisage how a mechanism that can control the charge on mucin polymers can directly effect the inherent hydration potential of the mucus gel. This may provide a rational link between the under-hydration of CF mucus and presence of CFTR in vacuolar membranes, defective functioning of which may affect vacuolar pH and thus enzyme activity, resulting in altered mucin charge [36]. In addition, the production of mucins from different *MUC* genes may result in mucins with different hydration potentials, particularly if differing gene products are expressed in CF and undergo altered patterns of glycosylation.

A further mechanism by which the hydration of mucus may be controlled is via the presence of large polyionic molecules in the swelling medium. Aitken and Verdugo demonstrated using video-microscopic techniques that the diffusivity of newly exocytosed mucins could be reduced by up to 90% when released into hydrating liquid containing serum albumin at physiological concentrations [37]. This is an important finding as albumin transport may be bidirectional and, together with the control of luminal pH and ionic concentration, may provide a physiological mechanism for the control of mucus hydration [37, 38]. It is also reasonable to assume that the presence of other polyions and large molecules such as DNA and alginate from mucoid strains of *Pseudomonas aeruginosa*, both of which are likely to be found in the CF lung, will effect the swelling process and may be contributing factors to mucus pathology.

It could be argued that viewing the hydration of mucus purely as a Donnan process suggests that the movement of ions and water into the gel simply results from equilibrium and neutrality requirements and does not necessarily take into account any direct interactions of the cations or water dipoles with the charged groups on the mucin oligosaccharides. Such interactions are known to exist in other hydrophilic polymers, so it is reasonable to suggest that similar interactions are present in the mucus network. It is possible, therefore, to envisage dynamic interactions between the polar groups on the oligosaccharide side chains and the cations and water dipoles. Under suitable conditions this may result in the formation of shells of hydration, which may themselves have a limited effect on the rheological proper-ties of the mucus. Such "microhydration" would probably be of

limited significance, however, in relation to the free water of hydration due to the Donnan effect.

2.6. Counterions and Mucus Hydration

A further consideration not previously taken into account is the associated hydration of counterions on the whole hydration of mucus in a situation analogous to that found in the glycosaminoglycan heparin. Grant and co-workers [39] revealed that the average number of water molecules bound to heparin is dependent upon counter cation identity, being maximal for Na^+-heparin complexes (16 water molecules per disaccharide repeat unit) and minimal for complexes with quaternary ammonium cations. It is, perhaps, reasonable to expect similar patterns to exist in the highly charged mucin molecules, although it is not clear how significant such hydration would be in a physiological situation.

It is clear that the role of charge in exocytosis and hydration of mucins from secretory granules provides a rationale for the presence of sialic acid and sulphate residues on the mucin side chains. This charge, together with indirect, direct and dynamic interaction with cations, anions and water dipoles may produce an extremely sensitive mechanism for the control of mucus swelling. This in turn may provide a fine control for alterating the rheological properties of airway mucus. It is also apparent that the exocytosis and hydration of mucus can be thought of as a three-stage process in which condensed mucins packed into granules are released via a phase transition into the airway lumen, where they undergo post-exocytotic swelling chiefly via a Donnan effect. In a situation where the hydration is limited, either by unfavourable ionic milieu, airway liquid pH or polymer charge, further hydration may depend on the relatively small osmotic effects of the mucin polymers themselves.

3. Principles and Measurement of Mucus Rheology

3.1. Principles of Rheology

Mucus behaves as a gel with an integrity thought to be reliant on non-covalent interactions between the predominantly anionic mucin polymers. These interactions are usually described as being molecular entanglements, although weak interactions such as hydrogen bonding are also likely. The possibility of ligand-like interactions between protein regions of adjacent molecules has also been suggested [40]. Such gels exhibit both solid-like or elastic, and liquid-like or viscous, properties simultaneously. The mechanical analysis of such materials has recently been lucidly discussed by King and Rubin [41] and will be considered here rather more briefly.

The mechanical characteristics of mucus are usually measured under conditions of shear. Some form of stress is applied and the resulting strain, or rate of strain, is measured (although some testing geometries reverse this). The process may usefully be thought of as providing an energy input to the system, and measuring the amount of energy stored (the elastic component) and the amount of energy dissipated (the viscous component). It is convenient to consider the viscous element as representing the mucus macromolecules undergoing translation relative to each other, that is, entanglements resolving and reforming (an energy-dissipating process), while the elastic component is represented by macromolecular deformation without relative translation. In reality, the situation is more complex, in that other components of the gel influence its behaviour. For instance, the movement of solvent molecules within the gel structure add a viscous component. This effect may be observed in covalently cross-linked hydrogels, which after deformation return to their original shape, but in an essentially damped fashion, dissipating energy as they do so. This loss of energy during a loading/unloading cycle may be seen in a plot of stress versus strain as the unloading path being different from the loading path, the area bounded by the two curves being a measure of the loss of energy and termed hysteresis. This behaviour is true viscoelasticity, as the term would be understood by an engineer. Mucus also displays a permanent deformation, or set, when deformed, due to relative motion of the macromolecules, and thus should also be termed plastic as a result. It is, however, now conventional to refer to such gels simply as viscoelastic.

There are three fundamental approaches to modelling such viscoelastic materials, the integral model derived from the Boltzmann superposition principle; the differential model, derived from modelling the system using Maxwell and Voigt elements; and what is referred to by authors such as Vincent [42] as the molecular model, based upon the empirical approach of dynamic testing. All three models are interrelated, and at some point all three have been considered with respect to mucus, but in practical terms it is the last of these which has proved most useful, as demonstrated by such workers as Lutz [43], King [44], Braga [45], James [46], Lethem [4] and others.

Boltzmann's superposition principle may be simply stated as being that subsequent step increases in load on a material will result in additive and independent increases in deformation, and also that the stress–relaxation behaviour of a material is dependent upon its deformation history. This may be generally stated as:

$$\gamma(t) = \int_{\infty}^{t} J(t-t_n)\, d\sigma(t_n)$$

where σ is the stress applied instantaneously at various times t_n, and J is the compliance of the material. γ is the immediate elastic response, and $J(t-t_n)$ is referred to as the creep compliance function.

While this approach suggests creep testing (see below) as appropriate, a more intuitively obvious approach is obtained by consideration of combinations of Voigt (a spring and dashpot in parallel) and Maxwell (a spring and dashpot in series) elements, the spring representing the elasticity in the system, the dashpot the viscosity. It is generally accepted that a Voigt element is a first approximation to creep test behaviour, although in real tests with mucus (see below) the immediate elastic response and permanent set suggest a small series Maxwell element is a useful addition.

The molecular model approach differs from the others in considering the behaviour of the material under dynamic conditions. The material is usually subjected to a sinusoidally oscillating strain (but sometimes stress) and the stress (or strain) in the material detected. For a purely elastic material the stress and strain are in phase, but for a purely viscous material the stress is proportional to the strain rate. The strain rate is maximum at zero strain, and thus the stress and strain will be 90° out of phase. A viscoelastic material like mucus will exhibit behaviour between these two extremes, having a frequency dependent phase lag (δ). The ratio of the maximum stress to the maximum strain is sometimes referred to as the amplitude ratio (A) and so at a sinusoidal frequency ω, a general description of events can be made such that a modulus characteristic of the material behaviour at that frequency may be established (G*, the complex modulus), thus

$$A = G^* \sin(\omega t + \delta) \tag{1}$$

G* may be considered a vector quantity resolvable into two components normal to each other, the real part being G′, the dynamic elastic or storage modules, and the imaginary part iG″ giving the dynamic viscous or loss modules, the relationship being that $G^* = G' + iG''$.
This means that Equation (1) can be expanded to the form

$$A = (G^* \cos \delta) \sin \omega t + (G^* \sin \delta) \cos \omega t$$

So

$$A = G' \sin \omega t + G'' \cos \omega t \tag{2}$$

but it is usually more useful to consider the coefficients of the real and imaginary parts of G* separately to give

$$G' = A \cos \delta \quad \text{and} \quad G'' = A \sin \delta \tag{3}$$

This relationship is used as the basis for the two major geometries used for the dynamic testing of mucus, that is the rheometers relying on the entrapment of the mucus sample between two surfaces which impart a controlled oscillating shear stress to it, on the one hand, and oscillating sphere rheometers on the other.

Interpretation of these derived variables, so that they may be of use in considerations of biological function, can become obscured by a (very reasonable) insistence that they may be described absolutely precisely only within a mathematical context. However, it is often useful to think of the complex modules G* as a measure of rigidity or stiffness of the gel, and G′ and G″ as representations of the elasticity and viscosity, respectively. The widest deviation is between G″ and viscosity; however the true dynamic viscosity η' may be obtained from the expression: $\eta' = G''/\omega$ (in fact, η' approaches η, the Newtonian viscosity, at very low frequencies). The ratio of G″ to G′, when considered from a vector perspective, can easily be seen to be tan δ (the loss tangent), and this can be viewed as the recoil capability of the material (i.e. the amount of energy dissipated to that stored upon deformation). J*, the complex compliance, may be derived in a way similar to G*, and is related such that $J^* = 1/G^*$; it may be thought of as the "sloppiness" of the mucus. It is perhaps worth noting that G* is a vector quantity and as such has magnitude and direction, the direction being defined in polar terms by the angle δ; it is thus not correct to speak of tan δ varying independently of G*, as the angle is, in fact, part of G*. Usually what is meant in this case is the magnitude of G*, or $|G^*|$.

Both creep and oscillatory testing assume the mucus is tested within its linear viscoelastic region. This is of considerable importance, both in terms of the theoretical basis of such testing, which takes this constraint to be axiomatic, and from the fairly practical viewpoint that is important when comparing results from different laboratories and, indeed, samples. The practical drawback is associated with small or valuable mucus samples, such that the testing for linearity may, in itself, render the sample unsuitable for analysis because of the considerable shear sensitivity of mucus. Both creep and oscillatory testing, and linearity testing, are performed at very low stresses and strains on mucus, in order to avoid such degradation.

3.2. Measurement of the Viscoelasticity of Mucus

Early attempts at the measurement of rheological descriptors of mucus include the use of pourability [47], simple capillary (Oswald) viscometers [48], and rotational cone-plate and parallel-plate viscometers [49]. These approaches usually measured only viscosity and, furthermore, would often degrade the fragile mucus structure, thus destroying the very thing they were attempting to measure. The capillary methods have been modified to a double-capillary geometry, in which a short length of capillary is placed in a length of wider-bore tubing connected to a vacuum device, thus allowing flow and recoil through a defined length of capillary to be observed through a microscope. While very useful for small mucus samples [50], this apparatus suffers from a variable shear rate within the capillary, making confirmation of linearity difficult.

Similarly, the thread-forming capacity (Spinnbarkeit or mucus cohesiveness) of mucus may be a reflection of certain of its rheological properties, and has been exploited in the Filancemeter. Here a sample of mucus is pulled out at constant elongation velocity until the thread of mucus, so formed, breaks. While this is a useful qualitative indicator of the mechanical behaviour of the mucus, it is difficult to define the process in basic rheological terms. It is also probably true that the mucin macromolecules become largely aligned along the thread, and the mucus in this condition may tend towards a crystalline rather than rubbery behaviour. Nevertheless, it reflects probably the second-oldest test of mucus rheology, the determination of phase of menstrual cycle by the Spinnbarkeit of cervical mucus tested between finger and thumb. It has also been shown to be negatively correlated with cough clearance [51, 52], although it tends to correlate poorly with viscoelasticity.

The original cone-plate and coaxial-cylinder rheometers have become modified to provide well-defined testing geometries for both creep-relaxation and dynamic testing. Machines employing a constant strain approach, and capable of measuring resultant stress, became popular in areas such as food rheometry and polymer testing. Such mechanical spectrometers sensitive enough to use with mucus were very expensive, and this somewhat limited their use. Third-generation controlled stress machines, together with microprocessor control and analysis, have now provided a device which can work at low-enough stresses, and with small-enough samples to be of routine use in mucus rheology. Cone-plate, parallel-plate and concentric-cylinder geometries may all be used, although the last of these tends to require rather large samples, and is also prone to the end-effect problem. Parallel-plate geometry is useful for inhomogeneous samples, but care must be taken to appreciate that the shear rate in the mucus varies across the plate diameter, making assurance of linearity important. Cone-plate geometry gives a constant shear rate across the face of the cone, and edge effects do not become significant at sample sizes above about 300–400 µl, but again, care must be taken with heterogeneous samples, since the gap between the truncated cone and the plate is, of course, very small at the axis.

Low-stress creep–relaxation curves can be carried out to give results similar to those in Figure 2. The instantaneous elastic response, conventionally referred to as J_0 in this context, and the residual shear (or equilibrium) viscosity η_0 can be derived from the first and last part of the curve, respectively, while most instruments can model the curved region as a series of Voigt units with increasing retardation times. While this approach is valid for mucus, it has become popular to test in the oscillatory mode, as behaviour over a range of frequencies from about 0.001 to 10–20 Hz is possible. Above this frequency inertial effects start to become rather difficult to correct. It is also the case that such testing yields results at frequencies directly comparable with ciliary beat frequencies and is intuitively attrac-

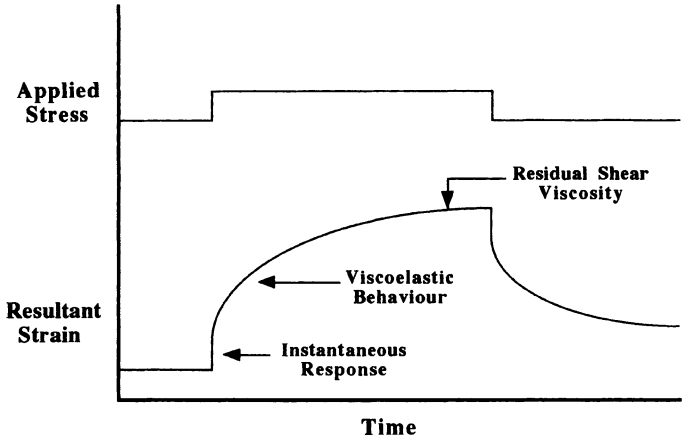


Figure 2. Typical creep–relaxation curve for mucus. Stress is applied to the sample as shown in the upper part of the figure, with the resultant strain in the mucus being shown by the lower line.

tive for that reason. As stated previously, the device generates G′ and G″ data from the ratio of stress to strain amplitude, and the phase lag, and such a result obtained in our laboratory for native human sputum is shown in Figure 3. A rather different approach has been adopted to rheological measurement by such workers as Lutz [43], King [44] and James [46], in the development of the magnetic microrheometer. Here, a microscopic steel sphere (50–100 μm diameter) is placed in the mucus sample and subjected to a sinusoidally oscillating magnetic field produced by two electromagnets. The resulting oscillation of the sphere is detected by an optical transducer fitted to a microscope, and the amplitude of movement of the sphere compared with the driving magnetic field. G′ and G″ may then be calculated by considering a force balance equation in which the magnetic force driving the sphere is balanced by the viscous drag as described by the Stokes-Einstein relationship. Transformation into the frequency domain leads to the equations derived by Lutz *et al.* [43], namely

$$G' = (F_0/6\pi r x_0) \cos \delta \quad (4)$$

and

$$G'' = (F_0/6\pi r x_0) \sin \delta \quad (5)$$

where F_0 is the maximum magnetic force acting on the sphere, r is the sphere radius and x_0 is the maximum amplitude of sphere oscillation.

Such a technique is valuable in that very small samples of mucus are measurable, perhaps a little as 5–10 μl, making measurements in non-hypersecretory states possible. Automation of such devices by computer

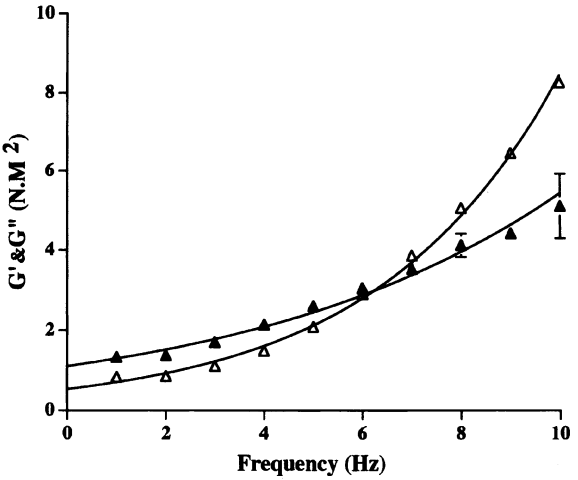


Figure 3. Rheological analysis of CF sputum by oscillatory testing using cone-and-plate geometry. Values are obtained for the G′, the storage modulus (△), and G″, the loss modulus (▲). At the lower frequencies G′ is relatively constant, the value being an indicator of cross-linking or equivalent processes in the gel. At higher frequencies the mucins have less translational freedom, and elasticity rises. G″ rises less rapidly in this sample, and indeed η' (G″/ω) would decrease, a characteristic phenomenon of mucus.

interfacing [53], or use of a frequency response analyser plus computer [44] make G′, G″ and tan δ results available in real time. Historically, this technique dates back to, at least, Seifritz in 1922 [54] who placed a small metallic sphere in the protoplasm of a cell and displaced it magnetically such that recoil data gave an estimation of the protoplasm elasticity. A similar, but far more sophisticated relaxation approach to testing mucus rheology, has been reported more recently by Edwards and Yeates [55].

In our experience, care must be taken in a number of specific areas when using such an approach. Because the sphere is so small, edge effects (for samples over 10 µl) and inertial effects tend to be insignificant. For example, in the derivation of Eqn. 4 and 5 an inertial term of $2/9\ \rho_s r^2 \omega^2$ arises, where ρ_s is the sphere density. This term is negligible at oscillation frequencies of less than about 20 Hz, but it does limit the upper-frequency range of the device. Second, the magnetic force term F_0 is, in practice, difficult to measure. It may be derived by calibration, either against a pure Newtonian fluid or by use of a standard viscoelastic material such as polyisobutylene in decalin, for which values of G′ and G″ are available in standard texts [56]. The use of a Newtonian fluid demonstrates the problem of the sphere sinking in a sample of insufficient elasticity and dragging on the bottom of the sample well. This is easily observed as a non-sinusoidal pattern of sphere displacement, and the problem has been overcome by

turning the device on its side and biasing the magnets to counteract gravity [57]. Optical clarity of the sample can also present difficulties if dealing with mucopurulent samples, although their small volume and sensitive optical detectors can usually overcome this problem. The major drawback associated with this type of device concerns inhomogeneity in native mucus samples (even when taken so as to avoid contamination with saliva). This manifests itself in two ways. First, small samples of 10 µl or so are just that, samples from an inhomogeneous material. No one sample can be termed inaccurate, since the G′ and G″ values obtained probably reflect the behaviour of that sample, but intersample variation is a true measure of the inhomogeneity of the whole sputum. Circumspection should thus be employed if quoting a mean average and estimation of error; a range value may be more appropriate. Second, and less obviously, the sample itself may not be homogeneous. In our experience this results in the sphere not moving in a pure sinusoidal fashion, or deviating from a linear path. Whilst this presents obvious problems when dealing with whole sputum, at least the problem is overt. The use of cone-plate and analogous geometries will mechanically average the variations in a sample in an unpredictable way, possibly leading to false confidence in the results. In summary, quoting a single value for G* for inhomogeneous sputum samples is analogous to describing a zebra as, on average, grey, but with a wide margin of error. Such difficulties do not arise when using purified (e.g. by isopycnic density centrifugation on caesium chloride), or even partially purified (e.g. by gel exclusion chromatography) mucin gels. Such gels provide a useful model for testing the effects of various additives when using either major type of rheometer.

Two very different approaches to the study of the rheological properties of mucus may be found in the compaction assay, and the excised frog palate. The compaction assay has to be regarded as semi-quantitative, but is quick and simple to perform [58]. Mucus is placed in a capillary tube and centrifuged. The compaction of the gel unrecoiled solids is measured. The authors claim that this method, at least for the measurement of drug-induced changes in sputum elasticity, compares well with results from cone-plate rheometry.

The frog palate model measures biological function rather than truly measuring rheological properties. However, it is a useful adjunct to rheological determinations, as it can indicate whether rheological changes in mucus are relevant to mucociliary clearance. The technique has been well described previously [59]. Briefly, the excised upper palate of a frog, e.g. *Rana temporaria*, is placed in a clear, humidified chamber and observed through a dissecting microscope. The cilia on the palate will continue to beat for up to 2 h and move antereoposterially either endogenous mucus, or exogenous mucus placed on a depleted palate. The movement of particles placed on the mucus allow its motion along the median paraspheroid to be followed and measured. The cilia beat frequency can be

obtained from the flickering of light reflected from the surface of the mucus, and captured electronically using a photodiode. The relationship between mucociliary transport and mucus rheology will be explored briefly later in this chapter.

3.3. Basic Principles Supporting the Alteration of Mucus Rheology

Whilst gel hydration is of paramount importance in the control of mucus rheology, other constituents of the mucus also influence its mechanical behaviour. The direct physiochemical interaction of these constituents with the mucin gel network has been an area of rather sporadic interest, but identification of key structural properties of mucolytic (mucus thinning) and mucospissic (mucus thickening) agents may provide an insight into the role of such constituents in altered mucus rheology. Furthermore, such principles could indicate where drugs used for therapeutic intervention in the airway may have potential mucus rheology altering effects.

Considerable interest has surrounded the idea of the reduction of sputum viscoelasticity by the use of compounds which are capable of reducing the disulphide bonds characteristic of mucin glycoproteins. The most obvious example of this type of compound is *N*-acetylcysteine, but *N*-carboxymethylcysteine, merceptoethane sulphonate and possibly Sepronin have all been implicated as this type of mucolytic. The common activity seems to be free thiol groups, or blocked thiols which can be metabolised to active forms, and thus disulphide bond reduction presents itself as the most attractive explanation. Care must be taken, however to assess this activity *in vitro*, since some of these compounds may have profound physiological effects *in vivo*. For instance, it has recently been shown that *N*-acetylcysteine affects ion transport in airway cells, possibly affecting the hydration state of secreted mucus [60]. Mucolytic activity as a result of endogenous mucus components is less well studied, the best examples probably being the effects of bile acids on gastric mucus [61]. The mode of action is suggested as being related to the anionic surfactant nature of the bile salts, suggesting disruption of weak bonds between the mucin glycoproteins; although it has recently been shown by electron microscopy that these salts are capable of reducing the size of the mucins, the mechanism of action is not clear [62].

In any event, strong hydrogen bond-disrupting agents such as urea, guanidinium chloride and sodium thiocyanate are routinely used to disrupt mucus gels, suggesting that breaking of hydrogen bonds between sugar residues on adjacent, entangled mucins is a significant mode of mucolytic action. Compared with mucolytics, even less attention has been paid to the modes of action of mucospissics. Furthermore, unlike mucolytics, most effort has been directed towards identifying endogenous mucospissics in pathological states, and very little towards identifying drugs

with mucospissic activity. This may prove problematic if therapeutic agents commonly used in the airway turn out, in time, to have such an effect.

The most obvious endogenous mucospissics are the highly charged polymers, in particular DNA [4] and bacterial alginate [5]. It is tempting to assume that these function by increasing the entanglement density in the gel network, but such highly hydrated molecules probably also serve to take water from the mucus gel, effectively dehydrating it. The most widely reported mucospissic drugs are the tetracycline family [7], but the biguanide molecules Vantocil and chlorhexidine have also been shown to be mucospissic when applied to cervical mucus [63]. It has, however, also been stated that chlorhexidine has no effect on gastric mucus gels [64]. The modes of mucospissic action of either the tetracyclins or the biguanides has never been fully elucidated. Chantler [63] has suggested that biguanides may exert their effect either by interaction of the guanidinium groups with the anionic groups of the mucin glycosylated regions, causing charge neutralisation and thus modification of the gel, or possibly by forcing the mucin anionic groups closer together by attraction to the cationic guanidinium groups. The latter concept is suggested by the importance of the spacing of the guanidinium groups being six methyl residues. This may also explain why mucins differing in sugar side-chain structure, cervical and gastric mucus react differently to chlorhexidine. Additionally, the biological polyamines spermine and spermidine have been reported as increasing mucus viscosity [49], although this effect was determined by rotational viscometry, an inappropriate method for mucus, as explained previously. We have tested a range of polyamines for effects on mucus rheology, using oscillating sphere microrheometry, and found them to be mucolytic [65] rather than mucospissic. Indeed, the common mucolytic feature of two terminal primary alkyl amines, in one case (1,6-diaminohexane) separated by six methyl residues, suggests that the mode of action of the biguanides is more complex than suggested above. It has also been reported that the diazo dye Congo Red is mucospissic, and in our hands similar dyes such as Evans Blue, Chicago Sky Blue and Trypan Blue are similarly so, although a variety of structurally unrelated dyes such as Coomazie Blue and Basic Fuchsin have no effect on mucus rheology (unpublished data). An inspection of these molecules suggests elements of commonality of form, including all being rigid planar molecules; similar size; all being bilaterally symmetrical chain-like molecules; and possessing terminal naphthalene residues with substituted amino and sulphonate groups. It can be argued that considerable electron mobility around the naphthalene residues, and the electronegative sulphonate groups, leads to strongly electron depleted amino groups. Depending on ambient pH, this would allow interaction with the mucin sugar residues either via bonding (at neutrality) or ionic interaction (at lower pH). Such a rigid bifunctional molecule could link adjacent mucin glycoproteins, or brace adjacent side chains in one macro-

molecule. Although chlorhexidene shares some structural similarities with these dyes, it is a highly flexible molecule, and also inductive dipoles are less easily formed, making Chantler's suggested mode of action more likely.

The mode of mucospissic action of the tetracyclins has, likewise, not been completely clarified. The hydroxyl group at the 12α position is important since 12α-dehydroxytetracycline has no mucospissic activity, whereas other marginal changes have little effect. The mucospissic agent BP101 [66] display keto/hydroxyl residues with electron mobility and spacial arrangement similar to tetracycline. This yields structures which can be predicted to be strongly water structuring. It is possible that these molecules function to remove free water from the gel, reducing the freedom of motion of the mucin macromolecules.

The effect of cations on mucus rheology is complex. Monovalent cations generally are mucolytic, whereas trivalent metal ions are usually mucospissic, divalent ions such as calcium having either no effect or a pH dependent effect [6]. It is very tempting to postulate ionic cross-links binding adjacent macromolecules together, but these workers point out that this is unlikely, as, for instance, the binding of calcium is mostly associated with the sugar residues, and ionic bridging between dipoles is unlikely. The mucolytic effect of monovalent ions is likely to be related to charge shielding counterions, and this action may also be involved in divalent ion action. As discussed elsewhere in this chapter, pH plays an important role in mucus hydration. It may well also control mucus rheology, although this is less well studied for airway mucus. Certainly, it has been reported that small intestinal mucus gels are disrupted at pH 1 [67], and it is likely that, as carboxylic residues on sugar side chains become protonated with changing pH, this will affect the rheology of the mucus.

It is clear that mechanisms of mucolytic and mucospissic action may range from direct interaction with the mucin glycoproteins to subtle alterations in water structure within the gel. It is also clear that the precise mechanisms involved are still poorly understood, and further work is required to provide a rational base for understanding altered rheology in disease states and to develop novel mucolytic agents.

4. Clinical Consequences of Altered Mucus Rheology

4.1. Rheological Effects on Mucus Clearance

The involvement of altered mucus rheology in respiratory disease arises principally from the requirement for the mucus to possess particular rheological properties in order for efficient clearance of the mucus from the airway to occur. Alteration of the mucus rheology such that mucociliary and cough clearance is impeded results in poor mucus clearance, thereby

predisposing the lung to obstructive airways disease and infection. Early studies of the rheological requirements for efficient mucociliary clearance suggested that the viscosity of the mucus had relatively little effect on mucociliary transport, but that the elasticity of the mucus had to lie within certain limits for optimal mucociliary transport to occur [68]. However, it should be noted that in this study the viscosity of the mucus was held relatively constant while the elasticity was varied, and hence the stiffness of the mucus and the loss tangent (tan δ) would have varied, raising the possibility that it is these parameters than are important in determining mucociliary clearance. Indeed, more recent studies of the transportability of rat nasal mucus [69] and synthetic polymers [70, 71] on the frog palate model ciliated epithelium have confirmed that this situation is probably the case. The extent to which these *in vitro* findings can be extrapolated to the *in vivo* situation has recently been called into question by Macchione and co-workers, who reported that the *in vitro* transport of rat nasal mucus on the frog palate was influenced by the mucus rigidity, while *in situ* transport on the rat nasal septum was associated with the viscosity/elasticity ratio [72].

In the case of cough clearance it is necessary not only to consider the rheological properties of the mucus sample at rest but also the changes in these properties which will occur as a result of shear thinning due to the high shear rates generated by the rapid velocity of air which occurs during coughing. This principle has been demonstrated by Zahm and co-workers who, using a simulated cough machine, showed a correlation between the thixotropic and shear thinning properties of a mucus simulant and cough clearability [73]. In an earlier review King and Rubin [41] suggested that, while viscosity represents the principal rheological determinant for cough clearance, other factors, e.g. spinnability and surface properties, may play a more important role in determining the cough clearance of mucus. A consistent finding has been that spinnability (cohesiveness), which correlates poorly with viscoelasticity but may be a reflection of polymer filament length (e.g. of DNA, F-actin and mucin) within the mucus, is negatively correlated with cough clearance [51, 52]. The importance of surface properties, e.g. adhesivity, has been suggested by several studies [74–76]. Changes in the surface properties of mucus will alter interactions between mucus and both cilia and epithelium, and hence are likely to affect the clearability of mucus. In addition, increases in adhesivity may affect cough clearance by reducing the ability of mucus to form waves at the mucus–air interface. Rapid movement of air across the surface of mucus, such as occurs during coughing, has been shown to induce the formation of waves in the surface of mucus [77, 78], thereby increasing the interaction between mucus and air. This wave formation, the extent of which may also be affected by the rheological properties of mucus, has been proposed as the initial event in the process that leads to mobilisation of mucus and its clearance from the airway [79]. From these studies and those on mucociliary clearance it is clear that mucus with the appropriate

physical properties is necessary to maintain a healthy airway and that alteration of these properties may play a central role in the progression of several respiratory diseases.

4.2. Cystic Fibrosis

Cystic fibrosis (CF) is probably the most intensively studied of the hypersecretory respiratory diseases with regard to mucus rheology. Early studies [48, 80] using rotational viscometry failed to find any rheological abnormality in CF sputum when compared with sputum from chronic bronchitis patients. However, it is likely that the high shear rates employed in these studies resulted in disruption of the sputum gel network with a consequent artifactual alteration in rheological properties. This suggestion is supported by studies using low-shear techniques which show a clear increase in both the viscosity and elasticity of CF sputum when compared with that from chronic bronchitis patients [4]. Nevertheless, the origin of this rheological abnormality remains unclear. In view of the well-documented defects in transepithelial chloride, sodium and water transport associated with the CF airway, it is tempting to assume that the rheological abnormality is a result of inadequate liquid for hydration of the freshly secreted mucus. Indeed, this hypothesis is the basis of attempts to treat CF respiratory disease with the sodium channel blocker amiloride, thereby reducing sodium and fluid absorption from the airway lumen and increasing the liquid available for mucus hydration. Trials of inhaled amiloride in CF patients have, however, produced inconclusive results. Two trials have reported a reduction in the sputum rigidity (G*) and improvements in lung function with chronic amiloride treatment [33, 81], while one study of acute amiloride treatment reported improvements in both mucociliary and cough clearance and a reduction in sputum spinnability [32]. Other trials, however, have failed to demonstrate significant clinical improvement or any alteration in sputum rheology with amiloride treatment [34, 35]. Furthermore, in both the trials of chronic amiloride treatment the reduction in sputum rigidity was not accompanied by any increase in the water content of the secretions, suggesting that if amiloride is having a beneficial effect, it is not through the proposed mechanism of increased mucus hydration. More recent studies have attempted to improve further the physical properties and clearance of CF airway secretions by combining amiloride with uridine 5′-triphosphate (UTP) in order not only to inhibit sodium absorption but also to increase chloride and fluid secretion into the airway lumen. Whilst there are no data on the effects of this approach on mucus rheology and hydration, it has been reported that this combination therapy improved mucociliary clearance in CF patients to near normal values [82].

An alternative approach to improving the clearance of airway secretions in CF has come from the belief that the abnormal rheological properties of

CF sputum are due, at least in part, to alterations in biochemical composition rather than a simple lack of hydration. The principal target of this approach has been the DNA content of the airway secretions, which has been shown to increase markedly in CF and, by interaction with the mucin gel network, to increase sputum viscoelasticity [4, 83]. Degradation of the sputum DNA treatment with recombinant human deoxyribonuclease (DNase) *in vitro* has been shown to reduce the viscoelasticity of CF sputum and in most cases to improve its *in vitro* transportability and surface properties [84, 85], raising the possibility that inhalation of the DNase may decrease the mucus viscoelasticity and improve clearance. Several trials of inhaled DNase have been completed (reviewed in Chapter 14 of this volume) and have generally reported a moderate clinical improvement in CF patients receiving DNase. Furthermore, these clinical improvements have recently been shown to be associated with a reduction in sputum viscoelasticity and an improvement in calculated cough clearance [86].

While DNase is the only current treatment aimed at altering mucus viscoelasticity by targeting biochemical changes in sputum composition associated with CF, several other components of CF sputum may lend themselves to such an approach. In particular, recent studies suggest that CF sputum contains a large amount of filamentous actin which interacts with DNA in the sputum to form large fibres which are at least partially responsible for the abnormal viscoelasticity of CF sputum [87]. The involvement of actin is further implicated in these abnormal rheological properties by the fact that addition of the actin filament-severing protein, gelsolin, causes a dramatic reduction in the viscoelasticity of CF sputum [88]. It has also been suggested that the alginate exopolysaccharide produced by mucoid strains of *Pseudomonas aeruginosa* which infect the CF lung is capable of increasing the viscoelasticity of CF airway secretions. However, unlike DNA and actin, which are well distributed throughout the secretions, the alginate is likely to be present mainly in the immediate vicinity of bacterial microcolonies, where its effect would be localised. Nevertheless, the addition of alginate lyase to CF sputum has been shown in some cases to reduce viscoelasticity; however, most sputum samples treated were unaffected due to inhibition of the alginate lyase activity by the high calcium and zinc concentrations in the sputum [89], suggesting that this particular therapeutic approach may be of limited effectiveness.

4.3. Asthma

For many years there have been suggestions of an abnormality in the rheological properties of asthmatic sputum, based largely on the jelly-like appearance. However, there are few reports of rheological studies of asthmatic mucus. Early studies of sputum viscosity in several hypersecre-

tory diseases suggested that, while there was considerable variation in viscosity in all diseases, the viscosity of asthmatic sputum was greater than that of the other diseases studied [48, 80]. However, these studies were conducted using a cone-and-plate viscometer, and hence the high shear rates employed are likely to have induced changes in the sputum rheology such that it is difficult to draw conclusions about differences between the diseases in terms of the original rheological properties.

As a result of the lack of low-shear studies, the best evidence for a rheological abnormality in asthmatic mucus remains the clinical features of asthma, the presence of mucus plugs in the airways of patients dying in *status asthmaticus*, the accumulation of mucus in the airways of patients with milder disease and the tenacious and jelly-like nature of the sputum. Indirect evidence for an abnormality can also be obtained from studies of the biochemical and structural properties of the mucins contained in asthmatic airway secretions. Recently, Sheehan and co-workers performed an exhaustive study on the mucins obtained from the mucus plugs from a patient who died in *status asthmaticus*. These workers reported that the plugs displayed a striking resistance to dissociation in 6 M guanidinium chloride (GuHCl), complete dispersion requiring extraction into greater than 200 volumes of GuHCl over a period of 7 days [90]. In addition, physical analysis indicated that the majority of the mucins purified from these plugs had a significantly higher molecular weight than mucins from "normal" and CF sputum, and appeared as complex networks in the electron microscope, unlike mucins from "normal" secretions. Further evidence for an abnormality in the composition of asthmatic airway secretions comes from studies which indicate that induced sputum from asthmatic subjects has a higher concentration of mucus glycoproteins and plasma-derived proteins than does induced sputum from healthy individuals [91, 92]. There is, therefore, substantial indirect evidence for a rheological abnormality in asthmatic mucus, but direct studies of asthmatic mucus rheology remain rare. While it is clear that there is a need for direct measurements of asthmatic mucus rheology, such studies are hindered by difficulties in obtaining adequate asthmatic samples and a lack of suitable control material.

4.4. Chronic Bronchitis and Chronic Obstructive Pulmonary Disease

Chronic bronchitis is usually the result of some chronic insult to the respiratory system, e.g. cigarette smoking, and is characterised by long-term mucus hypersecretion which is associated with sputum production, accumulation of mucus in the airways and episodes of respiratory infection. As with other hypersecretory diseases, the evidence for an abnormality in mucus rheology in chronic bronchitis is less definitive than might be expected. The principal difficulty in conducting such a study lies in the lack

of suitable control samples; healthy airways do not produce appreciable amounts of mucus, and disease controls are likely to be abnormal in themselves and may be the result of disease processes in which bronchitis is an element. Most studies comparing the rheology of chronic bronchitic sputum with that of other conditions were conducted before the advent of low-shear measurement techniques and hence suffer from the shear-related artifacts described earlier in this chapter.

Despite this problem, however, a consistent finding of these studies is the increase in sputum viscosity that occurs with purulence due to respiratory infection [48, 80]. The inflammatory processes triggered by such infections result in the release of large amounts of DNA from infiltrating inflammatory cells that cause increases in the DNA content of the sputum to levels approaching those found in CF [4, 93]. It is probable that the increased rheological properties of purulent sputum are at least in part due to the presence of these high concentrations of DNA, raising the possibility that treatment of infected bronchitic patients with inhaled DNase, as is used in CF, may be beneficial. This possibility is supported by recent studies indicating that incubation of bronchitic sputum with DNase results in a reduction in the viscoelasticity and contact angle (a measure of surface properties) of the sputum associated with an improvement in mucociliary and cough clearability [94]. Further evidence for an abnormality in chronic bronchitic mucus is provided by studies indicating that the amount of mucus in the lumen of immersion fixed lungs was significantly increased in chronic bronchitis [95]. While there are several possible explanations for the failure of the lung to clear this mucus, reduced clearance due to altered rheological properties remains a likely cause.

Attempts to improve clearance of airway secretions in chronic bronchitis by altering the rheological properties have met with varying degrees of success, and currently there is little clear clinical evidence for a benefit from mucolytic therapy. Trials of mucolytic therapy have most frequently involved sulphydryl-containing compounds which, it is thought, may reduce mucus viscoelasticity by a direct effect on the disulphide bridges of airway mucins. Carbocysteine-lysine and erdosteine have both been reported to reduce the viscosity of chronic bronchitic sputum while leaving the elasticity of the sputum unaffected [96, 97]. The specificity of erdosteine for viscosity was particularly surprising since the level of fucose, a mucin marker, and dry weight of the sputum were reported to decline with the treatment. This reduction in mucin content combined with an increase in the water content of the sputum would be expected to effect both viscosity and elasticity. The mechanism by which these sulphydryl compounds exert an effect has also been called into question by studies in rats with cigarette smoke-induced mucus hypersecretion. Two sulphydryl mucolytics, *N*-acetylcysteine (NAC) and *S*-carboxymethylcysteine (SCMC), were found to inhibit the mucus hypersecretion [98], while NAC was further found to be capable of reversing the smoke-induced mucous cell hyperplasia [99, 100].

The mechanism of this indirect effect on the airways is not clear; however, NAC is known to act as a precursor for the antioxidant glutathione, and it is possible that its "anti-bronchitic" effect is associated with protection of the airways from highly reactive oxidative radicals. Alternatively, it has been reported that both NAC and SCMC can stimulate chloride secretion in rabbit and human airway epithelia in a CFTR-dependent manner, and hence these compounds may facilitate hydration and clearance of mucus secretions [60, 101].

4.5. Acute Respiratory Distress Syndrome

Respiratory distress syndrome (RDS) is most commonly associated with premature babies in whom there is insufficient production of pulmonary surfactant, although an adult form of the condition also exists. Mucus plugging of the airways is common in the neonatal form, and while difficulty in obtaining control samples prevents studies comparing mucus from RDS babies with unaffected controls, treatment of RDS with exogenous surfactant is associated with a significant increase in the hydration and mucociliary transportability of the mucus and a decrease in mucus viscoelasticity [102]. Changes in the physical properties, particularly surface properties, induced by surfactant treatment may have a wider applicability than RDS, and further studies in dogs have suggested that surfactant can increase the clearability of mucus, although these changes seemed to be independent of alterations in the physical properties of mucus [103]. More recently, *in vitro* studies have suggested that surfactant can alter the surface properties of CF sputum in a manner conductive to increased mucus clearability but that these improvements in clearability do not extend to chronic bronchitic sputum [104]. Nevertheless, the possibility of using surfactant treatment to improve mucus clearance in several obstructive airways diseases warrants further consideration.

4.6. Fucosidosis

In all the diseases considered previously, poor clearance has been assumed to result from an increase in the rheological properties of the mucus. However, since efficient clearance requires the physical properties to lie within a particular range, it is possible for a disease process to result in mucus which is too thin to be cleared. Such a situation has been suggested in the rare condition fucosidosis, which results from an inherited absence of the enzyme α-L-fucosidase and is associated with recurrent respiratory infections. Fucose is a common terminal sugar on the oligosaccharide side chains of mucins, and hence a disturbance in fucose metabolism could affect the structure of the mucins, with potential consequences for mucus

rheology. This possibility has been studied in one patient, and the tracheal mucus was found to have very low levels of viscoelasticity [2]. However, due to the rare nature of this condition no other studies have been performed, and the occurrence and basis of this mucus abnormality has not been determined.

5. Conclusions

It is clear that the processes involved in the production of a mucus gel have a direct effect on the rheological properties of that gel. The release and hydration of mucins via phase transition and Donnan effects involves complex biophysical processes which are potentially sensitive regulators of mucus viscoelasticity. Hence, any failure in these processes due, for example, to alterations in the volume and composition of the luminal liquid or alterations in the charge and hydration potential of the mucin polymers, may result in mucus gels with rheological properties that fall outside the critical limits required for optimal clearance from the airway. The clinical consequences of such changes in mucus rheology and clearance are apparent in several obstructive airway diseases, including CF, chronic bronchitis and asthma. Although the precise roles of the different physical parameters in determining the clearability of mucus are not fully understood, the use of xenobiotics to manipulate these properties of airway mucus in order to maximise clearance may have some therapeutic benefit. The addition of xenobiotics to mucus may either increase (mucospissic) or decrease (mucolytic) its viscoelasticity. These effects may occur either via direct interaction with the macromolecular architecture of the gel (e.g. DNase) or via interaction with the water phase of the gel (e.g. BP101), effectively altering the hydration state of the gel. However, when considering the development of any novel therapeutic agent to aid mucus clearance, it is necessary to have regard for the impact such a treatment will have on the processes involved in the release and hydration of the mucus.

Acknowledgements

The authors would like to thank the Cystic Fibrosis Trust (UK), the Medical Research Council and the University of Brighton for financial support over the years.

References

1. Girod S, Zahm JM, Plotkowski C, Beck G, Puchelle E (1992) The role of the physiochemical properties of mucus in the protection of the respiratory epithelium. *Eur Respir J* 5: 477–487.
2. Rubin BK, Macleod PM, Sturgess J, King M (1991) Recurrent respiratory infections in a child with fucosidosis: Is the mucus too thin for effective transport? *Pediatr Pulmonol* 10: 304–309.

3. Shih CK, Litt M, Khan MA, Wolf DP (1977) Effect of nondialyzable solids concentration and viscoelasticity on the ciliary transport of tracheal mucus. *Am Rev Respir Dis* 115: 989–995.
4. Lethem MI, James SL, Marriott C (1990) The role of mucous glycoproteins in the rheologic properties of cystic fibrosis sputum. *Am Rev Respir Dis* 142: 1053–1058.
5. Lethem MI, Smedley Y, James SL, Burke J, Marriott C (1987) The contribution of non-mucin components to the increased viscoelasticity of cystic fibrosis sputum. *Pediatr Pulmonol* Suppl 1: 121.
6. Crowther RS, Marriott C, James SL (1984) Cation-induced changes in the rheological properties of purified mucus glycoprotein gels. *Biorheology* 21: 253–263.
7. Marriott C, Kellaway IW (1984) The effect of tetracyclines on the viscoelastic properties of bronchial mucus. *Biorheology* 21: 253–263.
8. Sleigh MA, Blake JR, Liron N (1988) The propulsion of mucus by cilia. *Am Rev Respir Dis* 137: 726–741.
9. Sturgess JM (1982) Morphological characterisations of the bronchial mucosa in cystic fibrosis. In: Quinton PM, Ricardo Martinez J, Hopfer U eds. *Fluid and electrolyte abnormalities in exocrine glands in cystic fibrosis.* San Francisco: San Francisco Press, 254–270.
10. Dearborn DG (1976) Water and electrolytes of exocrine secretions. In: Mangos JA, Talamo RC eds. *Cystic fibrosis: Projections into the future.* New York: Stratton, 179–191.
11. Wolf DP, Sokoloski J, Khan MA, Litt M (1977) Human cervical mucus. III. Isolation and characterisation of rheologically active mucin. *Fertil Steril* 28: 53–58.
12. Tam PY, Verdugo P (1981) Control of mucus hydration as a Donnan equilibrium process. *Nature* 292: 340–342.
13. Lee WI, Verdugo P, Blandau RJ (1977) Molecular arrangement of cervical mucus: A re-evaluation based on laser scattering spectroscopy. *Gynecol Invest* 8: 254–266.
14. Verdugo P, Tam PY, Butler J (1983) Conformational structure of respiratory mucus studied by laser correlation spectroscopy. *Biorheology* 20: 223–230.
15. Verdugo P, Deyrup-Olsen I, Aitken M, Villalon M, Johnson D (1987) Molecular mechanism of mucin secretion. I. The role of intragranular charge shielding. *J Dent Res* 66: 506–508.
16. Verdugo P, Aitken M, Langley L, Villalon MJ (1987 Molecular mechanism of product storage and release in mucin secretion. II. The role of extracellular Ca^{++}. *Biorheology* 24: 625–633.
17. Verdugo P (1984) Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: A new model. *Ciba Found Symp* 109: 212–225.
18. Verdugo P (1994) Molecular biophysics of mucin secretion. In: Takishima T, Shimura S eds. *Airway secretion. Physiological bases for the control of mucous hypersecretion.* New York: Marcel Dekker, 101–117.
19. Verdugo P (1986) Polymer gel phase transition: A novel mechanism of product storage and release in mucin secretion. *Biophys J* 49: 231a.
20. Izutsu K, Johnson D, Schubert M, Wang E, Ramsey B, Tamarin A, Truelove E, Ensign W, Young M (1985) Electron microprobe analysis of human labial gland secretory granules in cystic fibrosis. *J Clin Invest* 75: 1951–1956.
21. Almers W (1990) Exocytosis. *Annu Rev Physiol* 52: 607–624.
22. Monck J, Fernandez JM (1992) The exocytotic fusion pore. *J Cell Biol* 119: 1395–1404.
23. Forstner JF, Forstner GG (1975) Calcium binding to intestinal goblet cell mucin. *Biochim Biophys Acta* 386: 283–292.
24. Crowther RS, Marriott C (1983) Counter-ion binding to mucus glycoproteins. *J Pharm Pharmacol* 36: L21–26.
25. Tanaka T (1981) Gels. *Scientific American* 244: 124–138.
26. Vedugo P (1991) Mucin exocytosis. *Am Rev Respir Dis* 144: S33–S37.
27. Fernandez JM, Villalon M, Verdugo P (1991) Reversible condensation of mast cell secretory products *in vitro*. *Biophys J* 59: 1022–1027.
28. Donnan FG (1924) The theory of membrane equilibria. *Chem Rev* 1: 73–90.
29. Phillips G (1994) PhD Thesis. The hydration of mucus in cystic fibrosis. Brighton: University of Brighton.

30. Cheng PW, Boat TF, Cranfill K, Yankaskas JR, Boucher RC (1989) Increased sulfation of glycoconjugates by cultured nasal epithelial cells from patients with cystic fibrosis. *J Clin Invest* 84: 68–72.
31. Potter JL, Matthews LW, Spector S, Lemm J (1967) Studies on pulmonary secretions. II. Osmolality and the ionic environment of pulmonary secretions from patients with cystic fibrosis, bronchiectasis and laryngectomy. *Am Rev Respir Dis* 96: 83–87.
32. App EM, King M, Helfesreider R, Kohler D, Matthys H (1990) Acute and long-term amiloride inhalation in cystic fibrosis lung disease. *Am Rev Respir Dis* 141: 605–612.
33. Knowles MR, Church NL, Waltner WE, Yankaskas JR, Gilligan P, King M, Edwards LJ, Helms RW and Boucher RC (1990) A pilot study of aerosolised amiloride for the treatment of lung disease in cystic fibrosis. *N Engl J Med* 322: 1189–1194.
34. Bowler IM, Kelman B, Worthington D, Littlewood JM, Watson A, Conway SP, Smye SW, James SL and Sheldon TA (1995) Nebulised amiloride in respiratory exacerbations of cystic fibrosis – a randomised controlled trial. *Arch Dis Child* 73: 427–430.
35. Graham A, Hasani A, Alton EWFW, Martin GP, Marriott C, Hodson ME, Clarke SW and Geddes DM (1993) No added benefit from nebulised amiloride in patients with cystic fibrosis. *Eur Respir J* 6: 1243–1248.
36. Barasch J, Kiss B, Prince A, Saiman L, Gruenert D, Al-Awquati Q (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature* 352: 70–73.
37. Aitken ML, Verdugo P (1989) Donnan mechanism of mucin release and conditioning in goblet cells: The role of polyions. In: Chantler EN ed. *Mucus and related topics.* New York: Plenum Press, 73–80.
38. Widdicombe JG (1989) Airway mucus. *Eur Respir J* 2: 107–115.
39. Grant D, Long WF, Williamson FB (1990) The dependence on counter-cation of the degree of hydration of heparin. *Biochem Soc Trans* 18: 1283–1284.
40. Silberberg A (1989) Mucus glycoprotein, its biophysical and gel-forming properties. In: Chantler E, Ratcliffe NA eds. *Mucus and related topics.* Cambridge: Company of Biologists, 43–63.
41. King M, Rubin BK (1994) Rheology of airway mucus. In: Takashima T, Shimura S eds. *Airway secretion: Physiological bases for the control of mucous hypersecretion.* New York: Marcel Dekker, 283–314.
42. Vincent JFV (1990) *Structural biomaterials*, 2d ed. Princeton: Princeton University Press.
43. Lutz RJ, Litt M, Chakrin LW (1973) Physicochemical factors in mucus rheology. In: Gabelnick HL, Litt M eds. *Rheology of biological systems.* Springfield, II: Charles C. Thomas, 119.
44. King M, Macklem PT (1977) Rheological properties of microlitre quantities of normal mucus. *J Appl Physiol* 42: 797–802.
45. Braga PC, Allegra L, Dalloglio G, Angelini M, Mocchi A (1992) A new rheometer with special features designed for bronchial mucusanalysis in clinical practice. *Biorheology* 29: 285–293.
46. James SL, Marriott C (1981) A modified oscillating sphere magnetic microrheometer for use with biological secretions. *J Phys* (E) 15: 179–180.
47. Keal E, Reid L (1970) Méthodes d'étude des modifications de la sécrétion bronchique et de la viscosité. *Poumon Coeur* 26: 52–58.
48. Charman J, Reid L (1972) Sputum viscosity in chronic bronchitis, bronchiectasis, asthma and cystic fibrosis. *Biorheology* 9: 185–199.
49. Saga M, Hawada H, Nakamura RM, Darajan V, Allerton S (1979) Biorheology of human cervical mucus – interaction of purified cervical mucin with cationic charged polyelectrolytes. *St Marianna Med J* 7: 146–150.
50. Shake MP, Dresdner R, Gruenauer LM, Yates D, Irving MF (1987) A direct measuring capillary viscoelastometer for mucus. *Biorheology* 24: 231–235.
51. Agarwal M, King M, Shukla AB (1994) Mucus gel transport in a simulated cough machine – effects of longitudinal grooves representing spacings between arrays of cilia. *Biorheology* 31: 11–19.
52. Tomiewicz RP, Biviji A, King M (1994) Effects of oscillating air-flow on the rheological properties and clearability of mucus gel simulants. *Biorheology* 31: 511–520.
53. Silveira P, Bohm G, Yang H, Wen CL, Gurmaraes E, Parada M, King M and Saldiva PH (1992) Computer assisted rheological evaluation of microsamples of mucus. *Comp Meth Prog Biomed* 39: 51–60.

54. Seifritz W (1924) An elastic value of protoplasm with further observations on the viscosity of protoplasm. *Brit J Exp Biol* 2: 1.
55. Edwards PA, Yeates D (1992) Magnetic rheometry of bronchial mucus. *ACS Symposium Series* 489: 249–267.
56. Ferry JD (1970) Viscoelastic properties of polymers. New York: Wiley.
57. Majima Y, Hirata K, Takeuchi K, Hattori M, Sakakura Y (1990) Effects of orally administered drugs on dynamic viscoelasticity of human nasal mucus. *Am Rev Respir Dis* 141: 79–83.
58. Daugherty AL, Patapoff TW, Clark RC, Sinicropi DV, Mrsny RJ (1995 Compaction assay: A rapid and simple *in vitro* method to assess the responsiveness of a biopolymer matrix to enzymic modification. *Biomaterials* 16: 553–558.
59. King K, Gilboa A, Meyer F, Silberberg A (1974) On the transport of mucus and its rheological simulants on ciliated systems. *Am Rev Respir Dis* 110: 740–745.
60. Kottgen M, Busch AE, Hug MJ, Greger R, Kunzelmann K (1996) *N*-acetyl-L-cysteine and its derivatives activate a Cl^- conductance in epithelial cells. *Pflug Arch Eur J Physiol* 431: 549–555.
61. Martin GP, Marriott C, Kellaway IW (1978) Direct effect of bile salts and phospholipids on the physical properties of mucus. *Gut* 19: 103–107.
62. Heer JS, Roberts CJ, Tendler SJB, Davies MC, Martin GP, Marriott C (1995) The effect of bile salts on the primary structure of mucins. *J Pharm Pharmac* 47: 1125.
63. Chantler E, Sharma R, Sharman D (1989) Changes in cervical mucus that prevent penetration by spermatozoa. In: Chantler E, Ratcliffe NA eds. *Mucus and related topics.* Cambridge: The Company of Biologists, 325–336.
64. Marriott C (1989) Drug-mucus actions and interactions. In: Chantler E, Ratcliffe NA eds. *Mucus and related topics.* Cambridge: the Company of Biologists, 163–177.
65. Paget T, James SL (1994) The mucolytic effect of polyamines and mucosal invasion. *Biochem Soc Trans* 22: 394.
66. Marriott C, James SL, Ibrahim T, Baker J (1994) A mucus thickener. British Patent 8712948.
67. Sellers LA, Allen A, Morris E, Ross-Murphy S (1991) Rheology of pig small intestinal and colonic mucus – weakening of gel structure by non-mucin components. *Biochim Biophys Acta* 1115: 174–179.
68. Gelman RA, Meyer FA (1979) Mucociliary transference rate and mucus viscoelasticity: Dependence on dynamic storage and loss modules. *Am Rev Resp Dis* 120: 553–557.
69. Lorenzi G, Bohm GM, Guimaraes ET, Vaz MAC, King M, Saldiva PHN (1992) Correlation between rheologic propertis and *in vitro* ciliary transport of rat nasal mucus. *Biorheology* 29: 433–440.
70. Lin SY, Amidon GL, Weiner ND, Goldberg AH (1993) Viscoelasticity of anionic polymers and their mucociliary transport on the frog palate. *Pharmac Res* 10: 411–417.
71. Yu DM, Amidon GL, Weiner ND, Fleisher D, Goldberg AH (1994) The role of rheological properties in mucociliary transport by the frog palate ciliated model. *Pharmac Res* 11: 1785–1791.
72. Macchione M, King M, Lorenzi G, Guimaraes ET, Zin WA, Bohm GM, Saldiva PHN (1995) Rheological determinants of mucociliary transport in the nose of the rat. *Respir Physiol* 99: 165–172.
73. Zahm JM, King M, Duvivier C, Pierrot D, Girod S, Puchelle E (1991) Role of simulated repetetive coughing in mucus clearance. *Eur Respir J* 4: 311–315.
74. Agarwal M, King M, Rubin BK, Shukla JB (1989) Mucus transport in a miniaturized simulated cough machine: Effect of constriction and serous layer simulant. *Biorheology* 26: 977–988.
75. Girod S, Galabert C, Pierrot, D, Boissonade MM, Zahm JM, Baszkin A, Puchelle E (1992) Role of phospholipid lining on respiratory mucus clearance by cough. *J Appl Physiol* 71: 2262–2266.
76. King M, Zahm JM, Pierrot D, Vaquez-Girod S, Puchelle E (1989) The role of mucus gel viscosity, spinability and adhesive propertis in clearance by simulated cough. *Biorheology* 26: 737–745.
77. Kim CS, Abraham WM, Chapman GA, Sackner MA (1985) Influence of two-phase gas-liquid interaction on aerosol deposition in airways. *Am Rev Respir Dis* 131: 618–623.

78. Evrensel CA, Khan MRU, Elli S, Krumpe PE (1993) Viscous air-flow through a rigid tube with a compliant lining – a simple model for the air – mucus interaction in pulmonary airways. *J Biomech Eng – Trans ASME* 115: 262–270.
79. Basser PJ, McMahon TA, Griffith P (1989) The mechanism of mucus clearance by cough. *J Biomech Eng – Trans ASME* 111: 288–297.
80. Lopez-Vidriero MT, Reid L (1978) Chemical markers of mucous and serum glycoproteins and their relation to viscosity in mucoid and purulent sputum from various hypersecretory diseases. *Am Rev Respir Dis* 117: 465–477.
81. Tomkiewicz RP, App EM, Zayas JG, Ramirez O, Church N, Boucher RC, Knowles MR and King M (1993) Amiloride inhalation therapy in cystic fibrosis: Influence on ion content, hydration and rheology of sputum. *Am Rev Respir Dis* 148: 1002–1007.
82. Bennett WD, Olivier KN, Zeman KL, Hohneker KW, Boucher RC, Knowles MR (1996) Effect of uridine 5′-triphosphate plus amiloride on mucociliary clearance in adult cystic fibrosis. *Am J Respir Crit Care Med* 153: 1796–1801.
83. Marriott C, Beeson MF, Brown DT (1982) Biopolymer-induced changes in mucus viscoelasticity. In: Chantler EN, Elder JB, Elstein M eds. *Mucus in health and disease,* vol 2. New York: Plenum Press, 89–92.
84. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL (1990) Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc Natl Acad Sci USA* 87: 9188–9192.
85. Zahm JM, Debentzmann SG, Deneuville E, Perrotminnot C, Dabadie A, Pennaforte F, Roussey M, Shak S and Puchelle E (1995) Dose-dependent *in vitro* effect of recombinant human DNase on the rheological and transport properties of cystic fibrosis respiratory mucus. *Eur Respir J* 8: 381–386.
86. Shah PL, Scott SV, Knoght RA, Marriott C, Ranasinha C, Hodson ME (1996) *In vivo* effects of recombinant human DNase-1 on sputum in patients with cystic fibrosis. *Thorax* 51: 119–125.
87. Shiels CA, Kas J, Travassos W, Allen PG, Jamney PA, Wohl ME, Stossel TP (1996) Actin-filaments mediate DNA fibre formation in chronic inflammatory airway disease. *Am J Pathol* 148: 919–927.
88. Vasconcellos CA, Allen PG, Wohl ME, Drazen JM, Jamney PA, Stossel TP (1994) Reduction in viscosity of cystic fibrosis sputum *in vitro* by gelsolin. *Science* 263: 969–971.
89. Mrsny RJ, Lazazzera BA, Daugherty AL, Schiller NL, Patapoff TW (1994) Addition of a bacterial alginate lyase to purulent CF sputum *in vitro* can result in the disruption of alginate and modification of sputum viscoelasticity. *Pulmonary Pharmacol* 7: 357–366.
90. Sheehan JK, Richardson PS, Fung DCK, Howard M, Thornton DJ (1995) Analysis of respiratory mucus glycoproteins in asthma: A detailed study from a patient who died in *status asthmaticus*. *Am J Respir Cell Mol Biol* 13: 748–756.
91. Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, Boushey HA (1993) Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am Rev Resp Dis* 147: 1132–1137.
92. Fahy JV, Liu J, Wong H, Boushey HA (1993) Cellular and biochemical analysis of induced sputum from asthmatic and healthy subjects. *Am Rev Respir Dis* 147: 1126–1131.
93. Picot R, Das I, Reid L (1978) Pus, deoxyribonucleic acid and sputum viscosity. *Thorax* 33: 235–242.
94. Puchelle E, Zahm JM, Debentzmann, S, Grosskopf C, Shak S, Mougel D, Polu JM (1996) Effects of rhDNase on purulent airway secretions in chronic bronchitis. *Eur Respir J* 9: 765–769.
95. Aikawa T, Shimura S, Sasaki H, Takishima T, Yaegashi H, Takahashi T (1989) Morphometric analysis of intraluminal mucus in airways in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 140: 477–482.
96. Braga PC, Allegra L, Rampoldi C, Ornaghi A, Beghi G (1990) Long-lasting effects on rheology and clearance of bronchial mucus after short-term administration of high doses of carbocysteine-lysine to patients with chronic bronchitis. *Respiration* 57: 353–358.
97. Marchioni CF, Moretti M, Muratori M, Casadei MC, Guerzoni P, Scuri R, Fregnan GB (1990) Effects of erdostein on sputum biochemical and rheologic properties: Pharmacokinetics in chronic obstructive lung disease. *Lung* 168: 285–293.

98. Rogers DF, Turner NC, Marriott C, Jeffery PK (1989) Oral *N*-acetylcysteine or *S*-carboxymethylcysteine inhibits cigarette smoke-induced hypersecretion of mucus in rat larynx and trachea *in situ*. *Eur Respir J* 2: 955–960.
99. Rogers DF, Jeffery PK (1986) Inhibition by oral *N*-acetylcysteine of cigarette smoke-induced bronchitis in the rat. *Exp Lung Res* 10: 267–283.
100. Rogers DF, Godfrey RW, Majumdar S, Jeffery PK (1988) Oral *N*-acetylcysteine speeds reversal of cigarette smoke-induced mucous cell hyperplasia in the rat. *Exp Lung Res* 14: 19–35.
101. Colombo B, Turconi P, Daffonchio L, Fedele G, Omini C, Cremaschi D (1994) Stimulation of Cl^- secretion by the mucoactive drug *S*-carboxymethylcysteine-lysine salt in the isolated rabbit trachea. *Eur Respir J* 7: 1622–1628.
102. Rubin BK, Ramirez O, King M (1992) Mucus rheology and transport in neonatal distress syndrome and the effect of surfactant therapy. *Chest* 101: 1080–1085.
103. DeSanctis GT, Tomkiewicz RP, Rubin BK, Schurch S, King M (1994) Exogenous surfactant enhances mucociliary clearance in the anesthetized dog. *Eur Respir J* 7: 1616–1621.
104. Rubin BK (1996) Therapeutic aerosols and airway secretions. *J Aerosol Med* 9: 123–130.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 7
Goblet Cells: Physiology and Pharmacology

C. William Davis

Departments of Physiology and Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

1. Introduction

In healthy individuals, mucus plays a major role in lung defense by ensnaring the particulates and pathogens contained in the >8500 l of air inspired each day. These mucus–particulate complexes are propelled by incessantly beating cilia to the glottis over the luminal surface of epithelium lining the airways, and are cleared from the airways by swallowing. The principal component of mucus is the high molecular weight glycoconjugate (HMWG) mucin, which is secreted by goblet cells in the superficial epithelium and by mucous cells in the submucosal glands. In the obstructive pulmonary diseases (OPD, including asthma, and the *chronic* obstructive pulmonary diseases [COPD] cystic fibrosis [CF], chronic bronchitis and emphysema; see ref. [1] for a concise and informative review of lung diseases), both the mucociliary clearance and the respiratory functions of the lung may be compromised by hyperproduction of mucus which restricts

airflow and/or plugs the airways [2]. The various diseases in the OPD family have different causes and are characterized by different inflammatory conditions, but mucus hyperproduction, which presents a major challenge to the patient and the attending physician, is a principal characteristic of them all.

Submucosal gland hypertrophy is a prominent feature of diseased airways and, historically, submucosal glands have been a major focus in COPD research. In 1960, Reid proposed a simple morphometric for the degree of gland hypertrophy (the thickness of the glandular tissue region relative to wall thickness [3]), and the very ease of making this measurement has tended to emphasize the role of submucosal glands in both healthy and diseased airways.

The role of goblet cells in mucin production, however, is presently receiving more attention. First, submucosal glands are associated only with the large, cartilaginous airways, but the small, noncartilaginous airways (≤ 2 mm) are the paramount site of COPD, because, being flow-limited, these small airways cannot be cleared by coughing and consequently are the site of mucus-plugging [4, 5]. Since goblet cells are the principal source of mucin in the small airways, they are critically important in OPD. Second, goblet cells in the large airways may produce more mucin than the submucosal glands. Applying new morphometric methods to the airways, Plopper and associates have determined the relative *volume* of secretory material (total glycoconjugate) per unit surface area of airways basal lamina [6, 7]. Rather than simply determining relative cell numbers, this morphometric method determines the aggregate amount of secretory material contained within the two mucin-secreting cell types. When this method was applied to the cartilaginous airways of rhesus monkey, the amount of mucin in goblet cells was found to be three or more times greater than that stored in submucosal glands (Figure 1). While such measurements have yet to be made in human lungs, the studies in nonhuman primates are important because they suggest that goblet cells in the surface epithelium of the lung may be more important than realized previously in the physiology of the airways, both large and small.

At a time when appreciation of the role of goblet cells in the airways is heightening, our knowledge of their differentiation and regulation has suffered from the lack, until recently, of suitable experimental models. The goal of this chapter is to report on the remarkable progress that has been made in just the past few years toward understanding the regulation of mucin secretion by this rather elusive cell type.

2. Goblet Cell Morphology, Development and Distribution

In tissues that are chemically fixed for examination by light and electron microscopy, goblet cells frequently appear to be distended luminally

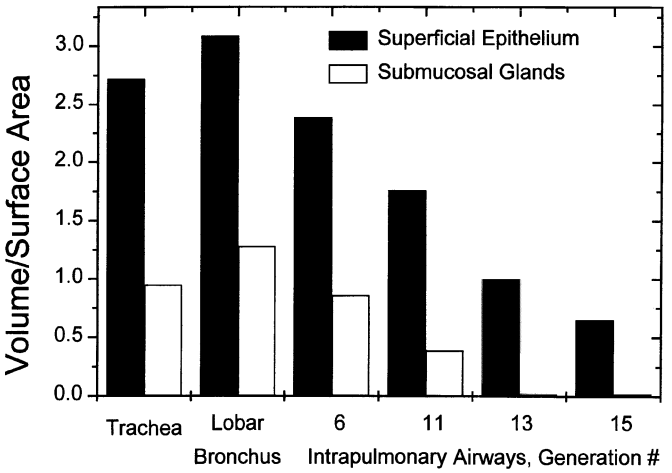


Figure 1. Aggregate cellular volume of stainable secretions in the superficial epithelium and submucosal glands of rhesus monkey airways. Morphometric techniques were used to quantify the total volume of Alcian Blue/PAS stained material in cells from the regions of the airways indicated. The data are expressed as the ratio of the volume of stained material ($mm^3 \times 10^{-3}$) per unit surface area of basal lamina (mm^2). After Plopper et al. [7], drawn from recalculated data.

(hence, the name) and to contain mucin secretory granules, many of which seem to have fused with neighboring granules. The appearance of "goblet cells" in the light microscope, however, may be an artifact of the fixation procedure employed. In nasal and gastrointestinal tract tissues prepared by rapid freezing followed by freeze-substitution fixation, goblet cells are columnar in form, and the mucin secretory granules are distinctly separated from their neighbors [8, 9]. Apparently, during chemical fixation mucins may hydrate, causing granules to swell and coalesce, possibly distorting the cell. Regardless of the type of fixation, mucin secretory granules fill most of the goblet cell, with the nucleus, numerous mitochondria and rough endoplasmic reticulum being restricted to a small volume in the basal aspect of the cell (e.g. see ref. [10]).

The development of the superficial epithelium in the airways is complicated and incompletely understood (see ref. 11). In the human fetus, airway epithelial cells are clearly undifferentiated through the first 11 weeks of gestation, following which, first, ciliated and, then, secretory cells appear [12]. This same progression was observed for the rhesus monkey; additionally, basal cells appeared after the differentiation of ciliated and secretory cells [13]. The numbers of secretory and ciliated cells progressively increase over the next 7–8 weeks in the human fetus, until at week 19 ciliated cells represent about 50% and goblet cells about 30% of all columnar cells. During weeks 20–23, ciliated cells increase in number of the expense of goblet cells until 80% of columnar cells are ciliated cells and 10–15%

are goblet cells – secretory cells were observed in the process of ciliogenesis during this period [12]. In an interesting experimental model utilizing denuded tracheal grafts implanted into syngeneic rat hosts to test the developmental potential of cells inoculated within [14, 15], populations of dissociated rat tracheal epithelial cells both depleted of, or highly enriched in, basal cells gave rise to epithelia similarly comprised of ciliated, goblet and basal cells. Thus, the developmental inter-relationships of epithelial cells in the airways are complicated, and the cells observed in the adult lung under control conditions may arise through multiple pathways. Goblet cells, specifically, appear to arise from an intermediate secretory cell [11, 16, 17].

Inflammation affects the number of goblet cells in airway epithelia. In healthy adult humans and most other larger adult mammals, goblet cells represent a minority (5–25%) of the columnar cells in the superficial epithelium of the cartilaginous trachea and bronchi, and the noncartilaginous bronchioles [18–21]. In healthy, distal human bronchioles, those with diameters ≤ 2 mm, goblet cells are rare; however, their numbers increase dramatically under conditions of inflammation [5, 22–28]. In laboratory rodents, goblet cells are rare in the cartilaginous airways of healthy individuals, but they may be increased under a variety of experimentally induced inflammatory conditions, apparently as a result of both hyper- and metaplastic processes [29–34]. An interesting observation in this regard concerns rat tracheal epithelial cells inoculated into denuded tracheas, which were incubated as xenografts in the backs of syngeneic rats. The tracheal xenografts developed epithelia containing numerous goblet cells, whereas the trachea of the host animal contained very few to none [15, 35]. Apparently, local inflammatory signals directed goblet cell differentiation in the developing xenograft epithelium. The role of inflammation in the induction of goblet cell hyper/metaplasia has been indicated by the inhibition of the process with dexamethasone, a corticosteroid [36].

3. Experimental Models

It has been difficult to establish rigorous experimental protocols for the study of the regulation of mucin secretion in airways. A major limitation of early studies, which employed intact tissues from larger mammals (e.g. canine trachea), was the inability to distinguish between the mucins secreted by submucosal glands and goblet cells, a problem appreciated by Florey as early as 1932 [37]. Studies of lung innervation and receptor distribution have demonstrated fundamental differences in the supply of neural elements to the submucosal glands versus the superficial epithelium, over and above differences between species (for reviews, see refs. [2, 39]). Thus, the results of histochemical investigations have generally shown that submucosal glands are richly innervated by the parasympathetic, sym-

pathetic and nonadrenergic noncholinergic nervous (NANC) systems, but have left unanswered how much control the nervous system exercises over goblet cells in the superficial epithelium. An initial histochemical indication of possible differences between submucosal gland cells and superficial epithelial goblet cells in the control of mucin secretion was the observation that adrenergic- and vasoactive intestinal peptide (VIP)-induced increases in cAMP occurred in the glands, but not in goblet cells [40, 41]. As a result of these and other differences between the control of submucosal glands and the superficial epithelium (see ref. [37]), it became imperative to develop experimental models which would separate the two sources of mucin so their regulation could be studied independently. Several cell culture techniques, which make this possible, have now been developed.

3.1. Primary Cell Cultures

Primary cell cultures from airway epithelia were developed as early as 1980 using culture media supplemented with fetal bovine serum [42]. The first serum-free culture system to support goblet cell differentiation, described by Wu and colleagues in 1985 [43], has given rise to the four airway primary cell culture systems in which goblet cell differentiation occurs, for which the secreted HMWG have been characterized biochemically as mucins and mucin-detection systems have been developed, and with which most of the work on the regulation of goblet cell-specific mucin secretion has been performed. These systems employ epithelial cells derived by protease digestion from the airways of hamster [43–47], guinea pig [48, 49], rat [50, 51] and cat [52]. Primary cultures of human airway epithelial cells have also been developed [53, 54], but have yet to be used extensively in studies of agonist regulation of mucin secretion. A presumption of all these culture techniques, one which has not been confirmed histologically, is that cells for primary culture are removed selectively from the superficial epithelium by enzymatic digestion, and that cells from submucosal glands are excluded.

3.2. Epithelial Explants

Our laboratory has developed an alternative culture system in which the superficial epithelium (containing goblet cells) is removed as intact sheets from cartilaginous airways following enzymatic digestion of the submucosal connective tissue. Small, $< 1\ cm^2$, pieces of the epithelium are explanted onto a nitrocellulose substratum to which the epithelial cells attach during an overnight incubation [55]. Goblet cells in these epithelial explants are easily recognizable by video microscopy and conveniently studied over a 1–3-day period following explantation. Explants have been obtained successfully from canine [55] and ovine trachea (see Figure 3),

as well as human turbinates [56]. This epithelial explant system has the advantage over primary cultures of allowing the study of "native" goblet cells, in the sense that they differentiate under the conditions of a normal host environment.

3.3. Passaged Primary Cell Cultures

Recently, the laboratories of Roussel and Nettesheim described cell culture systems in which cells from primary cultures of airway epithelial cells are

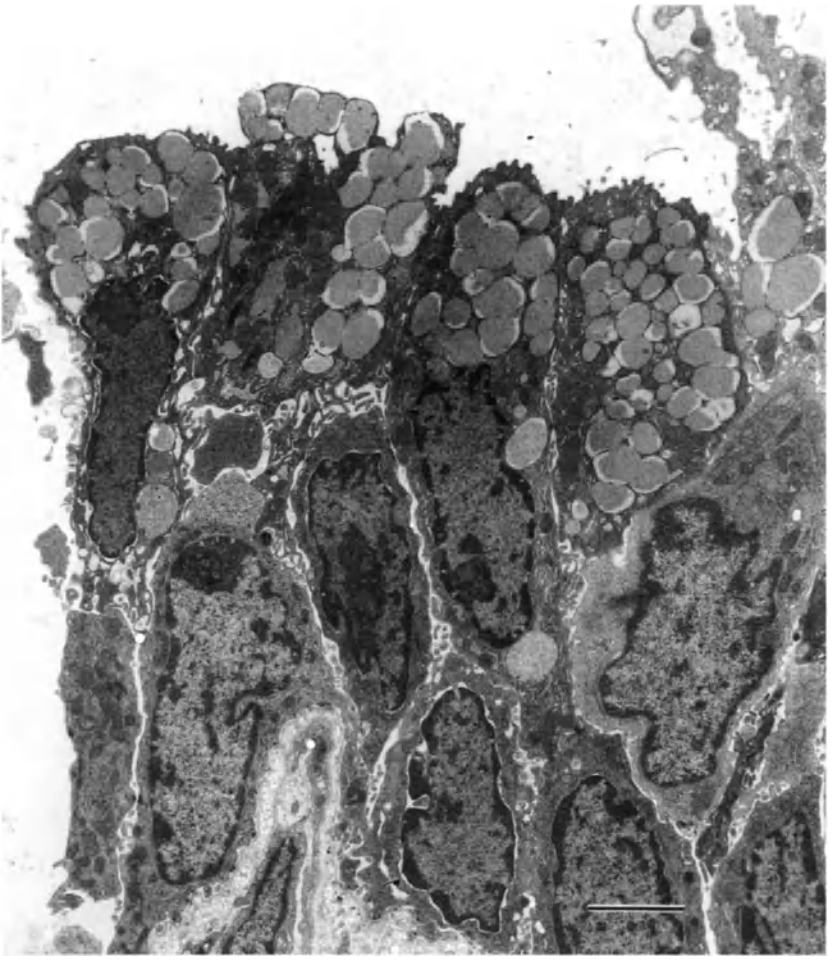


Figure 2. Electron micrograph of SPOC1 cells grown in a tracheal xenograft for 21 days. The specimen was stained with uranyl acetate-lead citrate. Bar = 2.5 μm. Published with permission from Randell et al. [59], and the *American Journal of Respiratory Cell and Molecular Biology.*

expanded in number by passaging and in which cells from passage 2 or later are then cultured under conditions in which they differentiate into ciliated and goblet cells [57, 58]. Since these culture systems have the distinct advantages of allowing the freezing and expansion of human airway epithelial cells, we may expect them to play an active role in our continued progress toward an understanding of airway cell and molecular biology, physiology and pharmacology. A limitation of passaged primary cell cultures, however, is that cell phenotypes may change from passage to passage. Gray *et al.* [58], for instance, found that the ability of the cells to differentiate into epithelia possessing a normal morphology and physiology declined with passage number. To acknowledge this limitation, these systems are occasionally termed "secondary cell cultures".

3.4. Cell Lines

To date, a single mucin-secreting cell line (SPOC1) has been developed from the epithelial cells of the superficial airway epithelium. The SPOC1 cell resulted from the spontaneous immortalization of passaged primary cell cultures of rat tracheal epithelial cells. When grown in tracheal xenografts, SPOC1 cells differentiate into a pseudostratified, columnar epithelium in which all of the columnar cells are goblet cells (Figure 2; [59, 60]). Characteristics of SPOC1 cells include minor alterations in chromosomes 1, 3 and 6, reduced requirements for growth factors, and in the fully-differentiated cells, a lack of keratin-18, which characterizes columnar epithelial cells in the airways. Nonetheless, SPOC1 cells in mature cultures produce and secrete an HMWG which has been identified as a mucin both biochemically [61] and by reaction with a mucin-specific monoclonal antibody [59]. Interestingly, older cultures of SPOC1 cells in tracheal xenografts or in culture on Matrigel, exhibit gland-like invaginations into the submucosal tissue or gel support [59, 60].

4. Mucin Gene Expression, Synthesis and Secretion

The biochemical and biophysical complexity of mucin, more than any other single factor, has impeded the development of our knowledge of goblet cell biology, physiology and pharmacology. Rather than being a homogenous, easily identifiable compound, mucins comprise a heterogeneous family of possibly unrelated, high molecular weight apoproteins hidden within dense, bristle-like coats of highly variable, heterogeneous carbohydrate. Those mucins usually found in airway mucus have molecular weights generally $>10^7$ Da. Produced by extensive end-to-end disulfide linkages of mucin monomers, they exist as linear polymers longer than 10 μm. Typically, mucins have a total Ser+Thr content on the order of

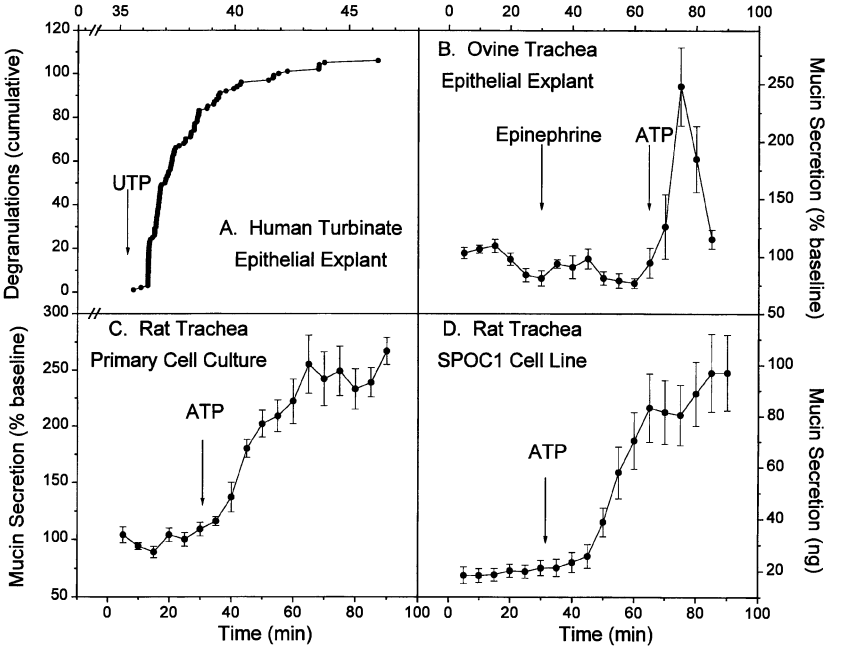


Figure 3. Time course of the mucin secretory response following purinergic stimulation. In each panel, the arrows indicate the time of addition of agonist to the luminal perfusion (except panel B, which was bilateral). *Panel A.* Response of an individual goblet cell in an epithelial explant removed from a human turbinate and monitored by DIC video microscopy, showing the time course of exocytosis – each point represents a single degranulation (C.W. Davis, unpublished data). *Panel B.* Mucin secretion from an epithelial explant isolated from an ovine trachea (K. Zhao and M.I. Lethem, unpublished data). *Panels C and D.* Mucin secretion from primary (*C*) and SPOC1 (*D*) cell cultures derived from rat tracheal epithelial cells (redrawn from Abdullah et al. [61]). In panels B and C, the data are presented as the mean ± SE, n = 6 cultures, and mucins were detected by enzyme-linked lectin assay (ELLA) using appropriate mucin-specific lectins.

≥25%, with these residues serving as O-glycosylation sites. The attached carbohydrate side chains account for 80–90% of the total weight of the protein. It is the combination of long polymeric strands and high carbohydrate content that gives rise to the unique biophysical properties of the mucus scaffold (for reviews of mucin biochemistry and biophysics, see refs. [62–69]).

4.1. Mucin Gene Expression in the Airways

As expected from the nature of the mucin protein, mucin genes are large and complex, and have proven resistant to analysis. Nearly everything we

know about these genes has come from efforts in the present decade, beginning with the cloning and sequencing of *MUC1*, a mucin which proved to be tethered to the apical plasma membrane [70, 71]. To date, *MUC1* is the only known membrane-associated mucin; the other known genes encode secretory mucins (for reviews, see refs. [69, 72, 73]). In both the superficial epithelium and submucosal glands, mRNAs for the secretory mucins *MUC2, 4, 5AC* and *5B* have been detected [73–76]. At the protein level, however, state-of-the-art separation methods and mucin-specific antibody probes recently showed that only *MUC5AC* and an unidentified species were the major mucins expressed in normal tracheobronchial mucus, whereas *MUC1, 2, 3* and *4* mucins were not detected [77]. The differences observed between the mucins identified at the mRNA and protein levels may reflect the higher sensitivities of the techniques for detecting mRNA, or post-translational regulation of protein expression. Studies such as these demonstrate that our understanding of the spatial and temporal patterns of mucin gene expression has advanced significantly over that past few years, but at present this understanding is still rudimentary. Nonetheless, it is encouraging that techniques relevant to the molecular biology and protein chemistry of mucins are now yielding provocative results.

4.2. Mucin Synthesis and Secretion

Synthesis of airway mucins has not been studied extensively due, primarily, to the lack of necessary immunochemical reagents. Available data indicate that mucins are synthesized in a manner similar to other exported proteins, with *N*-linked glycosylation occurring in the endoplasmic reticulum and *O*-linked glycosylation occurring in the Golgi apparatus as mucins progress through the secretory pathway (for review, see ref. [78]).

Mucin-secreting cells appear to exhibit every known mode of secretion. The slime glands of the hagfish, for instance, secrete mucin by holocrine secretion [79]. Goblet cells in the foot of the slug, *Ariolimax* [80, 81], and in the frog palate [82] exhibit apocrine secretion, in which individual membrane-bound mucin granules are released intact from the cell. Apocrine secretion has also been proposed recently for mucous cells in airway submucosal glands[1] [83]. Goblet cells in the mammalian intestinal tract feature compound exocytosis in which an apical pit develops following cell activation as mucin granules situated deeper in the cell fuse with the

[1] Potential apocrine secretion of submucosal gland mucins was deduced from the visualization by differential interference contrast (DIC) video microscopy of granule-like particles within gland lumina and their ejection to the airway surface through ciliated gland ducts [83]. The notion that these particles are mucin secretory granules has great appeal, since the airway, rather than the gland lumen, may be a more favorable site for mucin gel hydration.

membranes of adjacent, more proximal granules. Scanning electron micrographs indicate a stream of mucin emanating from such cells [84].

Observation of airway goblet cells are consistent with merocrine secretion. Using DIC video microscopy, our laboratory has observed goblet cell exocytosis in epithelial explants of canine trachea and human nasal epithelium [55, 56]. When we focused on the equators of the apical-most layer of granules, exocytosis was observed as the sudden disappearance of granules; shortly thereafter, new granules could be observed to migrate into the layer to fill the vacancy. Because we used a rapid perfusion system in these experiments, it was generally not possible to view the secreted material on the surface of the cells before the perfusion stream swept it away. Occasionally, however, a bolus of mucin could be observed to cling to the surface of the cell and to grow rapidly over a period of several seconds before being swept away. Similarly, using phase contrast microscopy, Verdugo observed boluses of mucin to appear spontaneously on the apical surface of cultured airway goblet cells from single exocytotic events, and to subsequently swell as the mucins hydrated [85].

Exocytosis involves the protein-assisted fusion of the secretory granule membrane and the apical plasma membrane, followed by expulsion of the granule contents to the extracellular domain (see refs. [86, 87]). Early notions of membrane fusion included the possibility that osmotic forces provided the driving force [88]. The advent of whole-cell patch-clamp capacitance measurements, in conjunction with video microscopy, however, provided the observation that swelling of the giant granules of beige mouse mast cells follows, rather than precedes, granule fusion [89, 90]. While the driving force behind granule fusion in exocytosis has not been explicitly demonstrated, existing data are consistent with a model of a fusion pore fabricated from a protein scaffolding which first bridges the two lipid bilayer membranes and then supports a fluidized lipid pore [87, 91]. This pore is reversible [92], but generally widens upon fusion to allow passage of granule contents.

By direct DIC video-microscopic observations of the ~1-μm mucin granules in canine tracheal goblet cells, we determined that the exocytotic process is completed in about 100 ms [55]. During this brief period, within the spatial and temporal limits of real-time video microscopy, no swelling of mucin granules was observed. In contrast, mast-cell secretory granules swell significantly following the opening of the exocytotic fusion pore [89, 90]. The granules of mast cells, chromaffin cells and neurosecretory cells contain proteoglycan matrices which apparently assist the condensation of histamine, catecholamines or peptides in these secretory cells, respectively (e.g. see refs. [93–95]). In chromaffin cells, direct measurements with carbon-fiber electrodes have revealed the diffusion of catecholamines from the granule interior to the extracellular domain immediately following the initial opening of the exocytotic fusion pore [96]. In mast cells, a similar dissipative release of histamine from granules through the exocytotic

fusion pore is indicated by its dose-dependent reversal by exogenous histamine application [97]. Isolated granule matrices of Beige mouse mast cells exhibit a reversible increase in size as histamine diffuses out of and Na^+ into the matrix, in a manner suggesting that the size of the matrix is determined by its ion-exchange properties [98]. More recent observations have supplemented this concept with the notion that the state of the matrix is dynamically altered, as the electrochemical potential between the outside and inside of the matrix changes with time (see ref. [91]).

In many secretory cells, an intragranule proteoglycan matrix aids condensation by providing closely-spaced, negative binding sites for positively-charged secretory materials. By virtue of their molecular size and numerous fixed, negative charges, mucins share many physical properties with these granule proteoglycans. Thus, the condensation of mucins most likely requires small cations to shield the negative electrostatic charges of their carbohydrate side chains. Such a charge-shielding role has been advocated for Ca^{2+} by Verdugo [62, 99], and several studies have shown that Ca^{2+} is elevated in goblet cell secretory granules in a variety of tissues and species [81, 100, 101]. Verdugo has studied the hydration of individual mucin boluses as they spontaneously appeared upon the apical surface of cultured epithelial cell cultures [85, 99]. The hydration process followed first-order kinetics, had a half-time on the order of seconds and showed the properties expected for those of a gel [62, 85, 99]. Additionally, Ca^{2+} was shown to affect the hydration (size) of the mucin boluses. Raising Ca^{2+} from 1 to 4 mM decreased the rate of bolus swelling by more than an order of magnitude, and at 6 mM Ca^{2+} swelling was arrested. Similarly, use of albumin as an inhibitory polyion diminished the hydration of mucin boluses in a dose-dependent and highly sensitive manner. Mucin bolus hydration was inhibited over 90% by 0.8 µM albumin [102]. In another model using bovine cervical mucin, Verdugo showed that hydration of the gel was dependent upon both the salt concentration and the pH [103, 104]. Thus, *after secretion*, mucin exhibits the properties of a polyionic gel in a Donnan equilibrium with its aqueous environment [62–64]; it is similar in this regard to the proteoglycan comprising the mast-cell granule matrix [97, 98]. It is important to note that these effects of Ca^{2+} and albumin on mucin gel hydration may be of more than academic interest, for these agents affect the overall physical properties of mucin and could have effects that are important clinically.

It has also been proposed that Ca^{2+} plays a role in the extrusion of mucin from the granule during exocytosis (see ref. [62]). Verdugo has observed mucin granules, secreted intact by apocrine secretion from *Ariolimax* goblet cells, to suddenly burst and the mucins within to undergo nearly explosive hydration. With the Ca^{2+} indicator arsenazo III in the bath, a Ca^{2+} halo was observed to appear around the granule ~100 ms prior to its bursting, suggesting a Ca^{2+} efflux. The mucins within the granule subsequently expanded their volume > 25-fold in 66 ms [62, 81]. Notably, this

rate of expansion was at least one order of magnitude faster than that observed for the hydration of mucin boluses described above. Perhaps, as Ca^{2+} diffused outward across the granule membrane, the repulsive forces between static charges within the condensed mucin gel increased to the point where the limiting membrane fractured, releasing the gel to undergo an explosive hydration. While the forces and time scales involved in the bursting of *Ariolimax* mucin granules might be involved in the extrusion of mucin from airway goblet cells during exocytosis, it is also possible that the *Ariolimax* phenomenon is specific to apocrine secretion, being necessary for bursting the granules to release the mucins held within. For the extrusion of granule contents during merocrine exocytosis, an alternative driving force might be the release of tension from the limiting membrane during the sphere-to-planar transformation the granule membrane undergoes in the process. This possibility was initially suggested for mast-cell granules after finding that granule swelling due to matrix hydration was some 20 times slower than the rate of fusion pore dilation during exocytosis [91, 105, 106].

5. Regulation of Mucin Secretion

Early histological studies established the importance of mucin-secreting cells as the primary source of mucus, but progress in understanding the regulation of mucin secretion has been hindered by the complex morphology and inaccessibility of the airways. Our knowledge base, however, is expanding rapidly now that suitable experimental models have become available (see Section 3 of this chapter).

5.1. Agonist Regulation

As secretory proteins are synthesized and processed along the secretory pathway, they are packaged into vesicles destined either for relatively immediate *constitutive* secretion, or for longer-term storage and *regulated* secretion. *Regulated secretion*, as discussed in this section, refers to the exocytosis of secretory granules in response to an extracellular agonist[2].

Few secretagogues have been demonstrated definitively to elicit mucin secretion from goblet cells in the superficial epithelium of the airways. In particular, there is little or no direct evidence that regulation of goblet cell function involves the participation of any of the major neural and

[2] Constitutive secretion is commonly stated to be unregulated, i.e. it is an activity which occurs in the absence of agonist. At the molecular level, however, constitutive secretion is likely to be highly regulated (e.g. see ref. 177).

inflammatory mediators that are known to be active in regulating pulmonary function, including acetylcholine, epinephrine, tachykinins, bradykinin, histamine etc. Although evidence exists that these factors may participate in the regulation of goblet cell function, the respective studies were conducted with tissue explants or intact animals where either the source of mucins measured not could be determined accurately or the reported effects on goblet cells in the superficial epithelium may have been indirect. Thus, this treatment is restricted to studies which used cultures of superficial epithelial cells, because in these studies the source of mucin is known and the possibility of indirect effects is minimal.

5.1.1. Purinergic regulation: In the epithelium of the lung, adenosine triphosphate (ATP) was first appreciated for an extracellular action in the alveolus, where it was shown to stimulate the secretion of surfactant by type II cells [107]. Subsequently, ATP and uridine 5′-triphosphate (UTP) were shown to elicit an increase in Cl^- secretion, in tissue and cultures derived from both normal patients and those with cystic fibrosis [108, 109]. ATP and UTP were equipotent in their effects on both ion transport [109] and inositol phosphate production in primary cultures of airway cells [110], suggesting the possibility of a novel purinoceptor. Similar results were reported for a purinoceptor in other cells, and the P_{2U} purinoceptor receptor was finally cloned by Julius from mouse neuroblastoma cells [111]. The human homolog of the receptor was cloned from an airway cell library and sequenced by Parr [112]. The P_{2U} purinoceptor has also been termed the P_{2N} and the P_{2Y2} purinoceptor [113, 114]. The latter and most recently proposed name results from assembly of all G protein-coupled, triphosphate nucleotide purinoceptors into one category, P_{2Yn}, where n is a number assigned to specific receptors as the genes are cloned. Because this categorization groups receptors which are not closely related, we have chosen to retain the original terminology. Consult Dubyak [115] and Harden [114] for recent reviews on P_2 receptors, and especially the former for an excellent discussion on the likely sources of nucleotides.

In terms of goblet cell regulation, ATP was initially shown by Kim to stimulate mucin secretion in primary cultures of hamster tracheal epithelial cells [116]. At the same time, our laboratory, working with the native goblet cells of epithelial explants from canine trachea, discovered that mucin granule exocytosis was stimulated by luminal applications of ATP and the nonhydrolyzable analog adenylyl imidodiphosphate (AMP-PNP). Adenosine diphosphate and adenosine, products of ATP hydrolysis, were without effect [55]. These findings were important in showing that it was extracellular ATP, and not its breakdown products, which interacted with a presumptive goblet cell purinoceptor. Subsequently, we showed that the P_{2U} purinoceptor agonists ATP and UTP stimulated exocytosis in goblet cells in epithelial explants from human nasal turbinates (Figure 2), but 2-methlythio-ATP, a hallmark agonist for P_{2Y} purinoceptors, did not [56].

Recent studies have also demonstrated purinergic stimulation of mucin secretion (see Figure 2) in passaged human bronchial primary cell cultures [58], the SPOC1 cell line and rat tracheal primary cell cultures [61], and in epithelial explants from ovine trachea. Other mucin-secreting cells that have been shown to respond to purinergic agonists are airway submucosal gland mucous cells [117], gastric mucous cells [118] and the colonic cell line HT29-Cl.16E [119].

The substantial evidence that ATP and, in at least some goblet cells, UTP elicit mucin secretion suggests that the purinoceptor mediating this response is a P_{2U} subtype, and recent, more rigorous pharmacologic studies support the involvement of a P_{2U} purinoceptor in the regulation of goblet cell mucin secretion. Dose–response studies showed that the purinergic agonists ATP and ATPγS are equipotent in activating mucin secretion with hamster trachea epithelial cells [116]. Similarly, in SPOC1 cells ATP, UTP and ATPγS are equipotent, as are ATP and UTP in rat tracheal primary cell cultures ([61]; apparent affinities were approximately 4 μM). Additionally, SPOC1 cells failed to respond to the P_{2Y} purinoceptor hallmark agonist, 2-methlythio-ATP, or the P_1 agonist, adenosine [61]. Thus both the selectivity of the mucin secretory response by hamster and rat tracheal primary cell cultures and SPOC1 cell cultures, and the apparent affinity for purinergic agonstis, are consistent with the presence of a P_{2U} purinoceptor in the apical membrane of goblet cells. Finally, the mRNA for the P_{2U} purinoceptor was identified by reverse transcriptase polymerase chain reaction (RT-PCR) in SPOC1 cells, consistent with pharmacologic data [61].

Being epithelial cells, goblet cells are polarized, i.e. their plasma membrane is divided into apical and basolateral domains separated by tight junctions (see ref. [120]). As a result, the distribution of receptors between these two domains of the plasma membrane may have important consequences in goblet cell physiology and pharmacology. An indication of this possibility is the asymmetric distribution of purinoceptors observed in canine goblet cells [55] and SPOC1 cells [61]. In both cases, selective perfusion of the luminal aspect of the cells with a P_{2U} purinoceptor agonist elicited a mucin secretory response, and no other purinergic agonist was effective.

The two cell types differed, however, in their response to perfusion of their submucosal (= serosal) aspects. In SPOC1 cell cultures, selective submucosal perfusion of the cultures with ATP, ATPγS, UTP, 2-methlythio-ATP or adenosine elicited no secretory response [61]. In canine epithelial explants, however, perfusion of the submucosal aspect with ATP, AMP-PNP, ADP and adenosine did elicit mucin secretion [55]. The pattern of responses to the submucosal challenge was highly variable from cell-to-cell, and some cells did not respond. There was no apparent difference from control in the mucin secretory response to submucosal ATP when hexokinase and glucose were present luminally to protect against indirect effects mediated by the apical membrane P_{2U} purinoceptor. Thus, the results of the submucosal purinergic challenges in epithelial explants are

complicated and are difficult to explain without postulating multiple receptors in the goblet cell basolateral membrane. Indirect effects are a likely hazard in studies with purinergic agents, given the large number of active nucleotides, the presence of ectonucleotidases and ectophosphatases on target tissues which rapidly hydrolyze nucleotides, and the growing numbers and apparent diversity of purinergic receptors [114, 115]. Thus, understanding the differences observed in secretory responses to submucosal purinergic challenges between native goblet cells and SPOC1 cells will require a considerable experimental effort to complete.

5.1.2. Inflammatory mediators and reactive oxygen species: The > 8500 l of air inspired each day contains various pollutants, the amounts of which may be considerable, depending upon locality. Some of these pollutants, such as ozone, nitrogen dioxide, cigarette smoke and automobile exhaust, contain oxidants which can interact with and severely damage the epithelial cells lining the airways. During inspiration, these pollutants interact first with the surface liquid layer that overlies the superficial epithelium. Cross *et al.* have proposed that some degree of detoxification occurs within this layer when the oxidants interact with mucin and other antioxidants [121], but numerous ozonation products and reactive oxygen species are produced, and these compounds may activate arachidonic acid metabolism and related lipid mediator pathways (see refs. [122–124]). Many of the compounds arising through theses pathways are also active under the conditions of inflammation in OPD (see refs. 125, 126).

Most of the work on the effects of these lipid mediators on mucin secretion from goblet cells has focused on platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine). Interestingly, PAF is frequently formed as the result of the action of phospholipase A_2 to liberate arachidonic acid from phosphotidylcholine. Hence, a single enzymatic reaction produces two powerful lipid mediators, PAF and arachidonic acid, and both PAF and the eicosanoid metabolites of arachidonic acid have wide-ranging effects in intact airways (see refs. [126, 126]) – including the hypersecretion of mucin [127–129]. The cellular mechanisms underlying these effects, however, are not well understood. In primary cell cultures of guinea pig and feline tracheal epithelial cells, PAF elicits mucin secretion [52, 130]. In both cases, PAF stimulated a dose-dependent increase in mucin release which was inhibited both by PAF receptor antagonists and nordihydro–guiaretic acid (NDGA), an inhibitor of both cyclo-oxygenase and lipoxygenase activities. In guinea pig cell cultures, PAF induced the production of HETEs. When added to cultures, mixtures of 5-, 12- and 15-HETEs stimulated mucin release, although, curiously, each HETE applied independently was without effect [130]. Lastly, Adler recently found that mucin release from primary cultures of guinea pig tracheae epithelial cells elicited by PAF, tumor necrosis factor-α or reactive oxygen species was inhibited by a competitive inhibitor of nitric oxide (NO) synthase [131]. Similarly,

both mucin secretion and guanosine 3′, 5′-monophosphate cGMP levels were stimulated by an NO donor in gastric mucosal cells, and both nitroprusside and dibutyryl-cGMP stimulated mucin secretion [132]. These studies thereby introduce the notion that NO regulates mucin secretion in goblet cells, or may mediate the effects of many inflammatory factors.

We have found in SPOC1 cells that PAF had no effect on mucin secretion at doses from 0.001 to 10 μM; subsequent challenges with 100 μM ATP in each case elicited a normal secretory response. Additionally, saturating doses of ATP, ionomycin, and phorbol-12-myristate-13-acetate (PMA) had no effect on the release of radiolabeled-arachidonic acid from SPOC1 cells, and no significant arachidonic acid metabolites were identifiable (M.C. Madden and C.W. Davis, unpublished observations). These results are interesting in light of the observations that PAF stimulates mucin release from isolated submucosal glands *only* in the presence of platelets, a response that is blocked by PAF receptor antagonists and inhibitors of thromboxane production [133]. Thus in both primary cell cultures and isolated submucosal glands, PAF exerts its primary effects on eicosanoid metabolism. Given the lack of response of isolated submucosal glands and SPOC1 cells to PAF, the direct effects of PAF apparently are not on mucin-secreting cells, but on one or more non-mucin-secreting cell types within the system. Clearly, we do not have sufficient information regarding identities, sources and interactions of inflammatory mediators to understand precisely their role in the regulation of mucin secretion in airways inflammation.

5.1.3. Other secretagogues: As noted above, there is little definitive evidence for the direct regulation of goblet cell function by the major neural and inflammatory mediators that are active in regulating pulmonary function. Two possibilities are that goblet cells are not regulated by these various factors, or that the effects attributed to a particular factor are in fact secondary, rather than primary, in nature. In support of this notion, note in Figure 3 B that epinephrine has no effect on epithelial explants from ovine trachea, whereas it does stimulate mucin release from submucosal glands (see ref. 134). Similarly, our laboratory found that several major agonists in the lung have no effect on mucin secretion in bilateral exposures to SPOC1 cells at doses which elicit maximal responses from their known receptor systems. The agonists tested were bethanechol (muscarinic receptors), isoproterenol (β-adrenergic receptors), histamine, and adenosine. In each case, the SPOC1 cells responded positively when challenged subsequently with ATP. These provocative results support the notion that goblet cells are not regulated directly by the nervous system.

That is not to say the nervous system does not influence goblet cells. Electrical stimulation of the vagus nerve, for instance, causes the loss of mucin granules from goblet cells in rats and guinea pigs [135–137], and there is a large body of evidence for the effects of the NANC nervous

system on mucin secretion in the lung (see refs. [2, 138–140]), including effects specific to goblet cells [141]. The primary problems hindering a clear understanding of the neural involvement in goblet cell function are: (1) a complex lung morphology; (2) complicated interactions involving, potentially, many different chemical mediators acting in multiple levels and on different cell types; and (3) differences between species. The species differences in lung innervation may be especially marked between humans and rodents [142, 143], and Rogers has proposed that goblet cells in species lacking extensive submucosal glands are under neural control, whereas they are independent in species with extensive gland systems [39]. Clearly, these are issues that need to be addressed for our understanding of goblet cell function to be solidified, and it is promising that the necessary studies may now be forthcoming given the robust *in vitro* and *in vivo* models for human and rodent airway epithelial cells now available.

5.2. Signal Transduction Mechanisms

5.2.1. Phospholipase C pathways: P_{2U} purinoceptors link through G proteins to phospholipase C (PLC) to activate the production of the intercellular messenger molecules, diacylglycerol (DAG) and inositol phosphates, and through the later, the mobilization of intracellular Ca^{2+} (Ca_i^{2+}; for reviews see refs. [114, 115, 144]). Harden and colleagues [110] showed that ATP and UTP elicited the production of inositol phosphates in primary cultures of airway epithelial cells, and Kim found similar responses in hamster trachea primary cell cultures containing goblet cells as the predominant columnar cell type [145]. Thus, purinergic activation of goblet cells most likely results in stimulation of PLC, with the subsequent production of inositol triphosphate (IP_3) and diacylglycerol (DAG) followed by the IP_3-induced mobilization of Ca_i^{2+}.

A mobilization of Ca_i^{2+} is probably involved in agonist-induced mucin secretion by airway goblet cells. Although no studies of agonist-induced changes in Ca_i^{2+} in airway goblet cells have been done, in gastric mucous cells, P_2 purinoceptor activation was accompanied by an increase in Ca_i^{2+} [118]. Using the ionophore ionomycin, our laboratory has recently shown in SPOC1 cells that mobilization of Ca_i^{2+} stimulates mucin secretion in a dose-dependent fashion, with a $K_{0.5}$ of 5 μM and a maximal response at 7.5 μM [146]. Mucin secretion in SPOC1 cells was also elicited by thapsigargin, an agent which effects Ca_i^{2+} mobilization by inhibiting the Ca^{2+} pump in the endoplasmic reticulum (see ref. [147]). Gastrointestinal goblet cells, in experiments using similar maneuvers, were also stimulated to secrete mucin when Ca_i^{2+} was increased (see ref. [148]). Thus, a role of intracellular Ca^{2+} in mucin secretion by airway goblet cells would be consistent with order secretory cells, including other mucin-secreting cells.

There is also evidence for the participation of protein kinase C (PKC, see refs. [149, 150]) in the regulation of goblet cell mucin secretion. Most of this evidence has been obtained using a DAG mimic, the phorbol ester PMA, to test the potential effects of PKC activation in eliciting secretion. In mucin-secreting cells, PMA elicits secretion in primary cell cultures from hamster and feline tracheas [151, 152], SPOC1 cells [146], airway submucosal glands [153], submandibular glands [154] and the colonic cell lines T84 [155, 156], HT29-18N2 [157] and LS174T [158]. The PKC family of isoforms, however, is numerous and diverse, with 12 known isoforms having been identified and assigned to three subfamilies (see refs. [149, 159, 160]). PKCα, β_1, β_2 and γ-comprise the *conventional*, or PMA-activated, Ca^{2+}-dependent (cPKC) subfamily. PKCδ, ε, η, θ and μ comprise the *novel*, PMA-activated, Ca^{2+}-independent (nPKC) subfamily. Lastly, PKCι, λ, and ζ comprise the *atypical*, PMA-independent, Ca^{2+}-independent (aPKC) subfamily, a unique group of isoforms possibly regulated by inositol lipids (see ref. [161]). Consequently, PMA treatment may force a full activation of all cPKC and nPKC isoforms in a particular cell type, and the observed effects may be inconsistent with the roles of the selective PKC isoforms in agonist-induced cellular responses. For instance, PMA activates both cPKCα and the nPKCδ and ε isoforms in cardiac myocytes, whereas agonist activation of these cells involves the selective participation of the nPKCs [160, 162]. We observed PMA to activate mucin secretion in a dose-dependent fashion ($K_{0.5} \cong 100$ nM) in SPOC1 cells; importantly, measured PKC activity in the membrane fraction of these cells was increased by both PMA (112%) and UTP (42%), thereby suggesting a physiologic role for PKC in P_{2U} purinoceptor-activated mucin secretion [146].

The actions of the Ca_i^{2+} and PKC pathways to effect exocytotic secretion appear to be independent in mucin-secreting cells. The effects of the Ca^{2+} ionophore A23187 and PMA are fully additive on mucin secretion in the colonic T84 cell line, and A23187 is effective in PKC-downregulated cells [155, 156]. Full additivity was also observed in SPOC1 cells for ionomycin and PMA (Figure 4), and downregulation of PKC by long-term exposure to a half-maximal dose of PMA had no effect on the subsequent ability of the cells to respond to ionomycin, even though the responses to maximal doses of UTP and PMA were abolished [146]. In the latter experiment, Ca^{2+} appeared to activate mucin granule exocytosis in the absence of PKC. Thus, PKC and Ca_i^{2+} appear to elicit mucin secretion independently of each other, but the exact relationships between these pathways during agonist stimulation remains to be determined.

The specific proteins activated by Ca^{+2} and PKC and responsible for effecting exocytosis in secretory cells are poorly known, especially in mucin-secreting cells. In other secretory cells, the targets of Ca^{+2}- and PKC-dependent activities which have received the most attention experimentally are actin cortical microfilaments: breakdown of these microfilaments generally precedes agonist-induced exocytosis (see refs. [163, 164]),

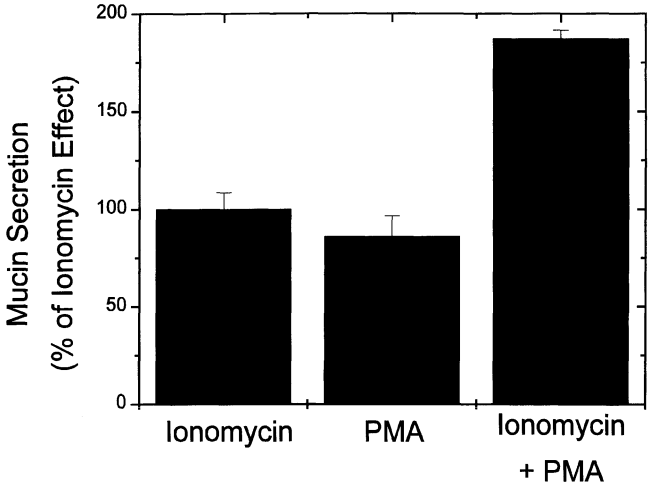


Figure 4. Effects of ionomycin (7.5 μm) and PMA (300 nM), applied separately or together, on mucin secretion by SPOC1 cells. Mucin secretion, over baseline, is expressed as a percentage of that elicited by ionomycin. Note that the two agents applied together are fully additive over the effects elicited when applied alone (redrawn from Abdullah et al. [146]).

and the experimental disruption or stabilization of microfilaments may stimulate or retard exocytosis, respectively (see ref. 165). In chromaffin cells, breakdown of the cortical cytoskeleton prior to exocytosis appears to result from the activity of a microfilament-severing enzyme, scinderin [166], and both Ca^{+2} and PMA cause a redistribution of scinderin from the cytosol to the cytoskeleton [167]. The only evidence for participation of the cytoskeleton in mucin secretion was the recent finding with the colonic LS180 cell line that microfilament disruption with cytochalasin D caused a release of stored mucin but had no effect on baseline mucin release, whereas disruption on microtubules with nocodazole stimulated the basal release of mucin but did not affect agonist-induced mucin secretion [168].

5.2.2. Cyclic nucleotides: Although cyclic adenosine monophosphate (cAMP) is a cellular mediator for regulated mucin secretion in salivary glands, stomach, gallbladder, small intestine and colon of the gastrointestinal tract (see ref. [148]), and submucosal glands of the airway (see ref. [134]), it does not appear to be a mediator of regulated mucin secretion in goblet cells of the airways superficial epithelium. For example, histochemical evidence exists for cAMP production during adrenergic and VIP regulation of the submucosal glands, but in the same studies no cAMP was detected in superficial epithelial goblet cells [40, 169]. Similarly, SPOC1 cells were recently shown to generate cAMP when stimulated by forskolin,

but neither forskolin nor cpt-cAMP stimulated mucin secretion [146]. Thus, cAMP does not appear to mediate regulated mucin secretion in airway goblet cells, and they may be unique in this regard amongst mucin-secreting cells.

Very little attention has been focused on the potential role of cGMP as a cellular messenger involved in regulated mucin secretion. This may change, however, given recent evidence implicating NO in the regulation of mucin release. As noted above (Section 5.1.2), NO was implicated in the stimulation of mucin secretion from airway primary cell cultures [131] and gastric mucosal cells [132], and NO is generally thought to activate soluble guanylate cyclase to produce cGMP (see ref. 170). In SPOC1 cells, however, 8-Br-cGMP was ineffectual in stimulating mucin release [146]. Thus, either goblet cells in primary cultures of guinea pig tracheal epithelial cells differ from SPOC1 cells with regard to cGMP effects, or the apparent action of NO to effect mucin release in the airways [131] is mediated by a novel, non-cGMP-based mechanism.

6. Mucin Secretion and Disease

The hyperproduction of mucin characterizes obstructive pulmonary diseases. It results from increases in the numbers of goblet and mucous cells by hyper- and metaplastic processes (see Section 2), and from increased release of mucin from those cells. Little is known regarding the molecular basis of either of these effects. Because CF has a known genetic defect, more work has been done on the problem of mucin hyperproduction in this disease than in the other obstructive diseases. The results of these efforts, however, are incomplete and somewhat inconsistent.

Submandibular glands [171] of CF patients were less responsive to β-adrenergic stimulation in terms of amylase and mucin release than from control, and CF bronchi [172] were similarly less responsive in terms of mucin release to cholinergic, β-adrenergic and peptidergic (substance P) stimuli than control, although the basal rates of secretion in both cases were similar. Cultured submucosal gland cells exhibited higher basal rates of SLPI, lysozyme and lactoferrin secretion that were insensitive to acetylcholine and norepinephrine [173]. In epithelial explants from CF and normal human turbinates, the basal rate of goblet cell exocytosis and the response to purinergic agonists were similar [56]. Last, CF gallbladder cells in passaged primary cell culture when transduced with a cystic fibrosis transmembrane regulator (CFTR) retroviral vector exhibited higher basal mucin secretory rates than did control cultures [174]. Thus, from these five studies the basal rate of secretion (mucin or otherwise) of CF cells or tissues has been reported to be the same as or higher than normal, whereas the agonist-stimulated rate of secretion from CF cells or tissues has been reported as being the same as or lower than normal.

Three possibilities might explain the variable results reported in these studies. Two of the potential difficulties are simply procedural. Namely, the cellular source of secretions was not always clearly identified, and in some studies there was no attempt to account for different number of secretory cells in the CF versus control tissues, making it difficult to distinguish differences in secretory rates from differences in cell numbers. The third and probably the most important potential difficulty with the reported studies on secretions in CF is biological in origin, namely, the effects of CFTR on secretory cells could be primary in CFTR-expressing cells and secondary in CFTR non-expressing cells. Thus, a critical question regarding mucin secretion in the *airways* may be whether goblet and mucous cells express CFTR; the weight of the available evidence suggests that either they do not express CFTR, or that they express very small amounts. *In situ* hybridization studies found CFTR to be expressed at high levels in submucosal gland serous cells, but a low levels in submucosal gland mucous cells and throughout the superficial epithelium [175]. Primary cultures of submucosal gland cells that exhibited a mixed seromucous cell phenotype expressed high levels of CFTR, whereas those that exhibited a mucous cell phenotype expressed negligible amounts [176]. Finally, SPOC1 cells express essentially undetectable amounts of CFTR mRNA and protein, but do express abundant amounts of a related ABC transporter, MDR1 [146]. Thus, CFTR may not be directly involved in mucin secretion by either airway goblet or mucous cells, making a direct causal relationship between CFTR and mucin hyperproduction unlikely. Mucin hyperproduction in CF therefore may be caused instead by complications secondary to the molecular defect in CFTR, perhaps the inflammatory processes associated with the disease.

7. Conclusions

During the past few years, the development of systems suitable for separating the different cellular components in the airways has spurred tremendous progress in the study of goblet cell mucin secretion. Even so, our understanding of mucin synthesis and secretion, and their regulation in the goblet cell, is woefully incomplete. I hope, however, that this discussion serves to consolidate the existing body of information relevant to goblet cells, and to stimulate the investigations necessary to complete the story.

Acknowledgements

My sincere appreciation goes to Drs. Michael Lethem and Kaicun Zhao, and Drs. Steven Ballard and Sarah Inglis, for sharing new or unpublished data used in this review. I also thank Drs. Scott Randell and Paul Nettesheim for introducing us to the SPOC1 cell line and for permission to use the electron micrograph depicted in Figure 3. Lastly, I am most grateful to Drs. Randell and Pedro Verdugo for taking the time to critically read the manuscript.

References

1. Welton AF, O'Donnell M, Morgan DW (1987) The physiology and biochemistry of normal and diseased lung. *Adv Clin Chem* 26: 293–383.
2. Lundgren JD (1992) Mucus production in the lower airways: A review of experimental studies. *Dan Med Bull* 39: 289–303.
3. Reid L (1960) Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. *Thorax* 15: 132–141.
4. Macklem PT, Thurlbeck WM, Fraser RG (1971) Chronic obstructive disease of small airways. *Ann Int Med* 74: 157–177.
5. Hogg JC, Macklem PT, Thurlbeck WM (1968) Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 278: 1355–1360.
6. Heidsiek JG, Hyde DM, Plopper CG, St. George JA (1987) Quantitative histochemistry of mucosubstance in tracheal epithelium of the macaque monkey. *J Histochem Cytochem* 35: 435–442.
7. Plopper CG, Heidsiek, JG, Weir AJ, George JA, Hyde DM (1989) Tracheobronchial epithelium in the adult rhesus monkey: A quantitative histochemical and ultrastructural study. *Am J Anat* 184: 31–40.
8. Yoshihara T, Kanda T, Yaku Y, Nagata H, Kaneko T Tatsuoka H (1986) An ultrastructural study of the nasal mucosa by rapid freezing and freeze-substitution. *Acta Oto-Larnygol* 101: 96–101.
9. Ichikawa M, Ichikawa A, Kidokoro S (1987) Secretory process of mucus-secreting cells in mouse colonic mucosa studied by rapid freezing and freeze-substitution. *J Elect Microsc* 36: 117–127.
10. Jeffery PK, Gaillard D, Moret S (1992) Human airway secretory cells during development and in mature airway epithelium. *Eur Respir J* 5: 93–104.
11. Randell SH (1992) Progenitor-progeny relationships in airway epithelium. *Chest* 101 (Suppl): 11S–16S.
12. Gaillard DA, Lallement AV, Petit AF, Puchelle ES (1989) *In vivo* ciliogenesis in human fetal tracheal epithelium. *Am J Anat* 185: 415–428.
13. Plopper CG, Alley JL, Weir AJ (1986) Differentiation of tracheal epithelium during fetal lung maturation in the rhesus monkey *Macaca mulatta. Am J Anat* 175: 59–71.
14. Randell SH, Comment CE, Ramaekers FC, Nettesheim I (1991) Properties of rat tracheal epithelial cells separated based on expression of cell surface alpha-galactosyl end groups. *Am J Respir Cell Mol Biol* 4: 544–554.
15. Shimizu T, Nettesheim P, Ramaekers FC, Randell SH (1992) Expression of "cell-type-specific" markers during rat tracheal epithelial regeneration. *Am J Respir Cell Mol Biol* 7: 30–41.
16. Randell SH, Shimizu T, Bakewell W, Ramaekers FC, Nettesheim P (1993) Phenotypic marker expression during fetal and neonatal differentiation of rat tracheal epithelial. *Am J Respir Cell Mol Biol* 8: 546–555.
17. Ayers MM, Jeffery PK (1988) Proliferation and differentiation in mammalian airway epithelium. *Eur Respir J* 1: 58–80.
18. Plopper CG, Mariassy AT, Wilson DW, Alley JL, Nishio SJ, Nettesheim P (1983) Comparison of nonciliated tracheal epithelial cells in six mammalian species: Ultrastructure and population densities. *Exp Lung Res* 5: 281–294.
19. Harkema JR, Mariassy A, St. George JA, Hyde DM, Plopper CG (1991) Epithelial cells of the conducting airways. In: Farmer SG, Hay DW eds. *The airway epithelium: Physiology, pathophysiology and pharmacology.* New York, Marcel Dekker, 3–39.
20. Mariassy AT (1992) Epithelial cells of trachea and bronchi. In: Parent RA ed. *Comparative biology of the normal lung.* Boca Raton: CRC Press, 63–76.
21. Plopper CG, Hyde DM (1992) Epithelial cells of bronchioles. In: Parent RA ed. *Comparative Biology of the normal lung.* Boca Raton: CRC Press, 85–92.
22. Lumsden A, McLean A, Lamb D (1984) Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax* 39: 844–849.
23. Jeffery PK (1992) Histological features of the airways in asthma and COPD. *Respiration* 59 Suppl 1: 13–16.

24. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T (1992) Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 101: 916–921.
25. Matsuba K, Thurlbeck WM (1973) Disease of the small airways in chronic bronchitis. *Am Rev Respir Dis* 107: 552–558.
26. Thurlbeck WM, Malaka D, Murphy K (1975) Goblet cells in the peripheral airways in chronic bronchitis. *Am Rev Respir Dis* 112: 65–69.
27. Kung TT, Jones H, Adams GK 3rd, Umland SP, Kreutner W, Egan RW, Chapman RW, Watnick AS (1994) Characterization of a murine model of allergic pulmonary inflammation. *Int Arch Allergy Immunol* 105: 83–90.
28. Jeffery PK (1994) Comparative morphology of the airways in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 150: Pt2: S6–13.
29. Huang HT, Haskell A, McDonald DM (1989) Changes in epithelial secretory cells and potentiation of neurogenic inflammation in the trachea of rats with respiratory tract infections. *Anat Embryol* 180: 325–341.
30. Breuer R, Christensen TG, Lucey EC, Stone PJ, Snider GL (1985) Quantitative study of secretory cell metaplasia induced by human neutrophil elastase in the large bronchi of hamsters. *J Lab Clin Med* 105: 635–640.
31. Cardozo C, Padilla ML, Choi HS, Lesser M (1992) Goblet cell hyperplasia in large intrapulmonary airways after intratracheal injection of cathepsin B into hamsters. *Am Rev Respir Dis* 145: 675–679.
32. Breuer R, Christensen TG, Lucey EC, Bolbochan G, Stone PJ, Snider GL (1993) Elastase causes secretory discharge in bronchi of hamsters with elastase-induced secretory cell metaplasia. *Exp Lung Res* 19: 273–282.
33. Steiger D, Hotchkiss J, Bajaj L, Harkema J, Basbaum C (1995) Concurrent increases in the storage and release of mucin-like molecules by rat airway epithelial cells in response to bacterial endotoxin. *Am J Respir Cell Mol Biol* 12: 307–314.
34. Breuer R, Christensen TG, Wax Y, Bolbochan G, Lucey EC, Stone PJ, Snider GL (1993) Relationship of secretory granule content and proliferative intensity in the secretory compartment of the hamster bronchial epithelium. *Am J Respir Cell Mol Biol* 8: 480–485.
35. Shimizu T, Nettesheim P, Eddy EM, Randell SH (1992) Monoclonal antibody (Mab) markers for subpopulations of rat tracheal epithelial (RTE) cells. *Exp Lunga Res* 18: 323–342.
36. Lundgren JD, Kaliner M, Logun C, Shelhamer JH (1988) Dexamethasone reduces rat tracheal goblet cell hyperplasia produced by human neutrophil products. *Exp Lung Res* 14: 853–863.
37. Florey H, Carleton HM, Wells AQ (1932) Mucus secretion in the trachea. *Brit J Exp Pathol* 13: 269–284.
38. Marin MG (1994) Update: Pharmacology of airway secretion. *Pharmacol Rev* 46: 35–65.
39. Rogers DV (1994) Airway goblet cells: Responsive and adaptable front-line defenders. *Eur Respir J* 7: 1690–1706.
40. Lazarus SC, Basbaum CB, Gold WM (1984) Localization of cAMP in dog and cat trachea: Effects of beta-adrenergic agonists. *Am J Physiol* 247 Pt1: C327–334.
41. Lazarus SC, Basbaum CB, Barnes PJ, Gold WM (1986) cAMP immunocytochemistry provides evidence for functional VIP receptors in trachea. *Am J Physiol* 251: C115–C119.
42. Goldman WE, Baseman JB (1980 Glycoprotein secretion by cultured hamster trachea epithelial cells: A model system for *in vitro* studies of mucus synthesis. *In Vitro Cell Dev Biol* 16: 320–329.
43. Wu R, Nolan E, Turner C (1985) Expression of tracheal differentiated functions in serum-free hormone-supplemented medium. *J Cell Physiol* 125: 167–181.
44. Kim KC, Rearick JI, Nettesheim P, Jetten AM (1985) Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture. *J Biol Chem* 260: 4021–4027.
45. Niles R, Kim KC, Hyman B, Christensen T, Wasano K, Brody J (1988) Characterization of extended primary and secondary cultures of hamster tracheal epithelial cells. *In Vitro Cell Dev Biol* 24: 457–463.
46. Wu R, Plopper CG, Cheng PW (1991) Mucin-like glycoprotein secreted by cultured hamster tracheal epithelial cells: Biochemical and immunological characterization. *Biochem J* 277: 713–718.

47. Kim KC, Zheng QX, Brody JS (1993) Effect of floating a gel matrix on mucin release in cultured airway epithelial cells. *J Cell Physiol* 156: 480–486.
48. Adler KB, Cheng PW, Kim KC (1990) Characterization of guinea pig tracheal epithelial cells maintained in biphasic organotypic culture: Cellular composition and biochemical analysis of released glycoconjugates. *Am J Respir Cell Mol Biol* 2: 145–154.
49. Li C, Cheng PW, Adler KB (1994) Production and characterization of monoclonal antibodies against guinea pig tracheal mucins. *Hybridoma* 13: 281–287.
50. Kaartinen L, Nettesheim P, Adler KB, Randell SH (1993) Rat tracheal epithelial cell differentiation *in vitro*. *In Vitro Cell Dev Biol Animal* 29A: 481–492.
51. Clark AB, Randell SH, Nettesheim P, Gray TE, Bagnell B, Ostrowski LE (1995) Regulation of ciliated cell differentiation in cultures of rat tracheal epithelial cells. *Am J Respir Cell Mol Biol* 12: 329–338.
52. Rieves RD, Goff J, Wu T, Larivee P, Logun C, Shelhamer JH (1992) Airway epithelial cell mucin release: Immunologic quantitation and response to platelet-activating factor. *Am J Respir Cell Mol Biol* 6: 158–167.
53. Wu R, Martin WR, Robinson CB, St. George JA, Plopper CG, Kurland G, Last JA, Cross CE, McDonald RJ, Boucher R (1990) Expression of mucin synthesis and secretion in human tracheobronchial epithelial cells grown in culture. *Am J Respir Cell Mol Biol* 3: 467–478.
54. Robinson CG, Wu R (1993) Mucin synthesis and secretion by cultured tracheal cells: Effects of collagen gel substratum thickness. *In Vitro Cell Dev Biol Animal* 29A: 469–477.
55. Davis CW, Dowell ML, Lethem M, Van Scott M (1992) Goblet cell degranulation in isolated canine tracheal epithelium: Response to exogenous ATP, ADP and adenosine. *Am J Physiol* 262: C1313–1323.
56. Lethem MI, Dowell ML, Van Scott M, Yankaskas JR, Egan T, Boucher RC, Davis CW (1993) Nucleotide regulation of goblet cells in human airway epithelial explants: Normal exocytosis in cystic fibrosis. *Am J Respir Cell Mol Biol* 9: 315–322.
57. Emery N, Place GA, Dodd S, Lhermitte M, David G, Lamblin G, Perini JM, Page AM, Hall RL, Roussel P (1995) Mucous and serous secretions of human bronchial epithelial cells in secondary culture. *Am J Respir Cell Mol Biol* 12: 130–141.
58. Gray TE, Guzman K, Davis CW, Abdullah LH, Nettesheim P (1996) Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am J Respir Cell Mol Biol* 14: 104–112.
59. Randell SH, Liu JY, Ferriola PC, Kaartinen L, Doherty MM, Davis CW, Nettesheim P (1996) Mucin production by SPOC1 cells – an immortalized rat tracheal epithelial cell line. *Am J Respir Cell Mol Biol* 14: 146–154.
60. Doherty MM, Liu J, Randell SH, Carter CA, Davis CW, Nettesheim P, Ferriola PC (1995) Phenotype and differentiation potential of a novel rat tracheal epithelial cell line. *Am J Respir Cell Mol Biol* 12: 385–395.
61. Abdullah LH, Davis SW, Burch L, Yamauchi M, Randell SH, Nettesheim P, Davis CW (1996) P2U purinoceptor regulation of mucin secretion in SPOC1 cells, an airway cell line. *Biochem J* 316: 943–951.
62. Verdugo P (1990) Goblet cells secretion and mucogenesis. *Ann Rev Physiol* 52: 157–176.
63. Carlstedt I, Sheehan JK, Corfield AP, Gallagher JT (1985) Mucous glycoproteins. A gel of a problem. *Essays Biochem* 20: 40–76.
64. Silberberg A (1989) Mucus glycoprotein, its biophysical and gel-forming properties. *Symp Soc Exp Biol* 43: 43–63.
65. Sheehan JK, Thornton DJ, Somerville M, Carlstedt I (1991) Mucin structure: The structure and heterogeneity of respiration mucus glycoproteins. *Am Rev Respir Dis* 144: S4–9.
66. Lamblin G, Lhermitte M, Klein A, Houdret N, Scharfman A, Ramphal R, Roussel P (1991) The carbohydrate diversity of human respiratory mucins: a protection of the underlying mucosa? *Am Rev Respir Dis* 144 Pt2: S19–24.
67. Jentoft N (1990) Why are proteins O-glycosylated? *Trends Biochem Sci* 15: 291–294.
68. Lamblin G, Aubert JP, Perini JM, Klein A, Porchet N, Degand P, Roussel P (1992) Human respiratory mucins. *Eur Respir J* 5: 247–256.
69. Rose MC (1992) Mucins: Structure, function, and role in pulmonary diseases. *Am J Physiol* 263 Pt1: L413–429.

70. Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EN, Wilson D (1990) Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem* 265: 15286–15293.
71. Lan MS, Batra SK, Qi WN, Metzgar RS, Hollingsworth MA (1990) Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J Biol Chem* 265: 15294–15299.
72. Gum JR (1992) Mucin genes and the proteins they encode: Structure, diversity and regulation. *Am J Respir Cell Mol Biol* 7: 557–564.
73. Gendler SJ, Spicer AP (1995) Epithelial mucin genes. *Ann Rev Physiol* 57: 607–634.
74. Finkbeiner WE, Carrier SD, Teresi CE (1993) Reverse transcription-polymerase chain reaction (RT-PCR) phenotypic analysis of cell cultures of human tracheal epithelium, tracheobronchial glands and lung carcinomas. *Am J Respir Cell Mol Biol* 9: 547–556.
75. Dohrman A, Tsuda T, Escudier E, Cardone M, Jany B, Gum J, Kim Y, Basbaum C (1994) Distribution of lysozyme and mucin (MUC2 and MUC3) mRNA in human bronchus. *Exp Lung Res* 20: 367–380.
76. Audie JP, Janin A, Porchet N, Copin MC, Grosselin B, Aubert JP (1993 Expression of human mucin genes in respiratory, digestive and reproductive tracts ascertained by *in situ* hybridization. *J Histochem Cytochem* 41: 1479–1485.
77. Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK (1996) Respiratory mucins: Identification of core proteins and glycoforms. *Biochem J* 316: 967–975.
78. Strous GJ, Dekker J (1992) Mucin-type glycoproteins. *Crit Rev Biochem Mol Biol* 27: 57–92.
79. Downing SW, Salo WL, Spitzer RH, Koch EA (1981) The hagfish slime gland: A model system for studying the biology of mucus. *Science* 214: 1143–1145.
80. Deyrup-Olsen I, Martin AW (1987) Processes in formation of mucus by the body wall of *Ariolimax columbianus*. Biorheology 24: 571–576.
81. Verdugo P, Deyrup-Olsen I, Aitken ML, Villalon M, Johnson D (1987) Molecular mechanism of mucin secretion. I. The role of intragranular charge shielding. *J Dent Res* 66: 506–508.
82. Puchelle E, Beorchia A, Menager M, Zahm JM, Ploton D (1991) Three-dimensional imaging of the mucus secretory process in the cryofixed frog respiratory epithelium. *Biol Cell* 72: 159–166.
83. Inglis SK, Corboz MR, Taylor AE, Ballard ST (1996) *In situ* visualization of bronchial submucosal glands and their secretory response to acetylcholine. *Am J Physiol.* In press.
84. Specian RD, Neutra MR (1980) Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. *J Cell Biol* 85: 626–640.
85. Verdugo P (1984) Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: A new model. *Ciba Found Symp* 109: 212–225.
86. Almers W (1990) Exocytosis. *Ann Rev Physiol* 52: 607–624.
87. Lindau M, Almers W (1995) Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr Opin Cell Biol* 7: 509–517.
88. Finkelstein A, Zimmerberg J, Cohen FS (1986) Osmotic swelling of vesicles: Its role in the fusion of vesicles with planar phospholipid bilayer membranes and its possible role in exocytosis. *Ann Rev Physiol* 48: 163–174.
89. Zimmerberg J, Curran M, Cohen FS, Brodwick M (1987) Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. *Proc Natl Acad Sci USA* 84: 1585–1589.
90. Breckenridge LJ, Almers W (1987) Final steps in exocytosis observed in a cell with giant secretory granules. *Proc Natl Acad Sci USA* 84: 1945–1949.
91. Monck JR; Oberhauser AF, Fernandez JM (1995) The exocytotic fusion pore interface: A model of the site of neurotransmitter release. *Mol Memb Biol* 12: 151–156.
92. Monck JR, Alvarez de Toledo G, Fernandez JM (1990) Tension in secretory granule membranes causes extensive membrane transfer through the exocytotic fusion pore. *Proc Natl Acad Sci USA* 87: 7804–7808.
93. Uvnas B, Aborg CH (1988) Catecholamines (CA) and adenosine triphosphate (ATP) are separately stored in bovine adrenal medulla, both in ionic linkage to granule sites, and not as a non-diffusible CA-ATP-protein complex. *Acta Physiol Scand* 132: 297–311.
94. Gowda DC, Hogue-Angeletti R, Margolis RK, Margolis RU (1990) Chromaffin granule and PC12 cell chondroitin sulfate proteoglycans and their relation to chromogranin A. *Arch Biochem Biophys* 281: 219–224.

95. Rosa P, Gerdes HH (1994) The granin protein family: Markers for neuroendocrine cells and tools for the diagnosis of neuroendocrine tumors. *J Endocrinol Invest* 17: 207–225.
96. Wightman RM, Schroeder TJ, Finnegan JM, Ciolkowski EL, Pihel K (1995) Time course of release of catecholamines from individual vesicles during exocytosis at adrenal medullary cells. *Biophys J* 68: 383–390.
97. Fernandez JM, Villalon M, Verdugo P (1991) Reversible condensation of mast cell secretory products *in vitro*. *Biophys J* 59: 1022–1027.
98. Curran MJ, Brodwick MS (1991) Ionic control of the size of the vesicle matrix of beige mouse mast cells. *J Gen Physiol* 98: 771–790.
99. Verdugo P, Aitken ML, Langley L, Villalon MJ (1987) Molecular mechanism of product storage and release in mucin secretion. II. The role of extracellular Ca^{++}. *Biorheology* 24: 625–633.
100. Izutsu K, Johnson D, Schubert M, Wang E, Ramsey B, Tamarin A, Truelove E, Ensign W, Young M (1985) Electron microprobe analysis of human labial gland secretory granules in cystic fibrosis. *J Clin Invest* 75: 1951–1956.
101. Wagner D, Puchelle E, Hinnrasky J, Girard P, Blossier G (1994) Quantitative X-ray microanalysis of P, Ca and S in the mucus secretory granules of the cryofixed frog palate epithelium. *Microsc Res Tech* 28: 141–148.
102. Aitken ML, Verdugo P (1989) Donnan mechanism of mucin release and conditioning in goblet cells: The role of polyions. *Symp Soc Exp Biol* 43: 73–80.
103. Tam PY, Verdugo P (1981) Control of mucus hydration as a Donnan equilibrium process. *Nature* 292: 340–342.
104. Tam PY, Verdugo P (1982) Mucus hydration: a Donnan equilibrium controlled process. *Adv Exp Med Biol* 144: 101–103.
105. Monck JR, Oberhauser AF, Alvarez de Toledo G, Fernandez JM (1991) Is swelling of the secretory granule matrix the force that dilates the exocytotic fusion pore? *Biophys J* 59: 39–47.
106. Chizmadzhev YA, Cohen FS, Shcherbakov A, Zimmerberg J (1995) Membrane mechanics can account for fusion pore dilation in stages. *Biophys J* 69: 2489–2500.
107. Rice WR, Singleton FM (1986) P2-purinoceptors regulate surfactant secretion from isolated rat alveolar type II cells. *Br J Pharmacol* 89: 485–491.
108. Mason SJ, Paradiso AM, Boucher RC (1991) Regulation of transepithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. *Br J Pharmacol* 103: 1649–1656.
109. Knowles MR, Clarke LL, Boucher RC (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N Engl J Med* 325: 533–538.
110. Brown HA, Lazarowski ER, Boucher RC, Harden TK (1991) Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5′-nucleotide receptor in human airway epithelial cells. *Mol Pharmacol* 40: 648–655.
111. Lustig KD, Shiau AK, Brake AJ, Julius D (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci USA* 90: 5113–5117.
112. Parr CE, Sullivan DM, Paradiso AM, Lazarowski ER, Burch LH, Olsen JC, Erb L, Weisman GA, Boucher RC, Turner JT (1994) Cloning and expression of a human P2U nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc Natl Acad Sci USA* 91: 3275–3279.
113. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) Nomenclature and classification of purinoceptors. *Pharmacol Rev* 46: 143–156.
114. Harden TK, Boyer JL, Nicholas RA (1995) P2-purinergic receptors: Subtype-associated signaling responses and structure. *Ann Rev Pharmacol Toxicol* 35: 541–579.
115. Dubyak GR, el-Moatassim G (1993) Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* 265 Pt1: C577–606.
116. Kim KC, Lee BC (1992) P2 purinoceptor regulation of mucin release by airway goblet cells in primary culture. *Br J Pharmacol* 103: 1053–1056.
117. Shimura S, Sasaki T, Nagaki M, Takishima T, Shirato K (1994) Extracellular ATP regulation of feline tracheal submucosal gland secretion. *Am J Physiol* 267: L159–164.

118. Ota S, Yoshiura K, Takahashi M, Hata Y, Kohmoto O, Kawabe T, Shimada T, Hiraishi H, Mutoh H, Terano A et al. (1994) P2 purinergic receptor regulation of mucus glycoprotein secretion by rabbit gastric mucous cells in a primary culture. *Gastroenterology* 106: 1485–1492.
119. Merlin D, Augeron C, Tien XY, Guo X, Laboisse CL, Hopfer U (1994) ATP-stimulated electrolyte and mucin secretion in the human intestinal goblet cell line HT29-Cl.16E. *J Membr Biol* 137: 137–149.
120. Brown D, Stow JL (1996) Protein trafficking and polarity in kidney epithelium: From cell biology to physiology. *Physiol Rev* 76: 245–297.
121. Cross CE, Halliwell B, Allen A (1984) Antioxidant protection: A function of tracheobronchial and gastrointestinal mucus. *Lancet* 1: 1328–1330.
122. Pryor WA, Squadrito GL, Friedman M (1995) The cascade mechanism to explain ozone toxicity: The role of lipid ozonation products. *Free Radical Biol Med* 19: 935–941.
123. Wright DT, Cohn LA, Li H, Fischer B, Li CM, Adler KB (1994) Interactions of oxygen radicals with airway epithelium. *Environ Health Perspect* 102 Suppl 10: 85–90.
124. Cross CE, van der Vliet A, O'Neill CA, Louie S, Halliwell B (1994) Oxidants, antioxidants and respiratory tract lining fluids. *Environ Health Perspect* 102 Suppl 10: 185–191.
125. Adler KB, Fischer BM, Wright DT, Cohn LA, Becker S (1994) Interactions between respiratory epithelial cells and cytokines: Relationships to lung inflammation. *Ann NY Acad Sci* 725: 128–145.
126. Shelhamer JH, Levine SJ, Wu T, Jacoby DB, Kaliner MA, Rennard SI (1995) NIH conference: *Airway inflammation. Ann Int Med* 123: 288–304.
127. Marom Z, Shelhamer JH, Kaliner M (1981) 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.
128. Adler KB, Schwarz JE, Anderson WH, Welton AF (1987) Platelet activating factor stimulates secretion of mucin by explants of rodent airways in organ culture. *Exp Lung Res* 13: 25–43.
129. Adler KB, Holden Stauffer WJ, Repine JE (1990) Oxygen metabolites stimulate release of high-molecular-weight glycoconjugates by cell and organ cultures of rodent respiratory epithelium via an arachidonic acid-dependent mechanism. *J Clin Invest* 85: 75–85.
130. Adler KB, Akley NJ, Glasgow WC (1992) Platelet-activating factor provokes release of mucin-like glycoproteins from guinea pig respiratory epithelial cells via a lipoxygenase-dependent mechanism. *Am J Respir Cell Mol Biol* 6: 550–556.
131. Adler KB, Fischer BM, Li H, Choe NH, Wright DT (1995) Hypersecretion of mucin in response to inflammatory mediators by guinea pig tracheal epithelial cells *in vitro* is blocked by inhibition of nitric oxide synthase. *Am J Respir Cell Mol Biol* 13: 526–530.
132. Brown JF, Keates AC, Hanson PJ, Whittle BJ (1993) Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. *Am J Physiol* 265 Pt1: G418–422.
133. Sasaki T, Shimura S, Ikeda K, Sasaki H, Takishima T (1989) Platelet-activating factor increases platelet-dependent glycoconjugate secretion from tracheal submucosal gland. *Am J Physiol* 257: Pt1: L373–378.
134. Shimura S, Takishima T (1994) Airway submucosal gland secretion. In: Takishima T, Shimura S eds. *Airway secretion: Physiological bases for the control of mucus hypersecretion*. New York: Marcel Dekker, 325–398.
135. McDonald DM (1988) Neurogenic inflammation in the rat trachea. I. Changes in venules, leucocytes and epithelial cells. *J Neurocytol* 17: 583–603.
136. Kuo HP, Rohde JA, Barnes PJ, Rogers DF (1992) Differential inhibitory effects of opioids on cigarette smoke, capsaicin and electrically-induced goblet cell secretion in guinea-pig trachea. *Br J Pharmacol* 105: 361–366.
137. Tokuyama K, Kuo HP, Rohde JA, Barnes PJ, Rogers DF (1990) Neural control of goblet cell secretion in guinea pig airways. *Am J Physiol* 259: L108–L115.
138. Shretton D (1991) Non-adrenergic, non-cholinergic neural control of the airways. *Clin Exp Pharmacol Physiol* 18: 675–684.
139. Barnes PJ, Baraniuk JN, Belvisi MG (1991) Neuropeptides in the respiratory tract. Part I. *Am Rev Respir Dis* 144: 1187–1198.
140. Ramnarine SI, Rogers DF (1994) Non-adrenergic, non-cholinergic neural control of mucus secretion in the airways. *Pulm Pharmacol* 7: 19–33.

141. Kuo HP, Rohde JA, Tokuyama K, Barnes PJ, Rogers DF (1990) Capsaicin and sensory neuropeptide stiumulation of goblet cell secretion in guinea-pig trachea. *J Physiol* (Lond) 431: 629–641.
142. Martling CR (1987) Sensory nerves containing tachykinins and CGRP in the lower airways: functional implications for bronchoconstriction, vasodilatation and protein extravasation. *Acta Physiol Scand* 563 Suppl: 1–57.
143. Persson CG (1991) Mucosal exudation in respiratory defence: Neural or non-neural control? *International Archives of Allergy & Applied Immunology* 94: 222–226.
144. Berridge MJ (1993) Inositol triphosphate and calcium signalling. *Nature* 361: 315–325.
145. Kim KC, Zheng QX, Van-Seuningen I (1993) Involvement of a signal transduction mechanism in ATP-induced mucin release from cultured airway goblet cells. *Am J Respir Cell Mol Biol* 8: 121–125.
146. Abdullah LH, Conway JD, Cohn JA, Davis CW (1996) Protein kinase C and Ca^{+2} activation of mucin secretion in airway goblet cells. *Am J Physiol.* In press.
147. Thastrup O (1990) Role of $Ca^{2(+)}$-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal $Ca^{2(+)}$-ATPase inhibitor, thapsigargin. *Agents Actions* 29: 8–15.
148. Forstner G (1995) Signal transduction, packaging and secretion of mucins. *Ann Rev Physiol* 57: 585–605.
149. Nishizuka Y (1986) Studies and perspectives of protein kinase C. *Science* 233: 305–312.
150. Azzi A, Boscoboinik D, Hensey C (1992) The protein kinase C family. *Eur J Biochem* 208: 547–557.
151. Kai H, Yoshitake K, Isohama Y, Hamamura I, Takahama K, Miyata T (1994) Involvement of protein kinase C in mucus secretion by hamster tracheal epithelial cells in culture. *Am J Physiol* 267 Pt 1: L526–530.
152. Larivee P, Levine SJ, Martinez A, Wu T, Logun C, Shelhamer JH (1994) Platelet-activating factor induces airway mucin release via activation of protein kinase C: Evidence for translocation of protein kinase C to membranes. *Am J Respir Cell Mol Biol* 11: 199–205.
153. Shimura S, Ishihara H, Nagaki M, Sasaki H, Takishima T (1993) A stimulatory role of protein kinase C in feline tracheal submucosal gland secretion. *Respir Physiol* 93: 239–247.
154. Fleming N, Bilan PT, Sliwinski-Lis E (1986) Effects of a phorbol ester and diacylglycerols on secretion of mucin and arginine esterase by rat submandibular gland cells. *Pflügers Arch – Eur J Physiol* 406: 6–11.
155. McCool DJH, Marcon MA, Forstner JF, Forstner GG (1990) The T84 human colonic adenocarcinoma cell line produces mucin in culture and release it in response to various secretagogues. *Biochem J* 267: 491–500.
156. Forstner G, Zhang Y, McCool D, Forstner J (1993) Mucin secretion by T84 cells: Stimulation by PKC, Ca^{2+}, and a protein kinase activated by Ca^{2+} ionophore. *Am J Physiol* 264: Pt 1: G1096–1102.
157. Phillips TE, Wilson J (1993) Signal transduction pathways mediating mucin secretion from intestinal goblet cells. *Dig Dis Sci* 38: 1046–1054.
158. Keller K, Olivier M, Chadee K (1992) The fast release of mucin secretion from human colonic cells induced by *Entamoeba histolytica* is dependent on contact and protein kinase C activation. *Arch Med Res* 23: 217–221.
159. Dekker LV, Parker PJ (1994) Protein kinase C – a question of specificity. *Trends Biochem Sci* 19: 73–77.
160. Steinberg SF, Goldberg M, Rybin VO (1995) Protein kinase C isoform diversity in the heart. *J Mol Cell Cardiol* 27: 141–153.
161. Divecha N, Irvine RF (1995) Phospholipid signaling. *Cell* 80: 269–278.
162. Puceat M, Hilal-Dandan R, Strulovici B, Brunton LL, Brown JH (1994) Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J Biol Chem* 269: 16938–16944.
163. Trifaro JM, Vitale ML (1993) Cytoskeleton dynamics during neurotransmitter release. *Trends Neurosci* 16: 466–472.
164. Trifaro JM, Vitale ML, Rodriguez Del Castillo A (1993) Scinderin and chromaffin cell actin network dynamics during neurotransmitter release. *J Physiol Paris* 87: 89–106.
165. Muallem S, Kwiatkowska K, Xu X, Yin HL (1995) Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J Cell Biol* 128: 589–598.

166. Vitale ML, Rodriguez Del Castillo A, Trifaro JM (1992) Loss and Ca(2+)-dependent retention of scinderin in digitonin-permeabilized chromaffin cells: correlation with Ca(2+)-evoked catecholamine release. *J Neurochem* 59: 1717–1728.
167. Rodriguez Del Castillo A, Vitale ML, Trifaro JM (1992) Ca^{2+} and pH determine the interaction of chromaffin cell scinderin with phosphatidylserine and phosphatidylinositol 4,5,-biphosphate and its cellular distribution during nicotinic-receptor stimulation and protein kinase C activation. *J Cell Biol* 119: 797–810.
168. McCool DJ, Forstner JF, Forstner GG (1995) Regulated and unregulated pathways for MUC2 mucin secretion in human colonic LS180 adenocarcinoma cells are distinct. *Biochem J* 312: 125–133.
169. Lazarus SC, Basbaum CB, Gold WM (1984) Prostaglandins and intracellular cyclic AMP in respiratory secretory cells. *Am Rev Respir Dis* 130: 262–266.
170. Ignarro LJ (1990) Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension* 16: 477–483.
171. McPherson MA, Dormer RL, Bradbury NA, Dodge JA, Goodchild MC (1986) Defective beta-adrenergic secretory responses in submandibular acinar cells from cystic fibrosis patients. *Lancet* 2: 1007–1008.
172. Rogers DF, Alton EW, Dewar A, Lethem MI, Barnes PJ (1993) Impaired stimulus-evoked mucus secretion in cystic fibrosis bronchi. *Exp Lung Res* 19: 37–53.
173. Merten MD, Figarella C (1993) Constitutive hypersecretion and insensitivity to neurotransmitters by cystic fibrosis tracheal gland cells. *Am J Physiol* 264 Pt 1: L 93–99.
174. Kuver R, Ramesh N, Lau S, Savard C, Lee SP, Osborne WR (1994) Constitutive mucin secretion linked to CFTR expression. *Biochem Biophys Res Comm* 203: 1457–1462.
175. Engelhardt JF, Yankaskas JR, Ernst SA, Yang Y, Marino CR, Boucher RC, Cohn JA, Wilson JM (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genetics* 2: 240–248.
176. Finkbeiner WE, Shen BQ, Widdicombe JH (1994) Chloride secretion and function of serous and mucous cells of human airway glands. *Am J Physiol* 267: L 206–210.
177. Miller SG, Moore HP (1991) Reconstitution of constitutive secretion using semi-intact cells, Regulation by GTP but not calcium. *J Cell Biol* 112: 39–54.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 8
Airway Submucosal Glands: Physiology and Pharmacology

Denis C. K. Fung[1] and Duncan F. Rogers[2,*]

[1] *Peptide Therapeutics, Cambridge Science Park, Cambridge, UK*
[2] *Thoracic Medicine, National Heart and Lung Institute (Imperial College), London, UK*

1. Introduction

Secretory processes in airway submucosal glands contribute to homeostasis and to first-line defence against inhaled insult. Secretion of high molecular weight mucous glycoproteins (mucins) and of water onto the internal surface of the airways are two important processes which contribute to regulation of

* Author for correspondence.

the volume and physicochemical properties of airway surface liquid. Absorption of water is also an important homeostatic mechanism. The secretion or absorption of water is linked to active transport of ions across the airway wall [1]. Secretion of mucins, electrolytes and water can be demonstrated in submucosal glands. Absorptive processes have not been formally studied, which does not mean to say that glands do not absorb ions and water. Increases in the rate of secretory processes will increase the volume of fluid, ions and macromolecules in the airway surface liquid. Mucous secretion and ion transport are processes controlled by specialised cells in the surface epithelium and submucosal glands. Chapter 7 of this volume will concentrate on the physiology and pharmacology of secretion from surface epithelial secretory cells (goblet cells). The present chapter will discuss the physiology and pharmacology of secretion by airway submucosal glands.

2. Gland Distribution and Structure

The submucosal glands of larger mammals, including humans, are complex structures comprising secretory tubules, a collecting duct and a ciliated duct which opens onto the airway surface [2] (Figure 1). The secretory tubules form acini which are lined with mucous and serous cells containing acid-staining and neutral-staining mucin, respectively [3]. In humans, glands are in greatest abundance in more proximal airways (trachea to basal bronchi), but may also be present in sub-segmental bronchi [4]. The glands of smaller animal species are more rudimentary than those of larger species [5, 6] and are confined to proximal (extrapulmonary) airways, or may be absent altogether [7, 8].

2.1. Mucous Cells

The mucous cells of the submucosal glands resemble the goblet cells of the surface epithelium. They contain numerous electron-lucent granules that enlarge towards the apex of the cell, where they appear to fuse with the luminal cell membrane before secretion (Figure 2). Their mucous content consists of acidic and neutral glycoproteins. Until recently it was difficult to study the secretions of these cells in isolation, as they were difficult to culture. However, gland-derived cells which show phenotypic characteristics of mucous cells can now be cultured [9, 10].

In terms of secretory product, submucosal gland mucous cells contain a qualitatively similar range of secretory products as those found in epithelial goblet cells. The main secretory products of both these cell types are mucins, which are the major glycoconjugates in normal airway surface liquid [11] (see Chapter 1 of this volume). Mucins are considered to be the chief molecules responsible for conferring the properties of viscosity and elasticity upon

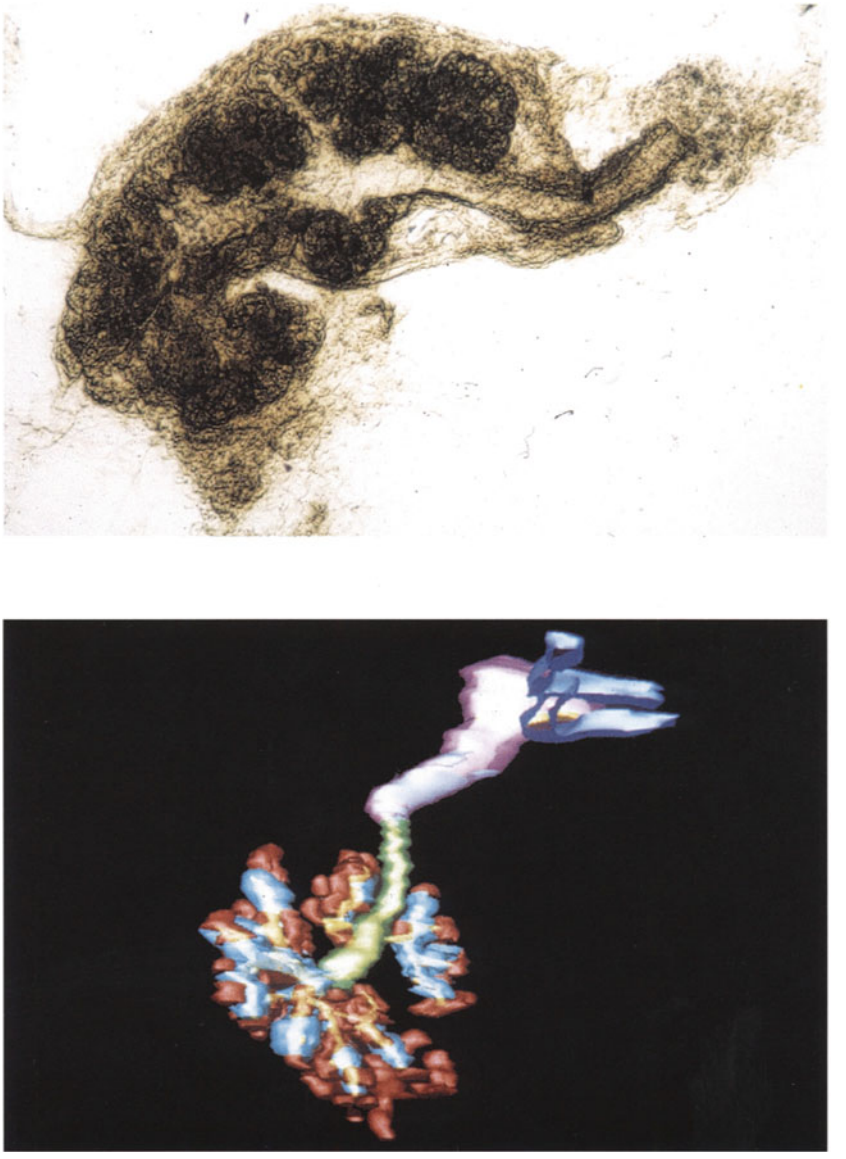


Figure 1. Appearance of single-airway submucosal glands. (Upper panel) Dog tracheal gland. Capillaries and some connective tissue are attached to the gland and mucus is secreted from the tip of the duct. Image kindly supplied by Dr. Sanae Shimura. (Lower panel) Computer-generated three-dimensional reconstruction of a human bronchial submucosal gland. Histological sections of the gland were reconstructed using a microcomputer 3D-shading package based on polygonal models. Violet, ciliated duct; green, collecting duct; orange, secretory tubules; dark blue, mucous acini; brown, serous acini. Image kindly provided by the late (sadly, recently deceased) Prof. William F. Whimster.

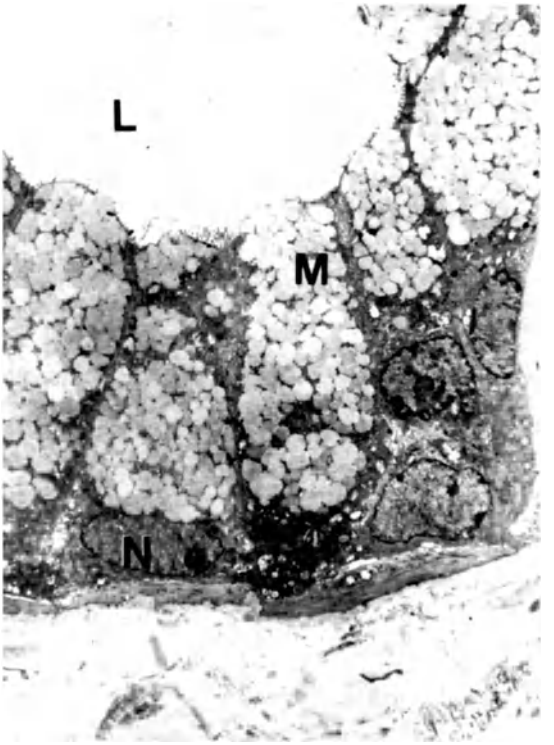


Figure 2. Mucous acinus in human bronchial submucosal gland. Mucous cells (M) packed with electron-lucent granules, many of which are coalescent with their neighbours, surround the acinar lumen (L). N = nucleus. Magnification ×2500.

airway surface liquid, with other molecules modifying viscoelasticity. It is thought that in humans and other large mammals the principal sources of typical mucins are the mucous cells in the glands and the goblet cells in the surface epithelium.

2.2. Serous Cells

The serous cells of the submucosal glands contain discrete electron-dense granules which are smaller and exhibit greater variety than those in the mucous cells (for review, see [12]). Unlike mucous cells, gland-derived serous cells have been cultured successfully for some time [13]. Immunocytochemical studies show that the serous cells contain lysozyme and lactoferrin [14, 15] as well as glycoconjugates, but appear to lack typical mucins. The main secretory products of serous cells appear to be non-mucin proteins including immunoglobulins, lysozyme, lactoferrin and other anti-bacterial enzymes, all

of which are important in host-defence mechanisms of the airways [16]. Thus, it appears that whilst the mucous cells supply the mucin components which control the overall viscoelastic properties of mucus, serous cells supply a series of non-mucin proteins which are primarily concerned with host-defence but which may also interact with mucin molecules to influence the physicochemical properties of airway surface liquid.

The serous cells, rather than the mucous cells, appear to be the cells primarily involved in providing the liquid component of gland secretions. The cystic fibrosis transmembrane conductance regulator (CFTR) [17] is an apical membrane chloride (Cl^-) channel [18]. Immunocytochemical studies have shown the highest concentration of CFTR in human airways is on the serous cells of the submucosal glands [19]. Studies in cultured human tracheal glands demonstrate that serous cells are indeed more involved in liquid secretion than are mucous cells [9]. Serous cells are sited distal to mucous cells in the glands and it might, therefore, be envisaged that the liquid serous secretions flow over the mucous cells on their way to the collecting ducts and help to flush out secreted mucins.

3. Quantification of Submucosal Gland Secretion

Many techniques are available for the collection of mucus under experimental conditions [20]. However, mucus collected from whole airway tissues, either *in vivo* in superfusates of animal tracheae or *in vitro* from Ussing chambers or organ cultures, includes secretions from secretory cells both in the submucosal glands and in the surface epithelium. The complex nature of the mucous glycoprotein molecule prevents direct measurement of the amount of mucus secreted, and indirect measurements are used. Use of radiolabelled mucin precursors, for example ^{35}S-sulphate [21], or of chemical markers such as fucose [22], which have some specificity for gland mucus, are not definitive markers of glandular secretion. Three techniques are specific for submucosal gland secretion: (1) micropipette collection of fluid directly from the gland duct *in situ*, (2) observation of "hillock" formation as secretions bubble through tantalum dust spread on the airway surface, and (3) collection of mucus from single submucosal glands isolated *in vitro* from the cat trachea [23]. None of these techniques differentiates effectively between secretions from serous or mucous acini. Neither the micropipette nor hillock technique accounts for the contribution of water secretion to the increased volume of liquid recorded.

An alternative to sampling mucus directly from submucosal glands is to measure mucus secretion in preparations which lack goblet cells. Measurement of concentrations of lysozyme, a marker for serous cell secretion [14], in fluid collected *in vitro* from the air-filled whole trachea of the ferret, a preparation containing virtually no goblet cells [24, 25], is a useful technique for studying secretion from serous acini [26]. Recent techniques of culturing

human bronchial mucosal cells appear to be able maintain cells with both mucous and serous phenotype in secondary culture [10], and may prove to be extremely useful for studying these cells *in vitro*.

In the following sections, where studies measuring airway secretion are discussed, attention will be drawn to the technique used so that an estimate of the contribution of secretion from submucosal glands can be made.

4. Basal and Stimulated Secretion

The precise sources of airway surface liquid under basal and stimulated conditions are ill-defined. There is also debate as to whether or not "basal" secretion exists under normal circumstances. Some animal species appear to have a moderate level of basal secretion, whilst others appear to have little or no mucous secretion under resting conditions. For example, in the ferret *in vitro* whole trachea, which contains abundant gland and virtually no goblet cells (see above), there was no detectable baseline secretion [26, 27]. In contrast, in the cat trachea, also with abundant gland [7], the basal rate of filling of a micropipette was 1–9 nl/min/gland [28, 29]. The *in vivo* cannulated air-filled cat trachea secretes 26 mg mucus/h under unstimulated conditions [30].

The basal rate of secretion from single cat tracheal glands is only slightly reduced by atropine [28, 29], which indicates that basal secretion does not rely greatly on tonic parasympathetic (cholinergic) discharge. This observation is consistent with the lack of effect of the neurotoxin tetrodotoxin on basal secretion in the cat trachea *in vivo* [31]. It is likely that products of cyclo-oxygenase metabolism of arachidonic acid, in particular prostaglandins, control basal secretion [32].

Microtubules and microfilaments appear to be important in the intracellular regulation of stimulated secretion *in vitro* by submucosal glands in human airways but are not involved to any great extent in basal secretion [33]. This indicates that stimulated secretion is an active process which possibly involves changes in cell shape that have to be regulated to limit their effects on cell homeostasis. This suggestion would be consistent with discharge of mucus from the secretory granules with consequent reduction in cell volume coupled with increased surface area of the apical membrane due to incorporation of secretory granule membrane. Why intracellular "skeletal" elements are not involved in basal secretion is unclear but indicates that basal secretion involves less cell deformation than does stimulated secretion.

The differences in physio-pharmacological control of basal and stimulated secretion discussed above are borne out by analysis of mucins from cat trachea which show biophysical and biochemical differences between "basal" and pilocarpine-stimulated secretions [34]. Stimulated secretions contained mucins, whereas basal secretion contained glycoconjugates with higher buoyant densities and higher resistance to enzymatic reduction than typical mucins. The origin of the latter material is unknown but may be related to the

epithelium-derived cell surface glycoconjugate released by dog cultured airway secretory cells [35], or may be derived from the apical border of the mucosa.

It is generally assumed that stimulated secretion is integral to airway defence and that the increased volume of airway surface liquid produced is required to provide a thicker barrier against inhaled insult, to facilitate mucociliary clearance and to facilitate cough. There is some experimental evidence to support this notion. In ferret trachea *in vivo*, the secretions elicited by either substance P, a neurotransmitter of "sensory-efferent" nerves (see below), or methacholine, a cholinomimetic, have an increased "transportability" on an experimental mucociliary preparation [36]. In addition, the viscoelasticity of the secretion produced by substance P in the latter study was consistent with a propensity for increased clearance by cough [37].

5. Neural Control of Submucosal Gland Secretion

Airway submucosal glands have a nervous supply [38–40], and stimulation of nerves or administration of neuromimetic drugs, alone or in combination, will affect secretion of mucins, ions and water from submucosal glands (Table 1). Three neural pathways are currently recognised in the airways: sympathetic (adrenergic), parasympathetic (cholinergic) and a non-adrenergic, non-cholinergic (NANC) system (Figure 3). It is clear that, rather than being separate, the three neural systems are intimately linked and undoubtedly interact.

Table 1. Neural humoral control of airway submucosal gland secretion

Stimulation	Mucus secretion	Ion/water flux
Cholinergic nerves	++ [28, 42–44, 46, 47, 56, 114–116]	np
Adrenergic nerves	0/+ [43, 44, 47, 64, 114]	np
Sensory-efferent nerves	0[a]/++ [42–44, 985, 98–100]	np
Cholinoceptor agonists	++ [36, 43–49, 53–55, 62]	++ [55, 57, 58, 60–62]
α-Adrenoceptor agonists	+ [28, 47, 63, 65, 68]	0/+ [57, 58, 70, 72]
β-Adrenoceptor agonists	+ [47, 63, 65, 66, 68, 69]	++ [57, 58, 70, 72]
PDE inhibitors	0/+[b] [69]	np
VIP[c]	+ [26, 88, 89, 91, 92]	+ [90]

Table 1 (continued)

Stimulation	Mucus secretion	Ion/water flux
PHI/PHM[c]	? [27]	np
NO	-ve[d] [94]	np
NPY[c]	? [27]	np
Substance P	++ [36, 101–103, 106–109, 111]	++ [110]
Neurokinin A	+ [106–109]	+ [110]
CGRP	0/+ [104]	np
Histamine	0/+ [48, 118, 119]	0/+ [120]
5-Hydroxytryptamine	0 [121]	np
Prostaglandins	0/+ [32, 122–126	+ [57, 128]
Leukotrienes	0/+ [32, 125, 127]	+ [129]
PAF	0/+ [130–132, 134]	+ [131]
Bradykinin	+ [136]	+ [57, 136]
Endothelin	0/+ [139, 140]	np
MMS	+ [142–144]	np
Proteinases	+++ [146–150]	np
Purine nucleotides	+ [153]	+ [153]
Reactive oxygen species	0/+[e] [141]	np

Scoring: +++ = highly potent, ++ = marked effect, + = lesser effect, 0 = minimal to no effect. np = not published. ? = effect alone not published. PDE = phosphodiesterase; VIP = vasoactive intestinal peptide; PHI/PHM = peptide histidine isoleucine/methionine; NO = nitric oxide; NPY = neuropeptide tyrosine; CGRP = calcitonin gene-related peptide; HETE = hydrosyeicosatetraeinoic acid; PAF = platelet activating factor; MMS = monocyte/macrophage-derived mucus secretagogue. a: difficult to demonstrate *in vitro* in human bronchi; b: only PDE IV inhibitor effective; c: modulation of stimulated secretion; d: inhibition of basal and stimulated secretion; e: dependent upon oxygen species.

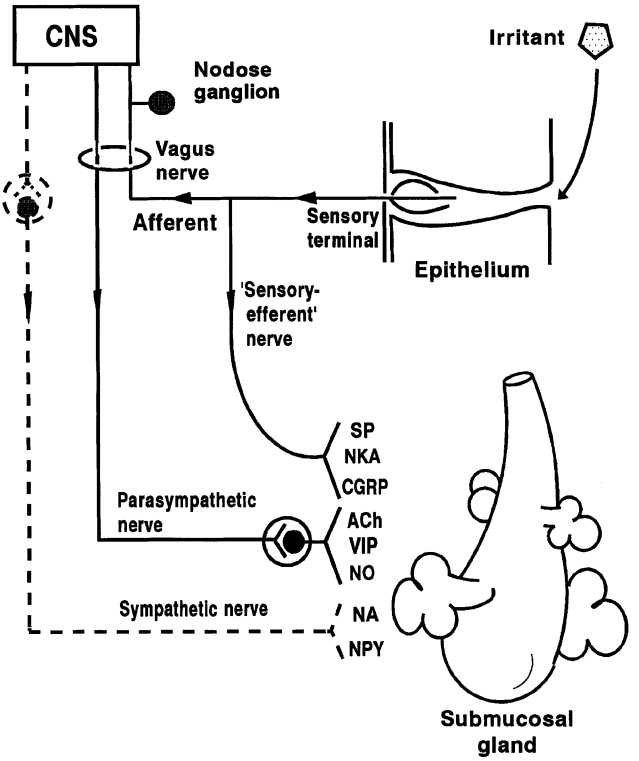


Figure 3. Innervation of airway submucosal glands. Parasympathetic (cholinergic) nerves constitute the dominant neutral pathway mediating neurogenic submucosal gland secretion. The "classical" neurotransmitter of the cholinergic nerves is acetylcholine (ACh). Co-localised with ACh in these nerves are vasoactive intestinal peptide (VIP), other VIP-related neuropeptides (for example peptide histidine), and nitric oxide (NO) synthase, the latter indicating that NO is a neurotransmitter affecting submucosal gland secretion. Parasympathetic (adrenergic) neural control of submucosal gland secretion is species-specific, being difficult to demonstrate in human airways. Co-localised with noradrenaline (NA) in these nerves is neuropeptide tyrosine (NPY). Sensory nerve terminals in the epithelium detect airway irritation, relay impulses via afferent pathways to the central nervous system (CNS), initiate reflex gland secretion (predominantly via cholinergic outflow). Local, or axonal, motor neurotransmission via collateral "sensory-efferent" pathways leads to release of sensory neuropeptides, including calcitonin gene-related peptide and the tachykinins substance P (SP) and neurokinin A (NKA). The figure is simplified in that it does not illustrate overlap between nerves in their neuropeptide content, only shows the major neuropeptides involved and gives no indication of any interaction between different components of the neural network.

5.1. Cholinergic Control

The parasympathetic nervous system is the dominant neural pathway in the airways. In animal and human airways, cholinergic nerve fibres are closely associated with submucosal glands [38], and autoradiographic mapping of muscarinic receptors demonstrates dense labelling over the glands [41]. Stimulation of cholinergic nerves induces marked airway mucus secretion in a number of animal species including ferret, cat and human [28, 42–44], where secretion is principally from submucosal glands. Similarly, muscarinic receptor agonists induce marked mucus secretion from airway submucosal glands of a number of animal species, including humans [43–49]. Activation of the inositol 1,4,5-trisphosphate (IP_3)/Ca^{2+} intracellular signal transduction pathway appears to be involved in the secretory response to cholinomimetics [50, 51].

Physiological responses to cholinergic nerve stimulation are mediated via cholinergic muscarinic receptors [52]. Three subtypes of muscarinic receptor (designated M_1–M_3), and possibly a fourth (M_4), can be demonstrated pharmacologically. In the airways, M_1 receptors are localised to parasympathetic ganglia, submucosal glands and, in humans, to alveolar walls [52]. M_2 receptors are localised to smooth muscle and to parasympathetic post-ganglionic nerve terminals, where they act as inhibitory autoreceptors to limit acetylcholine release. M_3 receptors exist on smooth muscle, submucosal glands, endothelial cells and epithelial cells. M_4 receptors are present in rabbit lung. In studies using muscarinic antagonists against glandular mucus secretion induced either by cholinoceptor agonists [53–55] or by electrical stimulation of cholinergic nerves [56] (Figure 4), the muscarinic receptor mediating mucus secretion from the glands is of the M_3 subtype. The M_1 receptor subtype, demonstrated on the glands by radioligand binding [41, 56], does not appear to be involved with mucus secretion [56] but may be involved in control of electrolyte (or liquid) secretion [55]. The M_2 receptor regulates the magnitude of cholinergic nerve-stimulated mucus secretion [56].

Cholinoceptor agonists also induce a matched flow of Na^+ and Cl^- from submucosa to mucosa in ferret, cat and human airways [57, 58]. The secretion of NaCl is considered to be via the glands because acetylcholine does not influence fluxes of these ions across rabbit trachea [59], a preparation lacking submucosal glands, and Na^+ efflux from isolated submucosal glands of the cat has been demonstrated directly [60]. Apically located IP_3 receptors comprise part of the Ca^{2+}-linked intracellular signal transduction mechanism underlying acetylcholine-induced Cl^- secretion by acinar cells of cat and human airway submucosal glands [61]. IP_3/Ca^{2+}-activated Cl^- conductances are also involved in methacholine-induced serous cell secretion in cultured sheep submucosal gland cells [62]. The observations above are consistent with the concept that cholinergic stimulation induces the "classic" secretory response of an outpouring of a large volume of a watery isosmotic "mucus" [36]. It should be noted, however, that unlike the response to exogenous administration of

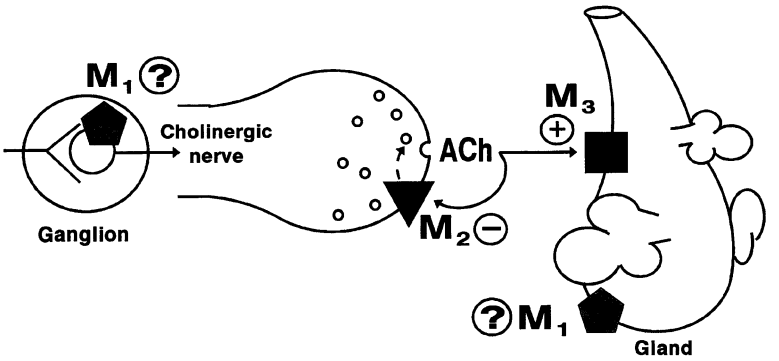


Figure 4. Cholinergic control of airway submucosal gland secretion. Traditionally, M_1 muscarinic (cholinergic) receptors are located on ganglia, where they facilitate neurotransmission. They are also localised to airway submucosal glands. M_2 receptors located on cholinergic nerve terminals are autoinhibitory to acetylcholine (ACh) release. Muscarinic M_3 receptors are localised to the end organ (in this case, the submucosal glands). Current information demonstrates that M_3 receptors mediate mucus secretion from the glands (+). The M_2 receptor is situated prejunctionally and regulates the magnitude of cholinergic mucus secretion (–). No evidence has been found for the ganglionic M_1 receptor in control of cholinergic gland secretion. The glandular-located M_1 receptor does not appear to be involved in control of mucus secretion. It may mediate ion and water secretion.

cholinomimetics, VIP and related peptides, co-localised with acetylcholine in cholinergic nerves, may modify the final secretory response to parasympathetic nerve activity (see Section 5.4 below).

5.2. Adrenergic Control

Although sympathetic nerves are associated with airway submucosal glands [38, 39], demonstration of functional adrenergic innervation is variable, and there are marked species differences. In human bronchi *in vitro*, no evidence has been found for the involvement of adrenergic nerves in inducing mucus secretion [43], and sympathomimetics have only weak stimulatory effects on secretion [63]. In contrast, electrical stimulation of adrenergic nerves induces mucus secretion in tracheae of ferret *in vitro* [44] and cat *in vivo* [64]. In cat isolated tracheal glands, α_1-adrenoceptor stimulation induces mucus secretion, whilst α_2-adrenoceptor stimulation may modulate the effect of β-adrenoceptor-mediated secretion [65]. It is likely that the initial steps in the intracellular signal transduction pathway leading to α-agonist-induced increases in gland mucus secretion are via IP_3 and Ca^{2+} [50, 51]. In contrast, in cat isolated tracheal glands, β-adrenoceptor stimulation did not alter intracellular Ca^{2+} [50]. β-adrenoceptor agonists stimulate mucus secretion in cat trachea *in vitro* via activation of protein kinase (PK) enzymes [66]. In cultured serous cells of bovine tracheal submucosal glands, activators of both PKA and PKC

stimulate mucus secretion [67], whilst PKC also modulates β-adrenoceptor-mediated cAMP accumulation [68]. Increasing the concentration of intracellular cAMP by phosphodiesterase (PDE) inhibitors (thereby increasing cAMP by limiting its degradation) stimulates mucus secretion in a preparation of rat trachea in which the principal source of secretion is the submucosal glands [69]. Non-selective PDE inhibitors (xanthines) had to be used at high concentrations (0.1–1 mM) to achieve a significant secretory effect, whereas an inhibitor of the PDE type IV isoform induced marked secretion in the nM concentration range.

Alpha- and β-adrenoceptor agonists stimulate Cl^- secretion by the airways of a number of animal species including dog, cat, ferret and human [57, 58, 70–72], all species with abundant submucosal gland. The Cl^- secretion may be via the glands because in the rabbit trachea, a preparation with sparse submucosal gland, neither α- nor β-adrenoceptor agonists alter ion transport [59], although the lack of effect of the β-agonist is probably a species peculiarity [71].

Thus, in a similar manner to that of cholinergic stimulation, adrenergic nerve activation or drugs which increase intracellular cAMP should increase the volume of airway surface liquid by inducing mucous and water secretion. However, neuropeptide tyrosine (NPY) may modify the final secretory response of sympathetic nerve discharge (see Section 5.4 below).

5.3. NANC Neural Control: General Considerations

The NANC neural system may, for convenience, be divided into two components [73]. The first component comprises an orthodromic system of sympathetic (adrenergic) and parasympathetic (cholinergic) nerves which have specific neuropeptides co-localised with the classical neurotransmitters. In cholinergic nerves, vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI; or PH-methionine, PHM in humans) and galanin are co-localised with acetylcholine [74–78]. Other VIP-related peptides including peptide histidine valine, helodermin and pituitary adenylate cyclase-activating peptide (PACAP-27) may also be co-localised in these "cholinergic/VIP-ergic" nerves. More recently, nitric oxide synthase has been localised to similar cholinergic/VIP-ergic nerves in human airways [79], which is consistent with the concept that nitric oxide (NO) is an NANC neurotransmitter [80]. For parasympathetic innervation, the distribution of neuropeptide tyrosine (NPY) is markedly similar to that of sympathetic nerve fibres [81], indicating that NPY is co-localised with noradrenaline in adrenergic nerves.

The second division of the NANC neural system comprises a discrete system of nerves which are neither cholinergic nor adrenergic. These form a population of C-fibre afferents (sensory nerves) which are susceptible to stimulation by capsaicin, the pungent extract of hot peppers of the *Capsicum* family [82]. In addition to their sensory function, these fibres also subserve a

"sensory efferent" or "local effector" function [83, 84]. The neurotransmitters of these C-fibres include calcitonin gene-related peptide (CGRP) and the tachykinins substance P (SP) and neurokinin (NK) A [85].

It should be noted that the above classification is a simplification and does not account for those sensory nerves which also contain VIP and galanin [86], nor for the proportion of NPY-containing adrenergic nerves which also contain VIP, galanin, somatostatin and enkephalin [87]. Thus, the concept of separate neural systems each controlling defined aspects of airway submucosal secretion is certain to prove a poor approximation to the truth. The close association of different nerve types, the co-localisation of different neurotransmitters within the same nerve and the interaction between certain neurotransmitters indicates that the final secretory product is the result of subtle and interacting activities of a variety of nervous mechanisms. Altered balance by one facet of the system, as part of a pathophysiological process, may lead to an inappropriate emphasis on another aspect which in turn may contribute to disease.

5.4. NANC Neural Control: Parasympathetic and Sympathetic Modulation

The co-localisation of classical neurotransmitters with other neurotransmitters (neuropeptides or NO) appears to lead to neuromodulation of parasympathetic and sympathetic mucus secretion. Thus, although VIP alone stimulates mucus secretion from ferret trachea *in vitro* [88] and isolated cat tracheal submucosal glands [89], as well as Cl^- secretion from canine trachea *in vitro* [90], it inhibits methacholine-stimulated mucus secretion from ferret and human airways [26, 91]. Similarly, PHI inhibits mucus secretion from ferret trachea [27]. We have found recently that endogenous VIP regulates the magnitude of cholinergic neurogenic mucus secretion in ferret trachea [92]. Whether VIP (or PHI)-induced inhibition of cholinergic neurogenic mucus secretion is prejunctional, via inhibition of acetylcholine release, or postjunctional, via an unidentified interaction with muscarinic receptors, remains to be determined [93]. Recently, endogenous NO has been found to regulate the magnitude of the total neurogenic mucus secretory response in ferret trachea *in vitro* [94]. Neurogenic secretion in the latter preparation is due to stimulation of both cholinergic nerves and sensory-efferent nerves (~60:40) [95]. The neuronal source of NO is not defined in the study above [94] but, in ferret trachea, includes a small population of cholinergic nerves, a small population of non-cholinergic NOS-containing nerves, a larger population of non-cholinergic nerves containing both NOS and VIP, and also some non-cholinergic nerves containing NOS, VIP and substance P [96]. Specific experiments are now required to determine whether NO regulates cholinergic or sensory-efferent neurogenic mucus secretion, or both.

Neuromodulation of sympathetic mucus secretion may also exist. NPY inhibits serous cell secretion *in vitro* in the whole ferret trachea [27], which

indicates that, in a manner similar to the regulation of cholinergic mucus secretion by VIP (and NO), co-release of NPY from sympathetic nerves may regulate the stimulatory effect of noradrenaline on adrenergic mucus secretion. The recent development of selective receptor antagonists for NPY [97] should serve in the future to clarify any regulatory role for NPY in adrenergic secretion.

5.5. Sensory-Efferent Neural Control

In the presence of adrenoceptor and cholinoceptor blockade, mucus secretion from submucosal glands induced either by capsaicin or by electrical field stimulation is due primarily to activation of capsaicin-sensitive sensory nerves and can be demonstrated *in vitro* and *in vivo* in a number of animal species including cat and ferret [42–44, 98, 99], preparations where the secretion is principally from the glands. In human bronchi *in vitro*, capsaicin induced an increase in mucus secretion [100], whereas the mucus secretion induced by electrical field stimulation is completely blocked by atropine, indicating that under these circumstances only cholinergic mucus secretion is present [43]. However, the increased mucus secretion in response to activation of sensory-efferent nerves is due to release of sensory neuropeptides, including SP, which may induce mucus release by activating cholinergic nerves [101–103]. Blockade of this neural pathway by atropine may, therefore, reduce the apparent effect of these sensory-efferent nerves on mucus secretion. In addition, release of VIP or NO from cholinergic nerves in response to electrical field stimulation may have inhibited the small stimulatory effects of sensory efferent-induced mucus secretion.

The tachykinins (SP and NKA) and CGRP are the principal sensory neuropeptides released from sensory-efferent nerves. CGRP is unlikely to be an important endogenous mediator of secretion, because it only weakly affects mucus secretion from submucosal glands [104]. However, CGRP may regulate the volume of respiratory tract fluid by inhibiting SP-induced mucus secretion [104]. Galanin and somatostatin are also capable of inhibiting mucus secretion stimulated by substance P [105] and by NKA and NKB [106]. The tachykinins appear to be the more likely candidates as the principal neurotransmitters mediating sensory-efferent airway submucosal gland mucus secretion. For example, SP potently induces submucosal gland secretion, either by causing myoepithelial cell contraction, which squeezes mucus out of the gland ducts [101, 102], or by direct induction of cellular secretion, predominantly from serous acini [103]. Both mechanisms may be mediated via cholinergic nerve activation. SP and NKA-induced increases in mucus secretion are associated with increases in intracellular Ca^{2+} [50, 107].

5.6. Tachykinins

Three classes of tachykinin receptor are currently recognised, denoted as NK_1, NK_2 and NK_3, which exhibit preferential affinity for SP, NKA and NKB, respectively. The order of potency of SP > NKA > NKB in inducing mucus secretion *in vitro* by human bronchi [108], cat trachea [109] and rat submucosal gland cells [106] indicates that the tachykinin receptor mediating mucus secretion is of the NK_1 type. Similar potency comparison studies indicate that the NK_1 receptor is the principal mediator of tachykinin-induced transepithelial flux of Na^+ and Cl^- [110]. More recent studies using selective tachykinin agonists [111, 112], or antagonist characterisation of neurogenic secretion [95], support the contention that it is the NK_1 receptor which mediates the tachykinin component of sensory-efferent neurogenic mucus secretion by airway submucosal glands. In contrast to the studies above, agonist studies show that in the isolated cat tracheal submucosal gland the NK_2 receptor mediates the mucus secretory response [107]. Although isolation of the gland may limit the "physiological" relevance of this observation, it is an interesting finding which merits further examination.

5.7. Reflexes

Inhalation of inert dusts through a tracheostomy in the cat induces tracheal mucus secretion via the combination of a direct local action on the secretory cells and a vagal reflex [113]. Similarly, irritation of the nasal passages, nasopharynx or larynx induces tracheal secretion via vagal (parasympathetic) and sympathetic motor pathways [114, 115]. Reflex gland secretion is linked to the cough reflex [114], which is consistent with the concept that it is more easy to expel irritants trapped in a large quantity of mucus. Similarly, in the cat, irritation of the stomach induces airway mucus secretion via a vagal reflex [116], presumably as a protection against potential aspiration of stomach fluids after vomiting. Thus, in the short term, increased production of respiratory tract fluid is a normal physiological response by the airways which is beneficial to the organism. However, when production is increased to a level whereby clearance mechanism are unable to cope, then the stage is set for respiratory tract fluid to lose its protective functions and to contribute to bronchial disease.

6. Inflammatory Mediators

A range of inflammatory cells produce mediators which will increase secretion by the airways (Figure 5). In many cases, the source of secretion is unspecified as either goblet cell, submucosal gland or both, for example for the effect of anaphylatoxins [117]. In the examples below, the source of secretion is principally the submucosal glands.

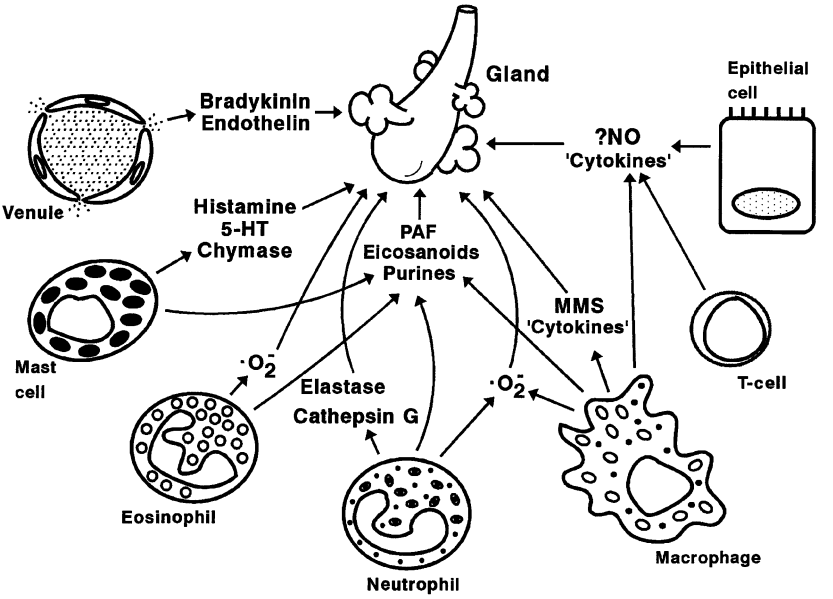


Figure 5. Inflammatory mediators and airway submucosal gland secretion. A wide variety of products from inflammatory cell and other cell types induce mucus secretion by submucosal glands. 5-HT, 5-hydroxytryptamine (serotonin); PAF, platelet activating factor; MMS, macrophage-/monocyte-derived mucous secretagogue; "cytokines", certain cytokines (for example TNF_α and IL-1_β) which have been shown recently to induce mucus secretion in mixed-cell population secretory systems, may be found to act on submucosal glands. The figure is simplified in that other mediators may be involved, and it gives no indication of the network of interactions between the different cell types.

6.1. Mast Cell-Derived Amines

Histamine and 5-hydroxytryptamine (5-HT, serotonin) are mast cell-derived amines which may affect airway submucosal gland secretion. The effects of histamine on secretion are variable and may be dependent upon species and preparation. In dog tracheal explants, histamine had no effect on mucus secretion [48], whereas in cat trachea *in vivo* it caused mucus secretion when administered directly into the tracheal segment but not when given as an aerosol [118]. In human explants, histamine causes mucus secretion via an action on H_2 receptors [119], whereas it weakly stimulates electrolyte secretion in dog trachea *in vitro* via H_1 receptors [120]. In bovine tracheal submucosal gland slices, activation of H_1 receptors induces IP_3 accumulation [51]. In contrast to histamine, 5-HT has no direct effect on mucus secretion but potentiates neural stimulation of hillock formation in canine trachea *in vivo* [121].

6.2. Bioactive Lipid Mediators

Leukotrienes (LT), prostaglandins (PG) and the hydroxyeicosatetraenoic acids (HETE) have comparatively weak and often contradictory effects on mucus secretion. Studies have shown PGE_2 to either decrease [122] or increase mucus secretion on human bronchi *in vitro* [123]. In dog trachea *in vivo* $PGF_{2\alpha}$ increases mucus secretion, whereas PGE_2 decreases secretion [124]. The difference in effect may lie in the technique used or in the different length of sampling times, although there is no altogether satisfactory explanation. Similarly, in human airways *in vitro* the order of potency in inducing secretion was LT > HETE > PG [32], whereas in cat trachea *in vivo* the order was PG > LT [125]. Again, the precise reasons for the different data are unclear but may be related to species, disease state of the human tissue or to the technique used. The PG used and its interaction with other secretory mechanisms, for example by altering cholinomimetic-induced submucosal gland secretion [126], and the possibility of LT receptor subtypes may contribute to the differences observed. In human bronchial explants, time course studies and the lack of lysozyme in the secretions indicate that LTC_4 and LTD_4 induce secretion from mucous cells in the submucosal glands [127]. Both PG's and LT's increase Cl^- secretion [128, 129].

Platelet activating factor (PAF) induces mucus secretion by cat isolated tracheal submucosal glands, but only in the presence of platelets [130]. This observation is consistent with the apparently weak effect of PAF alone *in vitro* in inducing mucus secretion by airway tissue segments from species with abundant submucosal glands, including human and cat [131, 132]. The weak direct effect of PAF on mucus secretion by human bronchi *in vitro* is also consistent with the relative lack of PAF receptors in the airways in general, including submucosal glands [133]. One anomaly is the weak effect of PAF in the ferret trachea *in vivo* [134], a preparation with abundant submucosal gland and available platelets. However, the dose of PAF used in the latter study (0.5 mg/kg) induced marked systemic affects which may have indirectly attenuated the secretory response. The effects of PAF are mediated via secondary release of thromboxanes [130] and lipoxygenase products [132].

6.3. Bradykinin

Autoradiographic localisation in human airways demonstrates less dense binding for bradykinin receptors over submucosal glands and other airway structures than for blood vessels [135]. However, bradykinin and related kinins may increase the volume of respiratory tract fluid by stimulating mucus and Cl^- secretion [136, 137].

6.4. Endothelin

The very low binding in human bronchi of ^{125}I-endothelin to submucosal glands [138], compared with significant binding to smooth muscle, alveoli and blood vessels, is consistent with the low potency of endothelin (ET) in inducing mucus secretion from isolated tracheal submucosal glands of the cat [139]. Similarly, ET-1 hat no effect on baseline secretion in the ferret whole trachea preparation, but inhibited methacholine- or phenylephrine-induced secretion from serous and mucous acini [140]. Inhibition may be due in part to activation of dihydropyridine-sensitive Ca^{2+} channels.

6.5. Reactive Oxygen Species

Reactive oxygen species are normal radical reactions in cellular homeostasis. Hydrogen peroxide (H_2O_2) weakly increases submucosal gland secretion in the ferret trachea *in vitro* [141]. Interestingly, in the latter study, H_2O_2 regulated the magnitude of glandular serous cell secretion induced by methacholine or phenylephrine and, as such, is one of the few molecules to inhibit secretion.

6.6. Macrophage-Monocyte-Derived Mucus Secretagogue

Macrophage-Monocyte-derived mucus secretagogue (MMS) oligopeptide is produced by the leucocytes in response to stimulation by opsonized zymosan, lipopolysaccharide or protein A-containing *Staphylococcus aureus* and induces comparatively small increases above controls (~40%) in mucus secretion by cultured human airways [142–144]. Thus, activated leukocytes produce a mediator(s) capable of inducing some degree of mucus secretion in human airway tissue. However, it is the proteinases produced by a number of inflammatory cells types which induce the most impressive increases in mucus secretion.

6.7. Proteinases

Proteinases are the most potent of the inflammatory cell products in stimulating mucus secretion in the airways, both by goblet cells [145] and by preparations in which the primary source of mucus is the submucosal glands, including the cat trachea *in vivo* and human bronchi *in vitro* [146]. The most impressive increases in secretion of macromolecules (predominantly chondroitin sulphate proteoglycan) are of over 1500% above controls induced in cultured bovine serous gland cells by dog mast cell chymase and human neutrophil elastase or cathepsin G [147, 148]. The responses were over 10-fold

greater than those to histamine or isoproterenol, were not cytotoxic, required catalytically active enzyme and could be blocked by a specific elastase inhibitor [149]. Proteinases also induce *in vivo* mucin release from cat trachea, as well as from human bronchi *in vitro*, preparations in which the source of the mucus is thought primarily to be glandular in origin [146]. They also stimulate secretion by cultured bovine tracheal gland cells [150].

6.8. Purine Nucleotides

Extracellular purine nucleotides, in particular ATP, are well known to induce mucus secretion by goblet cells [145] and to regulate transepithelial ion transport by human airways [151, 152]. The airway actions of the purines are via interaction with P_2 purinoceptors. In isolated cat tracheal submucosal glands, activation of these same receptors is associated with a rise in intracellular Ca^{2+} concentrations with concomitant marked increases in electrolyte and mucus secretion [153]. Thus, purine nucleotides increase liquid secretion into the airways.

7. Submucosal Glands and Bronchial Disease

The morbidity and mortality of a number of bronchial diseases are associated with abnormalities in airway surface liquid. The liquid may be increased in volume, or its composition may be changed, for example by an increase in mucus secretion at the expense of serous secretion. There may also be changes in the physiochemical properties of the fluid, for example by a change from a more neutral to a more acidic mucin. Changes in airway submucosal glands may contribute to the pathophysiology of bronchial diseases associated with mucus hypersecretion, including chronic bronchitis, asthma and cystic fibrosis (CF). In chronic bronchitis, the chronic coughing and sputum production which define the condition [154] are associated with mucus hypersecretion.

In asthma, the airways can become plugged with "mucus" comprising secreted mucins, plasma exudate and inflammatory cells [155, 156]. In CF, Although there may be bronchial mucus hypersecretion, it is the genetic abnormality in electrolyte transport with the consequent production of viscous dehydrated mucus, and the devastating lung infection, which are possibly of greater relevance in pathophysiology [157]. Nevertheless, the localisation of CFTR to the submucosal glands [19] indicates that the airway glands contribute to pathophysiology in CF.

Depending upon how much is present, increased liquid in the airway lumen may not significantly affect airflow. Larger quantities may still not affect airflow but may induce cough, which contributes markedly to patient morbidity. In the asthmatic patient, however, a small increase in luminal mucus could markedly potentiate the increase in airflow resistance induced by broncho-

constriction. Normal reflexes may also become pathophysiologic. For example, in the dog, hypoxaemia triggers a carotid body reflex which stimulates more tracheal submucosal glands to secrete [158], possibly as an adjunct to coughing out an obstruction by lubricating the airway. If this reflex is also present in patients with restricted airflow, and consequent hypoxia, it may exacerbate the symptoms of chronic bronchitis, asthma or cystic fibrosis by increasing the volume of luminal fluid.

7.1. Gland Hypertrophy

Enlargement of the submucosal glands (hypertrophy) can be demonstrated in the airways of patients with chronic bronchitis [159, 160] and asthma [156, 161], and in many patients with CF [162, 163] (Figure 6). In healthy humans, the volume of gland to goblet cells has been calculated as 40 : 1 [159]. Whether or not this ratio changes in disease is debatable, because although the glands undergo hypertrophy, the goblet cells increase in number (by a combination of hyperplasia and metaplasia; see Chapter 10 of this volume). Nevertheless, because the glands appear to occupy a greater volume of the airway than do the goblet cells, gland secretion is often considered to make the greater contribution to the mucus component of airway surface liquid. However, glands are generally confined to more proximal cartilaginous airways from which excessive secretions may be cleared by cough. Thus, it is may be considered that secretions from goblet cells in small airways, from which excessive secre-

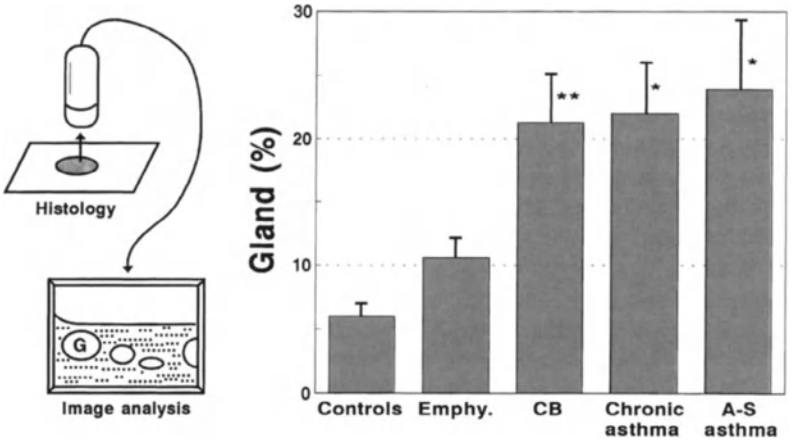


Figure 6. Submucosal gland hypertrophy in bronchial hypersecretory disease. Submucosal gland area in central airways of hospital patients dying of emphysema (Emphy.), chronic bronchitis (CB), chronic asthma or acute-servere (A-S) asthma was digitised and computer-analysed into a percentage of the airway wall.* $P < 0.05$, ** $P < 0.01$ compared with gland area in control subjects dying of causes other than lung disease. Adapted and redrawn after [160, 161].

tions are less easily cleared, may assume an important role in the pathophysiology of bronchial hypersecretion. However, the relative contribution to airway mucus hypersecretion of goblet cell and submucosal gland secretion is speculative and awaits formal experimentation, which in turn is dependent upon the discovery of selective mucus markers for goblet cell compared with gland secretion.

7.2. Physiology and Pharmacology of Hypertrophied Glands

In association with the increase in size of the submucosal glands in bronchial hypersecretory diseases, there are changes in the physiology and pharmacology of the hypertrophied glands. For example, precursor uptake, glycoprotein synthesis and mucus secretion occur at a higher rate in bronchial explants from patients with chronic bronchitis than in explants from patients without bronchitis [164]. Histological evidence suggests that mucous secretion is greater in the airways of cigarette smokers [165]. In addition, there is evidence not only that basal secretion is increase in chronic bronchitis but also that the response to stimuli is exaggerated. The increased secretion *in vitro* to acetylcholine by human airway tissue containing hypertrophied glands compared with normal-sized glands is less effectively blocked by atropine [166], which may explain in part the relatively poor response to anticholinergic therapy by patients with chronic hypersecretion [167]. VIP inhibits mucus secretion from normal human airways *in vitro* but has little or no effect on the airways of patients with chronic bronchitis [91]. A number of effects of cigarette smoke may explain the reduced activity of VIP, for example degradation of VIP by proteases produced by inflammatory cells recruited into the airways by cigarette smoke [168, 169]. These same proteases may themselves potently induce mucus secretion (see Section 6.7 above). Cigarette smoke induces mucus secretion from submucosal glands via nicotinic stimulation of ganglia and activation of cholinergic nerves [170]. Air pollution, respiratory infection and oxidants in cigarette smoke degrade neutral endopeptidase (NEP), which may result in the uncontrolled activity of neuropeptides such as SP and in hypersecretion from submucosal glands. The oxidants may also induce a degree of submucosal gland mucus secretion. In guinea pig trachea, NEP activity, demonstrated histochemically, is most prominent in the epithelium but is also localised to submucosal glands [171]. A functional role for NEP can be demonstrated by inhibiting its degradative activity, which potentiates a peptide-mediated response. For example, SP-induced mucus secretion *in vitro* in ferret trachea or in human bronchi is potentiated by phosphoramidon, a specific NEP inhibitor [108, 172]. In CF, bronchial segments obtained from CF patients appeared to be hyporesponsive towards stimulation by a cholinergic agonist as well as to substance P, which suggests that autonomic control of airway mucus secretion may be defective in CF patients [173]. Similarly, cultured glandular cells from CF airways showed a lack of responsiveness, as

assessed by their secretion of serous secretory markers after stimulation with a range of agonists [174].

8. Therapeutic Directions

General drug treatment for bronchial hypersecretion is discussed in Chapter 15 of this volume, with specific therapeutic approaches in the lung and problems in CF discussed in Chapter 14. The discussion below will, therefore, be limited to novel therapeutic approaches to hypersecretion by airway submucosal glands (Figure 7).

Most neural and humoral mechanisms induce secretory processes which will increase the volume of airway surface liquid in the airway lumen. In the short term it is probably inadvisable to inhibit secretion, because it is invariably a protective response by the airway to environmental insult or physiological stress. In the long term, suppression or rapid reversal of chronic airway hypersecretion may be a desirable goal. Experimental studies to date have concentrated upon models of irritant-induced goblet cell hyperplasia rather than submucosal gland hypertrophy. For example, in the rat model of chronic bronchitis (which may also act as a model of the "bronchitic" component of

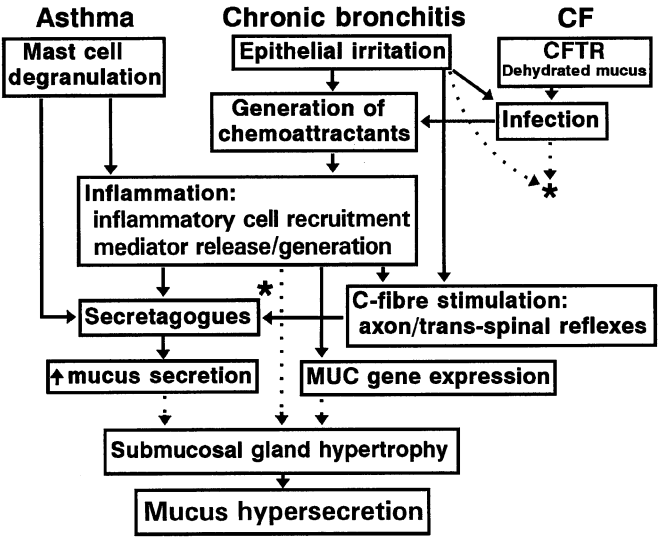


Figure 7. Hypothetical "flow chart" of the pathogenesis of submucosal gland hypertrophy in mucus hypersecretory airway disease. CF = cystic fibrosis; CFTR = CF transmembrane conductance regulator (abnormal functioning in CF transepithelial Cl^- secretion is considered to contribute to dehydration of airway mucus). Solid lines = experimental evidence available; broken lines = hypothetical scenarios. * Certain epithelially derived mediators and bacterial products can induce mucus secretion directly.

asthma), goblet cell hyperplasia induced by repeated exposure to irritants is inhibited by a range of non-steroidal and steroidal anti-inflammatory drugs, "mucoregulatory" compounds and flavenoids [145]. More important, however, after cessation of exposure to the irritant, anti-inflammatory agents speed reversal of cigarette smoke-induced goblet cell hyperplasia. It is obviously of interest to determine whether there is concomitant inhibition of submucosal gland hypertrophy in these models, and formal studies are obviously required. There are, however, indications that these drugs will affect the glands. For example, corticosteroid pretreatment for up to 24 h inhibits mucus hypersecretion by cultured airways from patients with bronchorrhea [142], whilst oral corticosteroids speed clearance of radiolabelled beads from the peripheral airways of asthmatics [175]. Taken together, the studies above indicate that reducing the inflammation associated with bronchial hypersecretory disease may prove beneficial in treatment and may explain, at least in part, the effectiveness of corticosteroids in the treatment of asthma [176, 177]. In addition, corticosteroids induce neutral endopeptidase gene expression in human tracheal epithelial cells [178], which may redress the balance of loss of NEP induced by oxidant and other damage.

Experimental studies also indicate a number of novel therapeutic options for suppressing airway hypersecretion. For example, potent and selective receptor antagonists have been invaluable in elucidating the contribution of different mediators and neural pathways in physiology and pathophysiology. The further development of, for example tachykinin antagonists, will allow the role of sensory neuropeptides in chronic bronchitis and asthma to be assessed [179]. However, it is unlikely that any one inflammatory or neurotransmitter will be responsibly solely for pathogenesis, and the more selective an antagonist, the less likely it may be useful in therapy. The development of antagonists which are potent and selective for more than one receptor is an interesting development. FK224 is a dual antagonist at tachykinin NK_1 and NK_2 receptors [180] and would, therefore, be expected to inhibit both secretory (i.e. NK_1-mediated) and contractile (i.e. NK_2-mediated) responses to sensory nerve activation. Thus, in contrast to a selective NK_1 receptor antagonist (CP-99,994) which has no inhibitory effect on hypertonic saline-induced bronchoconstriction or cough in asthmatic patients [181], FK224 inhibits bradykinin-induced bronchoconstriction and cough in asthmatic patients [182]. The airway responses to both the hypertonic saline and bradykinin are due in part to activation of sensory neural pathways. However, release of multiple mediators or neurotransmitters may confound even multiple receptor antagonists. A range of agonists inhibit prejunctional release of neurotransmitters and consequently inhibit a range of neurogenic airway responses, including mucus secretion [183]. There is now evidence that the final common pathway for the inhibition is via opening of large-conductance Ca^{2+}-activated potassium (K^+) channels (BK_{Ca} or maxi-K channels) [184]. Drugs targeted at opening the BK_{Ca} channel may, therefore, have a broad spectrum of inhibitory activity in the airways. One BK_{Ca}-opening drug, NS 1619, inhibits neurogenic

mucus secretion in ferret trachea *in vitro* [185]. Interestingly, activators of other K^+ channels (most probably of the ATP-sensitive type, K_{ATP} channels) inhibit neuromimetic-stimulated submucosal gland serous cell secretion *in vitro* in ferret trachea [62].

9. Conclusions

Neural and humoral influences on submucosal glands affect the volume and composition of airway surface liquid by changing the rates of mucus secretion and of electrolyte and fluid flux into the lumen. In the short term, production of an increased volume of airway liquid is a normal physiological response to environmental insult or physiological stress. Chronic overproduction of airway surface liquid as a component of a disease process will affect the calibre of the airways. However, even in disease, it is unlikely that a change in one component of the secretory system is important in pathophysiology, but rather that a combination of changes contributes to morbidity and mortality.

Experimental studies in human airways and extrapolation from data in other animals identify a number of specific areas in the pharmaco-physiological control of submucosal gland secretion which may contribute to the pathophysiology of certain bronchial diseases associated with hypersecretion, including chronic bronchitis, asthma, and possibly CF. Advances in understanding the relationship between the different processes that form airway surface liquid will eventually identify those changes in composition which are more relevant to normal physiology and in disease. This should lead to identification of signal transduction mechanisms and genomic control mechanisms specific for mucus secreting apparatus. Advances in techniques of mucus biochemistry will eventually identify whether there are relevant differences in the mucins from different cellular sources (goblet cells and gland cells) and between diseases. The identification of different mucins and the genes which encode them has allowed experimental studies which have demonstrated that mucin gene expression is increased in response to airway infection and irritation [186]. Studies on mucin gene expression to determine their involvement in the early stages of development of the hypersecretory state may indicate novel avenues for therapy at the nuclear level. New research into mucin genes, signal transduction mechanisms, receptor identification and mucin biochemistry should, in the future, increase our understanding of the processes underlying the production of airway surface liquid, which in turn should indicate more rational directions for therapy.

Acknowledgements

DFR thanks the Cystic Fibrosis Research Trust, the National Asthma Campaign, Pfizer Central Research, the Clinical Research Committee of Royal Brompton Hospital and A. Menarini Pharmaceuticals (Florence, Italy) for support over the years.

References

1. Nathanson I, Nadel J (1984) Movement of electrolytes and fluid across airways. *Lung* 162: 125–137.
2. Meyrick B, Sturgess JM, Reid L (1969) A reconstruction of the human bronchial gland. *Thorax* 24: 729–736.
3. Reid L (1977) Secretory cells. *Fed Proc* 36: 2703–2707.
4. Whimster WF, Lord P, Biles B (1984) Tracheobronchial gland profiles in four segmental airways. *Am Rev Respir Dis* 129: 985–988.
5. Goco RV, Kress MB, Branigan OC (1963) Comparison of mucus glands in the tracheal bronchial tree of man and animals. *Ann NY Acad Sci* 106: 555.
6. Gatto LA, Aiello E (1981) Mucus-secreting glands and goblet cells in the trachea of the deer mouse *Peromyscus leucopus*. *Trans Am Microsc Soc* 100: 355–365.
7. Breeze RG, Weeldon EB (1977) The cells of the pulmonary airways. *Am Rev Respir Dis* 116: 705–777.
8. Jeffery PK (1983) Morphologic features of airway surface epithelial cells and glands. *Am Rev Respir Dis* 128: S14–20.
9. Finkbeiner WE, Shen B-Q, Widdicombe JH (1994) Chloride secretion and function of serous and mucous cells of human airway glands. *Am J Physiol* 267: L206–L210.
10. Emery N, Place GA, Dodd S, Lhermitte M, David G, Lamblin G, Perini JM, Page AM, Hall RL, Roussel P (1995) Mucous and serous secretions of human bronchial epithelial cells in secondary culture. *Am J Respir Cell Mol Biol* 12: 130–141.
11. Thornton DJ, Davies JR, Kraayenbrink M, Richardson PS, Sheehan JK, Carlstedt I (1990) Mucus glycoproteins from "normal" human tracheobronchial secretion. *Biochem J* 265: 179–186.
12. Basbaum CB, Jany B, Finkbeiner WE (1990) The serous cell. *Annu Rev Physiol* 52: 97–113.
13. Finkbeiner WE, Nadel JA, Basbaum CB (1986) Establishment and characterisation of a cell line derived from bovine tracheal glands. *In Vitro* 22: 561–567.
14. Tom-Moy M, Basbaum CB, Nadel JA (1983) Localization and release of lysozyme from ferret trachea: effects of adrenergic and cholinergic drugs. *Cell Tissue Res* 228: 549–562.
15. Bowes D, Corrin B (1977) Ultrastructural immunocytochemical localisation of lysozyme in human bronchial glands. *Thorax* 32: 163–170.
16. Richardson PS, Fung DCK (1992) Mucus and mucus secreting cells in asthma. In: PJ Barnes, IW Rodger, NC Thomson (eds). *Asthma: Basic mechanisms and clinical management.* London: Academic Press: 157–190.
17. Riordan JR (1993) The cystic fibrosis transmembrane conductance regulator. *Annu Rev Physiol* 55: 609–630.
18. Anderson MP, Gregory R, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ (1991) Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253: 203–206.
19. Engelhardt JF, Yankaskas JR, Ernst SA, Yang Y, Marino CR, Boucher RC et al. (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genet* 2: 240–248.
20. Nadel JA, Borson DB, Basbaum CB, Widdicombe JH (1984) Technique for studying airway mucus secretion, ion transport and water movement. In: Otis AB, ed. *Techniques in the Life Sciences, Respiratory Physiology.* Shannon: Elsevier Scientific Publishers Ireland Ltd. 1–35.
21. Davies JR, Corbishley CM, Richardson PS (1990) The uptake of radiolabelled precursors of mucus glycoconjugates by secretory tissues in the feline trachea. *J Physiol* 420: 19–30.
22. Rogers DF, Godfrey RWA, Majumbdar S, Jeffery PJ (1988) Oral *N*-acetylcysteine speeds reversal of cigarette smoke-induced mucous cell hyperplasia in the rat. *Exp Lung Res* 14: 19–35.
23. Shimura S, Sasaki T, Sasaki H, Takishima T (1986) Contractility of isolated single submucosal gland from trachea. *J Appl Physiol* 60: 1237–1247.
24. Jacob S, Poddar S (1982) Mucous cells of the tracheobronchial tree in the ferret. *Histochemistry* 73: 599–605.
25. Robinson NP, Venning L, Kyle H, Widdicombe JG (1986) Quantitation of the secretory cells of the ferret tracheobronchial tree. *J Anat* 145: 173–188.
26. Webber SE, Widdicombe JG (1987) The effect of vasoactive intestinal peptide on smooth muscle tone and mucus secretion from the ferret trachea. *Br J Pharmacol* 91: 139–148.

27. Webber SE (1988) The effects of peptide histidine isoleucine and neuropeptide Y on mucus volume output from the ferret trachea. *Br J Pharmacol* 95: 49–54.
28. Ueki I, German VF, Nadel JA (1980) Micropipette measurement of airway submucosal gland secretion: autonomic effects. *Am Rev Respir Dis* 121: 351–357.
29. Quinton PM (1979) Composition and control of secretions from tracheal bronchial submucosal glands. *Nature* 279: 551–552.
30. Davies JR, Richardson PS (1989) Dense macromolecules in airway mucus from the anaesthetised cat: An artefact of the liquid-filled trachea? *J Physiol* 409: 45P.
31. Somerville M, Karlsson JA, Richardson PS (1990) The effects of local anaesthetic agents upon mucus secretion in the feline trachea *in vivo*. *Pulm Pharmacol* 3: 93–101.
32. Shelhamer JH, Marom Z, Kaliner M (1982) The effects of arachinoids and leukotrienes on the release of mucus from human airways. *Chest* 81: S36–S37.
33. Coles SJ, Reid L (1981) Inhibition of glycoconjugate secretion by colchicine cytochalasin B: An *in vitro* study of human airways. *Cell Tissue Res* 214: 107–118.
34. Davies JR, Gallagher JT, Richardson PS, Sheehan JK, Carlstedt I (1991) Mucins in cat airway secretions. *Biochem J* 275: 663–669.
35. Varsano S, Basbaum CB, Forsberg LS, Borson DB, Caughey G, Nadel JA (1987) Dog tracheal epithelial cells in culture synthesize sulfated macromolecular glycoconjugates and release them from the cell surface upon exposure to extracellular proteinases. *Exp Lung Res* 13: 157–184.
36. De Sanctis GT, Rubin BK, Ramirez O, King M (1993) Ferret tracheal mucus rheology, clearability and volume following administration of substance P or methacholine. *Eur Respir J* 6: 76–82.
37. King M (1987) Role of mucus viscoelasticity in cough clearance. *Biorheology* 24: 589–587.
38. Laitinen A, Partanen M, Hervonen A, Laitinen LA (1985) Electron microscopic study on the innervation of the human lower respiratory tract: Evidence of adrenergic nerves. *Eur J Respir Dis* 67: 209–215.
39. Pack RJ, Richardson PS (1984) The aminergic innervation of the human bronchus: A light and electron microscopic study. *J Anat* 138: 493–502.
40. Lundberg JM, Hokfelt T, Martling CR, Saria A, Cuello C (1984) Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell Tissus Res* 235: 251–261.
41. Mak JC, PJ Barnes (1990) Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. *Am Rev Respir Dis* 141: 1559–1568.
42. Fung DC, Beacock DJ, Richardson PS (1992) Vagal control of mucus glycoconjugate secretion into the feline trachea. *J Physiol* 453: 435–447.
43. Baker B, Peatfield AC, Richardson PS (1985) Nervous control of mucin secretion into human bronchi. *J Physiol* 365: 297–305.
44. Borson DB, Charlin M, Gold BD, Nadel JA (1984) Neural regulation of $^{35}SO_4$-macromolecule secretion from tracheal glands of ferrets. *J Appl Physiol* 57: 457–466.
45. Webber SE, Widdicombe JG (1987) The actions of methacholine, phenylephrine, salbutamol and histamine on mucus secretion from the ferret *in vitro* trachea. *Agents Actions* 22: 82–85.
46. Florey H, Carlton H, Wells SA (1932) Mucus secretion in the trachea. *Br J Exp Pathol* 13: 269–284.
47. Gallagher JT, Kent PW, Passatore M, Phipps RJ, Richardson PS (1975) The composition of tracheal mucus and the nervous control of its secretion in the cat. *Proc R Soc* (Biol) 192: 49–76.
48. Charkrin L, Baker AP, Christian P, Wardell J (1973) Effect of cholinergic stimulation on the release of macromolecules by canine trachea *in vitro*. *Am Rev Respir Dis* 108: 69–76.
49. Dwyer TM, Szebenzi A, Diveki K, Farley JM (1992) Transient cholinergic glycoconjugate secretion from swine tracheal submucosal gland cells. *Am J Physiol* 262: L418–426.
50. Ishihara H, Shimura S, Sato M, Masuda T, Ishide N, Miura M, Sasaki T, Sasaki H, Takishima T (1990) Intracellular calcium concentration of acinar cells in feline tracheal submucosal glands. *Am J Physiol* 259: L345–L350.
51. Hall IP (1992) Agonist-induced inositol phosphate responses in bovine airway submucosal glands. *Am J Physiol* 262: L257–L262.
52. Barnes PJ (1993) Muscarinic receptor subtypes in the airways. *Life Sci* 52: 521–527.
53. Gater PR, Alabaster VA, Piper I (1989) A study of the muscarinic receptor subtype mediating mucus secretion in the cat trachea *in vitro*. *Pulm Pharmacol* 2: 87–92.

54. Ishihara H, Shimura S, Satoh M, Masuda T, Nonaka H, Kase H, Sasaki T, Sasaki H, Takishima T, Tamura K (1992) Muscarinic receptor subtypes in feline tracheal submucosal gland secretion. *Am J Physiol* 262: L223–228.
55. Yang CM, Farley JM, Dwyer TM (1988) Muscarinic stimulation of submucosal glands in swine trachea. *J Appl Physiol* 64: 200–209.
56. Ramnarine SI, Haddad E-B, Khawaja AM, Mak JCW, Rogers DF (1996) On muscarinic control of neurogenic mucus secretion in ferret trachea. *J Physiol* 494: 577–586.
57. Corrales RJ, Coleman DL, Jacoby DB, Leikauf GD, Hahn HL, Nadel JA, Widdicombe JH (1986) Ion transport across cat and ferret tracheal epithelia. *J Appl Physiol* 61: 1065–1070.
58. Knowles M, Murray G, Shallal J, Askin F, Ranga V, Gatzy J, Boucher R (1984) Bioelectric properties and ion flow across excised human bronchi. *J Appl Physiol* 56: 868–877.
59. Jarnigan F, Davis JD, Bromberg PA, Gatzy JT, Boucher RC (1983) Bioelectric properties and ion transport of excised rabbit trachea. *J Appl Physiol* 55: 1884–1892.
60. Sasaki T, Shimura S, Ikeda K, Sasaki H, Takishima T (1990) Sodium efflux from isolated submucosal gland in feline trachea. *Am J Physiol* 258: L112A–L117.
61. Sasaki TA, Shimura S, Wakui M, Ohkawara Y, Takishima T, Mikoshiba K (1994) Apically localized IP_3 receptors control chloride current in airway gland acinar cells. *Am J Physiol* 267: L152–L158.
62. Griffin A, Newman TM, Scott RH (1996) Electrophysiological and ultrastructural events evoked by methacholine and intracellular photolysis of caged compounds in cultured ovine trachea submucosal gland cells. *Exp Physiol* 81: 27–43.
63. Phipps RJ, Williams IP, Richardson PS, Pell J, Pack RH, Wright N (1982) Sympathomimetic drugs stimulate the output of secretory glycoproteins from human bronchi *in vitro*. *Clin Sci* 63: 23–28.
64. Peatfield AC, Richardson PS (1982) The control of mucin secretion into the lumen of the cat trachea by alpha-beta-adrenoceptors, and their relative involvement during sympathetic nerve stimulation. *Eur J Pharmacol* 81: 617–626.
65. Culp DJ, McBride RK, Graham LA, Marin MG (1990) Alpha-adrenergic regulation of secretion by tracheal glands. *Am J Physiol* 259: L198–L205.
66. Liedtke CM, Rudolph SA, Boat TF (1983) Beta-adrenergic modulation of mucin secretion in cat trachea. *Am J Physiol* 244: C391–C398.
67. Shimura S, Ishihara M, Nagaki M, Sasaki H, Takishima T (1993) A stimulatory role of protein kinase C in feline tracheal submucosal gland secretion. *Respir Physiol* 93: 239–247.
68. Paul A, Mergey M, Veissiere D, Hermelin B, Cherqui G, Picard J, Basbaum CB (1991) Regulation of secretion in cultured tracheal serous cells by protein kinases A and C. *Am J Physiol* 261: L172–L177.
69. Wagner U, Bredenbröker D, Fehmann H-C, Schwarz F, Schudt C, von Wichert P (1996) Effects of selective and non-selective phosphodiesterase inhibitors on tracheal mucus secretion in the rat. *Eur J Pharmacol* 298: 265–270.
70. Al Bazzaz FJ, Cheng E (1979) Effects of catecholamines on ion transport in dog tracheal epithelium. *J Appl Physiol* 47: 397–403.
71. Liedtke CM (1986) Interaction of epinephrine with isolated rabbit tracheal epithelial cells. *Am J Physiol* 251: C209–C215.
72. Welsh MJ (1986) Adrenergic regulation of ion transport by primary cultures of canine tracheal epithelium: Cellular electrophysiology. *J Membrane Biol* 91: 121–128.
73. Ramnarine SI, Rogers DF (1994) Non-adrenergic, non-cholinergic neural control of mucus secretion in the airways. *Pulm Pharmacol* 7: 19–33.
74. Cheung A, Polak JM, Bauer FE, Cadieux A, Christofides ND, Springall DR, Bloom SR (1985) Distribution of galanin immunoreactivity in the respiratory tract of pig, guinea pig, rat and dog. *Thorax* 40: 889–896.
75. Dey RD, Hoffpauir J, Said SI (1988) Co-localization of vasoactive intestinal peptide- and substance P-containing nerves in cat bronchi. *Neuroscience* 24: 275–281.
76. Laitinen A, Partanen M, Hervonen A, Peto-Juikko M, Laitinen LA (1985) VIP-like immunoreactive nerves in human respiratory tract: Light and electron microscopic study. *Histochemistry* 82: 313–319.
77. Lundberg JM, Fahrenkrug J, Hokfelt T, Martling CR, Larsson O, Tatemoto K, Anggard A (1984) Co-existence of peptide histidine isoleucine (PHI) and VIP in nerves regulating blood flow and bronchial smooth muscle tone in various mammals including man. *Peptides* 5: 593–606.

78. Ghatei MA, Springall DR, Richards IM, Oostveen JA, Griffin RL, Cadieux A, Polak JM, Bloom SR (1987) Regulatory peptides in the respiratory tract of Macaca fascicularis. *Thorax* 42: 431–439.
79. Barnes PJ, Belvisi MG (1993) Nitric oxide and lung disease. *Thorax* 48: 1034–1043.
80. Rand MJ (1992) Nitrergic transmission: Nitric oxide as a mediator of non-adrenergic, non-cholinergic neuroeffector transmission? *Clin Exp Pharmacol Physiol* 19: 147–169.
81. Sheppard MN, Polak JM, Allen JM, Bloom SR (1984) Neuropeptide tyrosine (NPY): A newly discovered peptide is present in the mammalian respiratory tract. *Thorax* 39: 326–330.
82. Holzer P (1991) Capsaicin: Cellular targets, mechanisms of action and selectivity for thin sensory neurons. *Pharmacol Rev* 43: 143–201.
83. Maggi CA, Meli A (1988) The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen Pharmacol* 19: 1–43.
84. Szolcsanyi J (1988) Antidromic vasodilatation and neurogenic inflammation. *Agents Actions* 23: 4–11.
85. Barnes PJ, Baraniuk JN, Belvisi MG (1991) Neuropeptides in the respiratory tract. Part I. *Am Rev Respir Dis* 144: 1187–1198.
86. Luts A, Sundler F (1989) Peptide-containing nerve fibers in the respiratory tract of the ferret. *Cell Tissue Res* 258: 259–267.
87. Lindh B, Lundberg JM, Hokfelt T (1989) NPY-, galanin-, VIP/PHI-, CGRP- and substance P-immunoreactive neuronal subpopulations in cat autonomic and sensory ganglia and their projections. *Cell Tissue Res* 256: 259–273.
88. Peatfield AC, Barnes PJ, Bratcher C, Nadel JA, Davis B (1983) Vasoactive intestinal peptide stimulates tracheal submucosal gland secretion in ferret. *Am Rev. Respir Dis* 128: 89–93.
89. Shimura S, Sasaki T, Ikeda K, Sasaki H, Takishima T (1988) VIP augments cholinergic-induced glycoconjugate secretion in tracheal submucosal glands. *J Appl. Physiol* 65: 2537–2544.
90. Nathanson I, Widdicombe JH, Barnes PJ (1983) Effect of vasoactive intestinal peptide on ion transport across dog tracheal epithelium. *J Appl Physiol* 55: 1844–1848.
91. Coles SJ, Said SI, Reid LM (1981) Inhibition by vasoactive intestinal peptide of glycoconjugate and lysozyme secretion by human airways *in vitro*. *Am Rev Respir Dis* 124: 531–536.
92. Liu Y-C, Khawaja AM, Rogers DF (1997) Neuromodulation by vasoactive intestinal peptide of cholinergic mucus secretion in ferret trachea *in vitro*. *Respir Med.* 91: A46.
93. Stretton CD, Belvisi MG, Barnes PJ (1991) Modulation of neural bronchoconstrictor responses in the guinea pig respiratory tract by vasoactive intestinal peptide. Neuropeptides 18: 149–157.
94. Ramnarine SI, Khawaja AM, Barnes PJ, Rogers DF (1996) Nitric oxide inhibition of basal and neurogenic mucus secretion in ferret trachea *in vitro*. *Br J Pharmacol* 118: 998–1002.
95. Ramnarine SI, Hirayama Y, Barnes PJ, Rogers DF (1994) "Sensory-efferent" neural control of mucus secretion: Characterization using tachykinin receptor antagonists in ferret trachea *in vitro*. *Br J Pharmacol* 113: 1183–1190.
96. Dey RD, Altemius JB, Rodd A, Mayer B, Said SI, Coburn RF (1996) Neurochemical characterisation of intrinsic neurons in ferret tracheal plexus. *Am J Respir Cell Mol Biol* 14: 207–216.
97. Daniels AJ, Matthews JE, Slepetis RJ, Jansen M, Viveros OH, Tadepalli A, Harrington W, Heyer D, Landavos A, Leban J et al. (1995) High-affinity neuropeptide Y receptor antagonists. *Proc Natl Acad Sci USA* 92: 9067–9071.
98. Fung DC, Allenby MI, Richardson PS (1992) NANC nerve pathways controlling mucus glycoconjugate secretion into feline trachea. *J Appl Physiol* 73: 625–630.
99. Peatfield AC, Richardson PS (1983) Evidence for non-cholinergic, non-adrenergic nervous control of mucus secretion into the cat trachea. *J Physiol* 342: 335–345.
100. Rogers DF, Barnes PJ (1989) Opioid inhibition of neurally mediated mucus secretion in human bronchi. *Lancet* 1: 930–932.
101. Coles SJ, Neill KH, Reif LM (1984) Potent stimulation of glycoprotein secretion in canine trachea by substance P. *J Appl Physiol* 57: 1323–1327.
102. Shimura S, Sasaki T, Okayama H, Sasaki H, Takishima T (1987) Effect of substance P on mucus secretion of isolated submucosal gland from feline trachea. *J Appl Physiol* 63: 646–653.
103. Gashi AA, Borson DB, Finkbeiner WE, Nadel JA, Basbaum CB (1986) Neuropeptides degranulate serous cells of ferret tracheal glands. *Am J Physiol* 251: C223–229.

104. Webber SE, Lim JC, Widdicombe JG (1991) The effects of calcitonin gene-related peptide on submucosal gland secretion and epithelial albumin transport in the ferret trachea *in vitro*. *Br J Pharmacol* 102: 79–84.
105. Wagner U, Fehman H-C, Bredenbröker D, Yu F, Barth PJ, von Wichert P (1995) Galanin and somatostatin inhibition of substance P-induced airway mucus secretion in the rat. *Neuropeptides* 28: 59–64.
106. Wagner U, Fehman HC, Bredenröker D, Yu F, Barth PJ, von Wichert P (1995) Galanin and somatostatin inhibition of neurokinin A and B induced airway mucus secretion in the rat. *Life Sci* 57: 283–289.
107. Nagaki M, Ishihara H, Shimura S, Sasaki T, Takishima T, Shirato K (1994) Tachykinins induce a $[Ca^{2+}]_i$ rise in the acinar cells of feline tracheal submucosal gland. *Respir Physiol* 98: 111–120.
108. Rogers DF, Aursudkij B, Barnes PJ (1989) Effects of tachykinins on mucus secretion in human bronchi *in vitro*. *Eur J Pharmacol* 174: 283–286.
109. Lundgren JD, Weiderman CJ, Logun C, Plutchok J, Kaliner M, Shelhamer JH (1989) Substance P receptor-mediated secretion of respiratory glycoconjugate from feline airways *in vitro*. *Exp Lung Res* 15: 17–29.
110. Mizoguchi H, Hicks CR (1989) Effects of neurokinins on ion transport and sulfate macromolecule release in the isolated ferret trachea. *Exp Lung Res* 15: 837–848.
111. Meini S, Mak JCW, Rohde JAL, Rogers DF (1993) Rachykinin control of ferret airways: Mucus secretion, bronchoconstriction and receptor mapping. *Neuropeptides* 24: 81–89.
112. Geppetti P, Bertrand C, Bacci E, Huber O, Nadel JA (1993) Characterisation of tachykinin receptors in ferret trachea by peptide agonists and nonpeptide antagonists. *Am J Physiol* 265: L164–L169.
113. Peatfield AC, Richardson PS (1983) The action of dust in the airways on secretion into the trachea of the cat. *J Physiol* 342: 327–334.
114. Phipps RJ, Richardson PS (1976) The effects of irritation at various levels of the airway upon tracheal mucus secretion in the cat. *J Physiol* 261: 563–581.
115. German VF, Ueki IF, Nadel JA (1980) Micropipette measurement of airway submucosal gland secretion: laryngeal reflex. *Am Rev Respir Dis* 122: 413A–416.
116. German VF, Corrales R, Ueki IF, Nadel JA (1982) Reflex stimulation of tracheal mucus gland secretion by gastric irritation in cats. *J Appl Physiol* 52: 1153–1155.
117. Marom Z, Shelhamer J, Berger M, Frank M, Kaliner M (1985) Anaphylatoxin C3a enhances mucous glycoprotein release from human airways *in vitro*. *J Exp Med* 161: 657–668.
118. Richardson PS, Phipps RJ, Balfre K, Hall RC (1978) The roles of mediators, irritants and allergens in causing mucin secretion from the trachea. In: Porter R, Rivers J, O'Connor M, eds. *Respiratory tract mucus*. Amsterdam: Ciba Foundation Symposium 54, New Series, 111–131.
119. Shelhamer JH, Marom Z, Kaliner M (1980) Immunologic and neuropharmacologic stimulation of mucous glycoprotein release from human airways *in vitro*. *J Clin Invest* 66: 1400–1408.
120. Marin MG, Davis B, Nadel JA (1977) Effect of histamine on electrical and ion transport properties of tracheal epithelium. *J Appl Physiol* 42: 735–738.
121. Popovac D, Chinn R, Graf P, Nadel J, Davis B (1979) Serotonin potentiates nervous stimulation of mucus gland secretion in canine trachea *in vivo*. *Physiologist* 22: 102 (abstract).
122. Rich B, Peatfield AC, Williams AC, Richardson PS (1984) Effects of prostaglandins E1, E2, F2 alpha on mucin secretion from human bronchi *in vitro*. *Thorax* 39: 420–423.
123. Marom Z, Shelhamer JH, Kaliner M (1981) Effects of arachidonic acid, monohydroxyeicosatetraenoic acid and prostaglandins on the release of mucus glycoproteins from human airways *in vitro*. *J Clin Invest* 67: 1695–1702.
124. Yamatake Y, Yanaura S (1978) New method for evaluating bronchomotor and bronchosecretory activities: Effects of prostaglandins and antigen. *Jap J Pharmacol* 28: 391–402.
125. Peatfield AC, Piper PJ, Richardson PS (1982) The effect of leukotriene C_4 on mucin release into the cat trachea *in vivo* and *in vitro*. *Br. J Pharmacol* 77: 391–393.
126. Deffebach ME, Islami H, Price A. Webber SE, Widdicombe JG (1990) Prostaglandins alter methacholine-induced secretion in ferret *in vitro* trachea. *Am J Physiol* 258: L75–L80.
127. Coles SJ, Neill KH, Reid LM, Austin KF, Nii Y, Corey EJ, Lewis RA (1983) Effect of leukotrienes C_4 and D_4 on glycoprotein and lysozyme secretion by human bronchial mucosa. *Prostaglandins* 25: 155–170.

128. Al-Bazazz F, Yadava VP, Westen-Felder C (1981) Modification of Na and Cl transport in canine tracheal mucosa by prostaglandins. *Am J Physiol* 240: F101–F105.
129. Leikauf GD, Ueki IF, Widdicombe JH, Nadel JA (1986) Alteration of chloride secretion across canine tracheal epithelium by lipoxygenase products of arachidonic acid. *Am J Physiol* 250: F47–F53.
130. Sasaki T, Shimura S, Ikeda K, Sasaki H, Takishima T (1989) Platelet-activating factor increases platelet-dependent glycoconjugate secretion from tracheal submucosal gland. *Am J Physiol* 257: L373–L378.
131. Rogers DF, Alton EWFW, Ausudkij B, Boschetto P, Dewar A, Barnes PJ (1990) Effect of platelet activating factor on formation and composition of airway fluid in the guinea-pig trachea. *J Physiol* 431: 643–658.
132. Lundgren JD, Kaliner M, Logun C, Shelhamer JH (1990) Platelet activating factor and tracheobronchial respiratory glycoconjugate release in feline and human explants: involvement of the lipoxygenase pathway. *Agent Actions* 30: 329–337.
133. Goldie RG, Pederson KE, Rigby PJ, Paterson JW (1990) PAF receptors in guinea-pig and human lung. *Agents Actions Suppl* 31: 243–246.
134. Lang M, Hansen D, Hahn HL (1987) Effects of the PAF-antagonist CV-3988 on PAF-induced changes in mucus secretion and in respiratory circulatory and variables in ferrets. *Agents Actions Suppl* 21: 245–252.
135. Mak JC, Barnes PJ (1991) Autoradiographic visualization of bradykinin receptors in human and guinea pig lung. *Eur J Pharmacol* 194: 37–43.
136. Baker AP, Hillegas LM, Holden DA, Smith WJ (1977) Effect of kallidin, substance P and other basic polypeptides on the production of respiratory macromolecules. *Am Rev Respir Dis* 115: 811–817.
137. Smith JJ, McCann JD, Welsh MJ (1990) Bradykinin stimulates airway epithelial Cl-secretion via two second messenger pathways. *Am J Physiol* 258: L369–L377.
138. Goldie RG, Henry PJ, Paterson JW, Preuss JMH, Rigby PJ (1990) Contractile effects and receptor distributions for endothelin-1 (ET-1) in human and animal airways. *Agents Actions Suppl* 31: 229–232.
139. Shimura S, Ishihara H, Satoh M, Masuda T, Nagaki N, Sasaki H, Takishima T (1992) Endothelin regulation of mucus glycoprotein secretion from feline tracheal submucosal glands. *Am J Physiol* 262: L208–L213.
140. Yurdakos E, Webber SE (1991) Endothelin-1 inhibits pre-stimulated tracheal submucosal gland secretion and epithelial albumin transport. *Br J Pharmacol* 104: 1050–1056.
141. Morikawa T, Webber SE, Widdicombe JG (1991) The effect of hydrogen peroxide on smooth muscle tone, mucus secretion and epithelial albumin transport of the ferret trachea *in vitro*. *Pulm Pharmacol* 4: 106–113.
142. Marom Z, Shelhamer J, Alling D, Kaliner M (1984) The effects of corticosteroids on mucous glycoprotein secretion from human airways *in vitro*. *Am Rev Respir Dis* 129: 62–65.
143. Marom Z, Shelhamer JH, Kaliner M (1985) Human monocyte-derived mucus secretagogue. *J Clin Invest* 75: 191–198.
144. Sperber K, Goswami SK, Gollub E, Mayer L, Marom Z (1991) Mucus secretagogue production by a human macrophage hybridoma. *J Allergy Clin Immunol* 87: 490–498.
145. Rogers DF (1994) Airway goblet cells: Responsive and adaptable front-line defenders. *Eur Respir J* 7: 1690–1706.
146. Somerville M, Richardson PS, Rutman A, Wilson R, Cole JP (1991) Stimulation of secretion into human and feline airways by *Pseudomonas aeruginosa* proteases. *J Appl. Physiol* 70: 2259–2267.
147. Sommerhoff CP, Caughey GH, Finkbeiner WE, Lazarus SC, Basbaum CB, Nadel JA (1989) Mast cell chymase: A potent secretagogue for airway gland serous cells. *J Immunol* 142: 2450–2456.
148. Sommerhoff CP, Nadel JA, Basbaum CB, Caughey GH (1990) Neutrophil elastase and cathepsin G stimulate secretion from cultured bovine airway gland serous cells. *J Clin Invest* 85: 682–689.
149. Sommerhoff CP, Krell RD, Williams JL, Gomes BC, Strimpler AM, Nadel JA (1991) Inhibition of human neutrophil elastase by ICI 200,355. *Eur J Pharmacol* 193: 153–158.
150. Nadel JA (1991) Role of enzymes from inflammatory cells on airway submucosal gland secretion. *Respiration* (Suppl): 3–5.

151. Knowles MR, Clarke LL, Boucher RC (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N Engl J Med* 325: 533–538.
152. Mason SJ, Paradiso AM, Boucher RC (1991) Regulation of transepithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. *Br J Pharmacol* 103: 1649–1656.
153. Shimura S, Sasaki T, Nagaki T, Takishima T, Shirato K (1994) Extracellular ATP regulation of feline tracheal submucosal gland secretion. *Am J Physiol* 267: L159–L164.
154. Medical Research Council (1965) Definition and classification of chronic bronchitis for clinical and epidemiological purposes. *Lancet* i: 775–779.
155. Houston JC, de Navasquez S, Trounce JR (1953) A clinical and pathological study of fatal cases of asthmaticus. *Thorax* 8: 207–213.
156. Dunnill MS (1960) The pathology of asthma with special reference to changes in the bronchial mucosa. *J Clin Pathol* 13: 27–33.
157. Hodson ME (1993) Cystic fibrosis: The disease. *Monaldi Arch Chest Dis* 48: 647–652.
158. Davis B, Chinn R, Gold J, Popovac D, Widdicombe JG, Nadel JA (1982) Hypoxemia reflexly increases secretion from tracheal submucosal glands in dogs. *J Appl Physiol* 52: 1416–1419.
159. Reid L (1954) Pathology of chronic bronchitis. *Lancet* i: 275–278.
160. Aikawa T, Shimura S, Sasaki H, Takishima T, Yaegashi H, Takahasi T (1989) Morphometric analysis of intraluminal mucus in airways in chronic obstructive pulmonary disease. *Am Rev. Respir Dis* 140: 477–482.
161. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T (1992) Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 101: 916–921.
162. Sturgess J, Imrie JR (1982) Quantitative evaluation of the development of tracheal submucosal glands in infants with cystic fibrosis and control infants. *Am J Pathol* 106: 303–311.
163. Sobonya RE, Taussig LM (1986) Quantitative aspects of lung pathology in cystic fibrosis. *Am Rev Respir Dis* 134: 290–295.
164. Coles SJ, Reid L (1978) Glycoprotein secretion *in vitro* by human airway: Normal and chronic bronchitis. *Exp Mol Pathol* 29: 326–341.
165. De Poitiers W, Lord PW, Biles B, Whimster WF (1980) Bronchial gland histochemistry in lungs removed for cancer. *Thorax* 35: 546–551.
166. Sturgess J, Reid L (1972) An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland. *Clin Sci* 43: 533–543.
167. Mann JS, George CF (1985) Anticholinergic drugs in the treatment of airways disease. *Br J Dis Chest* 79: 209–228.
168. Koyama S, Rennard SI, Leikauf GD, Robbins RA (1991) Bronchial epithelial cells release monocyte chemotactic activity in response to smoke and endotoxin. *J Immunol* 147: 972–979.
169. Robbins RA, Nelson KJ, Gossman GL, Koyama S, Rennard SI (1991) Complement activation by cigarette smoke. *Am J Physiol* 260: L254–L259.
170. Peatfield AC, Davies JR, Richardson PS (1986) The effect of tobacco smoke upon airway secretion in the cat. *Clin Sci* 71: 179–187.
171. Kummer W, Fischer A (1991) Tissue distribution of neutral endopeptidase 24.11 ("enkephalinase") activity in guinea pig trachea. *Neuropeptides* 18: 181–186.
172. Borson DB, Corrales R, Varsano S, Gold M, Viro N, Caughey G, Ramachandran J, Nadel JA (1987) Enkephalinase inhibitors potentiate substance P-induced secretion of $^{35}SO_4$-macromolecules from ferret trachea. *Exp Lung Res* 12: 21–36.
173. Rogers DF, Alton EWFW, Dewar A, Lethem MI, Barnes PJ (1993) Impaired stimulus-evoked mucus secretion in cystic fibrosis bronchi. *Exp Lung Res* 19: 37–53.
174. Merten MD, Figarella C (1993) Constitutive hypersecretion and insensitivity to neurotransmitters by cystic fibrosis tracheal gland cells. *Am J Physiol* 264: L93–L99.
175. Agnew JE, Bateman JRM, Pavia D, Clarke SW (1984) Peripheral airways mucus clearance in stable asthma is improved by oral corticosteroid therapy. *Bull Eur Physiopathol Respir* 20: 295–301.
176. Cockcroft DW, Murdock KY (1987) Comparative effects of inhaled salbutamol, sodium cromoglycate and beclomethasone dipropionate on allergen-induced early asthmatic responses, late asthmatic responses and increased bronchial responsiveness to histamine. *J Allergy Clin Immunol* 79: 734–740.

177. Kraan J, Koeter GH, Mark TW, Sluiter HJ, de Vries K (1985) Changes in bronchial hyperreactivity induced by 4 weeks of treatment with antiasthmatic drugs in patients with allergic asthma: A comparison between budesonide and terbutaline. *J Allergy Clin Immunol* 76: 628–636.
178. Borson DB, Gruenert DC (1991) Glucocorticoids induce neutral endopeptidase in transformed human tracheal epithelial cells. *Am J Physiol* 260: L83–L89.
179. Khawaja AM, Rogers DF (1996) Tachykinins: Receptor to effector. *Int J Biochem Cell Biol* 28: 721–738.
180. Hirayama Y, Lei Y-H, Barnes PJ, Rogers DF (1993) Effects of two novel tachykinin antagonists, FK224 and FK888, on neurogenic airway plasma exudation, bronchoconstriction and systemic hypotension in guinea-pigs *in vivo*. *Br J Pharmacol* 108: 844–851.
181. Fahy JV, Wong HH, Geppetti P, Reis JM, Harris SC, Maclean DB, Nadel JA, Boushey HA (1993) Effect of an NK_1 receptor antagonist (CP-99,994) on hypertonic saline-induced bronchoconstriction and cough in male asthmatic subjects. *Am J Respir Crit Care Med* 152: 879–884.
182. Ichinose M, Nakajima N, Takahashi T, Yamauchi H, Inoue H, Takishima T (1992) Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. *Lancet* 340: 1248–1251.
183. Barnes PJ, MG Belvisi, DF Rogers (1990) Modulation of neurogenic inflammation: novel approaches to inflammatory disease. *Trends Pharmacol Sci* 11: 185–189.
184. Rogers DF (1996) Scorpion venoms: Taking the sting out of lung disease. *Thorax* 51: 546–548.
185. Ramnarine SI, Khawaja AM, Bryce P, Hicks PE, Barnes PJ, Rogers DF (1995) Effects of potassium channel openers on neurogenic airway mucus secretion in the ferret. *Am J Respir Crit Care Med* 151 (Suppl): A820.
186. Jany B, Gallup M, Tsuda T, Basbaum CB (1991) Mucin gene expression in rat airways following infection and irritation. *Biochem Biophys Res Commun* 181: 1–8.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 9
Mucus–Bacteria Interactions

Charlotte Rayner[1,*] and Robert Wilson[2]

[1] *Chest Clinic, St. George's Hospital, London, UK*
[2] *Host Defence Unit, Imperial College of Science, Technology and Medicine, London, UK*

1. Introduction

Human evolution has necessitated the development of an internal lung for gas exchange, which has developed from an adaptation of the primitive foregut. The surface area required for gas exchange is provided by large numbers of alveoli supplied by repeated branching of the bronchial tree. Through these blind-ending airways pass $1-2\times10^4$ l of air per 24 h. The respiratory mucosa is therefore continually exposed to a variety of noxious agents which include bacteria. To prevent infection, the respiratory tract possesses an elaborate array of host defences, whose co-ordinated action ensures sterility of the lung from the first bronchial division [1]. The initial

* Author for correspondence.

interaction of bacteria with the respiratory mucosa is with mucus, and this influences subsequent events that determine the success or failure of bacterial colonisation.

2. Host Defence Function of Mucus

Airway mucus serves a number of important functions of host defence. The mucus gel acts as a physico-chemical barrier to bacteria. Mucus floats on the extracellular (periciliary) fluid layer which exists between the epithelial surface and the tips of the cilia. Although mucus itself is rapidly transported to the pharynx and swallowed, the extracellular fluid layer is more stable and acts as a constant source of molecules which diffuse into and out of the mucus layer. Mucus contains a number of antibacterial factors, which include cytotoxic enzymes, antimicrobial proteins and antibodies [2]. Some of these factors are discussed below.

2.1. Neutrophil Proteinases

Neutrophil proteinases are cytotoxic enzymes, stored within and released from neutrophils. The production of proteinases facilitates clearance of bacteria from the respiratory tract and may stimulate mucus secretion [3]. However, an excessive inflammatory response may result from large numbers of neutrophils trafficking into the airway lumen in response to bacterial infection and/or inadequate inhibition of proteinase activity by the antiproteinases in mucus [4]. Neutrophil elastase is a serine protease which is present in the sputum sol of patients with chronic infection and bronchiectasis [5]. The concentrations that are found are able to degrade a wide range of extracellular matrix proteins and also to slow ciliary beat frequency and damage epithelial cells [4, 6].

2.2. Proteinase Inhibitors

A number of proteinase inhibitors are found in mucus which function to protect host tissues from the damaging effect of the proteolytic enzymes. These include alpha-1 proteinase inhibitor (alpha-1 PI), antichymotrypsin and the low molecular weight proteinase inhibitors antileukoproteinase and elafin.

Alpha-1 PI, a glycoprotein synthesised mainly in the liver and to a lesser extent in alveolar macrophages, is able to traverse the pulmonary endothelium and epithelium [7]. The reactive centre of alpha-PI binds rapidly and irreversibly to neutrophil elastase, resulting in its rapid inactivation [8].

Alpha-PI can be readily inactivated by oxidation, and therefore the release of oxidants from neutrophils may create a zone in which alpha-PI is inactive [7].

Elafin is a specific inhibitor of elastase. *In vitro*, both elafin and anti-leukoproteinase are secreted from type II pneumocyte cell lines [9]. There is evidence to suggest that, *in vitro*, the secretion of these proteinases is increased in the presence of interleukin (IL) 1-β and tumour necrosis factor α (TNF-α) [10].

2.3. Alveolar Macrophages

Alveolar macrophages are present on the epithelial surface of the human lung and are the resident phagocytes in the alveolar space [11]. A major function of alveolar macrophages is to maintain the sterility of the lung, particularly on the epithelial surface. The alveolar macrophage may be activated by non-specific phagocytosis of microorganisms, or by recognition of specific antibodies with or without the addition of complement proteins. Bacterial killing results from oxidative and non-oxidative processes both within and outside the cell [12, 13]. Non-oxidative killing methods include proteases, lysozyme, various acid hydrolases and defensins. Alveolar macrophages are also able to recruit and activate other inflammatory cells [14].

2.4. Lysozyme

Lysozyme, an enzyme which attacks the peptidoglycans in the cell walls of Gram-positive bacteria, was initially discovered in nasal secretions [15]. Lysozyme is a relatively small protein which is synthesised and released by neutrophils and serous cells of submucosal glands. It represents 15–30% of the protein normally found in nasal secretions.

2.5. Lactoferrin and Transferrin

Lactoferrin and transferrin are antimicrobial proteins secreted by serous cells. They bind iron, which is known to be important for bacterial growth. As a result they may be both bacteriostatic and bactericidal [1]. Lactoferrin is also known to be a major intermediate in oxygen-radical production by acting as a provider of iron to neutrophils.

2.6. Secretory Immunoglobulins

Immunoglobulins are important in the defence against bacterial colonization and invasion at the mucosal surface [16]. IgA, and to a lesser extent

IgG, are synthesised at the mucosal surface. IgA is linked to a protein produced by epithelial cells to form secretory IgA, which is the predominant antibody in mucus. The secretory immunoglobulins are thought to neutralise the action of bacterial toxins and to prevent bacterial adherence. Secretory IgA acts by binding bacterial surface antigens which mediate adherence, thereby blocking bacterial attachment. Binding to bacterial surface antigens may also facilitate complement activation and bacterial killing through direct lysis or by opsonophagocytosis [16].

Several bacterial species which colonize mucosal surfaces produce a protease which specifically cleaves IgA1 at the hinge region of the α-chain, releasing Fab and Fc fragments [17, 18]. One possible function of the IgA proteases is that by disabling IgA, bacteria are able to protect themselves with non-functioning antibody which would block opsonisation by effective antibody [19]. A second is that IgA protease expressed on the bacterial surface acts as a novel adhesin for bacteria to IgA and mucus [18, 20].

2.7. Cytokines

Cytokines have been shown to modify many of the host responses to bacterial infection [21]. Microorganisms associated with mucus in the bronchial lumen can activate both the transcription of cytokine mRNA and cytokine secretion from bronchial epithelium. Acute infective exacerbations of bronchiectasis are usually associated with increased volume of sputum expectoration. IL1, TNF-α and very high levels of IL8 have been found in the expectorated secretions of patients with bronchiectasis [22]. IL8 is a potent neutrophil chemoattractant and both activates and increases the adhesiveness of neutrophils. Cytokine-mediated recruitment of neutrophils from the circulation into mucus may underly the majority of bronchial damage that occurs in bronchiectasis [22].

3. Adherence of Bacteria to Mucus

The ability of bacteria to adhere to the mucosa is important in both colonisation of the respiratory tract and in the pathogenesis of respiratory tract infections [23, 24]. In health, adherence of bacteria to mucus is advantageous to the host in that they will be expelled on the mucociliary escalator. In situations where mucus clearance is impaired however, mucus adherence will be advantageous to the bacteria. Here, they can utilise a number of mechanisms, both to sabotage intact host defences and to multiply and spread, taking advantage of impairment of host defences. In this way they create an ecological niche for themselves in the airway lumen [25].

A number of respiratory pathogens demonstrate an affinity for mucus with preferential binding to mucus over normal ciliated epithelium. These pathogens include *Streptococcus pneumoniae*, non-typable *Haemophilus influenzae, H. influenzae* type b, *Staphylococcus aureus* and *Pseudomonas aeruginosa* [26–30]. Non-typable *H. influenzae and S. pneumoniae* are common commensals of the upper respiratory tract, present in up to 70% of normal adults [31]. These are also common causes of infection in patients with chronic obstructive pulmonary disease [32–34]. Several *in vitro* models have been used to study the adherence of *S. pneumoniae* and non-typable *H. influenzae* to the respiratory mucosa [27, 28, 35–38]. In a model using frog palate, *S. pneumoniae* has been shown to adhere rapidly to mucus but not to normal ciliated epithelium [26]. *In vitro, S. pneumoniae* has been shown to occupy a thick, gelatinous layer formed above the epithelial surface of immersed human nasal turbinate tissue [37]. In an organ culture of human respiratory tract epithelium with an air interface, *S. pneumoniae* was seen to adhere to and change the appearance of the mucus [38] (Figure 1).

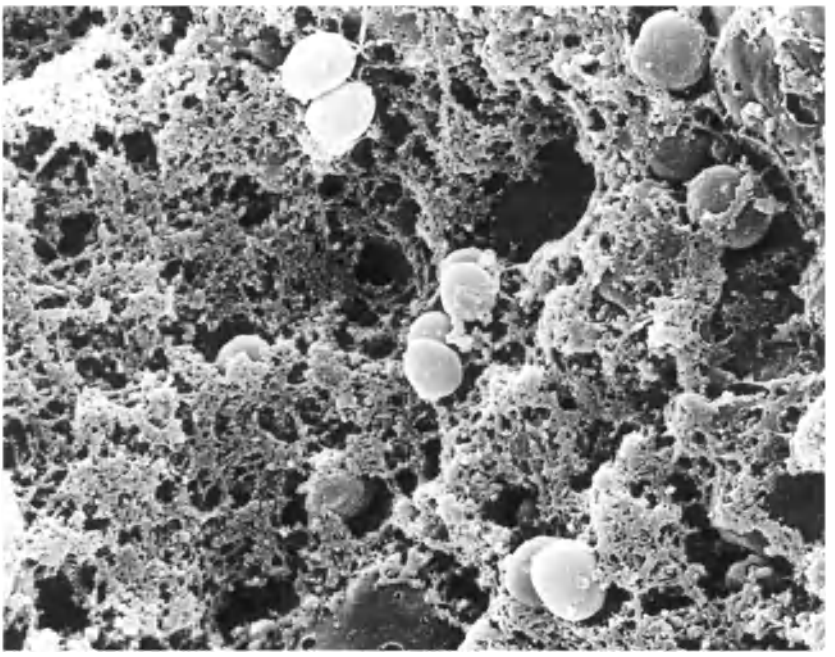


Figure 1. Scanning electron micrograph of human respiratory tract epithelium in organ culture infected by *S. pneumoniae*. The mucus appears fibrogranular, and diplococci are seen (magnification ×13,750).

Mucus from patients with cystic fibrosis contains chemotactic factors for *P. aeruginosa* [39]. Histopathological studies of *P. aeruginosa* in lung tissue from patients with cystic fibrosis demonstrate that bacteria are mainly associated with mucus intraluminally. *P. aeruginosa* only occasionally formed adherent microcolonies on the epithelium in areas of epithelial damage [40].

These studies suggest that bacteria adhere to mucus before adherence to other mucosal features. Thus, for these pathogens, mucus seems to be important in the pathogenesis of colonisation and of infection of the respiratory tract.

In contrast, of the bacteria studied, only one does not appear to adhere to mucus. *In vitro* models developed to study the pathogenesis of infection by *Neisseria meningitidis* have shown that bacteria are rarely seen in association with mucus, interacting preferentially with cells in areas where mucus is lacking [41, 42].

4. Mechanisms of Adherence of Bacteria to Mucus

A multitude of factors, some of which are discussed below, mediate the adherence of bacteria to mucus. The interaction between *P. aeruginosa* and mucus serves as an example of how these factors act together to enable adhesion to occur.

4.1. Physical and Electrostatic Mechanisms

The initial adherence of bacteria may be a simple physical interaction reflecting the tenacity of mucus, which has been shown to trap and transport inert particles such as carbon out of the respiratory tract [43]. The extent of the role played by this physical property in the adherence of bacteria to mucus has not been quantified. Electrostatic interactions between the bacteria and mucus may be important during initial stages of colonisation. The overall charge on both the bacteria and mucosal surface is negative, thus tending to favour repulsion. However, hydrophobic interactions between bacteria and mucus may favour bacterial adhesion [44]. For example, binding of *P. aeruginosa* to mucus can be inhibited by an agent which disrupts hydrophobic interactions, suggesting a non-specific hydrophobic interaction may mediate adherence of *P. aeruginosa* to mucin [45].

4.2. Interactions between Bacterial Fimbrae and Mucus

Bacterial fimbrae are filamentous proteins which project from the outer membrane of the bacterial cell wall. Fimbrae are hydrophobic structures

[46] and have been shown to mediate adherence to mucus. For example, fimbrae of *P. aeruginosa* have been reported to mediate adherence to mucin [47]. When compared with non-fimbriated strains *in vitro*, fimbriated strains of non-typable *H. influenzae* have increased adherence to sol phase mucus and purified respiratory tract mucin [48]. *Pseudomonas cepacia* is an opportunistic pathogen which colonises the respiratory tract in patients with cystic fibrosis. Its acquisition is not associated with a clinical deterioration in the patient [49]. *P. cepacia* binds to respiratory mucin, and adhesion is mediated by a protein located on fimbriae present over the surface of the bacterium [50]. The interaction of fimbrae and mucus is likely to be complex and to involve both non-specific and specific interactions.

4.3. Bacterial Adhesins

The surface adhesins of microorganisms have been shown to recognise several varied carbohydrate structures. When carbohydrate structures are exposed on the surface of human cells, they are possible sites for bacterial attachment. Potential carbohydrate sites for bacterial attachment have been described for a number of respiratory pathogens. For example, *S. pneumoniae* has been shown to bind the disaccharide unit Gal-β1-3-GlcNAcβ1-3Gal-β1-4Glc found in human milk [51]. *Mycoplasma pneumoniae* binds NeuAcA2-3Gal-β1-4GlcNAc found in glycoproteins and on ciliated cells [52].

4.4. Mucus Glycoproteins

Respiratory mucus contains a heterogeneous mixture of highly glycosylated glycoproteins [53]. Mucus glycoproteins (mucins) are accepted as the major constituent of mucus in hypersecretory conditions, although the exact composition of normal respiratory secretions is not known [54]. As large volumes of mucus are required to extract small quantities of oligosaccharides, most studies looking at the structure of mucin chains have been performed on sputum obtained from patients with underlying respiratory disease. These studies have shown that there is marked variation in the carbohydrate chains [55, 56]. It has also been demonstrated that glycopeptides obtained from normal subjects by bronchial lavage demonstrate some heterogenicity [57]. The multiple carbohydrate chains that cover mucin molecules may represent numerous and varied sites for bacterial attachment, although the precise mechanisms are still poorly understood.

4.5. Interaction of P. aeruginosa with Mucus

The interaction of *P. aeruginosa* with mucus has been extensively studied. Human respiratory mucins have an affinity for both mucoid and non-mucoid strains of *P. aeruginosa* [30]. The receptors for both strains are sensitive to oxidation by periodate and are therefore part of the carbohydrate chains of mucin. *P. aeruginosa* has an affinity for sialylated and neutral glycoproteins, but very low affinity for sulphated glycoproteins, suggesting carbohydrate specificity [58]. *In vitro*, adherence of *P. aeruginosa* to mucin can be inhibited by preincubation of respiratory mucins with purified carbohydrate chains obtained from β elimination of respiratory mucin [59]. This suggests the presence of specific receptors. To further elucidate the site of *P. aeruginosa* adherence, milk oligosaccharides, which have strong structural homology to the linear oligosaccharides of respiratory mucin, have been used [59]. *In vitro*, these milk oligosaccharides inhibit *P. aeruginosa* adherence to respiratory mucin. When derived as neoglycolipids, these milk oligosaccharides are also the site of *P. aeruginosa* adherence [60].

Both mucoid and non-mucoid strains of *P. aeruginosa* were shown to bind to type 1 (gal-β 1-3 *N*-acetylglucosamine) and type 2 (gal-β 1-4 *N*-acetylglucosamine) disaccharide units [60]. Respiratory mucin chains contain disaccharide units, which suggests that these units are the sites of *P. aeruginosa* adhesion. Although sialylation of neoglycolipids containing these two saccharide receptor units did not change *P. aeruginosa* adherence, α-2-6 linked sialic acid blocked adhesion [60]. The role of sialic acid in *P. aeruginosa* receptors may be to maintain conformation of the oligosaccharide chain or to increase the affinity of receptor/bacterial adhesion [60]. Adhesion of mucoid *P. aeruginosa* to mucin may be mediated by mucoid exopolysaccharide [61–63]. The gene involved in non-pilus-mediated adherence to mucin has recently been characterised. It has been shown to be involved both in flagella biosynthesis and mucin adherence [64].

P. aeruginosa adherence increases if mucin is in solution rather than in a microtitre plate, suggesting that adherence may be dependent on the structural conformation of mucin [45]. The same authors also assessed the binding of *P. aeruginosa* to mucins from patients with cystic fibrosis [45]. Once colonisation is established in patients with cystic fibrosis, bacteria are rarely eliminated. Persistence of *P. aeruginosa* in patients with cystic fibrosis could be explained by an increased affinity for a component of airway mucus unique to cystic fibrosis. However, mucin glycopeptides from patients with cystic fibrosis have reduced adhesion compared with patients with bronchitis [58]. In addition, adherence of *P. aeruginosa* to cystic fibrosis mucin is no greater than to structurally unrelated glycoproteins [45]. The same study demonstrated that non-specific hydrophobic interactions may mediate *P. aeruginosa* adherence. Colonisation of the respiratory tract

in cystic fibrosis patients by *P. aeruginosa* is likely to be multifactorial [65], but poor clearance of mucus containing large numbers of bacteria is likely to be very important.

5. Effect of Bacteria on Mucociliary Transport

Efficient mucociliary transport requires co-ordinated ciliary beating as well as the production of the correct quantity and quality of mucus and periciliary fluid [66]. Mucociliary clearance is delayed in conditions where bacterial infection is present and purulent secretions are produced. For example, tracheobronchial clearance has been shown to be delayed in patients with cystic fibrosis, bronchiectasis or serologically confirmed *M. pneumoniae* infection; and nasal mucociliary clearance is delayed in patients with chronic sinusitis [67]. One study, using mucus-depleted bovine trachea to investigate the transportability of sputum from patients with bronchiectasis, suggests that the altered transportability may be unrelated to the presence of infection [68].

There are a variety of mechanisms by which microbes can perturb mucociliary clearance: increased mucus production, altered rheology and transportability, altered ion transport, impaired ciliary function and epithelial cell damage. Some examples are discussed in the following sections. Again, information from the study of *P. aeruginosa* illustrates how various mechanism interact to disrupt mucociliary clearance.

5.1. Inhibition of Ciliary Activity

Inhibition of ciliary activity may reduce mucociliary transport. A number of bacteria have been shown to produce factors which interfere with normal ciliary activity, including *P. aeruginosa, M. pneumoniae, H. influenzae, Bordatella pertussis, S. aureus, S. pneumoniae* and *N. meningitidis.* Filtered broth culture supernates of *H. influenzae* caused significant ciliary slowing of nasal epithelium *in vitro* [69]. Further experiments have suggested that the ciliatoxin is a low molecular weight glycopeptide. Pneumolysin, a sulphydryl-activated haemolytic cytotoxin released by *S. pneumoniae* during autolysis, causes ciliary beat slowing [29]. *P. aeruginosa* produces the pigments pyocyanin and 1-hydroxyphenazine. At pathophysiological concentrations, these pigments cause ciliary beat slowing, ciliary dyskinesia and disorientation of human ciliated epithelium *in vitro* [67, 70, 71]. For example, 1-hydroxyphenazine has been shown to cause an immediate slowing and then recovery of the tracheal mucus transport velocity [TMV] in the guinea pig *in vivo* [72]. Pyocyanin has also been shown to produce slowing of TMV without recovery in the same model at pathophysiological concentrations [72].

5.2. *Qualitative and Quantitative Effects of Bacteria on Mucus*

Infection may alter the quality or quantity of mucus produced by the respiratory tract. The rheology of mucus may change during infection. Viral infection has been shown to reduce the elasticity of mucus, which results in impaired transportability, and studies have suggested that bacterial infection may increase mucus viscosity, thus reducing mucus transport. *P. aeruginosa, H. influenzae* and *S. pneumoniae* have been shown to stimulate the secretion of mucus glycoconjugates from explants of guinea pig trachea [73]. Infection of organ cultures of human respiratory epithelium with *S. pneumoniae* resulted in changes in the scanning electron microscopic appearances of the mucus layer, which were not typical of simple mucus [37, 38]. In these models, it has been suggested that the mucus is abnormal, containing bacterial products such as capsular components [37, 38]. Formation of an abnormal mucinous layer which is less well transported by cilia could thus be a mechanism for bacterial colonisation. The mechanism of mucus alteration in *S. pneumoniae* infection may be due to the action of the toxin neuraminidase. Neuraminidase is a pneumococcal toxin located in the cytoplasm, which cleaves terminal sialic acid residues from a variety of glycolipids, glycoproteins and oligosaccharides located on cell surfaces and in body fluids [19]. Cleavage of sialic acid residues in mucus may result in reduced mucus viscosity, thereby interfering with mucociliary transport.

5.3. *Effect of P. aeruginosa on Mucociliary Clearance*

P. aeruginosa utilises a number of mechanisms to impair mucociliary clearance. Clinical isolates of *P. aeruginosa* produce heat-stable glycolipids (rhamnolipids) during the stationary phase of growth. Rhamnolipids have a detergent-like structure with a polar head and non-polar tail. This surfactant-like property may account for their known haemolytic activity. Rhamnolipids interfere with sheep epithelial ion transport *in vitro* in a dose-dependent manner [74]. Abnormal epithelial ion transport may change the constituents of mucus and therefore interfere with mucociliary transport. In cats, *in vivo*, rhamnolipids act as mucus secretagogues, at pathophysiological concentrations [75]. Application of rhamnolipid to guinea pig tracheal mucosa *in vivo* reduced mucus velocity in a dose-dependent manner [76]. Rhamnolipid has also been shown to cause ciliary beat slowing and epithelial disruption in human ciliated epithelium *in vitro* [76].

P. aeruginosa proteases have been shown to stimulate mucus output *in vitro* from explants of guinea pig trachea [73]. Purified *P. aeruginosa* elastase stimulates mucus output from rabbit trachea *in vitro* [77]. Proteases have also been shown to stimulate mucus secretion from cat trachea *in vivo* and from human tracheal tissue *in vitro* [78]. The concentration of

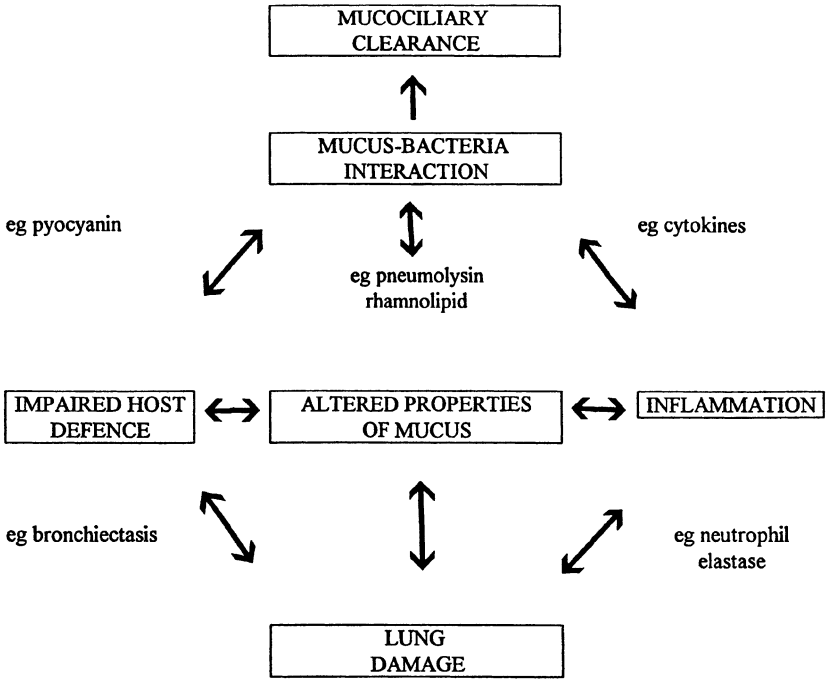


Figure 2. Shows the pivotal role of mucus–bacteria interaction in determining outcome of entry of bacteria into the respiratory tract. If bacteria are not removed by mucociliary clearance, their continued presence may trigger a cycle of events leading ultimately to lung damage. Mediators involved in this process may be bacterial products (pyocyanin, pneumolysin, rhamnolipid) or inflammatory mediators produced by host cells (cytokines, neutrophil elastase). The outcome is influenced not only by these factors but also by the presence of underlying lung pathology (e.g. bronchiectasis).

alkaline proteases and elastase in samples of bronchoalveolar fluid from patients with cystic fibrosis ranges from 8–25 ng/ml [79]. This concentration is 1% of that which has been found to stimulate mucus secretion in human epithelium *in vitro* and in feline epithelium *in vivo*. However, neither the degree to which secretions are diluted by lavage, nor the true local epithelial concentration of these enzymes, particularly near microcolonies of *P. aeruginosa,* is known. The effects of proteases on mucus secretion are likely to be manifold. The increased mucus load may enhance mucus clearance by increasing ciliary activity [43]. However, this mechanism may be ineffective if the mucus has abnormal rheological properties [80], if the increased mucus load alters the relationship between mucus and cilia [81] or if the ciliary beat is abnormal.

6. Conclusions

The mucociliary system provides a primary defence mechanism of the respiratory tract. Mucus is the first component of this system encountered by bacteria as they enter the respiratory tract. Although adherence of bacteria to mucus in health results in clearance of bacteria from the respiratory tract, abnormal mucus clearance may provide the initial niche in the pathological process of mucosal colonisation. The close proximity of bacteria to the epithelial surface may allow high local concentrations of toxins to reach the epithelium. The combined effect of various toxins may damage host cells and may further perturb host defences. Abnormal mucociliary clearance plays a pivotal role in this pathological process. The interaction between bacteria and mucus, as well as with cilia and epithelial cells, is therefore crucial in realising the potential of bacteria to colonise, proliferate and infect the respiratory tract. Future treatment strategies should aim to improve mucus clearance, since altering bacterial adherence to mucus may interfere with an important defence mechanism.

References

1. Newhouse M, Sanchis J, Bienstock J (1976) Lung defense mechanisms. *N Engl J Med* 295: 1045–1054.
2. Kaliner MA (1991) Human nasal respiratory secretions and host defense. *Am Rev Respir Dis* 144: S52–S56.
3. Nadel JA (1991) Role of mast cell and neutrophil proteases in airway secretion. *Am Rev Respir Dis* 144: S48–S51.
4. Tetley TD (1983) Proteinase imbalance: Its role in lung disease. *Thorax* 48: 560–565.
5. Stockley RA, Hill SL, Morrison HM, Starkie CM (1984) Elastolytic activity of sputum and its relation to purulence and to lung function in patients with bronchiectasis. *Thorax* 39: 408–413.
6. Amitani R, Wilson R, Rutman A, Read RC, Ward C, Barnett D, Stockley RA, Cole PJ (1991) Effects of human neutrophil elastase and bacterial proteolytic enzymes on human ciliated epithelium *in vitro*. *J Resp Cell Mol Biol* 4: 26–32.
7. Carrel RW (1986) Alpha-1-antitrypsin: Molecular pathology, leukocytes and tissue damage. *J Clin Invest* 78: 1427–1431.
8. Travis J, Salvesen GS (1983) Human plasma protein inhibitors. *Ann Rev Biochem* 52: 655.
9. Sallenave J-M, Silva A, Marsden ME, Ryle AP (1993) Secretion of mucus proteinase inhibitor and elafin by Clara cell and type II pneumocyte cell lines. *Am J Respir Cell Mol Biol* 8: 126–133.
10. Sallenave J-M, Shulmann J, Crossley J, Jordana M, Gauldie J (1994) Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am J Respir Cell Mol Biol* 11: 733–741.
11. Dubois RM (1986) The alveolar macrophage. *Thorax* 40: 321–327.
12. Klebanoff SJ (1988) Phagocytic cells: products of oxygen metabolism. In: Gallin JI, Goldstein IM, Snyderman R (eds.) *Inflammation: Basic principles and clinical correlates.* New York: Raven Press, 391–444.
13. Henson PM, Henson JE, Fittschen C, Kimani G, Bratton DL, Riches DWH (1988) Phagocytic cells: Degranulation and secretion. In: Gallin JI, Goldstein IM, Snyderman R (eds). *Inflammation: Basic principles and clinical correlates.* New York: Raven Press, 363–390.

14. Sylvester I, Rankin JA, Yoshimura T, Tanaka S, Leonard EJ (1990) Secretion of neutrophil attractant/activation protein by lipopolysaccharide-stimulated lung macrophages determined by both enzyme-linked immunosorbent assay and M-terminal sequence analysis. *Am Rev Resp Dis* 141: 683–688.
15. Fleming A (1922) On a remarkable bacteriolytic element found in tissues and secretions. *Proc R Soc Lond* (Biol) 93: 306– 317.
16. Brantzaeg P (1992) Humoral immune response patterns of human mucosae: Induction and relation ot bacterial respiratory tract infections. *J Infect Dis* 165 (Suppl 1): S167–S176.
17. Killian M, Mestecky J, Schrohenlohrer RE (1979) Pathogenic species of the genus *Haemophilus* and *Streptococcus pneumoniae* produce immunoglobulin A1 protease. *Infect Immun* 26: 143A–149.
18. Plaut AG (1983) The IgA proteases of pathogenic bacteria. *Am Rev Microbiol* 37: 603–622.
19. Paton JC, Andrew PW, Boulnois GJ, Mitchell TJ (1993) Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: The role of pneumococcal proteins. *Annu Rev Microbiol* 47: 79–115.
20. Moxon Er, Wilson R (1991) The role of *Haemophilus influenzae* in the pathogenesis of pneumonia. *Rev Infect Dis* 13(Suppl 6): S518–S527.
21. Hedges SR, Agace WW, Svanborg C (1995) Epithelial cytokine responses and mucosal cytokine networks. *Trends in Microbiology* 3(7): 266–270.
22. Eller J, Lapa e Silva JR, Poulter LW, Lode H, Cole PJ (1994) Cells and cytokines in chronic bronchial infection. *Annals of the New York Academy of Sciences* 725: 331–345.
23. Beachey EH (1981) Bacterial adherence: Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J Infect Dis* 143: 325–345.
24. Niederman MS (1989) Bacterial adherence as a mechanism of airway colonisation. *Eur J Clin Microbiol Infect Dis* 8: 15–20.
25. Cole P, Wilson R (1989) Host-microbial interrelationships in respiratory infection. *Chest* 95: 2175–2213.
26. Plotkowski MC, Beck G, Jacquot J, Puchelle E (1989) The frog palate mucosa as a model for studying bacterial adhesion to mucus coated respiratory epithelium. *J Comp Path* 100: 37–46.
27. Farley MM, Stephens DS, Mulks MH, Cooper MD, Bricker JV, Mirra SS, Wright A (1986) Pathogenesis of IgA1 protease-producing and non-producing *H. influenzae* on human nasopharyngeal organ cultures. *J Infect Dis* 154: 752–759.
28. Read RC, Wilson R, Rutman A, Lund V, Todd HC, Brain APR, Jeffery PK, Cole PJ (1991) Interaction of non-typable *Haemophilus influenzae* with human respiratory mucosa *in vitro*. *J Infect Dis* 163: 549–558.
29. Steinfort C, Wilson R, Mitchell T, Feldman C, Rutman A, Todd H, Sykes D, Walker J, Saunders K, Andrew PW et al. (1989) Effect of *Streptococcus pneumoniae* on human respiratory epithelium *in vitro*. *Infect Immun* 57: 2006–2013.
30. Vishwanath S, Ramphal R (1984) Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. *Infect Immun* 45: 197–202.
31. Murphy TF, Sethi S (1992) Bacterial infection in chronic obstructive pulmonary disease. *Am Rev Resp Dis* 146: 1067–1083.
32. Laurenzi GA, Potter RT, Kass EH (1961) Bacteriologic flora of the lower respiratory tract. *N Engl J Med* 265: 1273–1278.
33. Haas H, Morris JF, Samson S, Kilbown JP, Kim PJ (1977) Bacterial flora of the respiratory tract in chronic bronchitis: Comparison of transtracheal, fibrebronchoscopic and oropharyngeal sampling methods. *Am Rev Resp Dis* 116: 41–47.
34. Murphy TF, Apicella MA (1987) Non-typable *Haemophilus influenzae:* A review of clinical aspects, surface antigens and the human immune response to infection. *Rev Infec Dis* 9: 1–15.
35. Loeb MR, Connor E, Penney D (1988) A comparison of the adherence of fimbriated and non fimbriated *Haemophilus influenzae* type b to human adenoids in organ culture. *Infect Immun* 56: 484–489.
36. Van Alphen L, van den Berghe N, Geelen-van den Broek L (1988) Interaction of *Haemophilus influenzae* with human erythrocytes in oropharyngeal epithelial cells is mediated by common fimbrial epitope. *Infect Immun* 56: 1800–1806.
37. Feldman C, Read R, Rutman A, Jeffery PK, Brain A, Lund V, Mitchell PJ, Andrew PW, Boulnois GJ, Todd HC (1992) Interaction of *Streptococcus pneumoniae* with intact human respiratory mucosa *in vitro*. *Eur Respir J* 5: 576–585.

38. Rayner CFJ, Jackson AD, Rutman A, Dewar A, Mitchell TJ, Andrew PW, Cole PJ, Wilson R (1995) The interaction of pneumolysin sufficient and deficient isogenic variants of *Streptococcus pneumoniae* with human respiratory mucosa. *Infect Immun* 63: 442–447.
39. Nelson J, Tredgett M, Sheehan J (1990) Mucinophilic and chemotactic properties of *Pseudomonas aeruginosa* in relation to pulmonary colonization in cystic fibrosis. *Infect Immun* 58: 1489–1495.
40. Baltimore RS, Christie LDC, Walker Smith GJ (1989) Immunohistopathological localisation of *P. aeruginosa* in lungs from patients with cystic fibrosis. *Am Rev Resp Dis* 140: 1650–1661.
41. Rayner CFJ, Dewar A, Moxon Er, Virji M, Wilson R (1995) The effect of variations in the expression of pili on the interaction of *Neisseria meningitidis* with human nasopharyngeal epithelium. *J Infect Dis* 171: 113–121.
42. Read RC, Fox A, Miller K, Gray T, Jones N, Borrow R, Jones DM, Finch RG (1995) Experimental infection of human nasal mucosal explants with *Neisseria meningitidis*. *J Med Microbiol* 42: 353–361.
43. Spungin B, Silberberg A (1984) Stimulation of ciliary activity and transport in frog palate epithelium. *Am J Physiol* 247: C299–C308.
44. Rosenberg M, Kjelleberg S (1986) Hydrophobic interactions: Role in bacterial adhesion. *Adv Microb Ecol* 9: 353–393.
45. Sajjan US, Reisman J, Doig P, Irvin RT, Forstner G, Forstner J (1992) Binding of non-mucoid *Pseudomonas aeruginosa* to normal human interstitial mucin and respiratory mucin from patients with cystic fibrosis. *J Clin Invest* 89: 657–665.
46. Irvin RT (1990) Hydrophobicity of proteins and bacterial fimbrae. In: Doyle RJ, Rosenberg (eds). *Microbiol cell surface hydrophobicity.* Washington: American Society for Microbilogy, 137–177.
47. Ramphal R (1987) Fimbrinated strains of non-typable *Haemophilus influenzae* have increased adherence to sol phase of mucus and purified respiratory tract mucus *in vitro*. *Infect Immun* 55: 600–603.
48. Barsum W, Wilson R, Read RC, Rutman A, Todd HC, Houdret N, Roussel P, Cole PJ (1995) Interaction of fimbrinated and non-fimbrinated strains of non-encapsulated *Haemophilus influenzae* with respiratory tract mucin *in vitro*. *Eur Respir J* 8: 709–714.
49. Geddes DM, Hodson ME (1993) Cystic fibrosis. In: Barnes PJ (ed). *Respiratory medicine: Recent advances.* Oxford: Butterworth-Heinemann.
50. Sajjan US, Forstner JI (1992) Identification of the mucin-binding adhesin of *Pseudomonas cepacia* isolated from patients with cystic fibrosis. *Infect Immun* 60: 1434–1440.
51. Andersson B, Dahmen J, Frejd T, Leffler H, Magnusson G, Noori G, Svanborg-Eden C (1983) Identification of an active disaccharide unit of a glyconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells. *J Exp Med* 158: 559–570.
52. Krivan JC, Roberts DD, Ginsburg V (1988) Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. *Proc Natl Acad Sci USA* 85: 6157–6161.
53. Roussel P, Lamblin G, Degand P, Walker-Nasir E, Jeanloz RW (1975) Heterogeneity of the carbohydrate chains of sulphated bronchial glycoproteins isolated from a patient suffering from cystic fibrosis. *J Biol Chem* 259: 2214–2222.
54. Sheehan JK, Thorton DJ, Somerville M, Carlstedt I (1991) The structure and heterogenicity of respiratory mucus glycoproteins. *Am Rev Respir Dis* 144: S4–S9.
55. Feldhoff PA, Bhavanandan VP, Davidson EA (1979) Purification, properties and analysis of human asthmatic bronchial mucin. *Biochemistry* 18: 2430–2436.
56. Lamblin G, Boersma A, Klein A, Roussel P, Van Halbeek H, Vliegenthart JFG (1984) Primary structure determination of five sialylated oligosaccharides derived from bronchial mucus glycoproteins of patients suffering from cystic fibrosis. *J Biol Chem* 259: 9061–9068.
57. Lafitte JJ, Lamblin G, Lhermitte M, Humbert P, Degand P, Roussel P (1977) Etude des glycoproteines bronchiques humaines de type mucique obtenues par lavage de bronches macroscopiquement saines. *Carbohydr Res* 13: 383–389.
58. Ramphal R, Houdret N, Koo L, Lamblin G, Roussel P (1989) Differences in adhesion of *Pseudomonas aeruginosa* to mucin glycopeptides from sputa of patients with cystic fibrosis and chronic bronchitis. *Infect Immun* 57: 3066–3071.

59. Lamblin G, Lhermitte M, Klein A, Houdret N, Scharfman A, Ramphal P, Roussel P (1991) The carbohydrate diversity of respiratory mucins: A protection of the underlying mucosa? *Am Rev Respir Dis* 144: S19–S24.
60. Ramphal R, Carnoy C, Fievre S, Michalski JC, Houdret N, Lamblin G, Strecker G, Rousell P (1991) *Pseudomonas aeruginosa* recognizes carbohydrate chains containing type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) disaccharide units. *Infect Immun* 59: 700–704.
61. Ramphal R, Pier GB (1985) Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. *Infect Immun* 47: 1–4.
62. Hata JS, Fick RB (1991) Airway adherence of *Pseudomonas aeruginosa:* Mucoexopolysaccharide binding to human and bovine airway proteins. *J Lab Clin Med* 117: 410–422.
63. Marcus H, Baker NR (1985) Quantification of adherence of mucoid and non-mucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. *Infect Immun* 47: 723–729.
64. Simpson DA, Ramphal R, Lory S (1995) Characterization of *P. aeruginosa* fliU, a gene involved in flagellar biosynthesis and adherence. *Infect Immun* 63(8): 2950–2957.
65. Buret A, Cripps AW (1993) The immunoevasive activities of *Pseudomonas aeruginosa:* Relevance for cystic fibrosis. *Am Rev Respir Dis* 148: 793–805.
66. Wanner A (1977) Clinical aspects of mucociliary transport. *Am Rev Resp Dis* 116: 73–125.
67. Rayner CFJ, Rutman A, Dewar A, Cole PJ, Wilson R (1995) Ciliary disorientation in patients with chronic respiratory tract infection. *Am J Respir Crit Care Med* 151: 800–804.
68. Wills PJ, Garcia Suarez MJ, Rutman A, Wilson R, Cole PJ (1995) The ciliary transportability is slow on the mucus-depleted bovine trachea. *Am J Resp Crit Care Med* 151(4): 1255–1258.
69. Wilson R, Roberts D, Cole PJ (1985) Effect of bacterial products on human ciliary function *in vitro. Thorax* 40: 125–131.
70. Wilson R, Pitt T, Taylor GW, Watson D, MacDermott J, Sykes D, Roberts D, Cole PJ (1987) Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia *in vitro. J Clin Invest* 79: 221–229.
71. Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW, Cole JP (1988) Measurement of *Pseudomonas aeruginosa* phenazine pigments in and assessment of their contribution to sputum sol and toxicity for respiratory epithelium. *Infect Immun* 56: 2515–2517.
72. Munro NC, Barker A, Rutman A, Taylor G, Watson D, Macdonald-Gibson WJ, Towart R, Taylor WA, Wilson R, Cole P (1989) Effect of pyocyanin and 1-hydroxyphenazine on *in vivo* tracheal mucus velocity. *J Appl Physiol* 67: 316–323.
73. Adler KB, Hendles DD, Davis GS (1986) Bacteria associated with obstructive pulmonary disease elaborate extracellular products that stimulate mucus secretion by explants of guinea pig airways. *Am J Pathol* 125: 501–514.
74. Graham A, Steel D, Wilson R, Cole PJ, Alton EWFW, Geddes DM (1993) Effects of purified pseudomonas rhamnolipids on ion transport across sheep tracheal epithelium. *Exp Lung Res* 19: 77–89.
75. Somerville M, Taylor GW, Watson D, Rendell NB, Rutman A, Todd H, Davies JR, Wilson R, Cole PJ, Richardson PS (1992) Release of mucus glycoconjugates by *Pseudomonas aeruginosa* rhamnolipids into feline trachea *in vivo* and human bronchus *in vitro*. *Am J Resp Cell Mol Biol* 6: 116–122.
76. Read R, Roberts P, Munroe N, Rutman A, Hastie A, Shryock T, Hall R, McDonald-Gibson W, Lund V, Taylor G (1992) Effect of *Pseudomonas aeruginosa* rhamnolipids on mucociliary transport and ciliary beating. *J Applied Physiol* 72: 2271–2277.
77. Klinger JD, Tandler B, Liedke CM, Boat TF (1984) Proteinases of *P. aeruginosa* evoke mucin release by tracheal epithelium. *J Clin Invest* 74: 1669–1678.
78. Somervile M, Richardson PS, Rutman A, Wilson R, Cole PJ (1991) Stimulation of secretion into human and feline airways by *Pseudomonas aeruginosa* proteases. *J Appl Physiol* 70: 2259–2267.
79. Dorng G, Obermesser JH, Botzenhart K, Flehmig B, Holby N, Hofman A (1983) Proteases of *Pseudomonas aeruginosa* in patients with cystic fibrosis. *J Inf Dis* 147: 744–750.
80. Puchelle E, Zahm JM, Girard F, Bertrand A, Polu JM, Aug F, Sadoul P (1980) Mucociliary transport *in vivo* and *in vitro*: Relations to sputum properties in chronic bronchitis. *Eur J Resp Dis* 61: 254–264.
81. Sleigh MA, Blake JR, Liron N (1988) The propulsion of mucus by cilia. *Am Rev Respir Dis* 137: 726–741.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D.F. Rogers and M.I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 10
Experimental Induction of Goblet Cell Hyperplasia *In Vivo*

Dechun Li and Peter K. Jeffery*

Lung Pathology Unit, National Heart and Lung Institute, Imperial College and Royal Brompton Hospital, London, UK

* Author for correspondence.

1. Introduction

Experimental animal models have contributed to our understanding of the aetiology, pathogenesis and potential for therapy of many human diseases. Several human airway conditions, such as chronic bronchitis, asthma, cystic fibrosis and bronchiectasis have mucus-hypersecretion as a common feature [1–15]. The major sources of airway mucus are (1) luminal mucosubstance present on surface epithelium whose cellular source is presently unclear [16–19], (2) the surface epithelial mucous cell, also called the "goblet cell", and (3) the "mucous cells" of the submucosal glands [20–26]. The serous cells of the submucosal glands may also contribute to the glycoprotein component of mucus and in addition may secrete glycosaminoglycan, a small molecular weight antiprotease [27] and the secretory piece component of IgA. As we shall see, there is increasing evidence that other cell types including the ciliated cell may also contribute to the pool of airway mucus.

Goblet cell hyperplasia and submucosal gland hypertrophy constitute well-recognized and fundamental pathological changes seen in the airways of patients with mucus-hypersecretion [11, 26, 28–32]. Goblet cell hyperplasia is defined as an increase in the number of goblet cells in airways where they are present normally. In contrast, the appearance of newly formed goblet cells in airways where they are not normally present (e.g. the peripheral airways or bronchioles) is referred to as goblet cell or mucous metaplasia [25, 33, 34]. The observation of goblet cell hyperplasia and metaplasia in human airway hypersecretory disorders has prompted the development of animal models which simulate this aspect of pathological change. Distinct animal models involving a variety of animal species have been established in many laboratories around the world. Apart from their secretory function goblet cells may also serve as precursor cells for the regeneration of the airway epithelium and its several cell types. In the normal airway, particularly during injury and repair, the goblet cell may divide and differentiate to form ciliated cells and perhaps other epithelial cell types [35–38]. Inhaled organic and inorganic irritants, pathogens and their derivatives, pharmacological drugs and enzymes have been introduced to the airways in different ways to obtain a subacute or chronic effect which may mimic the pathogenic process in humans. The results of these studies have provided much new data and improved our understanding of the pathogenesis of experimentally induced goblet cell hyperplasia and metaplasia and the origin of the newly acquired goblet cells induced under these experimental conditions. Some of these studies will be reviewed in the following sections, which will begin by briefly considering goblet cell structure and anatomic distribution, followed by a short section on early studies and consideration of the distinct inducing agents used [39–42].

2. Morphology and Distribution of Airway Goblet Cells

2.1. Morphology of Goblet Cells

Goblet cells were first described using the light microscope (LM) by Knauff in 1867 and then by Schulze (1872) [43] and Frey (1874) as chalice or beaker-shaped cells each with a characteristic cup-shaped aspect swollen by secretory granules (Figure 1). Examination of thin sections by transmission electron microscopy (TEM) shows that, in the fixed state, human goblet cells contain large electron-lucent, coalescing granules (Figure 2). The secretory granules are on average 800 nm in diameter but range from 300 to 1800 nm in diameter. The electron-lucent secretory granules fill the cell and become closely packed and confluent probably due to imbibition of water and consequent swelling with loss of their surrounding membranes. Each goblet cell nucleus is compressed to the basal aspect of the cell, and is irregular in shape, often indented and contains much heterochromatin. The scanty cytoplasm contains a well-developed Golgi apparatus and much rough endoplasmic reticulum (RER). Tono- or intermediate filament bundles immunoreactive for keratin have been described, and occasionally dense-cored neurosecretory-like granules may be found [32]. Examination of the apical surface by scanning electron microscopy (SEM)

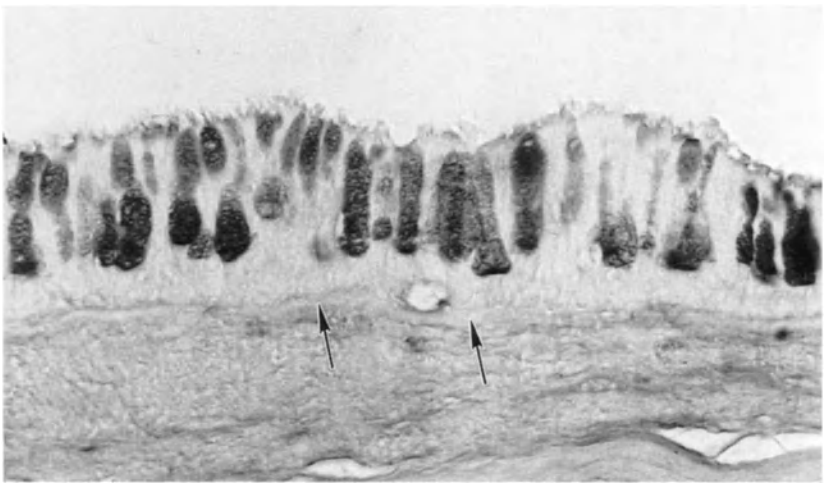


Figure 1. Human bronchial mucosa stained with a combination of Alcian Blue pH 2.5/periodic acid Schiff reagent to demonstrate the intracellular mucin of goblet cells present in the surface epithelium. The content of intracellular mucin gives the cell a characteristic beaker or chalice shape. An apical fringe of cilia is also visible. Arrows mark the position of the reticular basement membrane, the lower (outer) limit of the epithelium (×450).

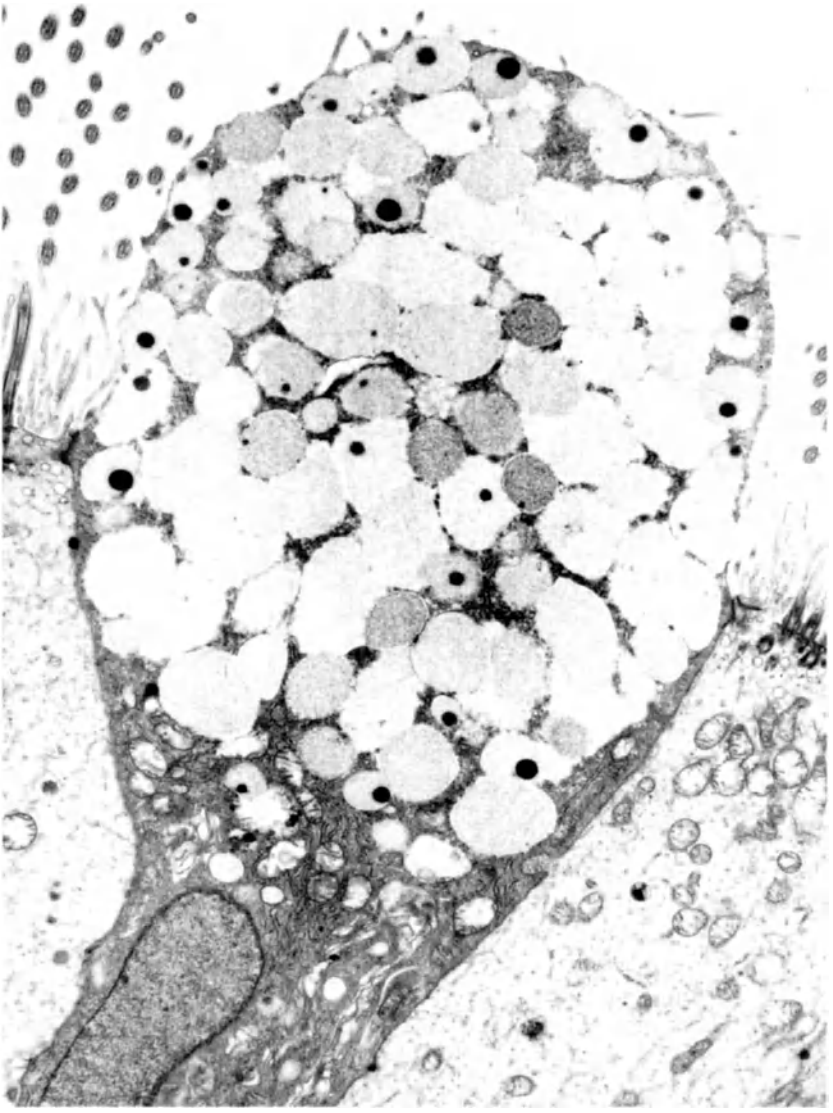


Figure 2. Transmission electron micrograph (TEM) illustrating the appearance of a goblet cell in human bronchus. The cell apex bulges beyond that of the adjoining ciliated cells. The secretory granules of the human goblet cell are normally electron-lucent and tend to confluency; they occasionally have electron-dense cores which may indicate their origin from the more compacted mucin characteristic of the discrete granules of the epithelial serous cell (see Figure 6A) (×13,000).

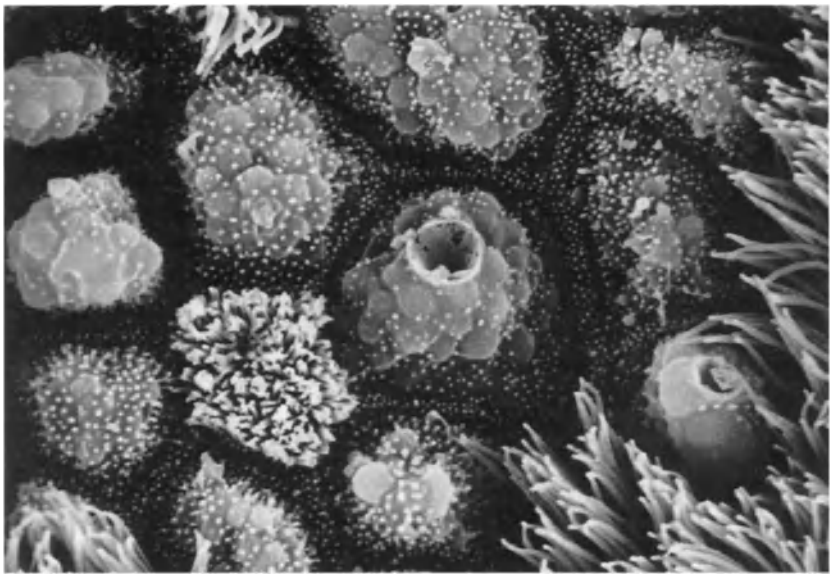


Figure 3. An electron micrograph taken using the scanning electron microscope which gives three-dimensional detail of the surface of the goblet cells. The cobblestone-like borders of each cell are outlined by numbers of short apical microvilli, and the outlines of intracellular secretory granules are seen bulging through the apical cell membrane (×8000).

reveals that the goblet cell has a dome-shaped surface often with secretory granules whose outline is seen protruding into the airway lumen (Figure 3).

Whilst the term "goblet cell" was originally used to describe its shape, in practice the term is applied to cells containing acidic or neutral glycoprotein whether they be thin or goblet in shape. Any acidity of the intracellular glycoprotein is conferred by sialic acid or sulphate moieties, and the extent to which this occurs depends airway level, stage of lung development, injury and species: a single cell may contain granules of only one type (e.g. either neutral or acidic) or a mixture [12, 13, 31, 44–50]. Histochemical staining of human airway goblet cells has revealed that the mucin of the secretory granules is usually acidic, and hence stains blue after sequential staining with Alcian Blue (pH 2.5) and periodic acid Schiff (PAS) (see Figure 1) owing to the presence of sulphate or sialic acid [51, 52]. In the cat [53, 54], dog [55], rabbit [56] and macaque monkey [57], sulphomucins predominate in goblet cells. In the sheep [58], like human [44], either sulphomucins or sialomucins predominate depending on the airway generation in which the cell is found [12, 31, 50].

2.2. Distribution and Development of Airway Goblet Cells

The distribution of goblet cells in the airways varies with species, airway generation, disease status and stage of lung development [59–62]. Histochemically, the presence of intracellular mucin is seen first in the human foetal trachea from the 13th week of gestation [63], when mature ciliated cells are already present. At this stage, goblet cells are sparse or gathered into small clusters, each having a centrally placed nucleus and containing sparse apically placed PAS-positive granules. Infrequently, there are goblet-shaped cells distended by their intracellular secretory granules. Goblet cells appear first in the proximal (central) airways and then develop in the more peripheral (distal) airways. Their number increases with time and peaks in the middle of gestation, when they represent approximately 30–35% of the cells lining the luminal surface [64]. Toward the end of the second term of gestation, there is a relative decrease in the number of goblet cells, these being replaced by an abundance of ciliated cells. Goblet cells that are present during the third term and at birth are less frequent than in the adult, but as in the adult they are more numerous in the proximal than in the peripheral airways [65]. In adult human trachea, the normal mean density of surface goblet cells is estimated at between 6000–7000/mm^2 surface epithelium [66], and in the bronchi the volume density of goblet cells is 6% [67]: they are present throughout the tracheobronchial tree but are rarely seen in bronchioles of less than 1 mm in diameter [68]. In other species the distribution of goblet cells in the trachea and bronchi varies greatly from about 20% in cat, 0.5% in rat, to virtually none in mouse and hamster: the terminal bronchioles of most mammalian species normally lack goblet cells, or they are sparse in number [60].

3. Experimental Induction of Goblet Cell Hyperplasia *In Vivo*

3.1. Early Studies

One of the earliest studies of experimentally induced goblet cell hyperplasia *in vivo* was reported in 1924 and later in 1927 by Carleton [69, 70], who observed the bronchi of guinea pigs subjected to dust inhalation and found that there was resultant increase in the number of goblet cells. In cases where "bronchitis" was marked, not only did the epithelium become composed largely of goblet cells, but many of them lay in plugs of debris present in the bronchial lumina. Several further studies were carried out in 1932 by Hilding [71] and Lord Florey [72]. Hilding's experiments involved suturing one nostril in dogs and rabbits, thereby preventing air movement in one nostril and doubling the movement of air in the other. After 3 weeks to 6 months, there were striking histologic changes in the epithelium. In the rabbit, on the patent side, where air flow was rapid, cilia were lost and the

epithelium took on a stratified form; on the closed side there was marked goblet cell hyperplasia at 3 weeks, and the epithelium became completely comprised of goblet cells 18 weeks after the operation. In the study by Florey, Carlton and Wells [72], 3 ml of formalin was injected daily into the cat trachea through a glass tube over periods of 5–14 days. These investigators observed that in some cats there was a greatly increased goblet cell number compared with normal unexposed cats: in others, the epithelium lost its cilia and its columnar shape, and became comprised of polygonal cells with a transitional form of epithelium resembling that of the bladder. Some submucosal glands contained more mucin than normal, whereas others appeared exhausted of their secretion. Many of the goblet cells were deeply situated in the epithelium abutting the basal layer, and this anatomic location led Florey and colleagues to conclude that the newly transformed goblet cells were probably derived from the undifferentiated basal cells present locally. These reports indicated that the goblet cell was not necessarily an end-stage cell, but it could alter its form and function experimentally. The conclusions of these early experiments emphasized the ease with which goblet cell hyperplasia and metaplasia could be induced by an altered local environment or by exogenously derived irritation, and demonstrated parallels with the changes observed in human hypersecretory disease. In this way these early studies encouraged further studies of experimental induction of goblet cell hyperplasia with a view to establishing the origin of newly formed goblet cells and to establish the systems which would allow investigation of potential therapies for humans.

3.2. Animal Species and the Range of Inducing Agents Used

A wide variety of species have been used for the experimental induction of goblet cell hyperplasia, and these include laboratory rodents such as rats, mice, hamsters, guinea pigs and rabbits, and domesticated animals such as cats, dogs, sheep and pigs. Primates, like bonnet monkeys (*Macaca radiata*) have also been used [73, 74]. The agents used in early studies were formalin [72], industrial dust [69], machining fluids [75], cotton dust [76] and then later, gases, including sulphur dioxide (SO_2) [38, 72, 77–82], nitrogen dioxide (NO_2) [83–86], chlorine (Cl_2) [87, 88] and ozone (O_3) [73, 74, 89–94]. Several of these gases are constituents of the polluted air we breathe in our everyday environment or workplace and are implicated in the etiology of hypersecretory diseases and acute or chronic damage to the lung and its airways [85]. Concentrations of these gases in air vary greatly from place to place and with time during the year, and safe limits are still debated. Tobacco smoke is a common self-inflicted pollutant, and experimentally it has been used to induce goblet cell hyperplasia. Many studies have concentrated not only on the effect of inducers of goblet cell hyperplasia/metaplasia but also on drugs which might prevent or reverse goblet

cell induction [95–110]. Tobacco emits a complex smoke of more than 2000 constituents in the gas and tar phases: some researchers have compared the distinct effects of the particulate and vapour phases [102] and found that most of the tobacco smoke-induced changes, including goblet cell hyperplasia, can be attributed to the particulate phase of the smoke [102].

There are also several reports of goblet cell hyperplasia following exposure to pathogens or their derivatives. These observations are of particular interest because infection such as that by *Pseudomonas aeruginosa* [111] in patients with cystic fibrosis is an important contributor to the development of goblet cell hyperplasia and metaplasia in such a hypersecretory disease [112]. As we shall see, lipopolysaccharide (LPS) (also referred to as endotoxin), released from the cell wall of some Gram-negative bacteria, can induce goblet cell hyperplasia and mucous metaplasia in several laboratory animals when instilled or inhaled into the trachea [113–120]. A prominent pathological change after intratracheal exposure of animals to LPS is a severe exudative bronchopneumonia comprised predominantly of neutrophils which infiltrate the epithelium of the conducting airways. The association of neutrophil infiltration and neutrophil elastase with goblet cell numbers is a strong one [120]. As a result crude neutrophil extracts and purified neutrophil elastase have been administered to the airways [121–125]. The finding was striking and expected and, in addition to the increase of goblet cell number in the conducting airways, the lung parenchyma also showed evidence of destruction (i.e. emphysema) in certain animal species. This has been of particular interest, as the coexistence of smoking-induced bronchitis and emphysema is the usual feature of human hypersecretory disease in which there is airflow obstruction, referred to as chronic obstructive pulmonary disease (COPD) [126].

The choice of animal species and method of administration of goblet cell hyperplasia inducers depends mainly upon the particular aim of the study. Some animals may be more susceptible or resistant to one particular inducer than another. For example, rats are particularly resistant to SO_2 (see Section 4.1.1). Consistency of administration is important as is consideration of the solubility of the gas (e.g. SO_2 is highly soluble and hence damages proximal airways, whereas NO_2 is less so and preferentially affects the distal airways). For exposure to agents such as SO_2 or NO_2, small laboratory animals remaining in their cages are usually placed in an exposure cabinet through which the irritant is passed at the required concentration. For tobacco smoke, the number of puffs, butt length, nicotine and tar content and airflow through the chamber should all be standardized. The type of tobacco leaf (blond or dark) and, more important, the manner in which it is cured (air or flue-dried) are critical to its efficacy in inducing the hypersecretory change. A blond flu-cured tobacco is high in sugar and provides a smoke of high acidity, whereas a black tobacco emits a more alkaline smoke. Armitage and Turner [127] have shown that the absorption of nicotine through the buccal mucosa is quicker in alkaline than in an acid

smoke, and hence the need to inhale with an alkaline smoke is reduced. For large animals, such as sheep, dog and monkey, tracheostomy may be the choice of delivery of either the tobacco smoke or the irritant gas [99, 104]. Some inducers such as LPS and neutrophil elastase can be given by intratracheal instillation, in which case the method of anaesthesia and its dose, volume, and the solvent carrier and pressure of instillation are factors to be considered. Using a nebulizer for delivery by inhalation may give rise to a better distribution of the agent to the lung and its airways. Atopic asthma models which show airway goblet cell hyperplasia use intratracheal instillation of ovalbumin given to sensitized animals [128]. During recent years, with the progress of gene-targeting techniques, animal models for cystic fibrosis have provided a new path for the exploring the pathogenesis of inherited diseases [92, 129]. Table 1 summarizes and references most of the animal species and goblet cell inducers used in the experimental induction of goblet cell hyperplasia. The following sections give further details of the results of such experiments.

Table 1. Animal species and goblet cell inducers used in the experimental induction of hypersecretion

Inducer	Route of Administration	Animal	Reference
Early Study			
Industrial dust	Inhalation	Guinea pigs	[41, 69]
Formalin	IT	Cats	[72]
Surgical suture	Surgery	Dogs and rabbits	[71]
Irritant			
SO_2	Inhalation	Rats	[77, 79, 80, 208]
		Mice	[77]
		Dogs	[38, 78, 81, 82]
		Hamsters	[130]
TS	Inhalation	Rats	[95–98, 101, 103, 108]
		Dogs	[99 141]
NO_2	Inhalation	Rats	[83, 84]
O_3	Inhalation	Rats, monkeys, rabbits	[73, 74, 89, 91, 92, 94]
Cl_2	Inhalation	Rats, mice	[87, 88]
Pathogens and their Derivatives			
Endotoxin	Intranasal instillation	Rats	[114, 120]
	IT	Rats	[117, 210, 211]
	Inhalation	Rats	[116, 209]
	IT	Hamsters	[118]
Mycoplasma hyorhinis	Intranasal inoculation	Pigs	[158, 159]
Cholera toxin	Intranasal instillation	Mice	[161]

TS = tobacco smoke; IT = intratracheal instillation.

Table 1 (continued)

Inducer	Route of Administration	Animal	Reference
Enzymes			
Neutrophil-derived enzymes	IT	Hamsters	[121–123, 125, 163, 165–168, 172]
	IT	Rats	[162]
Other enzymes	IT	Hamsters	[164]
Drugs			
Isoprenaline and salbutamol	im	Pigs	[177]
	Injection?	Rats	[173–175]
Pilocarpine	Injection	Rats	[173, 175]
Sex hormones	Endogenous	Rats	[180]
	Extrogenous	Guinea pig	[181]
Others			
Sensitization with OA	IT challenge	Mice	[128, 184]
Gene knockout (CFTR)	Transgenic	Mice	[92, 129]

IT = intratracheal instillation; im = intramuscular.

4. Induction of Airway Goblet Cell Hyperplasia

4.1. Irritation

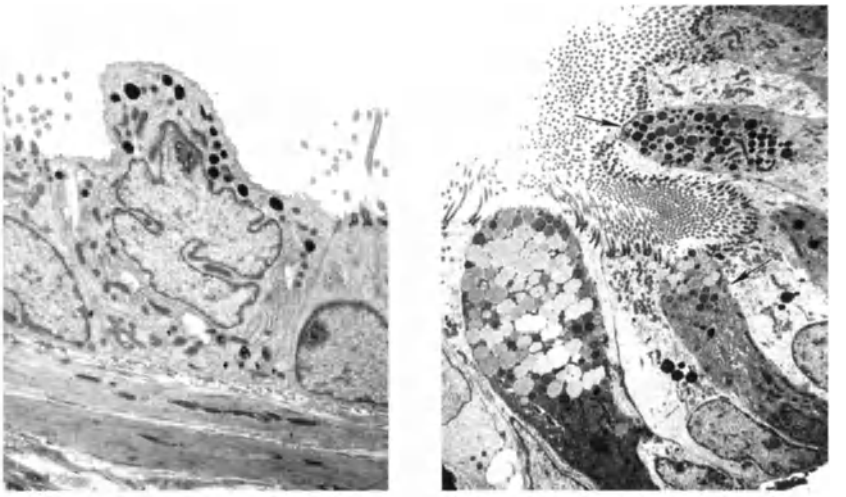
Irritant gases including SO_2, NO_2, O_3 and those present in tobacco smoke (e.g. acrolein, acetaldehyde) have been used in different species including mice [77, 90], hamsters [130], rats [77, 80, 84, 89, 100–103, 108, 109], dogs [81, 82] and sheep [104]. The pathological effects vary in these species.

4.1.1. Sulphur dioxide: Sulphur dioxide (SO_2) is a major pollutant of urban air. It reached particularly high levels during the UK smogs of the 1950s which contributed to the death of so many people. It was first used to induce experimental bronchitis by Reid in 1963 in mice and rats [77] exposed to an atmosphere of it passively within a chamber. Initially the dose administered was of the order of 40 parts per million (ppm, 1 ppm being considerably more than was recorded in the atmosphere during the London smogs). However, at this low dose (40 ppm), even after 3 months' exposure, no appreciable pulmonary change was found: the doses subsequently used were 300–400 ppm, as these rodents proved to be remarkably resistant to the effects of SO_2. In rats, exposure to SO_2 at these concentrations, 5 h daily, 5 days per week for 6 weeks, resulted in an increased concentration of

goblet cells in the airway epithelium. Goblet cell hyperplasia first appeared in large airways, and after 3–4 weeks goblet cells were found further out in the peripheral airways, where they were normally absent (i.e. there was mucous metaplasia). In the large bronchi, the number of cells containing neutral mucin was increased, and relatively more of these were cells distended with mucin. Goblet cell counts demonstrated there were significant increases in their numbers in the SO_2-treated rats, about three- to fourfold more than that found in the large bronchi of control sham-exposed rats, i.e. 10 goblet cells per high-power microscopic field in control rats compared with 34 in SO_2-treated rats. In the terminal bronchioli, normally free of goblet cells, the mean goblet cell count was 2.5 per high-power field, with the highest individual number being 11. The newly induced goblet cells contained a distinct histochemical mucin. At the periphery, they contained acidic mucin which was susceptible to digestion by sialidase, while proximally most of the acidic mucin remained after digestion by this enzyme, either because it was sulphated or comprised of sialidase-resistant sialomucin. Exposure of specific pathogen-free rats to SO_2 did not result in bacterial infection, so these changes were the result of irritation alone and not due to the superadded effects of infection on a compromised airway. These experimental results clearly implicated irritation by atmospheric pollutants as a cause of bronchitis without the requirement of infection.

Due to the high solubility of SO_2, there was ulceration of the surface epithelium and cell division associated with healing only in the proximal airways. The experimental goblet cell metaplasia of terminal bronchioli persisted for 3 months after cessation of exposure. Analysis of the patterns of SO_2-induced mitosis and goblet cell increase in distinct airway generations of rat lung for 2 or 4 days and for 3 or 6 weeks indicated that ulceration was not necessary for the goblet cell increase to occur and highlighted the potential for SO_2 to induce mucous metaplasia in the most distal airways of the lung. Electron microscopy demonstrated the phenotypic alteration from Clara cells, the normal secretory cell of the distal airway, to the newly-appearing goblet cell (Figures 4 A and B). These were the first studies to describe the induction by SO_2 of airway goblet cells and their appearance and increase in terminal bronchioli, a key of human chronic bronchitis associated with airflow obstruction (i.e. COPD).

Further studies using SO_2-exposed rats were carried out by Lightowler and Williams [80], and these authors confirmed the pathological changes described earlier by Lamb and Reid [131] and, in addition, found an association between the reduction of mucociliary clearance in the SO_2-exposed trachea with increasing severity of the pathological changes. They concluded that the reduction in flow of mucus was due to the combined effect of changes in the quantity and quality of the mucus as well as a reduction of cilia and replacement of the normal ciliated mucosa by atrophic and squamous epithelium. In association with the morphological changes reported by several researchers, Jany and colleagues [132] observed increased



(A)

Figure 4. TEM of distal (peripheral) airways in specific pathogen-free rats. (A) An exposed control showing the normal secretory cell of the distal airway: a Clara (non-ciliated bronchiolar) cell which bulges into the airway lumen and has few electron-dense secretory granules. Goblet cells are normally absent (×7000). (B) Following subacute exposure to SO_2, goblet cells appear and increase in number (i.e. mucous metaplasia). There are phenotypic forms showing features of both Clara and goblet cell (arrow) (×3000).

mucin gene expression by Northern blot analysis in rat airways following SO_2 irritation, but the effects were only marked in animals with superadded infection by Sendai (Parainfluenza I) virus. These authors suggested that the observed increase of mRNA transcripts for mucin gene would likely be the result of increased gene transcription rate or a decrease in the degradation rate of mucin mRNA.

In Syrian hamsters, exposure to SO_2 alone or SO_2 with papain for 19 to 74 days both resulted in goblet cell hyperplasia in the upper trachea and the proximal bronchi; the latter also resulted in the development of emphysema [130]. Dogs exposed passively to SO_2 for 2-h periods twice weekly for 4 to 5 months at concentrations of 500 and 600 ppm showed a decrease in the ratio of goblet cells to ciliated cells in the proximal portions of large (segmental) bronchi and an increased ratio at the distal ends of these bronchi and also in bronchioli. There was also an enlargement of bronchial submucosal glands throughout the airways similar to that seen in human chronic bronchitis [81, 82].

In the last-mentioned model, the changes to both surface goblet cells and gland make it a good model for human bronchitis. However, the animal numbers which can be used are limited by their size and cost. The rat and hamster models have the advantage of relatively low cost and homogeneity through inbreeding and the low initial numbers of goblet cells if specific pathogen-free animals are used. The mucous metaplasia of bronchioli

in response to SO_2 is likely a critical lesion to study experimentally, as it is this lesion rather than that seen in large airways which likely contributes to the development of COPD. The dose of SO_2 is difficult to chose: low concentrations of SO_2 do not induce goblet cell hyperplasia/metaplasia, and because high doses will result in ulceration of the bronchial mucosa, the results may be difficult to interpret.

4.1.2. Tobacco smoke: Tobacco smoke (TS) is a major cause and predisposing factor in the development of human chronic bronchitis [126]. The actions of TS on airway epithelium are many and include its ciliostatic effect and induction of goblet cell hyperplasia/metaplasia (the "bronchitic effect"), and stimulation of mitosis and squamous metaplasia (its "cancer effect") [100, 110, 133, 134]. Mellors [135] first observed airway epithelial changes in rats exposed to TS, and these light microscopic observations were followed by those of Passey (1967), Lamb (1969), Jones (1973) and others [101, 103, 108, 109, 136–138]. These authors found that experimental subacute inhalation of TS caused an increase in the total number of goblet cells in rat tracheal epithelium and enlargement of tracheal submucosal glands in exposures of up to 4 weeks [103, 108]. The increase was found to be dose-related and greatest in the largest intrapulmonary airways. In the extrapulmonary airways the first exposure to TS caused a discharge of intracellular mucin such that the absolute number of secretory cells apparently fell: their number was reduced to below the control value (i.e. that of unexposed or air-exposed animals) especially in the upper and mid-tracheal regions. The result is explained by the method of detection of goblet cells, which does not detect the secretory cell per se but instead its content of intracellular mucin. After 2 and 3 days of continued exposure, in each extrapulmonary region the number of goblet cells was markedly increased above control values. There was an increased goblet cell number throughout the axial intrapulmonary pathway following the first exposure, and after 3 days of exposure there was a goblet cell metaplasia in peripheral airways as well. The increase in proximal airways was approximately three times that of sham-exposed animals at 3 days and six times at 14 days. Rats given 1 day's break from the exposure again showed a discharge effect on re-exposure, but only in the extrapulmonary airways. These results suggest that tolerance develops with continued exposure to smoke.

Subacute exposure to TS increases the total number of secretory cells in rats exposed passively to it; the percentage containing acidic mucin increases, and that containing neutral mucin decreases at each of the airway generations examined (see Figure 5) [103, 106, 109]. Jeffery and Reid examined these changes by electron microscopy and quantified the relative proportions of secretory cells and other epithelial cell types in order to determine the origin of the newly acquired goblet cell [133]. In the unexposed or sham-exposed animals less than 1 % of epithelial cells were goblet: most of the secretory cells contained electron-dense granules and, due to their

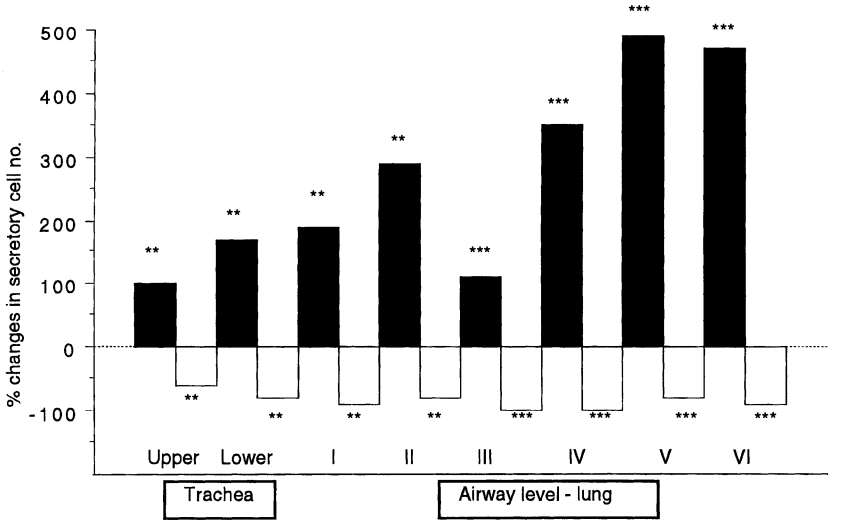


Figure 5. Experimental exposure of specific pathogen-free rats of an atmosphere of cigarette smoke, generated automatically from cigarettes and given for approximately 4 h daily for 2 weeks. Cigarette smoke increases the airway secretory cell number. Counting the numbers containing acidic (■) or neutral (□) glycoprotein shows that, at each successive airway level, cigarette smoke induces an increase in the percentage of the acidic and reduction in the neutral mucin-containing cells (expressed here as mean percentage change of control values ** $P = < 0.01$; *** $P < 0.001$ [106]).

morphological similarity with serous gland acinar cells, were referred to as epithelial "serous" cells [95]. Exposure to an atmosphere of TS resulted in a rapid thickening of the surface epithelium (by day 1) due to an increased cell height (cell hypertrophy or swelling). Cells were induced to multiply by mitotic division particularly between days 1 and 2 [100, 103], serous cells discharged their granules by day 3 and thereafter total secretory cell number increased with goblet cell hyperplasia evident between days 7 and 14 (Figures 6A and B). There was morphological evidence of transformation of serous to mucous cells (Figure 7), and the capacity of serous cells to divide was retained by the newly appearing goblet cells (Figure 8) [107, 139]. The authors concluded that the serous cells were a labile and dividing population which could rapidly change form to become goblet; subsequently goblet cell hyperplasia likely occurred by cell division. Unlike the changes induced by SO_2, there was no evidence of ulceration; ciliated cells were unaffected structurally, and also increased in number.

Coggins and colleagues [102] compared the effects of the particulate and vapour phases of TS in rats, and found that most of the changes produced by TS, including goblet cell hyperplasia, could be attributed to the particulate phase of the smoke. Interestingly, nicotine itself does not seem to be the pharmacological agent responsible for the TS effect [140].

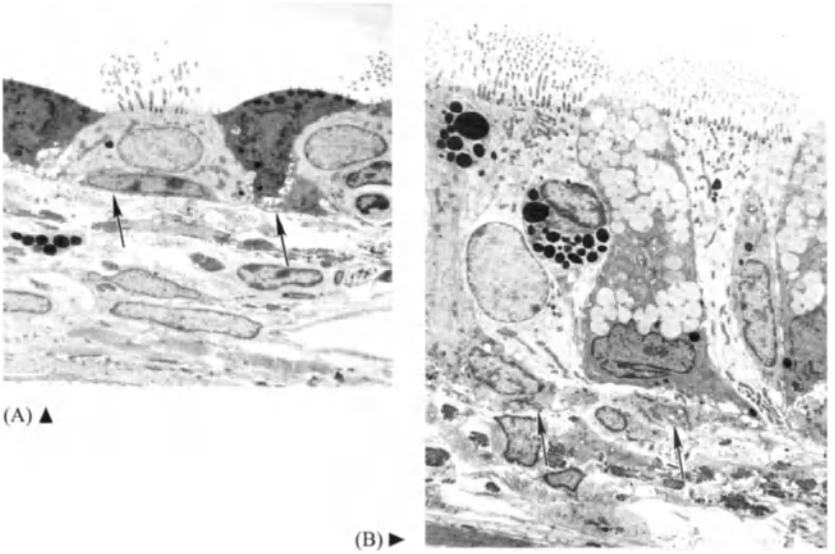


Figure 6. TEM of rat main bronchus. (A) Serous cells with discrete electron-dense secretory granules constitute the most frequently found secretory cells in unexposed animals [95] shown here adjacent to a ciliated cell and basal cell. Arrows mark the position of the basement membrane (×3250). (B) Following passive exposure to tobacco smoke for 2 weeks there is a thickening of the surface epithelium and hyperplasia of goblet cells with an abundance of confluent electron-lucent secretory granules resembling those found in the human bronchus (see Figure 2) [133]. Arrows mark the position of the basement membrane. The dense-granulated cells are globular leucocytes (×3250).

Whilst experimental inhalation of TS does normally induce squamous (epidermoid) metaplasia in the nose of rat [110], the combined effects of vitamin-A dietary deficiency and TS induce extensive squamous metaplasia of extrapulmonary airways [105]. Dogs and sheep have also been subacutely exposed to TS given via tracheotomy [99, 104, 141]; the results were similar to those seen in rats; except that there was also enlargement of the submucosal glands (normally sparse in rats). Chronic exposure of dogs has given rise to squamous metaplastic lesions and evidence of carcinoma *in situ* [141]. In each region the increase in goblet cells persisted after 7 and 14 days of exposure.

The inclusion of phenylmethyloxydiazole (PMO) (an anti-inflammatory agent) in the tobacco as 2% by weight prevented TS-induced goblet cell hyperplasia [100, 101, 103, 109, 133, 142], as did indomethacin, dexamethasone, prednisolone and hydrocortisone given i.p. concurrently with smoke exposure [96, 106]. *N*-acetylcysteine and *S*-carboxymethylcysteine taken orally (daily in the drinking water) inhibited TS-induced goblet cell hyperplasia and mucus-hypersecretion [97, 143–145]. Oral *N*-acetylcysteine and i.p. injection of the non-steroidal anti-inflammatory agents

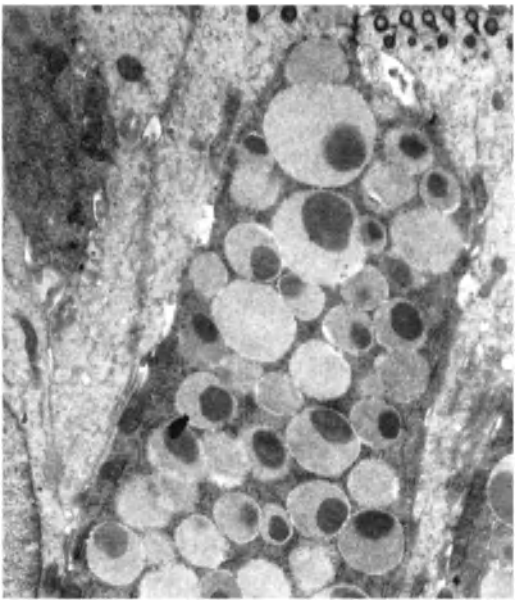
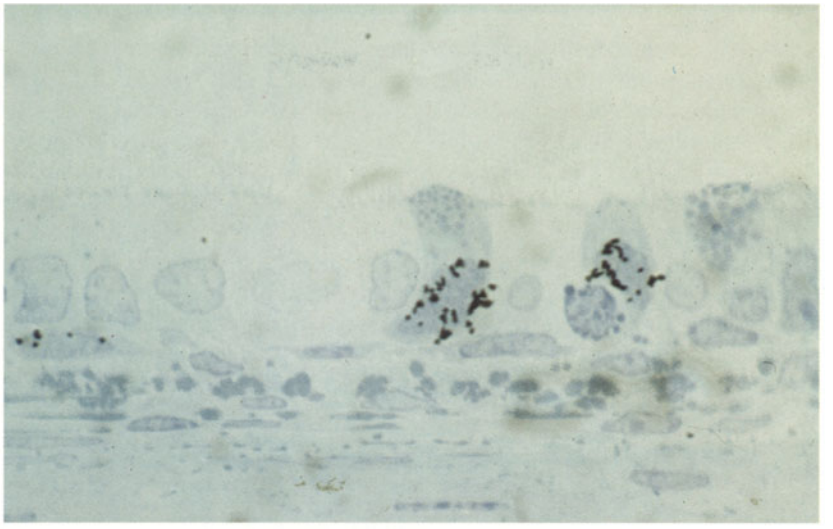


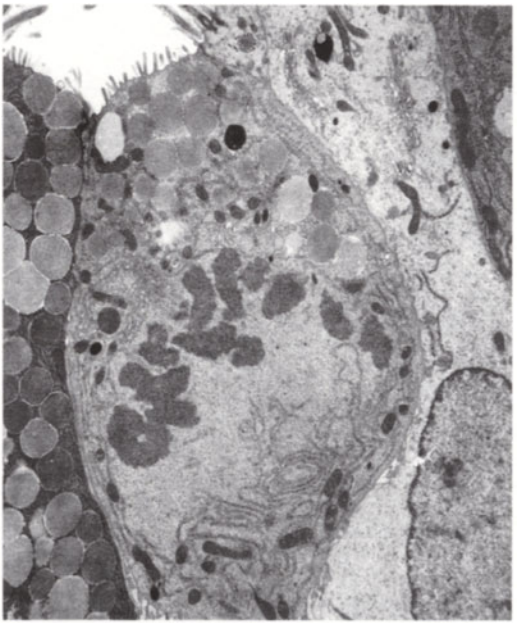
Figure 7. TEM of a phenotypic form frequently found following TS exposure showing features of both electron dense serous and electron lucent goblet cell granules. The morphological change may represent uptake of water and granule swelling due to TS-induced changes of cell and granule membrane permeability (×12,500).

indomethacin and flurbiprofen speed recovery of TS-induced goblet cell increase after cessation of exposure to the smoke [98, 105, 106, 143, 146].

TS-induced goblet cell hyperplasia in animals is a dose-related change which occurs at all levels of the conducting airways [41]. The changes in rats and dogs are similar to those reported in humans. The rat appears to provide a good model for examination of the pathogenesis of surface epithelial goblet cell hyperplasia and metaplasia in response to TS. Use of high-grade specific pathogen-free rats results in a very low background of goblet cells which can be made to increase in number (in large airways) or appear (in small airways), and this occurs without epithelial ulceration and in the absence of infection. However, there is little mucus-secreting submucosal gland in the rat, and gland enlargement (a feature of human bronchitis) is therefore difficult to study in the rat. This latter aspect is best modelled by studies of the dog, in which mucus-secreting glands are prominent. However, costs and public opinion may weigh heavily against such studies. Interestingly, the tobacco product currently available has had its content of nicotine and tar markedly reduced. Unless especially prepared cigarettes are used, the effects of today's cigarette smoke in the rat model are insufficient to induce a marked goblet cell hyperplasia (authors unpublished observations) and render the model less valuable than previously.



(A)



(B)

Figure 8. (A) Photomicrograph of rat main bronchus from an animal exposed to tobacco smoke. The animal was given tritiated thymidine which has been incorporated into the DNA of cells about to divide: thymidine incorporation is visualized as dense autoradiographic grains overlying serous cells cut in section (×2000). (B) TEM of a rat goblet cell complete with electron-lucent secretory granules and showing condensation of chromosomes and loss of nuclear membrane, characteristics of a dividing cell. The capacity of goblet cells to divide in response to injury is now widely accepted (×5000).

4.1.3. Nitrogen dioxide: In view of the characteristically protracted pathogenesis of chronic obstructive pulmonary disease in humans, experiments have been designed to model the bronchitis and emphysema of COPD. Nitrogen dioxide (NO_2) is an atmospheric pollutant with marked effects on airway epithelial cells [147]. As it is less water soluble than SO_2, it tends to affect small airways as well as proximal. Rats have been continuously exposed to inhalation of NO_2 at concentrations which cause no inflammation, edema or haemorrhage [83, 84]. Periods of respite have been interposed during an otherwise continuous regime to permit survival of the animals for the duration of their natural life (expectancy of 2 to 3 years). Rats exposed to 15 ppm of NO_2 have shown that the proximal airways (e.g. the trachea and bronchi) exhibit goblet cell hyperplasia and that there is an extension of their number into bronchioli (particularly the respiratory bronchioli) and even into alveolar ducts. The hyperplasia and metaplasia of surface epithelium are evident by light microscopy on approximately the fourth day of exposure, and these changes are detected even earlier by electron microscopy. Whilst the increase of goblet cell number particularly in the terminal bronchioles is characteristic, the main anatomical site for pathological changes is the alveolus. The end result is emphysematous enlargement of airspaces distal to the conducting airways which resembles the emphysema of humans.

The advantage of the NO_2-exposed animal model is that goblet cell hyperplasia/metaplasia extends to the bronchioli, and the bronchitic effect is combined with emphysema which mimics the changes in human chronic bronchitis and COPD. Unlike SO_2, NO_2 does not cause severe tissue damage, and the subacute/chronic effects can be studied over a longer time course. It is likely that the structural changes to small airways and alveoli are of key importance in the development of accelerated progressive loss of lung function associated with COPD, and the NO_2 model would seem to be a particularly valuable one to study further.

4.1.4. Ozone: Ozone (O_3) is a major component of photochemical smog, and high levels of this pollutant are found worldwide and threaten health. It is a potent respiratory irritant known to induce lung injury in both humans and experimental animals [73, 74, 89–91, 93, 94, 148], and is about 10 times more potent in terms of lung toxicity than NO_2. Bonnet monkeys exposed to 0.15, or 0.39 ppm O_3 for 6 or 90 days, 8 h per day, showed ciliated cell necrosis, shortened cilia, secretory cell hyperplasia and significant increases in both acidic and neutral mucus in the nasal mucosa [73, 74]. Similar results were also observed in the nose [73, 148] and tracheobronchial epithelium [91] of the rats treated with 0.12 and 0.80 ppm or 0.12, 0.5 and 1,0 ppm O_3, respectively. These results demonstrated that exposures to ambient levels of O_3 could induce significant epithelial lesions and changes in the stored secretory product in the nose and intrapulmonary airways. The extent of damage by ozone to the lung

depends upon its dose. Short-term ozone exposure (i.e. subacute exposure of 7–24 h daily for up to 3 weeks) results in ciliary damage and foci of ciliated cells which appear in the large airways of animals exposed to 1 ppm ozone [42]. In bronchioli, goblet cells discharge their granules during the first 24 h of exposure, followed by their hyperplasia up to 15 days. The long-term effects of ozone exposure (i.e. up to 18 months) include goblet cell metaplasia in bronchioli and bronchiolization of adjacent alveolar walls which may also become fibrotic. The tissue damage produced by concentrations of ozone below 1 ppm appears to be time-dependent. The adaptive response of the lung includes increased glutathione peroxidase and superoxide dismutase activity. Taurine, a detoxifiying antioxidant, given in drinking water, can significantly decrease ozone-induced inflammatory cell number both in the bronchoalveolar lavage and in the lung airway interstitium and reduce bronchiolar secretory cell metaplasia [89]. Low concentration O_3-induced tissue injury is focused mainly on bronchioli. In summary, the ozone model can be used to study the interrelationships between acute lung injury, inflammation and the induction of goblet cell hyperplasia.

4.1.5. Chlorine: Chlorine (Cl_2) is the most abundant naturally occurring halogen. In the elemental form it is highly reactive and moderately water soluble. Because of chlorine's activity, it is commonly employed in many industrial processes, in addition to its use as a household cleaner and disinfectant. Chlorine gas was used as a chemical warfare agent during World War I. Many soldiers who survived chlorine exposure developed respiratory symptoms and were considered severely impaired 5 years after exposure (see ref. [88]). In mice and rats exposed to 0.4, 1.0 or 2.5 ppm of chlorine gas for 6 h per day, 5 days/week or 3 alternate days/week for up to 2 years, there were severe olfactory epithelial degeneration, septal fenestration, mucosal inflammation, respiratory epithelial hyperplasia, secretory metaplasia and goblet cell hypertrophy and hyperplasia [88]. Exposure by inhalation of between 14 and 80 ppm of chlorine can induce goblet cell hyperplasia in rat airways [87]. These concentrations of chlorine gas cause acute ulcerative inflammation of the trachea and main bronchi of rats aged less than 20 weeks. Higher concentrations of chlorine have been given, and older animals appear to tolerate the higher concentrations better than younger animals. Necrosis of the entire mucosa with acute inflammation was seen in animals dying of chlorine exposure, whilst chronic damage was seen in the survivors and those dying towards the end of the exposure period. All those which survived the exposure had intraluminal mucus in the bronchi, much of which was aspirated to the alveoli. The excess of intraluminal mucus was associated with an increase in the numbers and extension of goblet cells to bronchioli. A severe centrilobular form of emphysema was also found in animals exposed to chlorine. The tendency of small laboratory animals and especially rodents and rabbits to spontaneous lung disease, usually due to infection by *Mycoplasma* sp., was exacerbated by exposure

to chlorine. The appearance of spontaneous respiratory disease makes interpretation of the effect of chlorine difficult, as spontaneously generated lung disease due to infection is, of itself, associated with marked goblet cell hyperplasia.

The end effects of a number of unrelated irritants, discussed above, include goblet cell hyperplasia which could be said to be a non-specific subacute/chronic response to persistent low-grade injury. Ulceration is not required for hyperplasia to occur, and it may reverse in time if the irritant is removed. Irritant-induced goblet cell hyperplasia appears to involve both phenotypic alteration together with cell division. It is the anatomic site of mucous metaplasia/hyperplasia which appears to be crucial to its contribution to airflow obstruction.

4.2. Pathogens and Their Derivatives

4.2.1 Endotoxin or Lipopolysaccharide: Derived from the walls of Gram-negative bacteria lipopolysaccharide (LPS) is highly toxic in low concentration [149, 150]. Issekutz and colleagues [151] and others [119, 152] have demonstrated that LPS can induce marked neutrophil emigration *in vivo.* Inhalational exposure to LPS even in low dose may be an important factor in several occupational diseases [116]. In addition, LPS is a ubiquitous "substance" found in our domestic environment. LPS has been detected in biologically active concentrations in domestic water (0.04–1.0 μg/ml) [153], in air humidification systems (0.13–0.39 $\mu g/m^3$) [154], in cotton dust [76], in machining fluids [75] and in house dust extracts (0.45–500 μg/ml) [154]. Thus people living and working in endotoxin-contaminated environments could potentially inhale enough of this deleterious agent to evoke airway responses [60, 75, 76, 115]. Airway infections induced by endotoxin-associated bacteria are often characterized by increased production and secretion of airway mucus and are associated with an influx of polymorphonuclear inflammatory cells, particularly neutrophils. Such infections are a characteristic of many bronchitic patients who experience exacerbations often during the winter months.

Endotoxin was first used experimentally to model bronchitis by Harkema et al. and Pauwels and colleagues *in vivo* in rat [114–116, 119, 120]. Following intranasal instillation to rats of 50 μl of 5 mg/ml of LPS in saline once each day for 3 consecutive days, there was a transient but marked inflammatory response in the surface mucosa observed 6 h after the last instillation. The cellular inflammatory response was composed of a diffuse distribution of neutrophils infiltrating the nasal mucosa. Goblet cell hyperplasia accompanied the neutrophil influx, and there was a marked increase of mucus in the surface epithelium 24 h after instillation. Rats given rabbit anti-rat neutrophil antiserum to deplete neutrophils, prior to the LPS, showed no significant neutrophil influx of the nasal mucosa, yet the increase of

goblet cells and AB/PAS stainable mucus compared with the saline-treated rats still occurred [155]. These authors postulated that intranasal endotoxin can induce an increase in the amount of intracellular mucin independent of neutrophils and that the endotoxin-induced neutrophil influx probably triggers the additional discharge of mucus from goblet cells [115, 120].

In contrast, LPS given as multiple doses to the lower airways by intratracheal instillation [114] or by inhalation [116] induced a significant increase in goblet cell numbers and intracellular mucin by 2 days after LPS exposure, and the effect persisted for at least 7 days. There was also an increase in the concentration of the epithelial cells after LPS instillation due mainly to an increase in the number of goblet cells: there was no increase in the number of basal cells in the larger airways. No epithelial "serous" cells were found in LPS-treated rat airways, and these authors suggested that serous cells were either destroyed by the LPS, or had been replaced by goblet (mucous) cells, or that LPS exposure stimulate serous cells to transform into goblet cells [114], as had previously been suggested to occur after TS (see Section 4.1.2 in this chapter). Saline instillation was used as a control procedure, and this induced no histologic alterations in nasal, tracheal or intrapulmonary airways of the control rats. Yet rats exposed by inhalation to 52.4 $\mu g/m^3$ LPS had mild to moderate goblet cell hyperplasia [116]. In animals exposed to medium and high concentrations of LPS, there were marked changes, including goblet cell metaplasia and epithelial cell hyperplasia in the surface epithelium lining the main axial airway of the left lung lobe. Similarly, epithelial cell hyperplasia with goblet cell metaplasia was evident in the peripheral bronchioles. Steiger et al. [117] demonstrated concurrent increases in the storage and release of mucinlike molecules in rat airway epithelial cells in response to a single intratracheal instillation of bacterial endotoxin. They first demonstrated reactivity of airway mucosubstances with antimucin antibodies A10G5 and B6E8 and then used the antibodies in an enzyme-linked immunosorbent assay (ELISA) to compare mucin release in bronchoalveolar lavage (BAL) fluid in LPS-treated vs control rats. They found that endotoxin treatment increased the amount of mucin released over that in controls 1.5-fold at 96 h and 2.5-fold at 168 h after instillation, and also increased the volume densities of intraepithelial mucins in rat pulmonary airways at 48, 96 and 168 h, 10-fold and 7-fold, respectively, after instillation. Goblet cell metaplasia with increases in stored and secreted mucins was evident at 24, 48, 96 and 168 h after the single instillation of endotoxin [117].

We have repeated the above experiments using a single relatively low dose of intratracheal instillation of LPS (100 µg in 200 µl of saline) and found that goblet cell hyperplasia and metaplasia are prominent both in proximal and peripheral airways. Tissue neutrophilia occurs by 4 h, and intraepithelial neutrophils peak at 16 h post LPS. There are associated degenerative changes and sloughing, particularly of ciliated cells, without epithelial ulceration. Goblet cell number and intracellular mucin (mostly

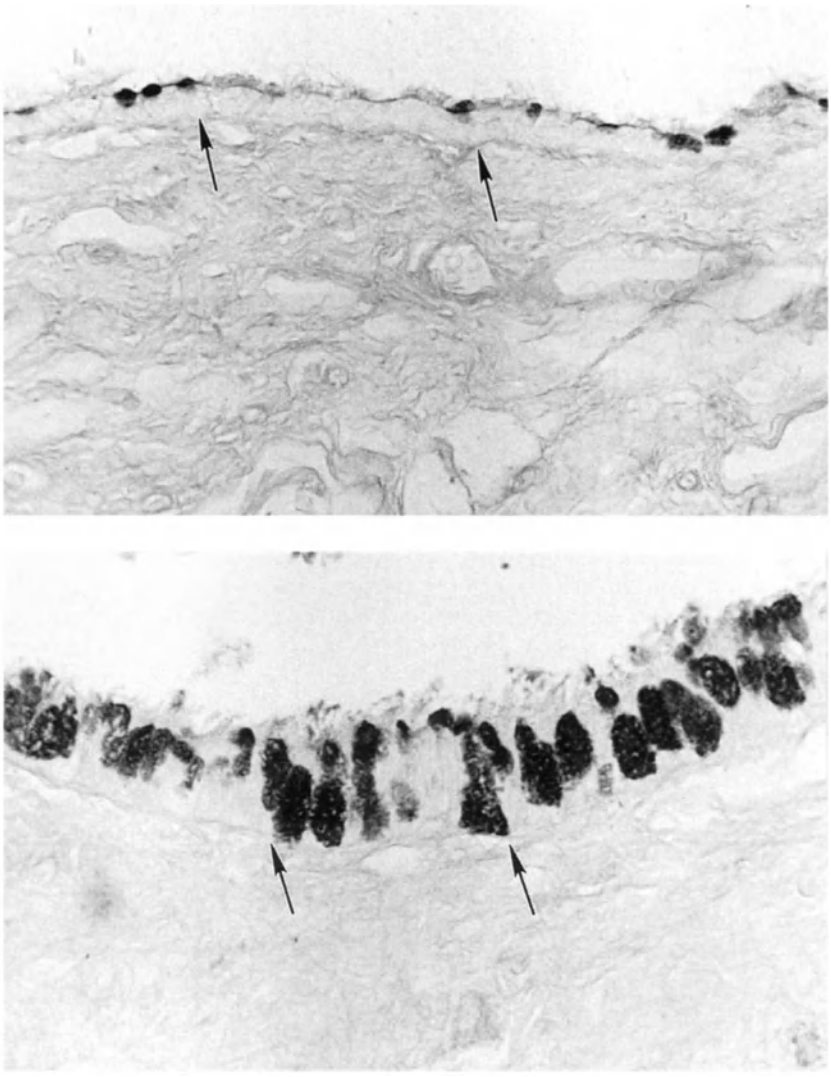


Figure 9. Photomicrographs of rat bronchus stained with Alcian Blue pH 2.5/periodic acid Schiff to demonstrate the intracellular mucus of goblet cells. (A) Unexposed (control) animals have few goblet cells and little intracellular mucin (×450). (B) Two days after a single intratracheal instillation of LPS (100 µg in 200 µl) there is a marked increase in the number of goblet cells and their content of mucin. Arrows mark the position of the basement membrane in A and B (×450).

Alcian Blue-positive and hence acidic) is increased and peaks at 2 days after LPS instillation (Figures 9a and b) and persists for 16 days, especially in the bronchioli. In contrast to the results of Harkema and colleagues we find that even saline-treated rats show a moderate increase of goblet cell number. Electron-microscopic examination at the 2-day time point reveals that there are marked increases of goblet (mucous) cell numbers and that 5–10% of epithelial cells show transitional phenotypic forms. As after TS [107, 133], cells with features of both serous and mucous cells were found, but were also present mucous-ciliated and serous-ciliated transitional cells (Figures 10a and b) in LPS-treated rat airways, as Harkema *et al.* described previously [114]. Northern blot analysis demonstrates that LPS induces upregulation of RAM7S mucin gene expression at between days 2 and 4. The timing of mucin gene upregulation indicates that mucin gene expression may have less to do with the newly and rapidly appearing goblet cells than was formerly thought [210, 211]. Mucin gene expression may have more to do with the control of induction of cell division and the repair phase of persistent injury.

Stolk and colleagues [118] have also exposed hamsters to LPS. In their study, LPS was instilled intratracheally twice a week for up to 5 weeks. After seven LPS instillations, the LPS-treated hamsters showed marked goblet cell hyperplasia which was most evident in the proximal intrapulmonary airways; this was associated with an emphysematous change. Interestingly, instillation of hamster neutrophil elastase inhibitor resulted in 35% inhibition of LPS-induced goblet cell hyperplasia and 50% inhibition of LPS-induced emphysema. As with NO_2, the combination of goblet cell hyperplasia and emphysema is of interest, as both changes are observed in COPD. Furthermore, the relevance of infection in the induction of airflow obstruction is uncertain, and the experimental findings in this model give clues as to how this might occur. In order to study the sequential response after a single LPS (intratracheal) instillation, the lungs of groups of four hamsters were lavaged: neutrophil recruitment peaked at 4 and 48 h after LPS instillation and returned to baseline values at 96 h. Simultaneous intratracheal instillation of LPS together with anti-tumour Necrosis Factor-α (TNFα) antiserum resulted in a considerable reduction of the neutrophil influx in the first 6 h after instillation.

Endotoxin instillation to rats provides a model of human bronchitis with relevance to infectious exacerbations and the associated marked neutrophilia observed in bronchoalveolar lavage in humans [156]. It appears to be repeatable by independent researchers, and goblet cell hyperplasia/metaplasia, mucin gene expression and mucus-hypersecretion are characteristic findings. The additional observations of emphysematous change in the hamster appear to parallel very well the changes observed in COPD in humans. The neutrophil dependency of the LPS effect and the mediators responsible for inducing giblet cell hyperplasia require further investigation.

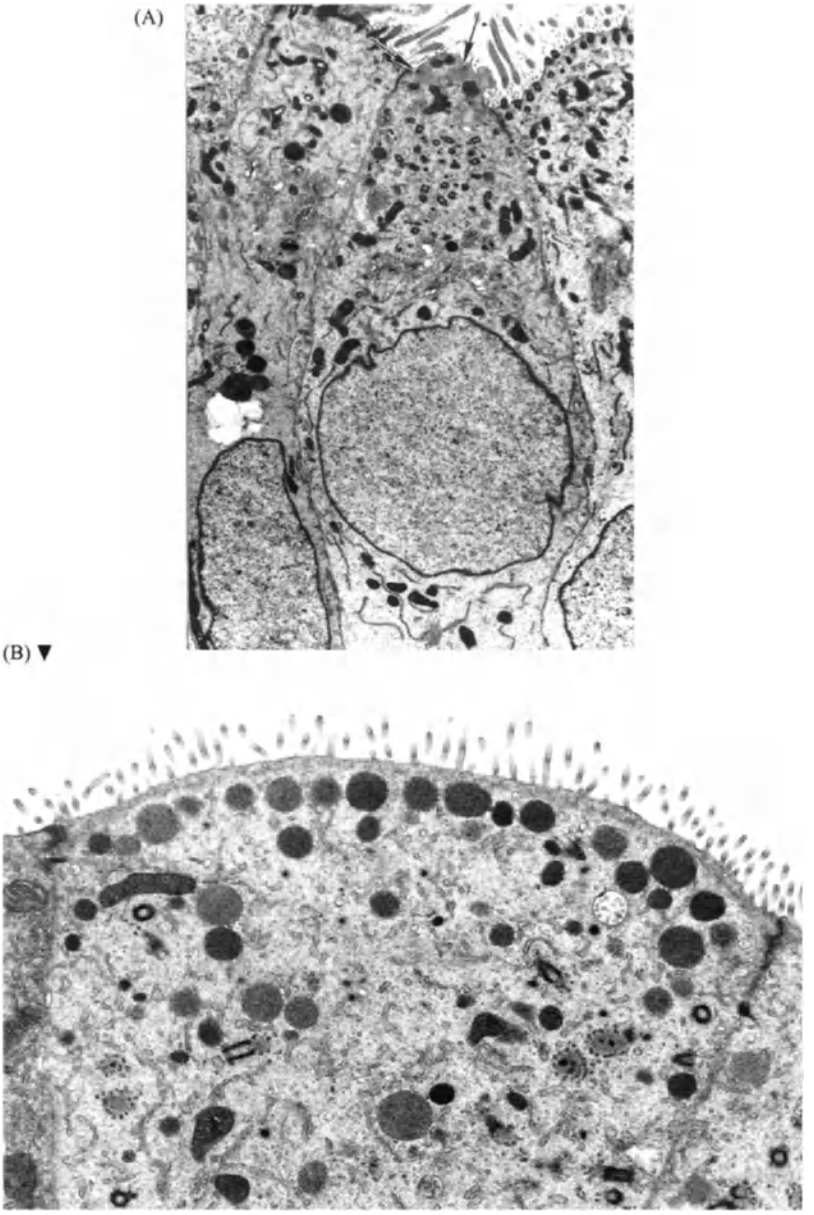


Figure 10. TEM of LPS-treated rat bronchus demonstrating two transitional cell forms. (A) A cell with a nucleus of smooth outline electron-lucent cytoplasm, centrioles and fibrogranular bodies characteristic of a ciliated cell but with a well-developed Golgi zone and mucous granules (arrow) indicative of goblet cell differentiation (×7000). (B) The elongated cell surface microvilli, electron-lucent cytoplasm, centrioles and fibrogranular bodies typical of the ciliated are joined with electron-dense secretory granules typical of the serous cells (×12,600).

4.2.2. Mycoplasma hyorhinis: A histologic comparison of the tracheobronchial submucosal glands of humans with those of seven other animal species has shown that the pig has submucosal glands which compare most closely in the degree of development and distribution with those of humans [59, 15]. Naturally occurring and experimentally induced enzootic pneumonia of pigs, a common disease caused by *Mycoplasma hyorhinis*, has characteristics of the changes of human bronchitis: i.e. goblet cell hyperplasia and enlargement of submucosal glands [158, 159].

In pigs inoculated intranasally with *M. hyorhinis*, the epithelium became thickened, goblet cells were depleted of their mucin (and thus were apparently reduced in number) and they became irregularly distributed around the airway internal circumference. Those that remained had little intracellular mucin, which was mostly neutral in type. Any acidic mucin found was sialidase-resistant sialomucin or sulphomucin. The submucosal glands became enlarged, and the intracellular mucin was mainly sulphomucin in type. This model has much to commend it, but the size of the animals (and hence the low numbers used) and the cost have probably restricted its widespread use.

4.2.3. Pseudomonas aeruginosa: Pseudomonas aeruginosa is a pathogen that can colonize the respiratory tract of patients with pre-existing lung disease, particularly cystic fibrosis, causing morbidity and hastening death. A *P. aeruginosa*-derived toxin, dirhamnolipid (DRL), can acutely increase the secretion of mucus in the cat trachea [113]. Cash and colleagues have established a rat model of chronic respiratory infection using *P. aeruginosa* [111], inoculating agar beads with *P. aeruginosa* into the distal bronchus through a bead-tipped needle. Histologically, the most consistent alteration was hyperplasia and metaplasia of goblet cells in airways close to the parenchyma, which was chronically inflamed. The bronchial epithelium became hyperplastic and was thrown into villous-like folds, often accentuated to form papillary intrusions into the airway lumen. Bronchiectasis was a common finding, particularly of the peripheral airways. Products of *P. aeruginosa* thought to be responsible for the tissue damage included exotoxin A, which blocks protein synthesis in mammalian cells, and a number of proteases which have been shown to lyse collagen and elastin. The release of LPS from the bacterial cell envelope may have contributed also to the pathogenicity and pathology of *P. aeruginosa*-induced bronchitis. Similar findings have been reported for other respiratory pathogens and their toxic products, which appear to be particularly effective in generating goblet cell hyperplasia when the airway lumen is partially restricted (by partial ligature) [160].

4.2.4. Cholera toxin and agents affecting intracellular cAMP: Cholera toxin (CT) was shown to induce goblet cell hyperplasia and stimulate mucin secretion by Nygren and colleagues in 1984 [161], who administered

a single dose of CT or prostaglandin E_1 (PGE_1) or N_6-O_9-dibutyryl-cAMP (DB-cAMP), each of them known to increase intracellular cAMP, to the nostrils of mice. They predicted the drugs would be inhaled and reach the lower airways, and demonstrated both increased secretion of goblet cells and goblet cell hyperplasia. A time- and dose-dependent appearance of new goblet cells was seen in response to each of the secretagogues. In the CT-treated animals, the increased number of goblet cells was evident at 72 h after instillation, but in the other two groups, given PGE_1 or DB-cAMP, the increase was apparent by 48 h after treatment. For the development of goblet cells, a relatively higher dose of CT was required than either of DB-cAMP or PGE_1. Moreover, the responses to DB-cAMP and PGE_1 were not only faster but more transient than CT, and a higher number of goblet cells was seen at the peak of response. ^{3}H-thymidine uptake, a marker of DNA synthesis in cycling and dividing cells, demonstrated that goblet cells could appear after CT without the need for DNA synthesis (hence division). However, increased numbers of ^{3}H-T-labelled cells were seen 24 h after treatment to the above inducers of cAMP. These researchers believed that the newly generated epithelial goblet cells appeared either via differentiation or transition of non-secretory cells to mucin synthesis or by rapid maturation of undifferentiated precursor cells present in the epithelium (see below for discussion of origin of goblet cells). The effects of β-agonists on goblet cell hyperplasia (see below) may also be due to their stimulation of intracellular cAMP.

4.3. Enzymes

4.3.1. Neutrophil-derived enzymes: There has been much interest in the capacity of inflammatory cell-derived enzymes to induce goblet cell hyperplasia. The neutrophil is a prominent inflammatory cell in both exacerbations of chronic bronchitis and cystic fibrosis.

Lysates prepared from normal human neutrophils, supernatants prepared from opsonized zymosan-activated neutrophils or purified neutrophil elastase are each capable of inducing goblet cell hyperplasia if instilled into the lower trachea of rats [162] and hamsters [121–125, 163–167]. In rats challenged with human neutrophil lysates or zymosan-activated neutrophils, rats were sacrificed between 1 and 5 weeks. There was a 50% and 42% increase, respectively, in the number of goblet cells in the tracheal epithelium of the rats after 3 weeks compared with control animals given buffer. Purified human neutrophil elastase (HNE) also induced a goblet cell hyperplasia, while the animals challenged with buffer alone, or with lysates of human mononuclear cells failed to increase their goblet cell number [162]. The increase in goblet cell number seen after neutrophil lysates or elastase alone was maximal by 3 weeks and persisted through 35 days. Some of the staining characteristics of the goblet cells induced in these animals differed

from control animals in that they stained more intensely with Alcian Blue than with PAS. The basement membrane appeared to be consistently thickened, and there was thickening of the epithelium with the presence of large Alcian Blue-positive (i.e. acidic mucin-containing) goblet cells. The subepithelial tissue usually contained increased numbers of macrophages and lymphocytes. In some treated animals, squamous metaplasia was found throughout the lower trachea, which lacked goblet cell or ciliated cell differentiation, and in other animals there was both squamous and pseudostratified columnar epithelium with goblet cell hyperplasia. There were no such changes in the anaesthetized animals that did not receive an instillation. Concurrent treatment of animals with glucocorticosteroids (e.g. addition of dexamethasone to the drinking water as 2 mg/l for 1 week and then 0.2 mg/l for 2 weeks) inhibited enzyme-induced goblet cell hyperplasia. None of the animals instilled with supernatant from lysed monocyte-lymphocyte preparations exhibited foci of squamous metaplasia or goblet cell hyperplasia. After instillation of neutrophil lysate supernatant, goblet cells constituted 42% of epithelial cells compared with a baseline of 38% 1 week after the instillation. By 3 weeks, goblet cells constituted 57% of epithelial cells ($p < 0.001$). The increased percentage of total cells that were goblet cells persisted through 5 weeks.

A single intratracheal injection of 300 µg of highly purified human neutrophil elastase in hamsters [163, 164, 168] causes an increase of goblet cells in large intrapulmonary airways. Quantification of goblet cell numbers in treated animals revealed that the increase of secretory cell frequency did not change the concentration of ciliated cells [42, 163]. Discharge of secretory granules occurred within 2 h of neutrophil elastase instillation, followed by their restoration to normal by day 3 and the development of goblet cell hyperplasia by day 8 with progression of the lesion to day 21. The number of granules per cell and the size of the cell increased between days 8 and 16 [124]. A phenotypic change showing transitions between Clara cells and goblet cells was observed in peripheral airways. With the increase of goblet cells after day 3 there was a decrease of Clara cells, and by day 16 goblet cells comprised 80% of all granulated secretory cells compared with 10% in the controls. As goblet cells increased, Clara cells decreased from 60% in controls to less than 10% in enzyme-treated animals [123]. The phenotypic change has also been observed in rats treated with sulphur dioxide or isoproterenol [22, 169, 170]. Lucey and colleagues [125] found that, after a single intratracheal instillation with 350 µg of human neutrophil elastase to hamsters, the increase of goblet cells persisted for 18 months and was considered a permanent feature of the altered airway.

Bronchial secretory cell metaplasia and emphysema can also be induced by other neutrophil products. The neutral serine protease cathepsin G (Cat-G) [122] is a weak inducer of secretory cell metaplasia: significant changes are induced by doses of 400 and 600 µg but not by 100 or 200 µg given to hamsters. Also, Cat-G at 100 or 200 µg did not potentiate the

secretory cell hyperplasia induced by 100 μg of human neutrophil elastase [122]. The proteolytic but not elastolytic activity of human neutrophil elastase appears to be necessary for the induction of secretory cell metaplasia. Crude or purified human neutrophil elastase treated with Suc-Ala-Pro-Val chloromethyl ketone, which inactivates elastolytic activity, does not affect the induction of secretory cells in the hamster airways [164]. Porcine pancreatic trypsin which does not possess elastolytic function can also induce secretory cell metaplasia in the same way as does human neutrophil elastase [164]. It is suggested that the induction of secretory cell metaplasia by serine proteases may be due to cell surface receptor injury, or alternatively the enzymes may penetrate the cell to affect the synthesis of secretory granules directly. Another possibility is that these enzymes might recruit inflammatory cells to the airways which release mediators of inflammation which in turn cause goblet cell hyperplasia. The suggested mechanism by which anti-inflammatory agents prevent goblet cell hyperplasia may be by preventing inflammatory cell influx or by inhibiting airway epithelial cell discharge [162, 171].

4.3.2. Other enzymes: Other enzymes used for induction of goblet cell hyperplasia include porcine pancreatic elastase [168, 172] and porcine pancreatic trypsin [164]. Doses of between 50 and 500 μg per 100 g of body weight of porcine pancreatic elastase instilled intratracheally to hamsters [172] induce goblet cell hyperplasia in a dose-dependent manner. Seventy-three percent of animals receiving 100 μg of elastase per 100 g of body weight or less do not develop lesions by 16 days. However, of those treated with 200 to 500 μg of elastase per 100 g of body weight, only 16% appear without a bronchial lesion. All animals examined at 3 months and later show goblet cell metaplasia, and this change does not appear to be associated with the development of emphysema. Hamsters treated with 500 μg of porcine pancreatic trypsin showed a severe goblet cell metaplasia 21 days after treatment, and the effects were similar to 300 μg of purified human neutrophil elastase [164]. In animals treated with trypsin-CMK, to eliminate residual active elastase, and trypsin inactivated by tosyl lysine chloromethyl ketone, and the effect was reduced and the results demonstrated that the proteolytic activity rather than the elastolytic activity played a very important role in the increase of goblet cell number.

Christensen and colleagues [168] have reported that the goblet cell metaplasia of elastase-treated animals is not only dose-dependent but also irreversible. They found that a single intratracheal dose of elastase could induce goblet cell metaplasia which persisted for more than 12 months. One possible mechanism is the continued release of enzymes from increased numbers of neutrophils or alveolar macrophages recruited to the airways over an extended period of time. This supposition is supported by the finding that airway lavage fluid has an elevated concentration of neutrophils for up to 60 days after a single exposure to elastase. These observations have

implications for our understanding of human emphysema, because they suggest a single insult can lead to a persistent and permanent lung injury. The persistence of the porcine elastase effect and the association of goblet cell metaplasia and emphysema is of particular relevance to the changes seen in the development of human smokers COPD, and it makes the hamster model a potentially valuable one.

4.4. Drugs

4.4.1. Isoprenaline and salbutamol: Isoprenaline (IPN) (syn. isoproterenol, isopropylnoradrenaline, isopropylarterenol) is a sympathomimetic agent which acts on β-adrenergic receptors and was much used, by inhalation, in the relief of asthma. IPN is a non-selective β-adrenergic agonist (i.e. it has mixed β_1 and β_2 effects). Salbutamol is a more selective β_2 agonist and relaxes airway smooth muscle with less effect on the heart. In 1973 Sturgess and Reid [173] reported for the first time the effects of chronic administration of IPN on airway bronchial secretory cells of rats, followed by other reports of its effects on rats [174, 175] and pigs [176, 177]. In the rat [173], following 6–12 i.p. injections of IPN at doses of 10 or 25 mg per animal, the number, staining characteristics and size of surface goblet cells was altered at all levels of airways. There was a significant increase in the total number of goblet cells present in all generations of airway, except for the midtrachea [174]. The increase was related to dose and number of injections. Using the combined AB/PAS staining technique, there was a fourfold increase in goblet cells staining for predominantly acidic mucin. There was no change in number in those staining for neutral mucin. After IPN, each goblet cell became more distinct, had more prominent secretory granules and a larger volume of intracellular mucin. There was also an increase in the mass of submucosal glands measured by their width, length and depth. Using the combined AB/PAS stain, a larger part of the mucous gland stained blue (i.e. for acidic mucin), and this was more intense than normal, while in the serous gland acini secretory granules stained more intensely for neutral mucin, and some granules also stained for acidic mucin.

The effects of salbutamol given i.p. at 10 or 25 mg per animal, i.e. the same doses as IPN, were compared. After the lower dose of salbutamol, the number of goblet cells was increased to a level significantly above the control value but only in the trachea and in one distal intrapulmonary region; after the higher dose, it was significantly higher only in the distal region. The effect overall was considerably less than that with IPN. Histochemical staining of mucin using the AB/PAS technique demonstrated that, after salbutamol, the increase in the trachea was of cells containing only neutral mucin, whilst within the lung it was of those containing acidic mucin.

In IPN-treated pigs [177] there was a significant increase in the total airway goblet cell number at 24 h: bronchial epithelium contained so many

distended goblet cells that ciliated cells were scarce. This histochemical change of mucin was similar to that described in rats after IPN. The initial effect of IPN on the goblet cell population in the pig was maintained until the 16th day, after which it gradually declined, but a significant increase over the control was still present on the 28th day. Higher than normal numbers of goblet cells persisted until about 2 months, when the difference was not significant statistically compared with the control animals. Baskerville [177] suggested that the increase in goblet cell numbers in the pig bronchial epithelium was at the expense of ciliated cells, the number of which was correspondingly greatly reduced. No increase in mitotic activity in the bronchial epithelium was observed, and the epithelium did not become hyperplastic. The implication was that there had been a shift in the pathway of differentiation so that virtually all cells were induced to differentiate into goblet cells. The studies with β-agonists are of interest in regard to understanding the mechanisms that control changes of goblet cell number and have implications for diseases such as asthma in which β-agonists are much used. However, in general, the doses used experimentally have been extremely high and raise questions about the applicability of this model.

4.4.2. Pilocarpine: Pilocarpine (PCP) is a parasympathomimetic agent with the muscarinic effects of acetylcholine. In 1932, Florey *et al.* [72] gave repeated intravenous injections of PCP to cats and studied the effects on respiratory airways histologically. They found that whilst the submucosal glands were stimulated to discharge to a stage of exhaustion, surface goblet cells did not. They thus concluded that the surface epithelium lacked innervation. The goblet cells were not, however, entirely unaffected, as they reported an increase in their intracellular mucin. In 1973 Sturgess and Reid [173] gave repeated subcutaneous injections to rats of 10 mg of PCP per rat. They found that PCP caused an increase in the total number of goblet cells in the trachea and the bronchial tree. After 6 injections both the small PAS (neutral mucin-containing) and AB-positive (acid mucin-containing) cells showed a slight increase; after 12 injections a larger increase was seen: all histochemical types of goblet cells were involved, particularly those containing neutral mucin. In all goblet cells the volume of intracellular mucin increased, and the secretory granules appeared larger, more conspicuous and had an increased affinity for PAS stain. PCP also produced an increase in submucosal gland size, its length, width and depth as in IPN-treated rats, and the changes appeared to be dose-dependent. After 12 injections of PCP the secretory cells of both mucous and serous acini were exhausted of mucin. Whilst of interest this model has not been pursued by further experimentation.

4.4.3. Sex Hormones: Lung cancer occurs among men at a rate six times that among women, and COPD is also more prevalent among men [178, 179]. These observations acted as a basis for studies by Hayashi and Huber

[180], who compared goblet cell number in the tracheal epithelium of male and female rat. They found that in the trachea of the adult normal male and female rat, "small" PAS-positive cells constituted the major population of the goblet cells. However, there were more "small" PAS-positive goblet cells in the trachea of female than male rats at most stages of the oestrous cycle, albeit the number in the dioestrous female was similar to that in the male. Among female rats, there was a fluctuation in the concentration of "small" secretory cells dependent upon the stage of the ovarian cycle. Proestrous and oestrous rats containing significantly more "small" PAS-positive cells than dioestrous animals. The occurrence of "small" AB-positive cells appeared to be inversely related to that of "small" PAS-positive cells and was consistently lower in females than in males. The total number of goblet cells was also significantly larger in proestrous and oestrous females than in dioestrous females or in males. The tracheal epithelium of the male rat was significantly thicker than that of the female rat in the measurements made both between and over cartilage plates. These findings indicated that the surface epithelium of the trachea was quantitatively different in male and female rats, and goblet cell numbers appeared to vary in the females with hormonal cycle.

Changes in the respiratory epithelia induced by oestrogen were studied by El-Ghazzawi and colleagues [181]. They administered synthetic oestrogen orally to guinea pigs at 10 mg/animal/day and then examined their lungs histologically at periods of 1, 2, 3, 4 and 8 weeks and 3, 6 and 12 months, respectively. After receiving oestrogen for 1 week, tracheal and bronchial goblet cell numbers increased and increased further over 2 weeks. The increase in goblet cell number reached its maximum after 3 weeks, to the extent that the epithelium was seen to be lined mainly by goblet cells. With continuous administration of oestrogen, goblet cells decreased in number until by the end of the 8th week they disappeared completely. The epithelium began to show signs of stratification, with disappearance of cilia followed by squamous metaplasia and mitosis of basal cells.

In animal models of experimentally induced goblet cell hyperplasia, the choice of gender must be considered. These differences may also exist in humans and may explain why hypersecretory conditions contributing to reduced lung function appear to be more prominent in the male.

4.5. Others

4.5.1. Ovalbumin challenge after sensitization: Atopic asthma models have been reported by Lukacs [182] and by Blyth and colleagues [128, 183, 184]. In the former model, the mice were immunized with *Schistosoma mansoni* eggs i.p. and were challenged intranasally and intratracheally with soluble *S. mansoni* egg antigen. They found eosinophil infiltration into the lungs began at 8 h and peaked between 48 and 72 h. Unfortunately, they did

not describe airway goblet cell changes in their model [182]. In mice challenged with intratracheally instilled ovalbumin (OA) (20 μg of OA in 10 μl of saline) after sensitization with i.p. injection of OA (10 μg of OA in 0.1 ml of saline), the lungs showed eosinophilia and neutrophilia. The airway epithelium was thickened, due largely to marked goblet cell hyperplasia, particularly in the larger airways but also in small and terminal bronchioles. The ratio of goblet cells to normal, columnar, ciliated cells was greatly increased compared with control groups: e.g. control airways (both small and large) had only the occasional goblet cell, but sections from OA-challenged lungs showed that 35% of small airways and 100% of large airways contained goblet cells as 50–100% of the total airway epithelial cells (qualitative five-point scoring system). Both acid and neutral mucins were seen, sometimes within the same goblet cell. In lungs that had not been lavaged or inflated with fixative, tenacious mucus could be seen in some airways, occasionally completely occluding the lumen. Cellular debris was enmeshed in these mucus plugs. Goblet cell hyperplasia was still a prominent feature of the epithelium 11 days after the third challenge, despite the disappearance of virtually all the eosinophils and lymphocytes from the airway lumen by 4 days after the third challenge. Administration of dexamethasone (1 mg/kg of body weight) intraperitoneally 30 min before each challenge only reduced the numbers of eosinophils, neutrophils, lymphocytes and macrophages in the airway lumen. However, the drug had no significant effect on the degree of goblet cell hyperplasia 24 h after a third OA-challenge. Treatment with TRFK5, an anti-IL-5 antibody, reduced blood eosinophil numbers to below those in sham-challenged control. Even when eosinophil infiltration into the airway lumen was virtually abolished, there was no suppression of goblet cell hyperplasia. They concluded that the development of goblet cell hyperplasia, and the apparent non-participation of transepithelial migration by eosinophils or lymphocytes, indicates that the initial appearance of this feature may be a consequence not of passage of inflammatory cells through the epithelium, but of direct contact of allergen with airway epithelial cells, macrophages, dendritic cells or mast cells [128].

4.5.2. Transgenic animals: Recent technology which allows the disruption of given genes in embryonic stem (ES) cells by homologous recombination (also called gene knockout) has been used to generate a murine model of cystic fibrosis (CF) [92, 129]. Snouwaert and colleagues constructed a targeting plasmid designed to disrupt the *CFTR* gene in ES cells. This plasmid consisted of a neomycin gene flanked by sequences homologous to the exon 10 region of the *CFTR* gene. Upon integration into the ES cell genome by homologous recombination, this plasmid interrupts exon 10 with the neomycin resistance gene preceded by an in-frame stop codon. The resulting mutation, named S489X, resulted in a truncated gene product similar to that seen with several types of human CF mutations. The

homozygous mice for the mutation (CFTR –/–) were bred from the heterozygous mice (CFTR +/–). The CFTR –/– mice which died 5 weeks after birth, showed dilation of the ducts in the upper airways and a moderate increase in the number of goblet cells in the proximal airways in 5 of 7 CFTR animals: but no acinar hyperplasia and no mucus plugs in the airway lumena were observed. The CFTR –/– mice appeared to be too young for respiratory deterioration to occur and they showed only a mild increase in goblet cells [92]. In the older animals the lungs and the upper airways, including nasal passages, sinuses, nasal pharynx and trachea, were examined. In several animals the glands were filled with eosinophilic materials, resulting in flattening of the gland epithelium. Examination of the lungs revealed a moderate increase in goblet cells. One animal sacrificed at 100 days of age showed extensive goblet cell hyperplasia extending to the peripheral airways. In addition, a number of airways in this animal were found to be obstructed by mucus. There was also evidence of mild pneumonia. Mucopurulent plugs consisting of large numbers of inflammatory cells, which were characteristic of the CF airways, were not present, nor did culturing of a portion of the lung revealed any bacteria [129]. This model demonstrated many pathological changes which were similar to those observed in human CF. The use of such transgenic or knockout mice may be helpful in understanding the pathogenesis of CF.

5. Origin of Newly Acquired Goblet Cells

The origin of the newly appearing airway goblet cell has been much studied and debated over the last 100 years. In spite of many studies it still remains unclear: several mechanisms may operate, dependent upon the stimulus, its persistence and the airway level affected. The basal cell has been traditionally considered as the pluripotential stem cell for the "superficial" secretory and ciliated cell phenotypes which were considered to be end-stage fully differentiated cells which had lost their capacity to divide [185, 186]. Whilst the basal cell certainly has the capacity to divide, their number reduces progressively in more peripheral airways, and they are absent from the bronchioli of most species, including humans. It is now recognized that serous secretory cells of the rat may form a substantial proportion (about 30%) of the dividing epithelial population (Figure 7) and that goblet cells, complete with their intracellular granules, may divide (Figure 11) and act as pluripotential cells which can differentiate to form most, if not all, other cell phenotypes found within the surface epithelium [187, 188]. It is likely that in response to experimental injury in the rat, the first goblet cells to appear in the proximal airways form as the result of a transition from pre-existing serous secretory cells, characterized by their discrete electron-dense granules, to goblet (mucous) cells with confluent electron-lucent granules. This process may involve alterations of gene expression, and

altered transcriptional and translational events, but its rapidity suggests that the first goblet cells to emerge may be merely the result of changes of apical cell and/or secretory granule membrane permeability to periciliary water: imbibition of water along a concentration gradient driven by osmosis would lead to swelling of secretory granules, loss of electron density and to the larger confluent granules characteristic of the newly appearing goblet cell. Discharge of secretion could also be the result of osmosis and the intracellular pressure generated by swelling granules. "True" goblet cell hyperplasia could then ensue in time as the result of goblet cell division.

Appearance of goblet cells in peripheral bronchioli would be expected to take longer, as pre-existing serous cells and basal cells are absent from these airways [186, 189, 190]. Clara cells are the secretory, dividing and stem cell population of bronchioli [190, 191]. There is evidence for their transformation (i.e. metaplasia) to the goblet phenotype following stimulation by TS, NO_2, sympathonemetic drugs and elastases. Recently we have found that LPS induces several transitional cell forms, including serous-mucous, Clara-mucous and, interestingly, serous-ciliated and mucous-ciliated cells (see Figures 9a and 9b). These forms appear at between 14 and 48 h in association with *MUC2* mucin gene expression in several cell forms, including the ciliated cell. Whether these findings implicate the ciliated cell as a precursor of the goblet cell or the goblet as the origin of the ciliated form is as yet unclear.

6. Prevention and Recovery from Goblet Cell Hyperplasia

The animal models used for experimental induction of goblet cell hyperplasia/metaplasia provide powerful tools for investigating the mechanism, prevention and potential for reversal of goblet cell hyperplasia. Several classes of anti-inflammatory agents, including corticosteroids and non-steroidal antiinflammatory drugs, have been used to prevent the increase in goblet cell number induced by the substances previously discussed [192, 193]. Phenylmethyloxadiazole (PMO) added into the tobacco as 2% by weight has been used to inhibit the effects of TS in rats [100, 101, 109, 133]. After 1 to 14 days of TS exposure PMO was found to prevent the observed increase in secretory cell number in extrapulmonary but not intrapulmonary airways [109]. The mitotic activity of airway epithelium was also reduced [100, 101]. Intraperitoneal treatment with corticosteroid and non-steroidal agents also inhibited the increase in goblet cell number induced by TS alone [96, 97], and dexamethasone inhibits the effect of human neutrophil lysates and elastase [162] in rats. *N*-acetylcysteine (given as 1% in drinking water) and *S*-carboxymethylcysteine can also inhibit TS-induced epithelial thickening and goblet cell hyperplasia in rats, and this so-called mucoregulatory effect may act via inhibition of cell division

[143] as well as by their known antioxidant activities [89, 105, 106]. Taurine, a ubiquitous amino acid present in most mammalian tissue and cells, protects rat bronchioles from acute O_3-induced lung inflammation and goblet cell hyperplasia [89].

Antineutrophil antibody given prior to LPS by intraperitoneal injection can reduce LPS-induced goblet cell hyperplasia by 50%. This demonstrates that inflammation is an essential lesion for LPS-induced goblet cell hyperplasia [155]. The discovery that irritants may stimulate airway epithelial cells to participate in the inflammatory reaction by producing inflammatory mediators and chemokines supports the hypothesis [194].

There are reports on the recovery of experimental induction of goblet cell hyperplasia. A single treatment with elastase produces an increase in concentration of goblet cells in hamster tracheobronchial epithelium that is maintained for at least a year [168, 172]. In contrast, in the TS-treated rat model, recovery of normal numbers of goblet cells takes between 9 and 84 days after smoking cessation, depending upon the airway level examined: non-steroidal antiinflammatory agents given i.p. and the mucoregulatory drug *N*-acetylcysteine given in drinking water can shorten the time taken to recover [98, 146]. This is a considerably shorter period than that claimed for the human airway, which is of the order of 2 years [195].

A single dose of LPS (100 µg/200 µl of saline) by intratracheal instillation into rats causes a marked increase of goblet cells in both extra- und intrapulmonary airways which last for at least 16 days before recovering to normal.

Recovery of the airway epithelium from subacute IPN administration in pigs was reported by Baskerville in 1975 [177].

7. Recent Developments

7.1. The Discovery of Mucin Genes

The isolation of a number of "mucin" genes (see Chapter 3 of this volume for details) which encode the protein backbone of the mucus glycoproteins has added a new dimension to the use of animal models to investigate the pathogenesis of hypersecretory disease.

Mucins constitute a heterogenous group of high molecular weight, polydisperse, richly glycosylated molecules (see Chapter 2 of this volume). There are secretory and membrane-associated forms of mucin [196]. Secretory mucins contribute to the viscid mucus of the tracheobronchial, gastrointestinal and reproductive tracts and typically form extremely large oligomers through linkage of their protein monomers by disulfide bonds [48, 135, 197, 198]. These proteins are secreted from the cell and form the mucous gel which becomes an integral part of the mucociliary escalator. In contrast, the membrane-associated mucins have a hydrophobic membrane-

spanning domain and have not been observed to form oligomer complexes. Mucins contain almost 80% sugars by weight and a very characteristic amino acid composition with a high content of threonine and serine in their backbone [198–200]. The mucin polypeptide backbone has proven to be difficult to sequence by conventional procedures due to the presence of numerous heterogenous O-linked glycan chains [135, 196, 201].

With the development of molecular biological techniques, the cDNA sequences of mucin genes can now be obtained and the amino acid sequences of mucins deduced. At the present time, there are at least eight human mucin genes (*MUC1* to *MUC4, MUC5B* and *MUC5AC, MUC6* and *MUC7*) one hamster mucin gene (*MUC1*) [202], one mouse mucin gene (*MUC-1*), three rat mucin genes (*RAM 7S* [203], *rMUC 2* [204], and *RMUC 176* [205]). Several other mammalian mucin genes from bovine submaxillary gland [206], canine tracheobronchial [201] and porcine submaxillary gland [207] have also been discovered. These discoveries have accelerated and intensified research into the extent of mucin gene expression in distinct airway conditions, yet the specific role of mucin genes is at present unclear.

7.2. Mucin Gene Expression in Experimental Animals

Jany and colleagues [132] observed human *MUC2* gene expression in SO_2-exposed rats. These authors used rats exposed to 400 ppm SO_2 for 3 h per day, 5 days a week, for 1–3 weeks, and found the airways contained increased numbers of goblet cells and visible mucinous secretions in the airway lumen. In parallel, they applied Northern blot analysis, using the total RNA extracted from the rat lung and hybridized it with human *MUC2* cDNA (SMUC 41) and demonstrated upregulation of *MUC2* gene in the SO_2-exposed rats: Sendai virus infection intensified mucin gene expression. These results suggested that pathogen- and irritant-induced goblet cell hyperplasia and hypersecretion may be partly controlled at the level of mucin mRNA.

In our experiment we found that expression of the "rat airway mucus" (*RAM* 7S) was increased four- to sixfold at 24–48 h after intratracheal instillation of LPS which lasted for 16 days post LPS. The first 24 h after LPS instillation was characterized by an increase of intracellular mucin, but increased gene expression was delayed. After 48 h intracellular mucin and mRNA were both increase. We speculate that:

1. Mucin discharge and synthesis within 24 h after LPS treatment does not require mucin gene expression, but relies upon an alteration of secretory cell phenotype (i.e. a metaplastic phase) in part due to an increase of cell permeability, uptake of water and swelling of the cell and its granules.
2. At 48 h after LPS, goblet cells express mucin gene and enter a phase of proliferation.

3. When the irritant is removed goblet cells are likely to reduce their mucin gene expression and their proliferative phase. The recovery phase is relatively slow and may last several weeks in the animal and several months or years in humans.

Although the specific mechanisms by which pathogens (and their derivatives) and irritants induce upregulation of mucin gene are unknown, such mechanisms would likely act either to increase the transcription rate or decrease the rate of degradation of mucin mRNA.

8. Summary and Future Prospects

Experimental induction of goblet cell hyperplasia *in vivo* has involved a wide variety of inducers and a variety of animal species. These animal models are an artificial but useful representation of usually only one aspect of human airway mucous hypersecretory disease. Each animal model has its advantages and disadvantages: SO_2 stimulation in rodents requires concentrations of SO_2 in excess of that tolerated by humans, and ulceration renders the pathogenetic mechanism in large airways difficult to interpret. NO_2 and O_3 are easily delivered to small airways, and the changes are similar to those observed in human chronic bronchitis, bronchiolitis and COPD. TS provides a more subtle model and is of relevance to investigation of the pathogenetic mechanisms associated with an all-too-common pollutant, but the model is becoming less easy to use due to the reduction of tar and nicotine in cigarettes. Elastase can not only cause goblet cell hyperplasia but also induce emphysema in hamsters. Interestingly, tolerance may occur with tobacco smoke and elastase, and the mechanism of the development of such tolerance requires investigation. LPS intratracheal instillation can induce marked goblet cell hyperplasia/metaplasia in rats, and the changes involve neutrophil influx and are repeatable; the response may not be homogeneous, and this may make quantitative analysis difficult. Rats, mice and hamsters, which do not have submucosal glands, seem to be amenable to studies of pathogenesis of goblet cell hyperplasia, but cough and spit are not produced, which differs from human chronic bronchitis. Monkeys, pigs and dogs have a similar airway mucosa to that of humans, but there are restrictions on their use due to size, cost and the social acceptability of their use in animal experimentation.

Future studies might address questions concerning:

1. the relationship between mucus secretion of airway epithelium and the inflammatory cell influx, i.e. the interaction between mucus-secreting epithelial cells and inflammatory cells;
2. cytokines and chemokines involved with inflammation of the tracheobronchial tree and the mechanism for their affect on mucin genes, secretory cell hyperplasia/metaplasia and secretion;

3. the relationship between mucin gene expression and goblet cell differentiation, division and secretion; mucin gene expression, and variation of distinct mucin genes in airway disorder such as chronic bronchitis, asthma and cystic fibrosis;
4. modulation of the goblet cell response and of mucin gene expression by distinct classes of drugs.

To achieve these aims molecular, biological, immunological and morphological technique should be combined. Collaboration and cooperation of different areas of biosciences will assist this research. The interpretation of experimental studies will need to be considered in the light of what is known about human airways from biopsy studies and the results of experiments designed to support the differentiated phenotype of human airway secretory cells in culture.

Acknowledgements

The studies of mucin gene expression were supported by Glaxo-Wellcome (UK).

References

1. Houston JC, De Navasquez S and Trounce JR (1953) A clinical and pathological study of fatal cases of status asthmaticus. *Thorax* 8: 207–213.
2. Dunnill MS (1960) The pathology of asthma, with special references to changes in the bronchial mucosa. *J Clin Pathol* 13: 27–33.
3. Glynn AA and Michaels L (1960) Bronchial biopsy in chronic bronchitis and asthma. *Thorax* 15: 142–153.
4. Lopez-Vidriero MT and Reid L (1985) Bronchial mucus in asthma. In: Weiss EB, Segal MS, Stein M (eds) *Bronchial asthma: mechanisms and therapeutics.* Boston: Little, Brown, 218.
5. De Haller R, Reid L (1965) Adult chronic bronchitis. *Medicina Thoracalis* 22: 549–567.
6. Rose MC (1988) Epithelial mucus glycoproteins and cystic fibrosis. *Horm Metabol Res* 20: 601–608.
7. Lamblin G, Lafitte JJ, Lhermitte M, Degand P, Roussel P (1977) Mucins from cystic fibrosis sputum. *Mod Probl Paediat* 19: 153–164.
8. Talamo RC, Rosenstein BJ, Beringer RW (1983) Cystic fibrosis. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein DS, Brown MS (eds) *Metabolic basis of inherited disease.* New York: McGraw Hill, 1189.
9. Geddes DM (1990) Cystic fibrosis: Epidemiology and pathology. In: Brewis RAL, Gibson GJ, Geddes DM (eds) *Respiratory medicine.* London: Bailliere Tindall, 760.
10. Lamb D (1968) Mucus secretion in hypersecretory states. *Bronchus* 18: 453–465.
11. Reid LM (1986) The pathology of obstructive and inflammatory airway diseases. *Eur J Respir Dis* 65 (Suppl 147): 26–37.
12. Jones R, Reid L (1978) Secretory cells and their glycoproteins in health and disease. *Br Med Bull* 34: 9–16.
13. Lopez-Vidriero MT, Reid L, Bronchial mucus in health and disease. *Br Med Bull* 34: 63–88.
14. Parke DV (1978) Pharmacology of mucus. *Br Med Bull* 34: 89–94.
15. Widdicombe JG (1978) Control of secretion of tracheobronchial mucus. *Br Med Bull* 34: 57–61.
16. Wardell JR, Chakrin LW, Payne BJ (1970) The canine tracheal pouch: A model for use in respiratory mucus research: *Am Rev Respir Dis* 101: 741–754.

17. Gallagher JT, Hall RL, Jeffery PK, Phipps RJ, Richardson PS (1978) The nature and origin of tracheal secretions released in response to pilocarpine and ammonia. *J Physiol* 275: 36–73P.
18. Jeffery PK (1978) The structure and function of the mucus-secreting cells of cat and goose airway epithelium. In: Porter R (ed.) *Respiratory tract mucus*, 56th CIBA Foundation Symposium. Oxford: Elsevier/Excerpta Med, 5.
19. Basbaum C, Carlson D, Davidson E, Verdugo P, Gail DB (1988) Cellular mechanisms of airway secretion. *Am Rev Respir Dis* 137: 479–485.
20. Jeffery PK, Reid L, Widdicombe JG (1976) Anatomical and physiological features of irritation of the bronchial tree. In: Aharonson EF, Ben-David A, Klinberg MA (eds) *Airpollution and the lung.* J Willey & Sons, 253.
21. Jeffery PK, Reid L (1977) The ultrastructure of the airway lining and its development. In: Hodson WA (ed.) *The development of the lung.* New York: Marcel Dekker, 87.
22. Jeffery PK (1987) Structure and function of adult tracheobronchial epithelium. In: McDowell EM (ed.) *Lung carcinomas.* London: Churchill Livingstone, 42.
23. Jeffery PK (1990) Microscopic structure of normal lung. In: Brewis RAL, Gibson GJ, Geddes DM (eds) *Textbook of respiratory medicine.* London: Balliere Tindall, 57.
24. Prescott E, Lange P, Vestbo J (1995) Chronic mucus-hypersecretion in COPD and death from pulmonary infection. *Eur Respir J* 8: 1333–1338.
25. Reid L (1954) Pathology of chronic bronchitis. *Lancet* i: 275–279.
26. Jeffery PK (1987) The origins of respiratory tract secretions. *Eur J Respir Dis* 71: 34–42.
27. Weller PF (1984) Eosinophilia. *J Allergy Clin Immunol* 73: 1–10.
28. Reid L (1970) Chronic bronchitis: A report on mucus research. *Proc Roy Instn Gt Br* 43: 438–463.
29. Reid L (1960) Measurement of the bronchial mucous gland layer: A diagnostic yardstick in chronic bronchitis. *Thorax* 15: 132–141.
30. Jany B, Basbaum CB (1991) Mucin in disease: Modification of mucin gene expression in airway disease. *Am Rev Respir Dis* 144: S38–S41.
31. Reid L (1965) Natural history of mucus in the bronchial tree. *Archives of Environmental Health* 10: 265–273.
32. Jeffery PK (1994) Comparative morphology of the airways in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 150: S6–S13.
33. Wright JL, Lawson LM, Pare PD; Kennedy F, Wiggs B, Hogg JC (1984) The detection of small airways disease. *Am Rev Respir Dis* 129: 989–994.
34. Wright JL, Cosio M, Wigg B, Hogg JC (1985) A morphologic grading scheme for membranous and respiratory bronchioles. *Arch Pathol Lab Med* 109: 163–165.
35. Ayers M, Jeffery PK (1982) Cell division and differentiation in the respiratory tract. In: Cumming G, Bonsignore G (eds) *Cell biology and the lung*, vol. 10. Ettore Majorana Intern Life Science Series. New York: Plenum, 33.
36. Jeffery PK (1991) Ultrastructure of airway epithelium with particular emphasis on secretory cells. In: Junod A, Olivieri D, Pozzi E (eds) *Endothelial and mucus secreting cells.* Milan: Masson, 157.
37. Jeffery PK, Corrin B (1984) Structural analysis of the respiratory tract. In: Bienenstock J, (ed) *Immunology of the lung and upper respiratory tract* (McGraw-Hill Book Company) 1–414.
38. Johnson N, Hubbs AF (1990) Epithelial progenitor cells in the rat trachea. *Am J Respir Cell Mol Biol* 3: 579–585.
39. Mawdesley-Thomas LE, Healey P (1969) The quantitative evaluation of experimental chronic bronchitis: A preliminary study. *Am Rev Respir Dis* 100: 231–233.
40. Reid L, Jones R (1983) Experimental chronic bronchitis. *Inter Rev Exp Pathol* 24: 335–382.
41. Reid L (1970) Evaluation of model systems for study of airway epithelium, cilia and mucus. *Arch Intern Med* 126: 428–434.
42. Snider GL (1992) Animal models of chronic airway injury. *Chest* 101: 74S–79S.
43. Schulze FE (1872) The lungs. I. The lungs of mammals. In: Stricker S (ed.) *Human and comparative histology.* London: New Sydenham Society, 49.
44. Lamb D, Reid L (1969) Histochemical types of acidic glycoprotein produced by mucous cells of the tracheobronchial glands in man. *J Pathol* 98: 213–229.

45. Jones R, Reid L (1973) The effect of pH on Alcian blue staining of epithelial acid glycoproteins I. Sialomucins and sulphomucins (singly or in simple combinations). *Histochem J* 5: 9–18.
46. Reid L, Jones R (1979) Bronchial mucosal cells. *Fed Proc* 38: 191–196.
47. Spicer SS, Schulte BA, Thomopoulos GN (1983) Histochemical properties of the respiratory tract epithelium in different species. *Am Rev Respir Dis* 128: S20–S26.
48. Roberts GP (1978) Chemical aspects of respiratory mucus. *Br Med Bull* 34: 39–41.
49. Clamp JR, Allen A, Gibbons RA, Roberts GP (1978) Chemical aspects of mucus. *Br Med Bull* 34: 25–41.
50. Reid L, Clamp JR (1978) The biochemical and histochemical nomenclature of mucus. *Br Med Bull* 34: 5–8.
51. Boat TF, Kleinerman JI, Carlson DM, Maloney WH, Matthews LW (1974) Human respiratory tract secretions. I. Mucous glycoproteins secreted by cultured nasal polyp epithelium from subjects with allergic rhinitis and cystic fibrosis. *Am Rev Respir Dis* 110: 428–441.
52. Boat TF, Cheng PW, Iyer RN, Carlson DM, Polony I (1976) Human respiratory tract secretions: Mucous glycoproteins of non-purulent tracheobronchial secretions, and sputum of patients with bronchitis and cystic fibrosis. *Arch Biochem Biophys* 177: 95–104.
53. Das RM, Jeffery PK, Widdicombe JG (1979) Experimental degeneration of intra-epithelial nerve fibres in cat airway. *J Anat* 128: 259–267.
54. Sherman JM, Cheng P, Tandler B, Boat TF (1981) Mucous glycoproteins from cat tracheal goblet cells and mucous glands separated with EDTA. *Am Rev Respir Dis* 124: 476–479.
55. Spicer SS, Charkin LW, Wardel JR, Kendrick W (1971) Histochemistry of mucosubstances in the canine and human respiratory tract. *Lab Invest* 25: 483–490.
56. Plopper CG, St. George JA, Nishio SJ, Etchison JR, Nettesheim P (1984) Carbohydrate cytochemistry of tracheobronchial airway epithelium of the rabbit. *J Histochem Cytochem* 32: 209–218.
57. St. George JA, Nishio SJ, Plopper CG (1984) Carbohydrate cytochemistry of the rhesus monkey tracheal epithelium. *Anat Rec* 210: 293–302.
58. St. George JA, Mariassy AT, Plopper CG (1983) Histochemical comparison of the airway epithelial secretory cells in ovine lung. *Anat Rec* 205: 172A.
59. Jeffery PK (1983) Morphology of airway surface epithelial cells and glands. *Am Rev Respir Dis* 128: S14–S20.
60. Harkema JR, Mariassy A, St. George J, Hyde DM, Plopper CG (1991) The epithelial cells of the conducting airways: A species comparison. In: Farmer SG, Hay DWP (eds) *Lung biology in health and disease*, vol. 55, *The airway epithelium.* New York: Marcel Dekker, 3.
61. Plopper CG, Halsebo JE, Berger WJ, Sonstegard KS, Nettesheim P (1983) Distribution of nonciliated bronchiolar epithelial (Clara) cells in intra- and extrapulmonary airways of the rabbit. *Exp Lung Res* 4: 79–89.
62. Plopper CG, Mariassy AT, Wilson DW, Alley JL, Nishio SJ, Nettesheim P (1983) Comparasion of nonciliated tracheal epithelial cells in six mammalian species: Ultrastructure and population densities. *Exp Lung Res* 5: 281–294.
63. Bucher U, Reid L (1961) Development of the mucus-secreting elements in human lung. *Thorax* 16: 219–225.
64. Gaillard DA, Lallemand AV, Petit AF, Puchelle ES (1989) *In vivo* ciliogenesis in human fetal tracheal epithelium. *Am J Anat* 185: 415–418.
65. Jeffery PK, Gaillard D, Moret T (1991) Human airway secretory cells during development and in mature epithelium. *Eur Respir J* 5: 93–104.
66. Ellefsen P, Tos M (1972) Goblet cells in the human trachea: Quantitative studies of a pathological biopsy material. *Arch Otolaryngol* 95: 547–555.
67. Soderberg M, Hellstrom S, Sandstrom T, Lungren R, Bergh A (1990) Structural characterization of bronchial mucosal biopsies from healthy volunteers. A light and electron microscopical study. *Eur Respir J* 3: 261–266.
68. Lumsden AB, McLean A, Lamb D (1984) Goblet and Clara cells of human distal airways: Evidence for smoking-induced changes in numbers. *Thorax* 39: 844–853.
69. Carleton HM (1927) The effects produced by the inhalation of haematite and dusts in guinea-Pigs. *J Hyg* 26: 227–234.
70. Carleton HM (1924) The pulmonary lesions produced by the inhalation of dust in guinea-pigs. *J Hyg* 22: 438–471.

71. Hilding A (1932) Experimental surgery of the nose and sinuses. *Arch Otolaryng* 15: 9–18.
72. Florey H, Carleton HM, Wells AQ (1932) Mucus secretion in the trachea. *Br J Exp Pathol* 13: 269–284.
73. Harkema JR, Plopper CG, Hyde DM, St. George JA, Wilson DW, Dungworth DL (1987) Response of the macaque nasal epithelium to ambient levels of ozone. *Am J Pathol* 128: 29–44.
74. Harkema JR, Plopper CG, Hyde DM, St. George JA, Dungworth DL (1987) Effects of an ambient level of ozone on primate nasal epithelial mucosubstances. *Am J Pathol* 127: 90–96.
75. Gordon T, Harkema JR (1995) Mucous cell metaplasia in the airways of rats exposed to machining fluids. *Fundament Appl Toxicol* 28: 274–282.
76. Gordon T, Harkema JR (1995) Cotton dust produces an increase in intraepithelial mucosubstances in rat airways. *Am J Respir Crit Care Med* 151: 1981–1988.
77. Reid L (1963) An experimental study of hypersecretion of mucus in the bronchial tree. *Br J Exp Pathol* 44: 437–445.
78. Seltzer J, Scanlon D, Drazen JM, Ingram RH Jr, Reid L (1984) Morphologic correlation of physiologic changes caused by SO_2-induced bronchitis in dogs. *Am Rev Respir Dis* 129: 790–797.
79. White R, Zoppi AL, Haroz RK, Broillet A (1986) Sulfur dioxide-induced bronchitis in rats. *Arch Toxicol Suppl* 9: 431–435.
80. Lightowler NM, Williams JRB (1969) Tracheal mucus flow rates in experimental bronchitis in rats. *Br J Exp Pathol* 50: 139–149.
81. Chakrin LW, Saunders LZ (1974) Experimental bronchitis: Pathology in the dog. *Lab Invest* 30: 145–154.
82. Spicer SS, Chakrin LW, Wardell JR Jr (1974) Effect of chronic sulfur dioxide inhalation on the carbohydrate histochemistry and histology of the canine respiratory tract. *Am Rev Respir Dis* 110: 13–24.
83. Freeman G, Haydon GB (1964) Emphysema after low-level exposure to NO_2. *Arch Environ Health* 8: 133–136.
84. Freeman G, Crane SC, Furiosi NJ, Stephens RJ, Evans MJ, Moore WD (1972) Covert reduction in ventilatory surface in rats during prolonged exposure to subacute nitrogen dioxide. *Am Rev Respir Dis* 106: 563–579.
85. Mustafa MG, Tierney DF (1978) Biochemical and metabolic changes in the lung with oxygen, ozone and nitrogen dioxide toxicity. *Am Rev Respir Dis* 118: 1061–1090.
86. Hoshi H, Yamauchi K, Sekizawa K, Ohkawara Y, Lijima H, Sakurai E, Maeda K, Okinaga S, Ohno I, Honma M et al. (1996) Nitrogen dioxide exposure increases airway contractile response to histamine by decreasing histamine *N*-methyltransferase activity in guinea pigs. *Am J Respir Cell Mol Biol* 14: 76–83.
87. Elmes PC, Bell D (1963) The effects of chlorine gas on the lungs of rats with spontaneous pulmonary disease. *J Path Bact* 86: 317–326.
88. Wolf DC, Morgan KT, Gross EA, Barrow C, Moss OR, James OR, Popp JA (1995) Two-year inhalation exposure of femal and male B6C3F1 mice and F344 rats to chlorine gas induces lesions confined to the nose. *Fundam Appl Toxicol* 24: 111–131.
89. Schuller-Levis GB, Gordon RE, Park E, Pendino KJ, Laskin DL (1995) Taurine protects rat bronchioles from acute ozone-induced lung inflammation and hyperplasia. *Exp Lung Res* 21: 877–888.
90. Zhang L-Y, Levitt RC, Kleeberger SR (1995) Differential susceptibility to ozone-induced airways hyperreactivity in inbred strains of mice. *Exp Lung Res* 21: 503–518.
91. Plopper CG, Chu F-P, Haselton CJ, Peake J, Wu J, Pinkerton KE. *Am J Pathol* 144: 404–421.
92. Snouwaert JN, Brigman KK, Latour AM, Malour NN, Boucher RC, Smithies O, Koller BH (1992) An animal model for cystic fibrosis made by gene targeting. *Science* 257: 1083–1088.
93. Schlesinger RB, Gorczynski JE, Dennison J, Richard L, Kinney PL, Bosland MC (1992) Long-term intermittent exposure to sulfuric acid aerosol, ozone and their combinations: Alterations in tracheobronchial mucocillary clearance and epithelial secretory cells. *Exp Lung Res* 18: 505–534.
94. Hotchkiss JA, Harkema JR, Henderson RF (1991) Effect of cumulative ozone exposure on ozone-induced nasal epithelial hyperplasia and secretory metaplasia in rats. *Exp Lung Res* 15: 589–600.

95. Jeffery PK, Reid L (1975) New observations of rat airway epithelium: A quantitative electron microscopic study. *J Anat* 120: 295–320.
96. Greig J, Ayers M, Jeffery PK (1980) The effect of indomethacin on the response of rat bronchial epithelium to tobacco smoke. *J Pathol* 132: 1–9.
97. Rogers DF, Jeffery PK (1986) Inhibition of cigarette smoke-induced airway secretory cell hyperplasia by indomethacin, dexamethasone, prednisoline or hydrocortisone in the rat. *Exp Lung Res* 10: 285–298.
98. Rogers DF, Jeffery PK (1986) Indomethacin and flurbiprofen speed recovery of rat bronchial epithelium after exposure to cigarette smoke. *Exp Lung Res* 10: 299–312.
99. Frasca JM, Auerbach O, Parks VR, Jamieson JD (1968) Electron microscopic observations of the bronchial epithelium of dogs. II. Smoking dogs. *Exp Mol Pathol* 9: 380–399.
100. Bolduc P, Jones R, Reid L (1981) Mitotic activity of airway epithelium after short exposure to tobacco smoke and the effect of the anti-inflammatory agent phenylmethyloxadiazole. *Br J Exp Pathol* 62: 461–468.
101. Jones R, Bolduc P, Reid L (1972) Protection of rat bronchial epithelium against tobacco smoke. *Br Med J* 2: 142–144.
102. Coggins CRE, Fouillet XLM, Lam R, Morgan KT (1980) Cigarette smoke-induced pathology of the rat respiratory tract: A comparison of the effects of the particulate and vapour phases. *Toxicology* 16: 83–101.
103. Jones R, Bolduc P, Reid L (1973) Goblet cell glycoprotein and tracheal gland hypertrophy in rat airways: The effect of tobacco smoke with or without the anti-inflammatory agent phenylmethyloxadiazole. *Br J Exp Pathol* 54: 229–239.
104. Mawdesley-Thomas LE, Healey P (1973) Experimental bronchitis in lambs exposed to cigarette smoke. *Arch Environ Health* 27: 248–250.
105. Jeffery PK (1992) Cigarette smoke-induced goblet cell hyperplasia and mucosal permeability in the rat: A synopsis of the effects of *N*-acetylcysteine, *S*-carboxymethylcysteine and budesonide. *Eur Respir J* 2: 23–26.
106. Rogers DF, Jeffery PK (1986) Inhibition by oral *N*-acetylcysteine of cigarette smoke-induced "bronchitis" in the rat. *Exp Lung Res* 10: 267–283.
107. Jeffery PK (1992) Chronic obstructive pulmonary disease and cigarette smoke-induced epithelial damage. *Eur Respir Rev* 2: 136–143.
108. Hayashi M, Sornberger GC, Huber GL (1979) Morphometric analysis of tracheal gland secretion and hypertrophy in male and female rats after experimental exposure to tobacco smoke. *Am Rev Respir Dis* 119: 67–73.
109. Jones R, Reid L (1978) Secretory cell hyperplasia and modification of intracellular glycoprotein in rat airways induced by short periods of exposure to tobacco smoke, and the effect of the antiinflammatory agent phenylmethyloxadiazole. *Lab Invest* 39: 41–49.
110. Tesfaigzi J, Th'ng J, Hotchkiss JA, Harkema JR, Wright PS (1996) A small proline-rich protein, SPRR1, is upregulated early during tobacco smoke-induced squamous metaplasia in rat nasal epithelia. *Am J Respir Cell Mol Biol* 14: 478–486.
111. Cash HA, Woods DE, McCullough B, Johanson WG Jr, Bass JA (1979) A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. *Am Rev Respir Dis* 119: 453–459.
112. Tager I, Speizer FE (1975) Role of infection in chronic bronchitis. *N Engl J Med* 292: 563–570.
113. Fung DCK, Somerville M, Richardson PS, Sheehan JK (1995) Mucus glycoconjugate complexes released from feline trachea by a bacterial toxin. *Am J Respir Cell Mol Biol* 12: 296–306.
114. Harkema JR, Hotchkiss J (1992) *In vivo* effects of endotoxin on intraepithelial mucosubstances in rat pulmonary airways. *Am J Pathol* 141: 307–317.
115. Harkema JR, Hotchkiss JA (1991) *In vivo* effects of endotoxin on nasal epithelial mucosubstances: Quantitative histochemistry. *Exp Lung Res* 17: 743–761.
116. Gordon T, Harkema JR (1994) Effect of inhaled endotoxin on intraepithelial mucosubstances in F344 rat nasal and tracheobronchial airways. *Am J Resp Cell Mol Biol* 10: 177–183.
117. Steiger D, Hotchkiss J, Bajaj L, Harkema J, Basbaum C (1995) Concurrent increases in the storage and release of mucin-like molecules by rat airway epithelial cells in response to bacterial endotoxin. *Am J Resp Cell Mol Biol* 12: 307–314.

118. Stolk J, Rudolphus A, Davies P, Osinga D, Dijkman JH, Agarwal L, Keenan KP, Fletcher D, Kramps JA (1992) Induction of emphysema and bronchial mucus cell hyperplasia by intratracheal instillation of lipopolysaccharide in the hamster. *J Pathol* 167: 349–356.
119. Pauwels RA, Kips JC, Peleman RA, van der Straeten ME (1990) The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. *Am Rev Respir Dis* 141: 540–545.
120. Harkema JR; Hotchkiss JA, Harmsen AG, Henderson RF (1988) *In vivo* effects of transient neutrophil influx on nasal respiratory epithelial mucosubstances: Quantitative histochemistry. *Am J Pathol* 130: 605–615.
121. Snider GL, Lucey EC, Christensen TG, Stone PJ, Calore JD, Catanese A, Franzblau C (1984) Emphysema and bronchial secretory cell metaplasia induced in hamsters by human neutrophil products. *Am Rev Respir Dis* 129: 155–160.
122. Lucey EC, Stone PJ, Breuer R, Christensen TG, Calore JD, Catanese A, Franzblau C, Snider GL (1985) Effect of combined human neutrophil cathepsin G and elastase on induction of secretory cell metaplasia and emphysema in hamsters, with *in vitro* observations on elastosis by these enzymes. *Am Rev Respir Dis* 132: 362–366.
123. Christensen TG, Breuer R, Hornstra LJ, Lucey EC, Stone PJ, Snider GL (1987) An ultrastructural study of the response of hamster bronchial epithelium to human neutrophil elastase. *Exp Lung Res* 13: 279–297.
124. Breuer R, Christensen TG, Lucey EC, Stone PJ, Snider GL (1987) An ultrastructural morphometric analysis of elastase-treated hamster bronchi shows discharge followed by progressive accumulation of secretory granules. *Am Rev Respir Dis* 136: 698–703.
125. Lucey EC, Stone PJ, Christensen TG, Breuer R, Snider GL (1988) An 18-month study of the effects on hamster lungs of intratracheally administered human neutrophil elastase. *Exp Lung Res* 14: 671–686.
126. Jeffery PK (1996) Cigarette smoke-induced damage of airway mucosa. In: Chretien J, Dusser D (eds) *Environmental impact on the airways*. New York: Marcel Dekker, 229.
127. Armitage AK, Turner DM (1970) Absorption of nicotine in cigarette and cigar smoke through the oral mucosa. *Nature* 226: 1231–1232.
128. Blyth DI, Pedrick MS, Savage TJ, Hassel EM, Fattah D (1996) Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol* 14: 425–438.
129. Snouwaert JN, Brigman KK, Latour AM, Iraj E, Schwab U, Gilmour MI, Koller BH (1995) A murine model of cystic fibrosis. *Am J Respir Crit Care Med* 151: 559–564.
130. Goldring IP, Greenburg L (1970) Pulmonary effects of sulfur dioxide exposure in the syrian hamster. *Arch Environ Health* 21: 32–37.
131. Lamb D, Reid L (1968) Mitotic rates, goblet cell increase and histochemical changes in mucus in rat bronchial epithelium during exposure to SO_2. *J Pathol Bacteriol* 96: 97–111.
132. Jany B, Gallup M, Tsuda T, Basbaum C (1991) Mucin gene expression in rat airways following infection and irritation. *Bioch Biophys Res Commun* 181: 1–8.
133. Jeffery PK, Reid L (1981) The effect of tobacco smoke with or without phenylmethyloxadiazole (PMO) on rat bronchial epithelium: A light and electron microscopic study. *J Pathol* 133: 341–359.
134. Rogers DF, Jeffery PK (1986) Inhibition by oral *N*-acetylcysteine of cigarette smoke-induced "bronchitis" in the rat. *Exp Lung Res* 10: 267–283.
135. Phelps DF (1978) Biosynthesis of mucus glycoprotein. *Br Med Bull* 34: 43–48.
136. Coles SJ, Levine LR, Reid L (1979) Hypersecretion of mucus glycoproteins in rat airways induced by tobacco smoke. *Am J Pathol* 94: 459–472.
137. Lamb D, Reid L (1969) Goblet cell increase in rat bronchial epithelium after exposure to cigarette and cigar tobacco smoke. *Br Med J* 1: 33–35.
138. Rogers DF, Turner NC, Marriott C, Jeffery PK (1987) Cigarette smoke-induced "chronic bronchitis": a study *in situ* of laryngo-tracheal hypersecretion in the rat. *Clin Sci* 72: 629–637.
139. Ayers M, Jeffery PK (1988) Proliferation and differentiation in adult mammalian airway epithelium. A review. *Eur Respir J* 1: 58–80.
140. Rogers DF, Williams DA, Jeffery PK (1986) Nicotine does not cause "bronchitis" in the rat. *Clin Sci* 70: 427–433.

141. Auerbach O, Hammond EC, Kirman D, Garfinkel L, Stout AP (1967) Histological changes of bronchial tubes of cigarette smoking dogs. *Cancer* 20: 2055–2066.
142. Rogers DF, Jeffery PK (1987) Experimental bronchitis: Prophylaxis and therapy. *Eur J Respir Dis* 71: 278–279.
143. Jeffery PK, Rogers DF, Ayers MM (1985) Effect of oral acetylcysteine on tobacco smoke-induced secretory cell hyperplasia. *Eur J Respir Dis* 139: 117–122.
144. Rogers DF, Jeffery PK, Turner NC, Marriott C (1987) The effects of oral *N*-acetylcysteine and *S*-carboxylmethylcysteine on experimental airway hypersecretion. *Eur J Respir Dis* 71: 279–280.
145. Rogers DF, Turner NC, Marriott C, Jeffery PK (1989) Oral *N*-acetylcysteine or *S*-carboxymethylcysteine inhibits cigarette smoke-induced hypersecretion of mucus in rat larynx and trachea *in situ*. *Eur Respir J* 2: 955–960.
146. Rogers DF, Godfrey RWA, Majumdar S, Jeffery PK (1987) Oral *N*-acetylcysteine speeds reversal of cigarette smoke-induced mucous cell hyperplasia in the rat. *Exp Lung Res* 14: 19–35.
147. Devalia JL, Sapsford RJ, Cundell DR, Rusznak C, Campbell AM, Davies RJ (1993) Human bronchial epithelial cell dysfunction following *in vitro* exposure to nitrogen dioxide. *Eur Resp J* 6: 1308–1216.
148. Harkema JR, Hotchkiss JA, Henderson RF (1989) Effects of 0.12 and 0.80 ppm ozone on rat nasal and nasopharyngeal epithelial mucosubstances: Quantitative histochemistry. *Toxicol Pathol* 17: 525–535.
149. Rietschel ET, Seydel U, Zahringer U, Schade UF, Wang M-H, Ulmer AJ, Flad H-D, Brandenburg K, Kirikae T, Grimmecke D et al. (1991) Bacterial endotoxin. Molecular relationships between structure and activity. *Infec Dis Clin North Am* 5: 735–779.
150. Freudenberg MA, Galanos C (1990) Bacterial lipopolysaccharides: Structure, metablism and mechanisms of action. *Intern Rev Immunol* 6: 207–221.
151. Issekutz A, Bhimji S (1982) Role for endotoxin in the leukocyte infiltration accompanying *Escherichia coli* inflammation. *Infection and Immunity* 36: 558–566.
152. Miotla JM, Williams TJ, Hellewell PG, Jeffery PK (1996) A role for the $\beta 2$ integrin cd11b in mediating experimental lung injury in mice. *Am J Respir Cell Mol Biol* 14: 363–373.
153. Muttari A, Rylander R, Salkinoja-Salonen M (1980) Endotoxin and bath-water fever. *Lancet* 2: 89.
154. Rylander R, Haglind P (1984) Airborne endotoxins and humidifier disease. *Clin Allergy* 14: 109–112.
155. Hotchkiss JA, Harkema JR (1994) Effect of neutrophil depletion on endotoxin-induced mucous cell metaplasia in pulmonary airways of F344 rats. *Am J Respir Crit Care Med* 149: A994.
156. Thompson AB, Daughton D, Robbins RA, Ghafouri MA, Oehlerking M, Rennard SI (1989) Intraluminal airway inflammation in chronic bronchitis: Characterization and correlation with clinical parameters. *Am Rev Respir Dis* 140: 1527–1537.
157. Goco RV, Kress MB (1963) Comparison of mucus glands in the trachealbronchial tree of man and animals. *Ann NY Acid Sci* 106: 555–571.
158. Jones R, Baskerville A, Reid L (1975) Histochemical identification of glycoprotins in pig bronchial epithelium: (a) Normal (b) hypertrophied from enzootic pneumonia. *J Pathol* 116: 1–11.
159. Baskerville A (1976) Animal model of human disease: Chronic bronchitis. *Am J Pathol* 82: 237–240.
160. Feldman C, Munro NC, Jeffery PK, Mitchell TJ, Andrew PW, Boulnois GJ, Guerriro D, Rohde JAL, Todd HC, Cole PJ et al. (1991) Pneumolysin induced the salient histologic features of pneumococcal infection in the rat lung *in vivo*. *Am J Respir Cell Mol Biol* 5: 416–423.
161. Nygren H, Lange S, Lonnroth I (1984) Development of mucous cells in mouse intrapulmonary airways induced by cholera toxin, dibutyryl cyclic AMP and prostaglandin E1. *Br J Exp Pathol* 65: 549–556.
162. Lundgren JD, Kaliner M, Logun C, Shelhamer JH (1988) Dexamethasone reduces rat tracheal goblet cell hyperplasia produced by human neutrophil products. *Exp Lung Res* 14: 853–863.

163. Breuer R, Christensen TG, Lucey EC, Stone PJ, Snider GL (1985) Quantitive study of secretory cell hyperplasia induced by human neutrophil elastase in the large bronchi of hamsters. *J Lab Clin Med* 105: 635–639.
164. Breuer R, Lucey EC, Stone PJ, Christensen TG, Snider GL (1985) Proteolytic activity of human neutrophil elastase and porcine pancreatic trypsin cause bronchial secretory cell metaplasia in hamsters. *Exp Lung Res* 9: 167–175.
165. Christensen TG, Breuer R, Lucey EC, Stone PJ, Snider GL (1989) Regional difference in airway epithelial response to neutrophil elastase: Tracheal secretory cells discharge and recover in hamsters that develop bronchial secretory-cell metaplasia. *Exp Lung Res* 15: 943–959.
166. Christensen TG, Breuer R, Haddad CE, Lucey EC, Stone PJ, Snider GL (1992) Resistance of hamster bronchiolar epithelium to neutrophil elastase: Investigation by cell surface lectin cytochemistry. *Exp Lung Res* 18: 115–129.
167. Breuer R, Christensen TG, Lucey EC, Bolbochan G, Stone PJ, Snider GL (1993) Elastase causes secretory discharge in bronchi of hamsters with elastase-induced secretory cell metaplasia. *Exp Lung Res* 19: 273–282.
168. Christensen TG, Korthy AL, Snider GL, Hayes JA (1977) Irreversible bronchial goblet cell metaplasia in hamsters with elastase-induced panacinar emphysema. *J Clin Invest* 59: 397–404.
169. Jeffery PK, Reid L (1977) The respiratory mucous membrane. In: Brain JD, Proctor DF, Reid L (eds) *Respiratory defence mechanisms. Lung biology in health and disease,* vol. 3, New York: Marcel Dekker, 193.
170. Jeffery PK, Ayers M, Rogers DF (1982) The mechanisms and control of bronchial mucous cell hyperplasia. In: Chantler E (ed.) *Second symposium on mucus in health and disease. Advances in experimental medicine and biology,* vol. 144, New York: Plenum Press, 399.
171. Nadel JA (1991) Role of mast cell and neutrophil proteases in airway secretion. *Am Rev Respir Dis* 144: S48–S51.
172. Hayes JA, Christensen TG, Snider GL (1977) The hamster as a model of chronic bronchitis and emphysema in man. *Lab Animal Sci* 27: 762–770.
173. Sturgess J, Reid L (1973) The effect of isoprenaline and pilocarpine on (a) bronchial mucus-secreting tissue and (b) pancreas, salivary glands, heart, thymus, liver and spleen. *Br J Exp Pathol* 54: 388–403.
174. Jones R, Reid L (1979) β-Agonists and secretory cell number of intracellular glycoprotein in airway epithelium. *Am J Pathol* 95: 407–421.
175. Bolduc P, Reid L (1978) The effect of isoprenaline and pilocarpine on mitotic index and goblet cell number in rat respiratory epithelium. *Br J Exp Pathol* 59: 311–318.
176. Pauwels R (1994) The role of airway epithelium in asthma: From cover to culprit. *Eur Respir Rev* 4: 380–381.
177. Baskerville A (1976) The development and persistence of bronchial gland hypertrophy and goblet-cell hyperplasia in the pig after injection of isoprenaline. *J Pathol* 119: 35–47.
178. Scheiderman MA, Levin DL (1972) Trends in lung cancer: Mortality, incidence, diagnosis, treatment, smoking and urbanization. *Cancer* 30: 1320–1326.
179. Milne JS, Williamson J (1972) Respiratory symptoms and smoke habits in older people with age and sex differences. *Respiration* 29: 359–363.
180. Hayashi M, Huber GL (1977) Quantitative differences in goblet cells in the tracheal epithelium of male and female rats. *Am Rev Respir Dis* 115: 595–599.
181. El-Ghazzawi IF, Mandour MA, Aziz MT, El-Heneidy AR (1979) Changes in the lower respiratory epithelia induced by oestrogen intake. *J Larnygol Otol* 93: 601–612.
182. Lukacs NW, Streiter RM, Chensue SW, Kunkel SL (1994) Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am J Respir Cell Mol Biol* 10: 526–532.
183. Gibson PG, Hargreaves FE, Girgis-Gabardo A, Morris M, Denburg JA, Dolovich J (1995) Chronic cough with eosinophilic bronchitis and examination for variable airflow obstruction and reponse to corticosteroid. *Allergy* 25: 127–132.
184. Hessel EM, Van Oosterhout AJM, Garsen J, Van Loveren H, Saveloul HFJ, Nijkamp FP (1993) Immediate asthmatic reactions and changes in airway responsiveness after single versus chronic ovalbumin inhalation in sensitized mice. *Eur J Allerg Clin Immunol* 48: 101.

185. Blenkinsopp WK (1967) Proliferation of respiratory tract epithelium in the rat. *Exp Cell Res* 46: 144–154.
186. Johnson NF, Hubbs AF, Thomassen DG (1990) Epithelial progenitor cells in the rat respiratory tract. In: Thomassen DG, Nettesheim P (eds) *Biology, toxicology and carcinogenesis of respiratory epithelium.* London: Hemisphere, 88.
187. McDowell EM, Ben T, Carnell Newkrik TB, Chang S, De Luca LM (1987) Differentiation of tracheal mucociliary epithelium in primary cell culture recapitulates normal fetal development and regeneration following injury in hamster. *Am J Pathol* 129: 511–522.
188. McDowell EM, Barratt LA, Harris CC, Trump BF (1978) The respiratory epithelium. I. Human bronchus. *J Natl Cancer Inst* 61: 539–549.
189. Plopper CG, St. George J, Pinkerton KE, Tyler N, Mariassy A, Wilson D, Wu R, Hyde DM, Evans MJ (1990) Tracheobronchial epithelium *in vivo*: composition, differentiation and response to hormones. In: Thomassen DG, Nettesheim P (eds) *Biology, toxicology and carcinogenesis of respiratory epithelium.* London: Hemisphere, 6.
190. Nettesheim P, Jetten AM, Inayama Y, Brody AR, Gray TE, Mahler JF, Hook GE (1990) The role of Clara cells and basal cells as epithelial stem cells of the conducting airways. In: Thomassen DG, Nettesheim P (eds) *Biology, toxicology and carcinogenesis of respiratory epithelium.* London: Hemisphere, 99.
191. Hook AGE, Gilmore LB, Gupta RP, Patton SE, Jetten AM, Nettesheim P (1990) The function of pulmonary Clara cells. In: Thomassen DG, Nettesheim P (eds) *Biology, toxicology and carcinogenesis of respiratory epithelium.* London: Hemisphere, 38.
192. Rogers DF (1994) Airway goblet cells: Responsive and adaptable front-line defenders. *Eur Respir J* 7: 1690–1706.
193. Jeffery PK (1993) Microscopic structure of airway secretory cells: Variation in hypersecretory disease and effects of drugs. In: Takishima T, Shimura S (eds) *Airway secretion: Physiological bases for the control of mucus-hypersecretion*, vol. 72. New York: Marcel Dekker, 149.
194. Smith SM, Lee DKP, Lacy J, Coleman DL (1990) Rat tracheal epithelial cells produce granulocyte/macrophage colony-stimulating factor. *Am J Respir Cell Mol Biol* 2: 59–68.
195. Betram JF, Rogers AW (1981) Recovery of bronchial epithelium on stopping smoking. *Br Med J* 283: 1567–1569.
196. Gendler SJ, Spicer AP (1995) Epithelial mucin genes. *Annu Rev Physiol* 57: 607–634.
197. Morris ER, Rees DA (1978) Principles of biopolymer gelation: Possible models for mucus gel structure. *Br Med Bull* 34: 49–53.
198. Sheehan JK, Thornton DJ, Somerville M, Carlstedt I (1991) The structure and heterogeneity of respiratory mucus glycoproteins. *Am Rev Respir Dis* 144: S4–S9.
199. Rose MC (1992) Mucins: Structure, function and role in pulmonary disease. *Am J Physiol* 263: L413–L429.
200. Gum JR (1992) Mucin genes and the proteins they encode: Structure, diversity and regulation. *Am J Respir Cell Mol Biol* 7: 557–564.
201. Verma M, Davidson EA (1993) Molecular cloning and sequencing of a canine tracheobronchial mucin cDNA containing a cysteine-rich domain. *Proc Natl Acad Sci USA* 90: 7144–7148.
202. Park H, Hyun SW, Kim KC (1996) Expression of MUC1 mucin gene by hamster tracheal surface epithelial cells in primary culture. *Am J Respir Cell Mol Biol* 15: 237–244.
203. Tsuda T, Gallup M, Jany B, Gum J, Kim Y, Basbaum C (1993) Characterization of a rat airway cDNA encoding a mucin-like protein. *Biochem Biophys Res Commun* 195: 363–373.
204. Ohmori H, Dorhman AF, Gallup M, Tsuda T, Kai H, Gum JR, Kim YS, Basbaum CB (1994) Molecular cloning of the amino-terminal region of rat MUC2 mucin gene homologue. *J Biol Chem* 269: 17833–17840.
205. Gum JR, Hicks JW, Lagace RE, Byrd JC, Toribara NW, Siddiki B, Fearney FJ, Lamport DTA, Kim YS (1991) Molecular cloning of rat intestinal mucin. *J Biol Chem* 266: 22733–22738.
206. Bhargava AK, Wotach JT, Davidson EA, Bhavanandan VP (1990) Cloning and cDNA sequence of a bovine submaxillary gland mucin-like protein containing two distinct domains. *Proc Natl Acad Sci USA* 87: 6798–6802.
207. Timpte CS, Eckhardt AE, Abernethy JL, Hill RL (1988) Porcine submaxillary gland apomucin contains tandemly repeated, identical sequences of 81 residues. *J Biol Chem* 263: 7686–7690.

208. Basbaum C, Gallup M, Gum J, Kim Y, Jany B (1990) Modification of mucin gene expression in the airways of rats exposed to sulfur dioxide. *Biorheol* 27: 485–489.
209. Gordon JT, Nadziejko C, Plant M, Rodger IW, Pon DJ (1996) One-month exposure to inhaled endotoxin produces a dose-dependent increase in stored mucosubstances in rat intrapulmonary airways. *Exp Lung Res* 22: 509–523.
210. Li D, Wang D, Godfrey R, Rogers AV, Majumdar S, English AF, Myles D, Jeffery PK (1996) Endotoxin-induced airway goblet cell hyperplasia and metaplasia in the rat: Increased (*RAMIS*) gene expression and intracellular mucin. *Am J Respir Crit Care Med* 153: A722.
211. Li D, Godfrey R, Rogers AV, Wang D, Majumdar S, English A, Myles D, Jeffery PK (1997) Endotoxin-induced neutrophil influx, intracellular mucin an mucous metaplasia in rat airways: A light and electron microscopic study. *Am J Respir Cell Mol Biol* (submitted).

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 11
Mucus Hypersecretion and Its Role in the Airway Obstruction of Asthma and Chronic Obstructive Pulmonary Disease

Ursula M. Wells and Paul S. Richardson

Department of Physiology, St. George's Hospital Medical School, London, UK

1. Introduction

Several distinct processes may narrow the airway lumen in the diseased lung: smooth muscle contraction, airway scarring and remodelling, encroachment of submucosal tissue swollen with oedema or engorged blood vessels, collapse of the airway walls under a pressure gradient and accumulation of mucus. Only in the last of these, the subject of this chapter, is the obstruction situated within the airway lumen itself; the remainder result from processes in or even outside the airway walls. It is rare to find that a single mechanism entirely accounts for airway obstruction in any patient, though one may predominate in a particular phase of his or her illness.

This chapter describes the role of airway mucus in blocking the airway lumen in asthma and chronic obstructive pulmonary disease (COPD).

2. Asthma

Asthma is common, affecting about 10% of the UK population. The main feature of the disease is intermittent airway obstruction which remits either spontaneously or with treatment, but the syndrome also includes cough, wheeze, hyperventilation and dyspnoea. In long-standing asthma airway obstruction may remit less [1] and the syndrome merges with COPD (see Section 8.1.3).

2.1. Airway Mucus Obstruction

Several pathogenic mechanisms lead to asthma, so it is not surprising that a particular form of airway obstruction, such as accumulation of mucus in the lumen, may dominate in one individual yet be trivial in another. Several lines of evidence demonstrate the presence of mucus obstruction in asthma.

Most compelling are autopsy studies on those who have died from the disease. Here it is common to find extensive mucus plugs which block or narrow one airway generation after another [2, 3]. Mucus accumulates in bronchi and bronchioles [2, 4, 5], though some individuals dying of asthma have normal amounts of mucus in their airways [6]. Recent attempts to measure airway mucus obstruction in eight asthmatics who came to autopsy [5] showed that the ratio of the cross-sectional area of airway blocked by mucus to the total airway cross-section, known as the mucus occupying ratio (MOR), was on average about 10% in both central and peripheral airways.

The airway plugs are not pure mucus, as they contain cells, DNA, proteoglycans and serum-type proteins as well as mucins [2, 7]. Dunnill describes them as *exudate* rather than *mucus* to emphasise this point [2]. However, in the few cases where the material has been analysed rigorously, mucins are the main gel-forming component [8–10]. Asthmatic mucins are difficult to dissolve, so unless samples are prepared with this in mind, they are easy to miss or underestimate [8].

Does mucus narrow the airway lumen in non-fatal cases of asthma? Evidence for this comes partly from autopsies of asthmatics who die from other causes. Their airways sometimes show patchy accumulations of a mucus-containing material similar to that found in those who died in status asthmaticus [3]. Sanerkin and Evans [11], who performed histology on the plugs of sputum, in some cases whole airway casts, coughed up by asthmatics, found that microscopically these resembled the post-mortem material described by Dunnill [2]. Some asthma sufferers are sputum producers, and the amounts they expectorate typically increase during and just after an attack, while others produce little sputum at any stage of the illness; it may be that the latter are the patients in whom mucus obstruction is unimportant. We do not yet know in how many asthma sufferers mucus obstructs the airway to an important extent.

2.2. How Important is Mucus Obstruction in Asthma?

In asthmatics who die from the disease, and who at autopsy have extensive airway plugging, accumulation of mucus plainly obstructs airflow enough to cause ventilatory failure. The role played by mucus in living asthmatics

is less certain, but the following are likely consequences of its presence in even limited amounts:

1. Any luminal obstruction of an airway will lead to airway hyper-responsiveness, for it takes less mural constriction to close a partially obstructed airway than one with a clear lumen. Application of Poiseuille's law to predict the increase in resistance caused by mural constriction shows that even modest luminal obstruction must increase responsiveness strikingly [12, 13].
2. Mucus obstruction is often patchy [3], reducing the ventilation to some groups of alveoli while diverting it to others thus producing ventilation-perfusion mismatch [14]. This mismatch causes arterial hypoxaemia and stimulates chemoreceptors which, in their turn, contribute to hyperventilation and dyspnoea.
3. Luminal mucus raises airway resistance and, consequently, the work of breathing.

Airway hyper-responsiveness, ventilation–perfusion mismatch and increased work of breathing are almost universal in asthma suffers but may result from causes other than mucus obstruction. Only when there is some way, so far unknown, of selectively relieving mucus obstruction, we will know the extent to which mucus is responsible in any given patient.

2.3. Why Does Mucus Obstruct Airways in Asthma?

Mucus obstructs the airway in asthma sufferers when its secretion outpaces its clearance. That much is simple to state, but the mechanisms which promote hypersecretion and slow clearance are many and their role in asthma complex. Sections 3 and 4 review some of the mechanisms which may be important.

3. Mucus Hypersecretion in Asthma

Submucosal glands hypertrophy in the asthmatic airway [15], and within them there is hyperplasia of mucous cells at the expense of serous cells [16]. Numbers of epithelial goblet cells increase as well [16]. This may be important, because goblet cells form the only clear reservoirs of secretion in the bronchioles. These structural changes are consistent with mucus hypersecretion.

3.1. Mediators Which Drive Secretion in Asthmatic Airways

Several mediators, thought to be present in the asthmatic airway, can drive airway mucus secretion. These include the following:

3.1.1. Histamine: Histamine, released from mast cells and basophils, acts on H_2 receptors to release mucus into the human airway [17].

3.1.2. Leukotrienes and platelet activating factor: Several cell types which are prominent in the asthmatic airway, including mast cells and eosinophils, release leukotrienes from antigen-challenged airways [18, 19], and the urine of sufferers contains increased amounts of leukotrienes during acute asthma attacks [20]. Leukotrienes release mucus from human airway *in vitro* [21]. Platelet activating factor (PAF) also releases mucus from human airways, but leukotriene antagonists block its action [22]. Thus the effect of PAF is probably secondary to that of leukotriene release.

3.1.3. Prostaglandins: Antigen-challenged airways release a number of cyclo-oxygenase products, including PGD_2 and $PGF_{2\alpha}$, mediators which release mucus from human airways *in vitro* [23]. PGE_1 may inhibit secretion, but this is disputed [23, 24].

3.1.4. Macrophage-derived mucus-stimulating protein: Marom and colleagues have identified a 68-kDa protein, released from airway macrophages, which stimulates airway mucus release [25]. Cultured macrophages from asthmatic airways release more MMS-68 than do those from controls, so it is possible that this protein drives secretion in the asthmatic airway [26].

3.1.5. Adenosine and other nucleotides: In the dog, adenosine, given on the serosal (though not the luminal) side of the airway, causes discharge from epithelial goblet cells, while adenosine triphosphate (ATP) and uridine 5′-triphosphate (UTP) given on the luminal aspect also result in goblet cell secretion, presumably via purinergic P_{2U} [27]. So far no one seems to have tested adenosine's action on goblet cell secretion in man; however ATP and UTP, given on the luminal aspect of human airway epithelium, cause goblet cell discharge [28]. There are adenosine A_2 receptors on cultured serous cells from submucosal glands [29]. Adenosine aerosols raise airway resistance in asthmatics [30], but it is not clear that airway secretion plays any part in this. Nor would adenosine application by aerosol to the luminal aspect of the airway cause secretion if human airways resemble their canine counterparts.

3.1.6. Cytokines: Cytokines may play an important part in the pathogenesis of asthma, though so far we know little of how they affect airway secretion. TNF_α increases mucus secretion [31, 32], but other cytokines need to be tested too.

3.1.7. Endothelins: Endothelins, which may be released in asthma, drive mucus secretion from airway submucosal glands in the cat [33].

3.2. Nerves and Reflexes

Autonomic nerves regulate mucus secretion into the airways. There is indirect evidence that nervous drives to secretion increase during asthma attacks. This would contribute to the hypersecretion of mucus, at least in the large airways where it is clear that the submucosal glands are innervated.

3.2.1. Cholinergic pathways: Cholinergic parasympathetic nerves innervate the submucosal glands of the airway in man [34] and their activity drives mucus secretion [35, 36]. In species where it has been tested, stimulation of the rapidly adapting receptors and of non-myelinated receptors reflexly triggers secretion via this pathway [37–39]. Stimuli which provoke coughing generally cause secretion, presumably via these afferent nerves and partly via the cholinergic efferent pathway. Acute hypoxia also stimulates peripheral chemoreceptors which drive secretion by the same efferent pathway [40].

3.2.2. Adrenergic pathways: While airway smooth muscle has few sympathetic nerves in man, the submucosal glands have a moderately rich noradrenergic innervation [34]. Extrinsic noradrenaline causes mucus secretion, but evidence for a functional sympathetic innervation remains indirect [36, 41].

3.2.3. Non-adrenergic, non-cholinergic innervation: In several non-human species stimulation of airway nerves and reflexes releases mucus even in the presence of high doses of atropine and adrenoceptor antagonists [42–44]. Non-adrenergic, non-cholinergic (NANC) nerves probably mediate these effects via both sympathetic and parasympathetic pathways [42, 45]. The part of these effects which survives ganglion blockade, given to prevent orthodromic autonomic conduction, is best explained by antidromic conduction in afferent nerves [43, 46].

There is little information on the equivalent pathway in humans. Rogers and Barnes [47], however, showed that capsaicin stimulates mucus output from human airway removed at lung resection, but that this effect was small unless they simultaneously gave naloxone. Their explanation was that capsaicin stimulates non-myelinated and small myelinated afferent fibres which send collateral branches to secretory cells. Antidromic spread of action potentials releases transmitters like substance P which in turn drive secretion. Opiate premedication of donor patients inhibits transmitter release unless naloxone is given to antagonise this effect. Thus there is indirect evidence for NANC secretory pathway in humans. Barnes argues that these, and the axon reflexes which drive them, are important in asthma [46].

3.3. Drugs

It is possible that the drugs which asthma sufferers take to ameliorate their symptoms may increase secretion. Most asthmatics take β-adrenoceptor agonist aerosols to relieve wheezing and dyspnoea. Both β_1 and β_2 agonists drive airway secretion in some non-human animals and probably also in man [45, 48]. In the ferret, β-agonists stimulate mucous cells more than serous cells, and the resulting secretions have a correspondingly high viscosity [49–51]. Thus it is conceivable that these drugs encourage secretion of a viscid mucus which is particularly prone to obstruct the airway. Accumulations of airway mucus with a high viscoelasticity may explain the epidemics of asthma deaths which have coincided with prescription of high-dose β-adrenoceptor agonist aerosols.

3.4. Antigens, Proteins and Proteases

3.4.1. Antigens: Antigen exposure of actively or passively sensitised airways releases mucus [17, 52]. A large part of this effect depends on mediator release.

3.4.2. Proteins: Presence of proteins, including exudate, in the airway lumen stimulates mucus secretion [53, 54].

3.4.3. Proteases: Several proteases release mucus from the airway [55, 56]. In asthmatic airway, activation of mast cells releases tryptase, and the influx of motile cells such as neutrophils, eosinophils and macrophages are all potential sources of proteolytic enzymes.

A number of mediators, reflexes, drugs, antigens and other agents which are present in the asthmatic airway can release mucus from the hypertrophied mucus apparatus in the asthmatic airway. There is the potential for asthmatic airways to secrete copious material. Any slowing of airway clearance which coincides with this hypersecretion makes for mucus obstruction.

4. Mucus Transport Mechanisms

Three mechanisms usually propel mucus from the airways towards the pharynx or mouth: mucociliary clearance, two-phase flow and cough. Mucociliary clearance depends on the co-ordinated beating of the cilia lining the airways. During its power stroke, a cilium engages the overlying mucus with its tip and pushes it towards a larger airway. During the recovery stroke the cilium disengages and returns to its original position without contacting the mucus raft, and the next power stroke begins. Thus each cilium pushes the overlying mucus in one direction. Whole fields of

cilia are co-ordinated so that their activity travels on waves across the airway surface [57].

Two-phase flow depends on the tug exerted on mucus by the air flowing over it. During inspiration intrapleural pressure falls to bring air into the chest, while in expiration intrapleural pressure rises slightly. This rise and fall in intrapleural pressure, and hence the pressure surrounding the airways, ensures that they widen in inspiration and narrow during expiration. This in turn means that expiratory air velocity is slightly faster than that during inspiration. The ebb and flow of air tugs at mucus lining the airway, but the expiratory force will slightly exceed the inspiratory, so mucus travels in the expiratory direction, from smaller to larger airways and eventually out of the lungs altogether. This effect becomes stronger as mucus accumulates, for example when mucociliary clearance has failed [58, 59].

Coughing is a special case of two-phase flow where a forced expiration follows a preparatory inspiration [60]. The forced expiration creates a high intrapleural pressure, sufficient to narrow the larger airways. Expired air then travels through the narrowed airways at a velocity high enough to shear mucus from the airway walls. Narrowing is confined to the larger airways, and it is in these that cough is most effective. Both two-phase flow and cough are more effective when the mucus layer is thick and of low viscoelasticity [58, 61, 62].

4.1. Mucus Transport in Asthma

Tracheal mucus velocity is slow in asthmatics. When radio-opaque particles are placed on the tracheal surface of asthmatic patients in remission, cilia move them at little more than half the velocity seen in normal subjects [63]. Subsequent airway challenge with an antigen to which the patient is sensitive slows ciliary transport further [63, 64].

Less direct evidence, based on clearance of radiolabelled aerosol particles deposited in the lower airways, suggests that bronchial mucociliary clearance is also slow in asthmatics, even those in remission [65, 66]. Such evidence is difficult to interpret, because radioactive particles tend to deposit in the more central airways of asthmatics than healthy controls. A recent study largely overcame this difficulty by getting more severely bronchoconstricted subjects to inhale more slowly, thus ensuring that all subjects, irrespective of the severity of their disease, had a similar distribution of particles in their lung fields [67]. On comparison of mucociliary clearance of radiolabelled particles deposited in the larger airways of two groups of asthmatic subjects, one with severe obstruction causing flow limitation at rest and the other with less severe disease, the more severely afflicted group showed consistently slower clearance [67].

Recently Svartengren and colleagues developed a technique for depositing radiolabelled particles mainly in the bronchioles [68]. In health, clear-

ance from bronchioles is much slower than from the bronchi. However, clearance from the bronchioles of well-controlled asthmatics occurred at a rate similar to that in the healthy controls. It is now important to test whether bronchiolar clearance is slowed when asthma is more active [68, 69].

4.2. Why is Mucociliary Clearance Slow in Asthmatic Bronchi?

4.2.1. Epithelial damage: Epithelial damage, which includes shedding of ciliated cells, is common in the airways of asthma sufferers [70, 71]. This is likely to slow mucociliary transport.

4.2.2. Abnormalities of mucus: Cilia only transport mucus efficiently if its viscosity, elasticity and adhesiveness lie within certain limits [62, 72]. Mucus from asthma sufferers appears often to have unusually high visco-elasticity, but physical measurements are still needed to establish this clearly. It is possible that the β-adrenoceptor agonist drugs, used to treat asthmatics, may contribute to the physical abnormality of secretions [51].

4.2.3. Tethering of mucus: Goblet cells in the surface epithelium secrete part of the mucus which then anneals with mucus from other sources (e.g. the submucosal glands) in the airway lumen [73]. Thurlbeck noted that, in the airways of asthmatics who had died of the disease, mucus in the lumen was continuous with that inside airway goblet cells and submucosal glands and appeared to tether it [16]. Recently Shimura has extended this finding to quantitative observations on the airways in a series of patients who had died, some from asthma, others from COPD [5]. It appears that a process of cleavage, which normally separates extracellular from intracellular mucus, had failed in the asthmatic airway. This failure left intraluminal mucus tethered by multiple strands to the secretory cells of the epithelium. Similar tethering was less evident in COPD [5]. This could help explain slowing of mucus transport by cilia, two-phase flow and cough, especially if asthmatic mucus has a high tensile strength.

4.2.4. Mediators which slow mucus transport: Most mediators, such as histamine, which stimulate airway mucus secretion, also accelerate mucociliary transport [74]. One exception to this rule is leukotriene D_4, which slows mucociliary transport, at least in allergic sheep [75]; another is PAF [76].

4.2.5. Allergen exposure: As noted above (Section 4.1), allergen inhalation slows tracheal mucociliary transport. Leukotriene inhibitors such as FPL 55712 reverse this effect [63, 64].

5. Mucus in Asthma: Conclusions

Accumulation of mucus in the airway lumen is one of several mechanisms which obstruct the airways of asthma sufferers. It plays an important part in many asthmatics at certain stages of their disease, and its role increases with the severity of the asthma. Most asthmatics who die from the disease have airway obstruction with mucus which appears to have a high viscoelasticity, but its role in mild asthma is less certain. Asthma sufferers often manifest both mucus hypersecretion and unusually slow mucus transport; together these result in airway mucus obstruction. We know various causes for both hypersecretion and slowed mucus transport, but not yet which are the most important contributors to airway mucus obstruction.

6. Mucus Hypersecretion in Chronic Obstructive Pulmonary Disease

6.1. Mucus and COPD

Sputum expectoration in COPD ranges from a few millimetres in the early morning to daily production of more than 100 ml produced around the clock [77]. There are three clinical reasons for the interest in mucus secretion in COPD. First, mucus may contribute to airway obstruction and cause ventilation-perfusion mismatch, impairing gas exchange (Section 7.2). Second, failure of mucus clearance may allow bacterial infection which in turn exacerbates bronchitis, with the hypersecretion this involves, and further damages mucus transport mechanisms. This putative sequence has the potential to encourage yet further infection and so on in a vicious circle. Finally, coughing up large volumes of sputum embarrasses and exhausts patients.

6.2. Definition of COPD

The classification of inflammatory airway diseases which lead to irreversible airflow obstruction and the nature of the obstructive lesions responsible have been debated since the 1950s [77–82]. The overlap in signs and symptoms of the variety of pathogenic mechanisms leading to similar syndromes have been largely responsible for the difficulties; but disagreement about the role of mucus accumulation in airflow obstruction has also contributed.

Chronic bronchitis has been defined in the UK as chronic cough and sputum production not explained by focal lung disease [79]. This typically goes with hypertrophy of submucosal glands in the trachea and bronchi and hyperplasia/metaplasia of lower airway goblet cells [83]. Emphysema is

defined anatomically as destruction of lung tissue and enlargement of alveolar spaces [82]. The structural changes in the airway wall that lead to mucus hypersecretion are therefore strictly a feature only of bronchitis, but emphysema and bronchitis often co-exist and are difficult to distinguish clinically [84]. However, some patients with the stigmata of chronic bronchitis fail to expectorate [85]. Consequently, COPD has been defined more recently without reference to hypersecretion, as a disorder characterised by abnormal tests of respiratory flow that do not change markedly over periods of several months' observation [86].

This definition is intended to distinguish COPD from asthma, in which airflow obstruction is reversible, but it has two drawbacks. First, in many asthmatic patients, particularly those with long-standing disease, there is an element of irreversibility to airflow obstruction. Second, airflow limitation does not occur in all cases of emphysema or chronic bronchitis. Thus, COPD has also been described "as a *process* characterised by chronic bronchitis or emphysema that may lead to the development of airway obstruction; the airway obstruction may be accompanied by airway hyperreactivity and may be partially reversible" [77].

6.3. Structural Changes in the Airway Wall

Changes in the airway wall in chronic bronchitis include hypertrophy and hyperplasia of the submucosal glands (with dilation of gland ducts), an increase in goblet cell number (hyperplasia in the large airways and metaplasia in the bronchioles) and hypertrophy of smooth muscle [83]. Increased transudation, epithelial damage and ciliary abnormalities may be present. Plugging of bronchioles with mucus and distortion of bronchioles due to loss of alveolar attachments or fibrosis can also occur [77].

Not all of these changes are specific to chronic bronchitis. For example, gland hypertrophy also occurs in other diseases, such as cystic fibrosis. There is also an overlap in gland size between normals and bronchitics, and between bronchitics with and without sputum production [87, 88].

7. Mucus and Airway Obstruction in COPD

Most airflow obstruction in COPD occurs in small airways of less than 3 mm diameter [89], and may be accompanied by the presence of mucus plugs, fibrosis, narrowing and obliteration of airways. Mucus plugs can partly or completely occlude the lumen of some bronchioles. The part mucus plays in airflow obstruction in COPD remains controversial, and the evidence is conflicting.

7.1. Epidemiological Evidence

Most epidemiological studies of the role of chronic mucus hypersecretion in airflow obstruction have sought an association between sputum production and either age-related decline in FEV_1 or mortality. The findings which have emerged are contradictory.

An early and influential study on the initial stages of development of COPD in working men in London concluded that mucus hypersecretion did not cause the irreversible airflow obstruction that developed in a subset of smokers, nor did it cause chronic obstruction to develop more rapidly [104]. Other studies in France [90] and in the USA [91] also found no association between chronic hypersecretion and FEV_1 decline. Furthermore, mortality from COPD in British men was not found to be related to the level of mucus hypersecretion at the start of the study [92]. Similar findings were obtained from the Whitehall study of male civil servants [93].

On the other hand, evidence for an association between chronic hypersecretion and FEV_1 decline has been found amongst men (but not women) in the USA Six Cities study [94], and in Copenhagen [95]. A weak association between mortality and chronic sputum production was found in French workers [96]. Sputum production has also been found to be related to mortality from COPD in the Six Cities study [97] and in Copenhagen [98].

There are two general problems in interpreting these types of studies. The first is that hypersecretion cannot adequately be quantitated since we lack measurements of baseline secretions in normal subjects for comparison [85]. Second, sputum production is a poor indicator of mucus plugging in small airways, the main site of airflow obstruction in COPD.

7.2. Mucus Plugging of Small Airways

Small airways ($<2-3$ mm diameter) normally contribute 20–25% of total airway resistance. In COPD, resistance in small airways increases, but measurements of total airways resistance are insensitive to this change unless airflow obstruction is very severe. Thus "it is entirely possible for a patient with chronic cough and sputum to have considerable small airway obstruction and an increase in peripheral resistance and yet have virtually normal total airway resistance" [89]. The main deleterious effect of small airway obstruction is to cause ventilation-perfusion inequalities and thus inefficient oxygenation of the blood.

The degree of airflow obstruction in COPD is strongly related to the severity of emphysema [99], which in turn may largely reflect irreversible structural changes in the airways. However, there is also evidence that mucus plugging is more extensive in airways with emphysema or with both emphysema and chronic bronchitis than in those with just bronchitis [100].

The extent to which mucus plugs increase resistance and cause ventilation-perfusion inequalities in these diseases has not been measured adequately, but it is difficult to see why they should not contribute to these changes [101].

Bacteria proliferate in stationary mucus, causing further inflammation and secretion. During these acute exacerbations mucus may reversibly obstruct the bronchioles [89, 100, 102]. Direct evidence for this is lacking, although bronchographs have shown that mucus plugs disappear in those who quit smoking and that their bronchitis improves [103]. The most telling argument against a large role for mucus in the obstruction of COPD is that giving up smoking improves lung function only slightly [104].

8. Overgrowth of Secretory Cells in COPD

8.1. Causes

8.1.1. Cigarette smoke: Smoking is the main cause of COPD [103] although most smokers do not develop the disease, so genetic factors may determine individual susceptibility. Chronic exposure to tobacco smoke results in hyperplasia of goblet cells and hypertrophy of submucosal glands in humans [105–107] and in experimental animals [108, 109].

8.1.2. Pollutant dusts and gases: Chronic exposure to air pollutants, e.g. coal dust [110] is another risk factor for COPD [111, 112]. Prolonged exposure to the common pollutant gases sulphur dioxide (SO_2), ozone and nitrogen dioxide (NO_2) causes hyperplasia/metaplasia of goblet cells and hypertrophy of submucosal glands [113–117] and epithelial damage [118, 119] in experimental animals.

8.1.3. Asthma: Asthmatics, particularly those who smoke or breathe other irritants, often develop irreversible airflow obstruction (COPD) and chronic productive cough (chronic bronchitis) [112]. Thus, while some asthmatics suffer simply from reversible airway obstruction, others – particularly those with long-standing disease – go on to develop features of other obstructive diseases.

8.1.4. Protease–antiprotease balance: A genetically determined deficiency of the antiprotease α_1-antitrypsin (α_1-AT) is clearly associated with emphysema [120, 121], although it accounts for only a very small number of cases (0.1% cases of COPD in the USA) [77]. An association with chronic bronchitis is less clear, but in one study half of the α_1-AT-deficient non-smokers had chronic bronchitis [122].

8.2. Intermediate Mechanisms

8.2.1. Neutrophils: The common intermediate step between chronic irritation of the airways and the structural changes of chronic bronchitis may be the accumulation of neutrophils at the site of injury. Neutrophil numbers increase in sputum and bronchoalveolar lavage (BAL) fluid from smokers and chronic bronchitics [123–125], and also in the airway wall during acute exacerbations of chronic bronchitis [126]. Neutrophils release proteases, oxidants and free oxygen radicals, all of which promote mucus secretion (Sections 9.1 and 9.2).

8.2.2. Bacterial infections: COPD patients often suffer exacerbations, with wheezing and increased sputum production. These typically occur in response to bacterial infections, either primary or in the aftermath of viral bronchitis [127].

Many respiratory pathogens – including *Streptococcus pneumoniae* and *Haemophilus influenzae*, which are frequently associated with exacerbations of COPD – can bind to glycoproteins in mucus by adhesins on the bacterial cell membrane [128]. Release of proteases and toxins from the bacteria cause hypersecretion of mucus [55, 129] and epithelial damage (Section 10.1.1). Adherence of bacteria to damaged epithelial cells can lead to mucosal invasion and an inflammatory response, including neutrophil infiltration [128].

9. Control of Secretion

9.1. Proteases and Antiproteases

9.1.1. Proteases: Protease activity is low in normal secretions but increased in sputum and BAL in chronic bronchitis [130] and in smokers [125, 131]. Proteases, including neutrophil elastase and cathepsin G, are potent secretagogues [56] and also cause goblet cell metaplasia and/or hyperplasia [132, 133], epithelial damage and reduced mucociliary clearance (Section 10.1.1) when applied exogenously.

Human neutrophil elastase (HNE) is the most potent endogenous secretagogue known [56]. It stimulates release of cell surface glycoproteins [133, 134], and ^{35}S-labelled macromolecules from submucosal glands *in vitro* [56, 135]. The local concentrations of free HNE in airways of chronic bronchitics are not known, but in sputum from cystic fibrosis patients 3.4×10^{-6} M of unbound HNE was reported [136]. Thus HNE may be the major stimulus of mucus secretion in inflamed airways i.e. during exacerbations of COPD. However, sputum from patients with stable bronchitis does not contain free elastase activity [130].

Bacterial proteases may also be important during infective episodes. Elastase and alkaline protease from *Pseudomonas aeruginosa*, a common

pathogen in cystic fibrosis and bronchiectasis [137], increase mucin secretion from goblet cells [138], and from surface epithelium and submucosal glands [55].

9.1.2. Antiproteases: Antiproteases in secretions and in the airway wall limit protease activity, but in inflamed airways this capacity may be overwhelmed by the release of proteases from the multitude of neutrophils. The most important inhibitors of HNE are probably α_1-AT and antileukoproteinase ALP, also known as secretory leukoprotease inhibitor (SLPI). Plasma contains α_1-AT but monocytes and macrophages also synthesise it locally, whilst goblet cells, serous cells and Clara cells can all secrete ALP [139].

A genetically-determined deficiency of α_1-AT is associated with development of COPD (Section 8.1.4). α_1-AT activity can also be decreased by oxidation (by cigarette smoke or neutrophils [140, 141] or by proteolytic cleavage [142, 143]. This may account for the reduced α_1-AT activity in sputum from smokers and from cystic fibrosis patients [136]. Such a reduction in activity may contribute to mucus hypersecretion in COPD.

9.2. Oxidants and Antioxidants

9.2.1. Source of oxidants: The main sources of oxidants and free oxygen radicals in the airway are cigarette smoke, neutrophils and bacteria; their relative importance in causing mucus hypersecretion is unknown. In addition, air pollutants such as SO_2, NO_2 and ozone can generate free oxygen radicals in solution and may, during periods of high exposure, exacerbate mucus secretion. There has been much interest recently in the role of the oxidant-antioxidant balance in the pathogenesis of COPD [144].

9.2.2. Cigarette smoke: Direct evidence that inhaled cigarette smoke causes mucus secretion acutely in humans is lacking, although it is often said that the first cigarette of the day is an effective expectorant. Acute exposure to cigarette smoke *in vivo* in experimental animals increases secretion from submucosal glands [145], goblet cell discharge [44], and plasma exudation [146].

Cigarette smoke contains over 4700 chemical compounds, including a high concentration of oxidants and free oxygen radicals in both the vapour and particulate phases of the smoke [147]. A single inhalation of cigarette smoke contains about 10^{14} free oxygen radicals [148]. Despite this, it has not been established whether oxidants play any role in the hypersecretion generated by cigarette smoke. Low smoke concentrations increase gland and goblet cell secretions via stimulation of autonomic ganglia by a component of the particulate phase, probably nicotine [44, 149]. Higher concentrations of smoke stimulate secretion from goblet cells and plasma

exudation via sensory nerve activation by an unknown component of the vapour phase [44, 146].

9.2.3. Oxidative metabolism: Oxidative metabolism by neutrophils and bacteria can convert molecular oxygen to hydrogen peroxide (H_2O_2) as well as to highly reactive unstable intermediates including superoxide (O_2^-) and hydroxyl ($\cdot OH$) radicals [150]. Exogenous H_2O_2 increases mucus secretion from glands *in vitro* [151], but we do not know whether local levels of H_2O_2 are high enough to do so in inflamed airways.

9.2.4. Air pollutants: Chronic exposure to ozone (0.5 ppm) [115] and to SO_2 (50–250 ppm) [117, 152] *in vivo* increases glycoprotein secretion in experimental animals. Reflexes may contribute to the effect (Section 9.5).

9.2.5. Antioxidants: The actions of oxidants and free oxygen radicals on the airway epithelium and mucosa are limited by antioxidants present in mucus (either in the gel layer or in periciliary fluid). These include low molecular weight molecules (e.g. glutathione, uric acid, ascorbic acid), metal-binding proteins either from plasma (albumin, transferrin, caeruloplasmin) or locally secreted (lactoferrin), and enzymes such as catalase and superoxide dismutase. In the gel layer, mucins [153] and possibly a glycosylated peroxidase [154] act as antioxidants. Thus, mucus hypersecretion may increase the protection of the underlying mucosa from oxidant-induced damage.

The distribution of antioxidants varies between different parts of the respiratory tract, between species and between individuals. The latter may partly explain the variation in susceptibility to cigarette smoke and air pollutants. Smoking and acute exacerbations of COPD also reduce the antioxidant capacity of plasma [155]. The effects on airway surface antioxidant levels are not yet clear [144]. Differences in these may help determine the variability of hypersecretion among those who inhale equal quantities of cigarette smoke.

9.3. Mediators

During inflammation, mediators may be released from many sources, including neutrophils and other inflammatory cells, epithelium, endothelium and sensory nerves. Many promote mucus secretion, either directly or by stimulation of sensory nerve endings (Section 9.5). Many of the mediators involved have already been described in Section 3.1 on asthma.

9.4. Dust particles

Charcoal and barium sulphate particles increase glycoprotein secretion from submucosal glands and epithelium [37] by both direct and reflex mechanisms (Section 9.5).

9.5. Reflexes

Many stimuli which do or may result in development of COPD, such as cigarette smoke, pollutant gases and particle deposition, can activate C-fibres and/or rapidly adapting receptors (RARs) in the airway wall. Activity in these afferent nerves can lead to both local axon reflexes [46] and central vagal reflexes [156], which in turn cause mucus secretion, microvascular leakage, bronchoconstriction and cough (Section 10.2).

Vasoactive intestinal peptide (VIP), which is co-localised with acetylcholine in cholinergic nerve endings, inhibits ^{35}S-glycoconjugate release from normal human bronchial cultures but not from bronchi from chronic bronchitics [157]. This suggests that hypersecretion in bronchitis may partly be due to a lack of a normal inhibitory process. However, evidence from *in vivo* studies to support this view is lacking.

10. Airway Clearance

10.1. Mucociliary Clearance

General observations on mucus transport made in Section 4 also apply here. Mucociliary transport rate is decreased in smokers and in chronic bronchitics [158–160]. This may result from either a defective clearance mechanism (due to epithelial or ciliary damage) or the secretion of abnormal mucus or both. Stagnant mucus predisposes to infection with bacteria, leading to further hypersecretion and/or production of mucus with abnormal viscoelasticity, with greater potential for airway plugging.

10.1.1. Epithelial and ciliary damage: Epithelial and ciliary damage are caused by many of the factors implicated in COPD. Smoking disrupts ciliary structure in humans [161] and other animals [162], alters ciliary beat frequency, leads to loss of ciliary co-ordination [163, 164] and denudes the epithelium [165].

Other factors which cause damage include chronic exposure to NO_2 (3–30 ppm) [166, 167], ozone (0,5–1 ppm) [168, 169], exogenous hydrogen peroxide [170–172], proteases [173–176] and products from *Streptococcus pneumoniae* and *Haemophilus influenzae* [137].

10.1.2. Abnormal mucus: Healthy humans do not cough up mucus, so sputum production is by definition pathological. However, no clear diagnostic changes have been found in the physical and chemical characteristics of mucus in COPD [85, 177–180]. This is perhaps not surprising, since many of the pathophysiological factors which influence its composition (e.g. infection, tissue damage, increased transudation) are both common to and variable in extent in several lung diseases. In addition, the low secretory volume in normal airways makes baseline comparison difficult [85].

For effective mucociliary clearance, the viscoelasticity of mucus must lie within a certain range (see Section 4.2.2). When patients with chronic bronchitis suffer exacerbations, the physical properties of their mucus may lie outside this range [180].

10.2. Cough

Cough is a prominent symptom in COPD, particularly with chronic bronchitis; many of the stimuli which promote mucus secretion also cause coughing. It may result from chronic stimulation of RARs in the large airways including the larynx (Section 9.5) by exposure to cigarette smoke and other irritants, mediator release during inflammation and by particle deposition.

Coughing helps to remove from the larger airways abnormal mucus that cannot be shifted by ciliary action. However, it is ineffective in smaller airways, and it is there that obstruction by mucus may be important pathologically in COPD. Chronic severe coughing may also cause fatigue and social difficulties.

Providing that mucus does not become too solid and that it does not become fixed to the airway wall, two-phase flow should continue to move it from the airway, preventing complete stagnation (see Section 4).

11. Conclusions

In asthma, mucus often obstructs the airways to an important degree, and in those who die from the disease, it is usually a major contributor to respiratory failure. In COPD, on the other hand, the role of mucus obstruction is less clear. This apparent difference is puzzling, for in both diseases mucus hypersecretion is coupled with slowed mucus transport. The divergence may be more apparent than real if the main site of mucus obstruction, predominantly the small airways in COPD versus the medium-sized airways in asthma, makes its presence more difficult to demonstrate in the former. An alternative explanation is that the difference in airway obstruction by mucus between the two diseases is real, with the highly viscoelastic phys-

ical properties of asthmatic mucus and its tethering to the airway wall making its clearance more resistant to two-phase flow and cough. Thus, in severe asthma, stagnant mucus gathers until it plugs airways with the danger of ventilatory failure, while in COPD two-phase flow can generally keep the airways open.

References

1. Sobonya S (1984) Quantitative structural alterations in long-standing allergic asthma. *Am Rev Respir Dis* 130: 289–292.
2. Dunnill MS (1960) The pathology of asthma, with special reference to changes in the bronchial mucosa. *J Clin Pathol* 13: 27–33.
3. Dunnill MS (1975) The morphology of the airways in bronchial asthma. In: Stein M (ed.) *New directions in asthma.* Park Ridge. IL: American College of Chest Physicians, 213–221.
4. Saetta M, Di Stefano A, Rosina C, Thiene G, Fabbri LM (1992) Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *Am Rev Respir Dis* 143: 138–143.
5. Shimura S, Andoh Y, Haraguchi M, Shirato K (1996) Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma. *Eur Respir J* 9: 1395–1401.
6. Keal EE, Reid L (1975) Pathological alterations in mucus in asthma within and without the cell. In: Stein M (ed.) *New directions in asthma.* Park Ridge, IL: American College of Chest Physicians, 223–239.
7. Bhaskar KR, O'Sullivan DDF, Coles SJ, Kozakevich H, Vawter GP, Reid LM (1988) Characterization of airway mucus from a fatal case of status asthmaticus. *Pediat Pulmonol* 5: 176–182.
8. Sheehan JK, Richardson PS, Fung DCK, Howard M, Thornton DJ (1995) Analysis of respiratory mucus glycoproteins in asthma: A detailed study from a patient who died in status asthmaticus. *Am J Respir Cell Mol Biol* 13: 748–756.
9. Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, Boushey HA (1993) Markers of mucus secretion and DNA levels in induced sputum from asthmatic and healthy subjects. *Am Rev Respir Dis* 147: 1132–1137.
10. Feldhoff PA, Bhavanandan VP, Davidson EA (1979) Purification, properties and analysis of human asthmatic bronchial mucin. *Biochemistry* 18: 2430–2436.
11. Sanerkin NG, Evans DMD (1965) The sputum in bronchial asthma, pathognomic patterns, *J Path Bact* 89: 535–541.
12. James AL, Paré PD, Hogg JC (1989) The mechanics of airway narrowing in asthma. *Am Rev Respir Dis* 139: 242–246.
13. Freedman BJ (1971) The functional geometry of the bronchi. *Bull Physiopathol Resp* 8: 545–552.
14. Wagner PD, Hedenstierna G, Rodriguez-Roisin R (1996) Gas exchange, expiratory flow obstruction and the clinical spectrum of asthma. *Eur Respir J* 9: 1278–1282.
15. Dunnill MS, Massarella GR, Anderson J (1969) A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis and emphysema. *Thorax* 24: 176–179.
16. Thurlbeck WM (1976) Chronic airflow obstruction in lung disease. In: Bennington JL (ed.) *Major problems in pathology,* vol. 5. Philadelphia: W.B. Saunders, Chap. 3.
17. Shelhamer JH, Marom Z, Kaliner M (1980) Immunologic and neuropharmacologic stimulation of mucous glycoprotein release from human airways *in vitro. J Clin Invest* 66: 1400–1408.
18. Weller PF, Lee CW, Foster DW, Corey EJ, Austen KF; Lewis RA (1988) Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: Predominant production of leukotriene C4. *Proc Natl Acad Sci USA* 80: 7626–7630.

19. Fox CC, Kagey-Sobotna A, Schleimer RP, Peters SP, MacGlashan DW, Lichtenstein LM (1985) Mediators released from human basophils and mast cells from lung and intestinal mucosa. *Int Arch Allergy Appl Immunol* 77: 130–136.
20. Taylor G, Black P, Turner N (1989) Urinary leukotriene E_4 after antigen challenge and in acute asthma and allergic rhinitis. *Lancet* 1: 584–588.
21. Marom Z, Shelhamer JH, Bach MK, Morton DR, Kaliner M (1982) Slow reacting substances, leukotrienes C4 and D4 increase the release of mucus from human airway *in vitro, Am Rev Respir Dis* 126: 449–451.
22. Goswami SK, Ohashi MO, Stathas P, Marom ZM (1989) Platelet-activating factor stimulates secretion of respiratory glycoconjugate from human airways in culture. *J Allergy Clin Immunol* 84: 726–734.
23. Marom Z, Shelhamer JH, Kaliner M (1981) 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.
24. Rich B, Peatfield AC, Williams IP, Richardson PS (1984) Effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ on mucin secretion from human bronchi *in vitro*. *Thorax* 39: 420–423.
25. Marom Z, Shelhamer JH, Kaliner M (1985) Human monocyte-derived mucus secretagogue. *J Clin Invest* 75: 191–198.
26. Sperber K, Gollub E, Goswami S, Kalb TH, Mayer F, Marom Z (1991) *In vivo* detection of a novel macrophage-derived protein involved in the regulation of mucus-like glycoconjugate secretion. *Am Rev Respir Dis* 146: 1589–1597.
27. Davis CW, Dowell ML, Lethem M, Van Scott M (1992) Goblet cell degranulation in isolated canine tracheal epithelium: Response to exogenous ATP, ADP and adenosine. *Am J Physiol* 262: C1313–C1323.
28. Lethem MI, Dowell ML, Van Scott M, Yankaskas JR, Egan T, Boucher RC, Davis CW (1993) Nucleotide regulation of goblet cells in human airway epithelial explants: Normal exocytosis in cystic fibrosis. *Am J Respir Cell Mol Biol* 9: 315–322.
29. Merten MD, Kammouni W, Figarella C (1995) Evidence for, and characterisation of, a lipopolysaccharide-inducible adenosine A2 receptor in human submucosal tracheal gland serous cells. *FEBS Lett* 369: 202–206.
30. Cushley MJ, Tattersfield AE, Holgate ST (1983) Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. *Br J Clin Pharmacol* 15: 161–165.
31. Levine SJ, Larivée P, Logun C, Angus CW, Ognibene FP, Shelhamer JH (1995) TNFα induces mucin hypersecretion and MUC-2 gene expression by human airway epithelial cells. *Am J Respir Cell Mol Biol* 12: 196–204.
32. Fischer BM, Krunkorsky TM, Wright TD, Dolan-O'Keefe M, Adler KB (1995) TNFα stimulates mucin secretion and gene expression in airway epithelium *in vitro. Chest* 107: 133S–135S.
33. Shimura S, Ishihara H, Satoh M, Masuda T, Nagaki N, Sasaki H, Takishima T (1992) Endothelin regulation of mucus glycoprotein secretion from feline tracheal submucosal glands. *Am J Physiol* 262: L208–L213.
34. Pack RJ, Richardson PS (1984) The aminergic innervation of the lower respiratory tract: A light and electron microscopic study. *J Anat* 138: 493–502.
35. Florey H, Carleton HM, Wells AQ (1932) Mucus secretion in the trachea. *Br J Exp Path* 13: 269–284.
36. Baker B, Peatfield AC, Richardson PS (1985) Nervous control of mucin secretion into human bronchi. *J Physiol* 365: 297–305.
37. Peatfield AC, Richardson PS (1983) The action of dust in the airways on secretion into the trachea of the cat. *J Physiol* 342: 327–334.
38. Phipps RJ, Richardson PS (1976) The effects of irritation at various levels of the airway upon mucus secretion into the cat trachea. *J Physiol* 261: 563–581.
39. Davis B, Roberts AM, Coleridge HM, Coleridge JC (1982) Reflex tracheal gland secretion evoked by stimulation of bronchial C-fibers in dogs. *J Appl Physiol* 53: 985–991.
40. Davis B, Chinn R, Gold J, Popovac D, Widdicombe JG, Nadel JA (1982) Hypoxaemia reflexly increases secretion from tracheal submucosal glands in dogs. *J Appl Physiol* 49: 1416–1419.
41. Pack RJ, Richardson PS, Smith ICH, Webb SR (1988) The functional significance of the sympathetic innervation of mucous glands in the bronchi of man. *J Physiol* 403: 211–219.

42. Peatfield AC, Richardson PS (1983) Evidence for non-cholinergic, non-adrenergic nervous control of mucus secretion into the cat trachea. *J Physiol* 342: 335–345.
43. Fung DCK, Allenby MI, Richardson PS (1992) NANC nerve pathways controlling mucus glycoconjugate secretion into feline trachea. *J Appl Physiol* 73: 625–630.
44. Kuo HP, Rohde JA, Barnes PJ, Rogers DF (1992) Cigarette smoke-induced airway goblet cell secretion: Dose-dependent differential nerve activation. *Am J Physiol* 263: L161–L167.
45. Peatfield AC, Richardson PS (1982) The control of mucin secretion into the lumen of the cat trachea by α- and β-adrenoceptors, and their relative involvement during sympathetic nerve stimulation. *Eur J Pharmacol* 81: 617–626.
46. Barnes P (1986) Asthma as an axon reflex. *Lancet* 1: 242–244.
47. Rogers DF, Barnes PJ (1989) Opioid inhibition of neurally mediated mucus secretion in human bronchi. *Lancet* 1: 930–932.
48. Phipps RJ, Williams IP, Richardson PS, Pell J, Pack RJ, Wright N (1982) Sympathomimetic drugs stimulate the output of secretory glycoproteins from human bronchi *in vitro*. *Clin Sci* 63: 23–28.
49. Basbaum CB, Ueki I, Brezina L, Nadel JA (1981) Tracheal submucosal gland serous cells stimulated *in vitro* with adrenergic and cholinergic agonists: A morphometric study. *Cell Tiss Res* 220: 481–498.
50. Basbaum CB (1984) Regulation of secretion from serous and mucous cells in the trachea. *Ciba Found Symp* 109: 4–19.
51. Leikauf GAD, Ueki IF, Nadel JA (1984) Autonomic regulation of viscoelasticity of cat tracheal gland secretions. *J Appl Physiol* 56: 426–430.
52. Phipps RJ, Denas SM, Wanner A (1983) Antigen stimulates glycoprotein secretion and alters ion fluxes in sheep trachea. *J Appl Physiol* 55: 1593–1602.
53. Peatfield AC, Hall RL, Richardson PS, Jeffery PK (1982) The effect of serum on the secretion of radiolabeled mucous macromolecules into the lumen of the cat trachea. *Am Rev Respir Dis* 125: 210–215.
54. Williams IP, Rich B, Richardson PS (1983) Action of serum on the output of secretory glycoproteins from human bronchi *in vitro*. *Thorax* 38: 682–685.
55. Somerville M, Richardson PS, Rutman A, Wilson R, Cole PJ (1991) Stimulation of secretion into human and feline airways by *Pseudomonas aeruginosa proteases*. *J Appl Physiol* 70: 2259–2267.
56. Sommerhoff CP, Nadel JA, Basbaum CB, Caughey GH (1990) Neutrophil elastase and cathepsin G stimulated secretion from cultured bovine airway gland serous cells. *J Clin Invest* 85: 682–689.
57. Sleigh MA, Blake JR, Liron N (1988) The propulsion of mucus by cilia. *Am Rev Respir Dis* 137: 726–741.
58. Kim CS, Rodriguez CR, Eldridge MA, Sackner MA (1986) Criteria for mucus transport in the airways by two-phase gas-liquid flow mechanism. *J Appl Physiol* 60: 901–907.
59. Kim CS, Greene MA, Sankaran S, Sackner MA (1986) Mucus transport in the airways by two-phase gas-liquid flow mechanisms: continuous flow model. *J Appl Physiol* 60: 908–917.
60. Leith DE (1977) *Cough*. In: Brain JD (ed.) *Lung biology in health and disease*, vol. 5. New York: Marcel Dekker, 545–592.
61. Richardson PS, Peatfield AC (1981) Reflexes concerned in the defences of the lung. *Bull Europ Physiopath Respir* 17: 979–1012.
62. King M (1980) Rheological requirements for optimal clearance of secretions: Ciliary transport versus cough. *Eur J Respir Dis* 61: (Suppl 110), 39–45.
63. Mezey RJ, Cohn MA, Fernandez RJ, Januszkiewicz AJ, Wanner A (1978) Mucociliary transport in allergic patients with antigen-induced bronchospasm. *Am Rev Respir Dis* 188: 677–684.
64. Ahmed T, Greenblatt DW, Birch S, Marchette B, Wanner A (1981) Abnormal mucociliary transport in allergic patients with antigen-induced bronchospasm: Role of slow reacting substance of anaphylaxis. *Am Rev Respir Dis* 124: 110–114.
65. Pavia D, Bateman J, Clarke S (1980) Deposition and clearance of inhaled particles. *Bull Europ Physiopathol Resp* 16: 335–366.
66. Bateman JRM, Pavia D, Sheahan NF, Agnew JE, Clarke SW (1983) Impaired tracheobronchial clearance in patients with mild stable asthma. *Thorax* 38: 463–467.

67. O'Riordan TGZ, J. Smaldone GC (1992) Mucociliary clearance in adult asthma. *Am Rev Respir Dis* 146: 594–603.
68. Svartengren K, Philipson K, Svartengren M, Anderson M, Camner P (1996) Tracheobronchial deposition and clearance in small airways in asthmatic subjects. *Eur Respir J* 9: 1123–1129.
69. Agnew JE (1996) Bronchiolar deposition and clearance. *Eur Respir J* 9: 1118–1122.
70. Jeffery PK, Wardlaw A, Nelson FC, Collins JV, Kay AB (1989) Bronchial biopsies in asthma. An ultrastructural quantification study and correlation with hyper-responsiveness. *Am Rev Respir Dis* 140: 1745–1753.
71. Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T (1985) Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 131: 599–606.
72. King M, Gilboa A, Meyer FA, Silberberg A (1974) On the transport of mucus and its rheologic simulants in ciliated systems. *Am Rev Respir Dis* 110: 740–745.
73. Verdugo P (1990) Goblet cell secretion and mucogenesis. *Ann Rev Physil* 52: 157–176.
74. Garrard CS, Mussatto DJ, Loutenço RV (1989) Lung mucociliary transport in asymptomatic asthma: Effects of inhaled histamine. *J Lab Clin Med* 113: 190–195.
75. Russi W, Abraham WM, Chapman G, Stephenson J, Codias E, Wanner A (1985) Effects of leukotriene D_4 on mucociliary and respiratory function in allergic and non-allergic sheep. *J Appl Physiol* 59: 1416–1422.
76. Abraham WA, Stefenson JS, Garrido R (1989) A possible role for PAF in allergen-induced late response: Modification by a selective antagonist. *J Appl Physiol* 66: 2351–2357.
77. Snider GL, Faling LJ, Rennard SI (1994) Chronic bronchitis and emphysema. In: Murray JF, Nadel JA (eds). *Textbook of respiratory medicine.* Philadelphia: WB Saunders, pp 1331–1397.
78. Ciba GS 1959 Terminology, definitions and classification of chronic pulmonary emphysema and related conditions. *Thorax* 14: 286–299.
79. MRC (1965) Definition and classification of chronic bronchitis for clinical and epidemiological purposes. *Lancet* 2: 775–779.
80. Fletcher CM, Pride NB (1984) Definitions of emphysema, chronic bronchitis, asthma and airflow obstruction: 25 years on from the Ciba symposium. *Thorax* 39: 81–85.
81. Sluiter HJ, Koeter GH, de Monchy JGR, Postma DS, de Vries K, Orie NGM (1991) The Dutch Hypothesis (chronic non-specific lung disease) revisited. *Eur Respir J* 4: 479–489.
82. Nadel JA (1994) Obstructive diseases: General principles and diagnostic approach. In: Murray JF, Nadel JA (ed.) *Textbook of respiratory medicine.* Philadelphia: WB Saunders, 1245–1258.
83. Reid L (1954) Pathology of chronic bronchitis. *Lancet* 1: 275–278.
84. Burrows B, Bloom JW, Traver GA, Cline MG (1987) The course and prognosis of different forms of chronic airways obstruction in a sample from the general population. *New Eng J Med* 317: 1309–1314.
85. Widdicombe JG (1990) A critical look at mucus markers. In: Persson CGA, Brattsand R, Laitinen LA, Venge P (eds.) *Inflammatory indices in chronic bronchitis.* Basel: Birkhäuser Verlag, 269–279.
86. ATS (1987) Standards for the diagnosis and care of patients with chronic obstructive pulmonary diseases (COPD) and asthma. *Am Rev Respir Dis* 135: 225–244.
87. Thurlbeck WM, Angus GE (1964) A distribution curve for chronic bronchitis. *Thorax* 19: 436–442.
88. Hayes JA (1969) Distribution of bronchial gland measurements in a Jamaican population. *Thorax* 24: 619–622.
89. Hogg JC, Macklem PT, Thurlbeck WM (1968) Site and nature of airway obstruction in chronic obstructive lung disease. *New Eng J Med* 278: 1355–1360.
90. Kauffmann F, Drouet D, Lellouch J, Brille D (1979) Twelve years spirometric changes among Paris area workers. *Int J Epidem* 8: 201–212.
91. Higgins MW, Keller JB, Becker M, Howatt W, Landis JR, Rotman H (1982) An index of risk for obstructive airways disease. *Am Rev Respir Dis* 125: 144–151.
92. Peto R, Speizer FE, Cochrane AL, Moore F, Fletcher CM, Tinker CM (1983) The relevance in adults of air-flow obstruction, but not of mucus hypersecretion, to mortality from chronic lung disease. *Am Rev Respir Dis* 128: 491–500.

93. Ebi-Kryston KL (1988) Respiratory symptoms and pulmonary function as predictors of 10-year mortality from respiratory disease, cardiovascular disease and all causes in the Whitehall study. *J Clin Epidemiol* 41: 251–260.
94. Sherman CB, Xu X, Speizer FE, Ferris BG, Weiss ST, Dockery DW (1992) Longitudinal lung function decline in subjects with respiratory symptoms. *Am Rev Respir Dis* 140 (Suppl): S49–S55.
95. Vestbo J, Prescott E, Lange P (1996) Association of chronic mucus hypersecretion with FEV_1 decline and chronic obstructive pulmonary disease morbidity. *Am J Respir Crit Care Med* 153: 1430–1535.
96. Annesi I, Kaufmann F (1986) Is respiratory mucus hypersecretion really an innocent disorder? *Am Rev Respir Dis* 134: 688–693.
97. Speizer FE, Day ME, Dockery DW, Ferris BG (1989) Chronic obstructive disease mortality in six U.S. cities. *Am Rev Respir Dis* 140: S49–55.
98. Lange P, Nyboe J, Appleyard M, Jensen G, Schnohr P (1990) Relation of ventilatory impairment and of chronic mucus hypersecretion to mortality from obstructive lung disease and from all other causes. *Thorax* 45: 579–585.
99. Mitchell RS, Stanford RE, Johnson JM, Silvers GW, Dart G, George MS (1976) The morphologic features of the bronchi, bronchioles and alveoli in chronic airway obstructions: A clinicopathologic study. *Am Rev Respir Dis* 114: 137–145.
100. Matsuba K, Thurlbeck WM (1973) Disease of the small airways in chronic bronchitis. *Am Rev Respir Dis* 107: 552–558.
101. Anthonisen NR, Bass H, Heckscher T, Oriol A, Bates DV (1967) Recent observations on the measurement of regional V/Q ratios in chronic lung disease. *J Biol Nuclear Med* 11: 73–79.
102. Wanner A (1990) The role of mucus in chronic obstructive pulmonary disease. *Chest* 97: 11S–15S.
103. Gregg I, Trapenll DH (1969) The bronchographic appearances of early chronic bronchitis. *Brit J Radiol* 42: 132–139.
104. Fletcher C, Peto R (1977) The natural history of chronic airflow obstruction. *Brit Med J* 1: 1645–1648.
105. Lumsden AB, McLean A, Lamb D (1984) Goblet and Clara cells of human distal airways: Evidence for smoking induced changes in their numbers. *Thorax* 39: 844–849.
106. Ryder RC, Dunnill MS, Anderson JA (1971) A quantitative study of bronchial mucous gland volume, emphysema and smoking in a necropsy population. *J Pathol* 104: 59–71.
107. Tos M, Moller K (1983) Goblet-cell density in human bronchus in chronic bronchitis. *Arch Otolaryngol* 109: 673–676.
108. Lamb D, Reid L (1969) Goblet cell increase in rat bronchial epithelium after exposure to cigarette and cigar smoke. *Br Med J* 1: 33–35.
109. Rogers DF, Jeffery PK (1986) Inhibition by oral *N*-acetylcysteine of cigarette smoke-induced "bronchitis" in the rat. *Exp Lung Res* 10: 267–283.
110. Marine WM, Gurr D, Jacobsen M (1988) Clinically important respiratory effects of dust exposure and smoking in British coal miners. *Am Rev Respir Dis* 137: 106–112.
111. Becklake MR (1989) Occupational exposure: Evidence for a causal association with chronic obstructive pulmonary disease. *Am Rev Respir Dis* 140: S85–S91.
112. Buist AS, Vollmer WM (1994) Smoking and other risk factors. In: Murray JF, Nadel JA (eds.) *Textbook of respiratory medicine.* Philadelphia: WB Saunders, 1259–1287.
113. Chakrin LW, Saunders LZ (1974) Experimental chronic bronchitis: Pathology in dog. *Lab Invest* 30: 145–154.
114. Drazen JM, O'Cain CF, Ingram RH (1982) Experimental induction of chronic bronchitis in dogs: Effects on airway obstruction and responsiveness. *Am Rev Respir Dis* 126: 75–79.
115. Phipps RJ, Denas SM, Sielczak WM, Wanner A (1986) Effect of 0.5 ppm ozone on glycoprotein secretion, ion and water fluxes in sheep trachea. *J Appl Physiol* 60: 918–927.
116. Lamb D, Reid L (1986) Mitotic rates, goblet cell increase and histochemical changes in mucus in rat bronchial epithelium during exposure to sulphur dioxide. *J Pathol Bacteriol* 96: 97–111.
117. Scanlon PD, Seltzer J, Ingram RH Jr, Reid L, Drazen JM (1987) Chronic exposure to sulfur dioxide: Physiologic and histologic exposure of dogs exposed to 50 or 15 ppm. *Am Rev Respir Dis* 135: 831–839.

118. Evans MJ, Johnson LV, Stephens RJ, Freeman G (1976) Renewal of the terminal bronchial epithelium in the rat following exposure to NO_2 or O_3. *Lab Invest* 35: 246–257.
119. Lum H, Schwartz LW, Dungworth DL, Tyler WS (1978) A comparative study of cell renewal after exposure to ozone or oxygen: Response to terminal bronchiolar epithelium in the rat. *Am Rev Respir Dis* 118: 335–345.
120. Laurell C-B, Eriksson S (1963) The electrophoretic alpha$_1$-globulin pattern of serum in alpha$_1$-antitrypsin deficiency. *Sand J Clin Lab Invest* 15: 132–140.
121. Kueppers F, Black LF (1974) Alpha$_1$-antitrypsin and its deficiency. *Am Rev Respir Dis* 110: 176–194.
122. Black LF, Kueppers F (1978) Alpha$_1$-antitrypsin deficiency in nonsmokers. *Am Rev Respir Dis* 117: 421–428.
123. Lacoste JY, Bousquet J, Chanez P, Van Vyve T, Simony-Lafontaine J, Lequeu N (1993) Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 92: 537–548.
124. Martin TR, Raghu G, Maunder RJ, Springmeyer SC (1985) The effects of chronic bronchitis and chronic airflow obstruction on lung cell populations recovered by bronchoalveolar lavage. *Am Rev Respir Dis* 132: 254–260.
125. Thompson AG, Daughton D, Robbins RA, Ghafouri MA, Oehlerking M, Rennard SI (1989) Intraluminal airway inflammation in chronic bronchitis: Characterization and correlation with clinical parameters. *Am Rev Respir Dis* 140: 1527–1537.
126. McCusker KT, Hoidal J (1988) Leukocyte function and chronic bronchitis. *Seminars in Resp Infections* 3: 5–13.
127. Murphy TF, Sethi S (1992) Bacterial infection in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 146: 1067–1083.
128. Widdicombe JG (1995) Relationships among the composition of mucus, epithelial lining liquid and adhesion of microorganisms. *Am J Respir Crit Care Med* 151: 2088–2093.
129. Somerville M, Taylor GW, Watson D, Rendell NB, Rutman A, Todd H, Richardson PS, Cole PJ (1992) Release of mucus glycoconjugates by *Pseudomonas aeruginosa* rhamnolipids into feline trachea *in vivo* and human bronchus *in vitro*. *Am J Respir Cell Mol Biol* 6: 116–122.
130. Stockley RA, Burnett D (1979) Alpha$_1$-antitrypsin and leukocyte elastase in infected and noninfected sputum. *Am Rev Respir Dis* 120: 1081–1086.
131. Janoff A, Raju L, Dearing R (1983) Levels of elastase activity in bronchoalveolar lavage fluids of healthy smokers and nonsmokers. *Am Rev Respir Dis* 127: 540–544.
132. Snider GL, Lucy EC, Christensen TG, Stone PJ, Calore JD, Catanase A (1984) Emphysema and bronchial secretory metaplasia induced in hamsters by human neutrophil products. *Am Rev Respir Dis* 129: 155–160.
133. Breuer R, Christensen TG, Lucey EC, Stone PJ, Snider GL (1985) Quantitative study of secretory cell metaplasia induced by human neutrophil elastase in the large bronchi of hamsters. *J Lab Clin Med* 105: 635–640.
134. Kim KC, Wasano K, Niles RM, Schuster JE, Stone PJ, Brody JS (1987) Human neutrophil elastase releases cell surface mucins from primary cultures of hamster tracheal epithelial cells. *Proc Natl Acad Sci USA* 84: 9304–9308.
135. Schuster A, Ueki I, Nadel JA (1992) Neutrophil elastase stimulates tracheal submucosal gland secretion that is inhibited by ICI 200,355. *AM J Physiol* 262: L86–L91.
136. Goldstein W, Doring G (1986) Lysosomal enzymes from polymorphonuclear leukocytes and proteinase inhibitors in patients with cystic fibrosis. *Am Rev Respir Dis* 134: 49–56.
137. Wilson R, Cole PJ (1988) The effect of bacterial products on ciliary function. *Am Rev Respir Dis* 138: S49–S53.
138. Klinger JD, Tandler B, Liedtke CM, Boat TF (1985) Proteinases of *Pseudomonas aeruginosa* evoke mucin release by tracheal epithelium. *J Clin Invest* 74: 1669–1678.
139. De Water R, Willems LNA, Van Muijen GNP, Franken C, Fransen JA, Dijkman JH (1986) Ultra-structural localization of bronchial antileukoprotease in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies. *Am Rev Respir Dis* 133: 882–890.
140. Carp H, Janoff A (1978) Possible mechanisms of emphysema in smokers. *In vitro* suppression of serum elastase inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *Am Rev Respir Dis* 118: 617–621.

141. Carp H, Janoff A (1979) *In vitro* suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. *J Clin Invest* 63: 793–797.
142. Johnson D, Travis J (1977) Inactivation of human alpha$_1$- proteinase inhibitor by thiol proteinases. *Biochem J* 163: 639–641.
143. Morihara K, Tsuziki H (1979) Protease and elastase of *Pseudomonas aeruginosa:* Inactivation of human plasma alpha$_1$- proteinase inhibitor. *Infect Immun* 24: 188–193.
144. Rahman I, MacNee W (1996) Oxidant imbalance in smokers and chronic obstructive pulmonary disease. *Thorax* 51: 348–350.
145. Peatfield AC, Davies JR, Richardson PS (1986) The effect of tobacco smoke upon airway secretion in the cat. *Clin Sci* 71: 179–187.
146. Lei Y, Barnes PJ, Rogers DF (1995) Mechanisms and modulation of airway plasma exudation after direct inhalation of cigarette smoke. *Am J Respir Crit Care Med* 151: 1752–1762.
147. Jones JG, Lawler P, Crawley JCW, Minty BD, Hulands G, Veall N (1980) Increased alveolar epithelial permeability in cigarette smokers. *Lancet* 1: 66–68.
148. Pryor WA, Stone K (1993) Oxidants in cigarette smoke: Radicals, hydrogen peroxides, peroxynitrate and peroxynitrite. *Ann NY Acad Sci* 686: 12–28.
149. Peatfield AC, Richardson PS, Wells UM (1986) The effects of airflow on mucus secretion into the trachea of the cat. *J Physiol* 380: 429–439.
150. Cheeseman KH, Slater TF (1993) An introduction to free radical biochemistry. *Br Med Bull* 49: 481–493.
151. Morikawa T, Webber SE, Widdicombe JG (1991) The effect of hydrogen peroxide on smooth muscle tone, mucus secretion and epithelial albumin transport of the ferret trachea *in vitro*. *Pulmon Pharmacol* 2: 106–113.
152. Shore SA, Kariya ST, Anderson K, Skornik W, Feldman HA, Pennington J (1987) Sulfur dioxide-induced bronchitis in dogs: Effects on airway responsiveness to inhaled and intravenously administered metacholine. *Am Rev Respir Dis* 135: 840–847.
153. Cross CE, van der Vliet A, O'Neill CA, Louie S, Halliwell B (1994) Oxidants, antioxidants and respiratory tract lining fluids. *Envrion Health Perspect* 102: 185–191.
154. Salathe M, Wanner A, Conner GE (1995) Hydrogen peroxide scavenging properties of sheep airway mucus. *Am J Respir Crit Care Med* 151: 1543–1550.
155. Cross CE, O'Neill CA, Reznick AZ, Hu ML, Marcocci L, Packer L (1993) Cigarette smoke oxidation of human plasma constituents. *Ann NY Acad Sci* 686: 72–90.
156. Widdicombe JG, Wells UM (1994) Vagal reflexes. In: Raeburn D, Giembycz MA (ed.) *Airways smooth muscle: Structure, innervation, and neurotransmission,* vol. 1. Basel: Birkhäuser 279–307.
157. Coles SJ, Said SI, Reid LM (1981) Inhibition by vasoactive intestinal peptide of glycoconjugate and lysozyme secretion by human airways *in vitro*. *Am Rev Respir Dis* 124: 531–536.
158. Goodman RM, Yergin BM, Landa JF, Golinvaux MH, Sackner MA (1978) Relationship of smoking history and pulmonary function tests to tracheal mucous velocity in nonsmokers, young smokers, ex-smokers and patients with chronic bronchitis. *Am Rev Respir Dis* 117: 205–214.
159. Matthys H, Vastag E, Koehler K, Daikeler G, Fisher J (1983) Mucociliary clearance in patients with chronic bronchitis and bronchial carcinoma. *Respiration* 44: 329–337.
160. Santa Cruz R, Landa J, Hirsch J (1974) Tracheal mucous velocity in normal man and patients with obstructive lung diseases. *Am Rev Respir Dis* 109: 458–463.
161. McDowell EM, Barrett LA, Harris CC, Trump BF (1976) Abnormal cilia in human bronchial epithelium. *Arch Pathol Lab Med* 100: 429–436.
162. Auerbach O, Hammond EC, Kirman D, Garfinkel L, Stout AP (1967) Histologic changes in bronchial tubes to cigarette-smoking dogs. *Cancer* 20: 2055–2066.
163. Iravani J, van As A (1972) Mucus transport in the tracheobronchial tree of normal and bronchitic rats. *J Pathol* 106: 81–93.
164. Iravani J, Melville GN (1974) Long-term effect of cigarette smoke on mucociliary function in animals. *Respiration* 31: 358–366.
165. Jones NL (1981) The pathophysiological consequences of smoking on the respiratory system. *Can J Public Health* 72: 388–390.

166. Ohashi Y, Nakai Y, Sugiura Y, Ohno Y, Okamoto H (1993) Nitrogen dioxide-induced eosinophilia and mucosal injury in the trachea of the guinea-pigs. *J Oto-Rhino-Laryngol* 55: 36–40.
167. Heller RF, Gordon RE (1986) Chronic effects of nitrogen dioxide on cilia in hamster bronchioles. *Exp Lung Res* 10: 137–152.
168. Hiroshima K, Kohno T, Ohwada H, Hayashi Y (1989) Morphological study of the effects of ozone on rat lung. II. Long-term exposure. *Exp Molec Path* 50: 270–280.
169. Mellick PW, Dungworth DL, Schwartz LW, Tyler WS (1977) Short-term morphologic effects of high ambient levels of ozone on lungs of rhesus monkeys. *Lab Invest* 35: 62–90.
170. Kobayashi KM, Salathe M, Pratt MM, Cartagena NJ, Soloni F, Seybold ZV (1992) Mechanism of hydrogen peroxide-induced inhibition of sheep airway cilia. *Am J Respir Cell Mol Biol* 6: 667–673.
171. Jeppsson AB, Sundler F, Luts A, Waldeck B, Widmark E (1991) Hydrogen peroxide-induced tracheal epithelial damage increases terbutaline transport in guinea-pig tracheal wall: Implications for drug delivery. *Pulmon Pharmacol* 4: 73–79.
172. Burman WG, Martin W (1986) Oxidant-mediated ciliary dysfunction: Possible role in airway disease. *Chest* 89: 410–413.
173. Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D (1991) Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol* 4: 26–32.
174. Honda I, Shimura S, Sasaki T, Sasaki H, Takishima T, Nakamura M (1988) Airway mucosal permeability in chronic bronchitics and bronchial asthmatics with hypersecretion. *Am Rev Respir Dis* 137: 866–871.
175. Smallman LA, Hill SL, Stockley RA (1984) Reduction of ciliary beat frequency *in vitro* by sputum from patients with bronchiectasis; a serine proteinase effect. *Thorax* 39: 663–667.
176. Tegner H, Ohlsson K, Toremalm NG, Von Mecklenberg C (1979) Effect of human leukocyte enzymes on tracheal mucosa and its mucociliary activity. *Rhinology* 17: 199–206.
177. Lopez-Vidriero MT, Reid L (1978) Bronchial mucus in health and disease. *Br Med Bull* 34: 63–74.
178. Lopata M, Barton MD, Lourenco RV (1974) Biochemical characteristics of bronchial secretions in chronic obstructive pulmonary diseases. *Am Rev Respir Dis* 110: 730–739.
179. Boat T, Cheng PW (1980) Biochemistry of airway secretions. *Fed Proc* 39: 3067–3074.
180. Sadoul P, Puchelle E, Girard F (1978) Criteria for evaluating mucus functions and their disorders in chronic bronchitis. In: *Respiratory tract mucus.* Ciba Foundations Symposium 54 New Series. Amsterdam: Elsevier, 277–295.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 12
Mucus and Airway Epithelium Alterations in Cystic Fibrosis

Edith Puchelle*, Jean-Marie Zahm, Sophie de Bentzmann and Dominique Gaillard

Unité INSERM 314, Université de Reims, Reims, France

1. Introduction

Cystic fibrosis (CF) is a multiorgan lethal inherited disease, affecting about 2000–5000 children and adults of Caucasian origin. Most of the major manifestations of the disease are related to a primary defect in the protein product of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which is normally present in the apical membrane of the airway surface epithelium and submucosal glands [1]. The pulmonary complications, characterized by airway mucus accumulation, recurrent bacterial infections in the lower respiratory tract and bronchial obstruction are the major causes of the morbidity and mortality in CF disease [2].

The primary cellular dysfunction in CF airways is believed to be due to abnormal traffic of CFTR to the apical membrane of airway epithelium, associated to a lack or severe defect in CFTR channel activity. CFTR

* Author for correspondence.

functions both as a Cl^- channel and as a regulator of Cl^- and Na^+ conductance pathways. The defective Cl^- secretion and increased Na^+ absorption [3–6] are considered to result in the dehydration of mucus secretions and impairment of mucociliary transport. Other primary defects in mucous sulphation, sialylation, glycosylation and phospholipid content may also contribute to abnormal rheological and physical properties of mucus [7–8] and be involved in the predisposition of bacterial colonization. The specific role of CFTR in the physicochemical alterations of airway mucus in CF is difficult to assess because of the permanent inflammatory state of the mucosa. Serum-derived proteins, DNA released by the inflammatory and desquamated epithelial cells, and membrane-associated lipids may greatly influence mucus properties. All these alterations also complicate the understanding of the chronological steps involved in the cycle of infection/inflammation and airway obstruction, characteristic of CF.

The recent development of animal models such as transgenic CF mice or CF human bronchial xenografts have markedly improved the validity of mucus analyses, since they are carried out in experimental conditions which do not involve the secondary effects of airway infection. Actually, in most of the studies reported in the literature, CF mucus is collected by expectoration, from infected patients, which limits the interpretation of the data.

In CF patients, impaired mucociliary clearance may predispose to bacterial colonization and infection, but we still do not know clearly whether the physicochemical properties of airway mucus are abnormal before infection. Due to chronic bacterial colonization, the virulence factors produced by bacteria and the inflammatory mediators released in the lumen induce a marked remodeling of the airway epithelium demonstrated by mucous cell hyperplasia, squamous cell metaplasia and areas of epithelial shedding. Such changes of the airway epithelium may also secondarily alter the physicochemical properties of airway secretions and airway epithelial functions.

In this chapter we describe airway mucus abnormalities in CF, including the biochemical, physical, rheological and transport properties of mucus. We also review *in vivo* airway epithelium histological alterations in CF patients. The last part of the review attempts to analyze the respective role of primary and secondary CF epithelial and mucus alterations in airway bacterial colonization by *Pseudomonas aeruginosa.*

2. Biochemical Composition of Airway Mucus in CF

2.1. Mucin and Glycoconjugate Secretion

In CF, the mutation of the gene coding for CFTR membrane transport protein not only results in dysfunction of Cl^- and Na^+ permeability and regulation [3–6] but also induces a defective adenosine 3′-5′-cyclic mono-

phosphate (cAMP)-stimulated secretion of macromolecules. McPherson et al. [9] have reported a defective β-adrenergic response of glycoconjugates in CF submandibular acini cells. Rogers et al. [10] found that CF bronchial tissue secretory response to cholinoceptor and β_2 adrenoceptor stimulation, as well as the neuropeptide substance P, is markedly reduced. A constitutive hypersecretion and hyporesponsiveness to agonists that act *via* the cAMP pathway have been also reported in CF human tracheal gland cells [11].

Evidence for a role of CFTR in glycoconjugate secretion was provided by several recent studies [12–13]. Engelhardt et al. [12] have suggested that CFTR is involved in the coupled secretion of Cl^- and mucus in *Xenopus laevis* skin. Increased secretion of mucin and glycoconjugates has been reported after infection of CF epithelial cells with a vector recombinant for CFTR [14]. All these data suggest a defect in autonomic control of bronchial secretion in CF, not in the basal rate but in its response to receptor stimulation. The mechanism by which CFTR contributes either directly or indirectly to the regulation of mucin and glycoconjugate secretion is still unclear.

Biochemical abnormalities in CF mucous glycoproteins have been described in the gastrointestinal tract [15] and in sputum collected in CF patients in the early 1980s. The main alterations of the biochemical properties of CF airway secretions are summarized in Table 1. Biochemical and histo-

Table 1. Main alterations of the biochemical properties of CF airway secretions

Biochemical constituents	References
Mucins and other glycoconjugates	
Increased level of sulphation Decreased level of sialylation	[16–18, 21]
Proteins	
Increase in serum-derived proteins	[22]
Protease-antiprotease imbalance	[23]
Marked collagenase activity	[27]
Decrease in antibacterial lactoferrin activity	[22]
Lipids	
Increase in total phospholipids (PL)	[31, 33, 53]
Imbalance between surface active PL and rigidifying PL fractions	[31, 34, 52]
Ion and water content	
Chloride and sodium concentration	
– Increased	[40, 90]
– Decreased	[36, 37]
Water decrease	[8, 38–40, 42]
DNA	
High concentration of leucocyte and bacterial DNA	[45, 50]

chemical studies of CF bronchial mucins have indicated an increase in their sulphate content [16, 17]. Chace et al. [18] isolated a CF respiratory mucin with a marked increase in sulphate content, particularly in most acidic mucin species, which appeared to correlate with the severity of the disease. Thornton et al. [19] demonstrated that although CF mucins are heterogenous in both size and buoyant density, the various populations have the same macromolecular properties and architecture and are, in this respect, similar to mucins from normal respiratory secretions. It is generally admitted that increased sulphation is associated with a decreased level of sialylation in CF airway mucus. Some discrepancies appear in the literature which could be related to differences in CF genotype. The fundamental hypothesis of intracellular glycoprotein processing defects comes from the work of Barasch et al. [20], who observed a defect in Golgi pH in CF as compared with non-CF cells which would decrease the activity of enzymes such as sialytransferase.

Recently, the group of Engelhardt published interesting results on the genotypic analysis of respiratory mucus sulphation defects in CF [21]. They compared the sulphation patterns of radiolabeled mucin purified from CF and non-CF paired human bronchial xenografts. This model has the advantage of excluding the secondary effect of airway epithelium remodeling due to chronic bacterial infection and therefore allows a better physiologic evaluation of the primary defect in CF mucus biochemistry. Cumulative results of xenografts generated from independent CF tissue samples demonstrated a significantly higher level of sulphation as compared with non-CF tissue. These data substantiate the findings of other investigators who have reported in bronchial secretions and explants a two- to threefold higher level of sulphation. Nevertheless, a marked variability in mucus sulphation was found in genotypically different groups of CF patients, and more interesting, one ΔF 508/ΔF 508 patient showed no increase in the level of mucus sulphation, suggesting the provocative speculation that as yet unidentified mutant alleles in such patients could provide some level of functional intracellular CFTR capable of facilitating normal mucus glycoprotein processing.

2.2. Protein and Lipid Composition

In CF, no specific primary abnormality of protein or glycoprotein other than mucins has been reported. Actually, there is evidence that the chronic bronchial infection characteristic of CF is associated with a severe local inflammation resulting in a marked increase in serum-derived proteins such immunoglobulins G and M, serum albumin and α1 antitrypsin [22]. The IgM concentration in CF sputa was shown to significantly increase with the severity of the disease assessed by the Shwachman score. Among the other main proteins with potential antibacterial activity, lactoferrin, an

iron-binding glycoprotein of the transferrin family, was significantly and negatively correlated with the severity of the disease, suggesting that local secretory immunity against bacteria may be impaired in CF.

There is clear evidence that, due to chronic airway inflammation, there is a marked protease-antiprotease imbalance in the lungs of patients with CF [23]. Neutrophil elastase, a serine protease capable of damaging epithelial cells, is also able to induce the cleavage of molecules implicated in the respiratory host-defense system. Several reports by Birrer et al. [24], Konstan [25] and Kahn et al. [26] clearly show that CF neutrophil-dominated inflammation begins very early in a patients life. Birrer *et al.* [24] have shown that the chronic imbalance between the neutrophil elastase and the antineutrophil protective screen may be observed in CF by 1 year of age with resultant potential lung damage. Neutrophil elastase and collagenases can act in synergy, and their combined action can cause considerable airway destruction. Such an association has been demonstrated in patients with CF where high levels of active elastase and collagenases have been observed in sputum in close association with the severity of the disease [27]. Infected sputum from CF patients contains high concentrations of DNA released from degenerating polymorphonuclear leukocytes, bacteria and cell debris [28, 29]. The effects of DNA present in purulent sputum are numerous: it binds proteins and possibly also mucins and phospholipids and, therefore, may directly control the rheological and physical properties of sputum; due to its polyanionic properties, DNA attracts polycationic antibiotics such as aminoglycosides. Tobramycin and amikacin bind to DNA and mucin glycoproteins present in CF sputum, and it has been shown that, after treatment with rhDNase, the binding of aminoglycosides to the sputum decreased [30]. The effect of DNA on CF sputum rheology is discussed in Section 3.1.

Lipids, which constitute up to 3–5% of bronchial secretions, contribute to the physical and transport properties of airway secretion [31] and in the defense mechanism against bacteria [32]. Slomiany et al. [33] and Girod et al. [31] reported an increase in total phospholipids in CF secretions compared either with normal secretions or with secretions collected in chronic obstructive pulmonary diseases. It is particularly interesting that these two groups of workers have noted that the phospholipid content of CF moves towards a profile of poorly lubricant airway secretions characterized by an increased concentration of typically rigidifying fractions such as sphingomyelin, phosphatidylserine, and a decrease in phosphatidylglycerol and phosphatidylcholine. These changes may possibly result in an increased surface tension at airway and alveolar levels. These data are in agreement with the results of Gilljam et al. [34] who reported a decrease in phosphatidylcholine associated with an increased surface tension of total lipids in CF patients. The origin of the alteration in the phospholipid profile in CF airway secretions is still unknown. In CF, the chronic airway inflammation leads to the accumulation of cellular membranes rich in phosphatidyl-

ethanolamine and sphingomyelin and containing low levels of phosphatidylglycerol. Bacterial enzymes such as phospholipases from *P. aeruginosa* may also contribute to the lowering in the concentration of surface-active phospholipids such as phosphatidylcholine. It can be also hypothesized that, due to CFTR abnormality, the defective acidification of intracellular organelles may alter phospholipid synthesis pathways and trafficking.

2.3. Ion and Water Composition of CF Airway Secretions

Due to increased Na^+ absorption and the defect in cAMP-regulated Cl^- secretion, the water content of airway secretions in CF is markedly reduced, although large variations may be observed from one patient to another [35–39].

The ionic composition of the airway secretions exhibits large variations according to their origin and method of collection (Table 2). In most studies, airway secretions are collected during expectoration [36–39]. These data show that the infected airway secretions are hyperosmolar (300–466 mOsm) compared with plasma. Using ultramicroanalytic techniques applied to microsamples collected in human airways *in vivo*, Joris et al. [40] have shown that airway surface fluid is significantly hypotonic (220 mOsm) compared with plasma and extracellular fluid. Direct sampling and a cryofixation technique should be developed to minimize local airway irritation and hypersecretion. When comparing CF and non-CF secretions, it appears from most of the results that the Na^+ and Cl^- concentrations are lower in CF secretions (likely due to the higher Na^+ absorption and relative Cl^- impermeability). Nevertheless, the results from Joris

Table 2. Ion and water composition of normal and CF airway secretions

Origin and method of collection	Na^+ mmol/l	Cl^- mmol/l	K^+ mmol/l	% Water	Ref.
Normal secretions (laryngectomy)	165.0 ± 42	162.20 ± 60	132 ± 5.4	94.8 ± 1.9	36
CF sputum	101.0 ± 27	75.0 ± 12	28.0 ± 8.2	89.4 ± 2.2	
Normal secretions	211.1 ± 33.8	156.7 ± 24.6	16.6 ± 3.4	ND	37
CF sputum	131.1 ± 10.8	77.6 ± 14.4	35.4 ± 9.2	ND	
Normal secretions (tracheal pouch)	121.3 ± 11.9	102.5 ± 1.6	29.4 ± 2.6	ND	44
CF sputum	68.9 ± 3.9	55.6 ± 2.5	57.0 ± 3.5	ND	
CF sputum	94.8 ± 16.4	64.4 ± 11.8	14.5 ± 3.6	92.4 ± 1.6	39
Normal secretions (bronchoscopy)	82.0 ± 6.0	84.0 ± 9.0	29.0 ± 5.0	ND	40
CF	121.0 ± 3.5	129 ± 5.3	23.0 ± 9.4	ND	

ND = not determined.

et al. [40] suggest that in CF human airway surface liquid, the ClNa content may be increased airway surface compared to non CF. Such alterations in the ion content are associated with a decrease water content, although large variability has been reported in CF sputum samples [8].

A method for stimulating Cl^- secretion in CF is to use adenosine triphosphate (ATP) or uridine 5′-triphosphate (UTP), which act via a class of cell-surface nucleotide receptors. ATP stimulates outward rectifying chloride channels through a P_{2u} purinergic receptor. The amount of fluid transported by human surface respiratory epithelial cells under ATP or UTP stimulation is $\sim 2.4\ \mu l\ h^{-1}\ cm^{-2}$. This amount of fluid appears large enough to hydrate the surface of epithelial cells and to reconstitute the pool of water of the periciliary sol layer [41–43]. It can also facilitate the hydration and swelling of the mucins and therefore improves the rheological properties of airway secretions and mucociliary transport. The ionic composition and osmolality of the airway secretions are very potent stimuli for mucin release [44]; they can also directly affect the phagocytic and bactericidal properties of neutrophils. A range of Na^+ concentration from 68.9 mM [44] to 118.1 mM [37] reported in CF sputa has been demonstrated to be critical to the phagocytosis and killing of *P. aeruginosa* by white blood cells *in vitro* [45].

3. Rheological and Physical Properties of CF Airway Secretions and Related Mucociliary and Cough Transport Abnormalities

The airway secretion is a very complex biological material which possesses both flow and deformation properties (viscoelastic) characterized by non-linear (non-Newtonian viscosity) and time-dependent behaviour (thixotropy). Surface properties have been recently shown to affect the efficiency of mucociliary transport. Surface properties are analyzed by determining the wettability or measuring the adhesivity (or work of adhesion) of the airway secretions. These surface properties, which are not directly dependent on viscoelastic properties, determine the capacity of airway secretion to protect and lubricate the underlying airway mucosa. It is generally described in the literature [46] that the periciliary fluid forms a lubricating layer that enables the mucus to be transported on the tips of the cilia, whereas the viscoelastic properties of the gel mucus present at the apex of the cilia are critical for an optimal interaction between the ciliary stroke and the mucus.

3.1. Rheological Properties of CF Airway Secretions

The mucus gel is a highly non-Newtonian viscoelastic material. Under discontinuous stress, induced by the active ciliary beating or by coughing, the mucus deforms and then relaxes once the stress is removed. Using airway secretions from different patients or standardized preparations of

biopolymers as simulants of airway secretions, we could demonstrate that an intermediate apparent viscosity of 15 Pa.s, measured at a shear rate of 0.1 s^{-1}, represents an optimal viscosity for mucociliary transport. A low ($10 \leq$ Pa.s) or very high (> 20 Pa.s) viscosity is inefficient for mucus transport. Fifteen years ago, King [47] asked a very relevant question: Is cystic fibrosis mucus abnormal? In other words, do CFTR mutations per se induce an alteration of CF airway secretions? Abnormalities in the CF viscoelastic properties of sputum appear to be mainly dependent on the purulence degree of the sputum samples [48]. King [47] showed that for mucoid and mucopurulent CF sputum samples, the viscoelastic properties were remarkably similar to those observed for control canine tracheal mucus samples. For purulent CF sputum samples, elasticity and viscosity were higher and the viscosity/elasticity ratio lower than for either non-purulent sputum or canine tracheal mucus. In CF, due to the *CFTR* gene mutation, ion and water secretion dysfunctions may contribute to a dehydrated and hyperviscous mucus. The release of large amounts of DNA contributes to increased viscosity of the purulent respiratory mucus [49].

Nevertheless, some controversy exists in the literature about the specific role of DNA. A lack of significant correlation between DNA content and viscosity was reported by Picot et al. [28]. In a recent study carried out on purulent sputum samples from patients with CF [50], we observed that in the range of 2.4–19.5 mg ml^{-1} the DNA content was significantly and closely associated with the viscosity and elastic modulus. In addition, the higher the DNA content, the more marked the decrease in viscosity after rhDNase treatment, suggesting that at least in CF purulent secretions, DNA is a major factor of mucus hyperviscosity. Moreover, in addition to DNA, the actin filaments identified in CF mucus may also contribute to its viscosity [51].

In CF, the content and profile of lipids identified in airway secretions are also major determinants of mucus rheology. Lipids identified in airway secretions include a variety of neutral lipids, phospholipids and glycolipids. Several of these components, namely sphingomyelin, phosphatidylethanolamine, cholesterol and glycosphingolipids are considered to be factors which increase the viscosity of lipid-lipid/lipid-protein assemblies [52]. In CF, infection is associated with markedly abnormal rheological and transport properties of airway secretions, which may be responsible for the severity of the disease. Houdret et al. [53] reported that the very frequent infections observed in CF produce increased amounts of lipids that stick to the respiratory mucins.

Galabert et al. [52] analyzed the relationship between total lipid content, as well as the proportion of the different fractions of lipids, and the rheological properties of CF airway secretions, taking into account the degree of infection, as judged by the leucocyte count in the expectorated secretions. It was demonstrated that the total lipid content was higher in the superinfected CF secretions and, in particular, that the content in glycosphingolipid fractions and in cholesterol was markedly increased when the

CF sputum samples were highly purulent. Glycolipids and sphingomyelin increased, as well as the viscosity. Hyperviscosity and low spinnability, both unsuitable for an efficient mucus transport by ciliary beating, were in CF sputa associated with a high content of neutral lipids and glycosphingolipids. Galabert et al. [52] and Girod et al. [31] have shown that the contribution of phospholipids to the rheological profile of CF sputum may be different, and even quite the opposite, according to the degree of saturation of their fatty acid chains or the nature of the polar groups. For example, phosphatidylethanolamine, sphingomyelin and lysophosphatidylcholine are phospholipids which contribute to the increase in viscosity of CF sputa, whereas phosphatidylglycerol is negatively correlated with the viscosity.

The primary CF origin of the abnormalities in the viscosity may of course be directly related to the water paucity of the secretions. Mislocation or absence of CFTR expression at the apical plasma membrane of surface airway epithelium is responsible for a defective chloride secretion and increased sodium absorption resulting in lowering of the depth of the periciliary layer, change in the osmolality of the secretions and inadequate mucin hydration [54].

The inability of the mucins packed inside the secretory granules to swell and pass from the condensed phase (inside the secretory granules) to the expanded gel phase (normally present at the apex of the cilia) is likely partly responsible for decreased CF mucus transportability and accumulation of mucins in the lumen of the glandular acini and ducts.

3.2. Surface Properties of CF Airway Secretions

CF airway secretions have been widely described in terms of rheological properties, but less is known about their surface properties. According to Pillai [44], the predominant forces shearing the mucus occur in a lateral rather than a normal direction. This is due to the small mucus depth atop the cilia compared with its lateral dimension and to the small shearing forces from the cilia which are applied to one side of the mucus layer and which are not significantly opposed by the mucus–air interface. This clearly means that interfacial interaction between the mucus and cilia is critical to the effectiveness of mucociliary transport.

The interfacial interaction can be characterized by the work of adhesion, which is defined as the work per unit area required to separate two phases initially in contact.

The work of adhesion, W_A, is related to the interfacial tension by

$$W_A = \gamma (1 + \cos \theta)$$

where γ is the interfacial tension of mucus in contact with air (surface tension) and θ is the equilibrium contact angle formed by a mucus droplet on a solid surface (i.e. the respiratory mucosa). The work of adhesion is

therefore dependent on the properties of both the mucus and the substrate and results from the local surface free energy of the two phases initially in contact. In CF patients, the surface tension of respiratory mucus exhibits large variations (50–165 mN m^{-1}) which are closely related to the water content of sputum samples [32]. Purulent sputum samples, with a low water content, are generally characterized by a high surface tension and, in parallel, a low mucociliary transport rate.

In addition to being a function of hydration, the work of adhesion can be predicted to be related to the hydrophobic nature of the mucus surface. Phospholipid fractions at the interface of the respiratory mucosa, and the mucus gel may be important in governing the work of adhesion of mucus. Girod et al. [55, 56] have demonstrated that a surface-active phospholipid fraction such as phosphatidylglycerol is a major factor in mucus surface properties. In CF airway secretions, the deficient phosphatidylglycerol fraction contributes together with dehydration to increased adhesion of mucus to the respiratory mucosa [31].

Apart from mucus gel phase surface properties, mucus sol phase properties are also largely involved in mucus-mucosa interactions. King et al. [57–58] and Zahm et al. [59] have shown in an experimental model that the addition of surface-active liquids as sol phase simulants significantly enhanced the clearance of mucus gel simulants by cough. In addition, Girod et al. [32, 55, 56] have demonstrated that the work of adhesion of CF mucus was significantly decreased by the addition of a sol phase containing distearoyl phosphatidylglycerol. This decrease in work of adhesion was accompanied by a significant improvement in mucus transport by cough and by ciliary activity. Deneuville et al. [60] reported a strong association in CF patients between increased viscosity and decreased water content of CF sputum and the lowering of mucociliary transport. In addition, they demonstrated that the impairment in CF mucus transport by coughing was associated with abnormal surface properties. Recently, Zahm et al. [49] have shown that the addition of recombinant human DNase to CF respiratory mucus induced a significant improvement in mucus surface properties which could be related to the recovery of surface active lipids which might be dissociated from DNA.

In conclusion, the surface properties of the mucus are abnormal in CF patients and appear to be important physical determinants to modify in order to improve CF mucus transport by ciliary activity and cough.

4. Airway Epithelium Inflammation, Damage and Remodeling in CF

4.1. Early Histological Alterations of the Airway Mucosa before Infection

We recently addressed the question whether the impairment in mucociliary clearance frequently reported in CF patients could be associated with air-

way inflammation, and whether it could precede the bacterial infection or was a *primum movens* in mucociliary clearance impairment [61]. In order to answer this question, we studied transgenically generated mice, developed by Dorin et al. following targeted insertional mutagenesis into exon 10 of the murine *CFTR* gene in embryonal stem cells [62]. We quantified in a blind trial the mucociliary transport velocity, the histological state and the degree of inflammation of tracheal mucosa in $cftr^{m1HGU}/cftr^{m1HGU}$ transgenic mice and in control littermates housed in pathogen-free conditions. The nasal and tracheal transepithelial potential difference (PD), measured in basal conditions, was significantly more negative in the $cftr^{m1HGU}/cftr^{m1HGU}$ mutant mice as compared with control mice. In the $cftr^{m1HGU}/cftr^{m1HGU}$ mice, a decrease in mucociliary transport velocity and an increased number of inflammatory cells in the lamina propria were observed, compared with control mice. These findings support the concept that the inflammation is associated, although not correlated, with decreased mucociliary transport and suggest that in the $cftr^{m1HGU}/cftr^{m1HGU}$ mice, prior to any infection, the mucosal inflammation can be associated with increased mucus production which in turn will induce the impairment of the mucociliary transport rate. This does not mean that the higher the number of inflammatory cells in the lamina propria, the lower the mucociliary transport velocity. In fact, we could not demonstrate any significant correlation between these two parameters, suggesting that the decrease in mucus transport is likely not directly related to the release of inflammatory mediators. The origin of this local inflammation is totally unknown. According to Standiford et al. [63] and Nakamura et al. [64], airway epithelial cells may represent a major source of cytokines, such as interleukin 8 (IL-8) which have potent neutrophil chemotactic properties.

A study made by Ruef et al. [65] has reported, in a tracheal cell line (homozygous for the deletion of the Phe 508), the production of IL-8 at a concentration able, even in unstimulated conditions, to induce a neutrophil chemotactic activity. The downregulation of IL-10, an anti-inflammatory cytokine, in CFTR defective airway epithelial cells could also be implicated [66]. Based on the work of Ruef et al. [65], Standiford et al. [63] and Bonfield et al. [67], it may be proposed that the regulation of airway inflammation is the result of the interactions of multiple pro- and anti-inflammatory signals. The origin of the signal initiating the recruitment of inflammatory cells in the lamina propria is totally unknown. The absence of an increased influx of inflammatory cells in the airway surface epithelium of the $cftr^{m1HGU}/cftr^{m1HGU}$ mice may be due to the fact that, in the absence of bacterial infection, the migration of neutrophils towards the airway lumen is normally interrupted by counter-regulatory mechanisms. The cycle of inflammation of the airway surface epithelium could therefore require an additional signal, like bacterial adherence and colonization, to complete the vicious cycle of inflammation. The primary decrease of mucociliary clearance in this CF mouse model may explain the impaired

capacity of the *cftr*^{m1HGU} mutant mouse to clear *Staphylococcus aureus* and *Burkholderia (Pseudomonas cepacia)* and the appearance after repeated bacterial exposure of a marked mucous hyperplasia with extensive mucus retention associated with a mixed population of polymorphonuclear and lymphocytic cells [68].

In CF patients, impaired mucociliary clearance and neutrophil-dominated inflammation is thought to result for a large part from bacterial colonization by *P. aeruginosa.* Our observation that in CF mice airway inflammation can occur without any *P. aeruginosa* infection reinforces the idea that airway mucosal inflammation may be the primary cause of the decreased mucociliary transport. The inflammation of the airway surface epithelium secondary to bacterial infection most likely represents a further grave evolutionary step in CF disease.

Recent studies suggest that inflammation begins very early in the course of lung disease. Bronchoalveolar lavages carried out in infants without clinically apparent lung disease showed endobronchial bacterial infection with associated inflammation predominated by neutrophils. Interestingly, some infants had inflammation in the apparent absence of infection, leading to the speculation that inflammation may precede infection [69]. Moreover, in the bronchoalveolar lavage of CF infants as young as 4 weeks, Kahn et al. [26] confirmed, that the number of neutrophils and level of IL-8 were already increased, although they had negative cultures for common CF-related pathogens and viruses. No direct linkage has ever been established between the CF basic defect and systemic immunologic or neutrophil dysfunction. However, recent studies focusing on epithelial cells have suggested ways in which defects in CFTR function may influence the infectious and inflammatory processes in the lung. Studies of cytokines in the CF airways show that in addition to dramatically increased concentrations of proinflammatory cytokines like IL-1, tumour necrosis factor α (TNFα) and IL-8, CF airways are relatively deficient in the anti-inflammatory cytokine IL-10 [66, 67]. In contrast, CF epithelial cells produce little or no IL-10 but express large amounts of intercellular adhesion molecule I (ICAM-I) and IL-8.

4.2. Histological Remodeling of the Airway Epithelium in CF

Airway obstruction and pulmonary lesions are the major factors contributing to morbidity and mortality in CF. The usual course of CF is a progressive pulmonary disease with severe bronchitis and bronchiectasis with overdistension emphysema. According to Chow [70] the absence of mucous gland hyperplasia at birth suggests that mucous obstruction of airways may not be primarily responsible for the increased susceptibility to respiratory tract infection. Nevertheless, in early life, mucopurulent plugging of airways and inflamed bronchi have been reported [71]. A number

of histological and electron-microscopic studies have been carried out in infected CF airways obtained post-mortem from patients dying of CF [72–74]. In patients undergoing heart/lung transplantation, it has been clearly shown that most of the airways are filled with purulent secretions markedly adherent to the airway surface. The main pathologic changes summarized in Figure 1 are observed early in life. Inflammatory infiltrates and mucopurulent plugging of airways are identified in most of the patients who died in the first 4 months of life [74]. Mucous cell hyperplasia, basal cell hyperplasia and focal areas of squamous metaplasia and cell sloughing represent characteristic features of the remodeled airway surface epithelium. Acquired and non-specific ultrastructural lesions such as compound cilia, and disorganization of the cilia and of the tight junctional complexes, are commonly identified [75]. The inflammatory infiltrate of the airway surface epithelium is the characteristic feature of CF (Figure 2). Neutrophils infiltrate the airway epithelium and subsequently enter the airway lumen, where they release an arsenal of oxidants, proteases and DNA which in turn are responsible for airway remodeling and epithelial shedding when the excess of elastase overwhelms the antiprotease screen.

At the submucosal level, gland hyperplasia is very often observed, although the increase in the Reid index of submucosal gland hyperplasia is

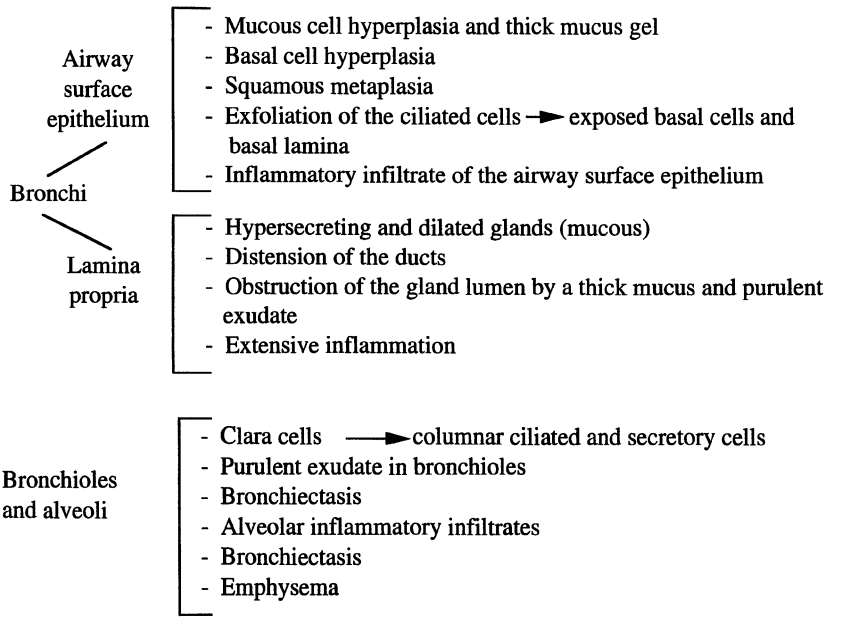


Figure 1. Main histological alterations in CF-infected airway.

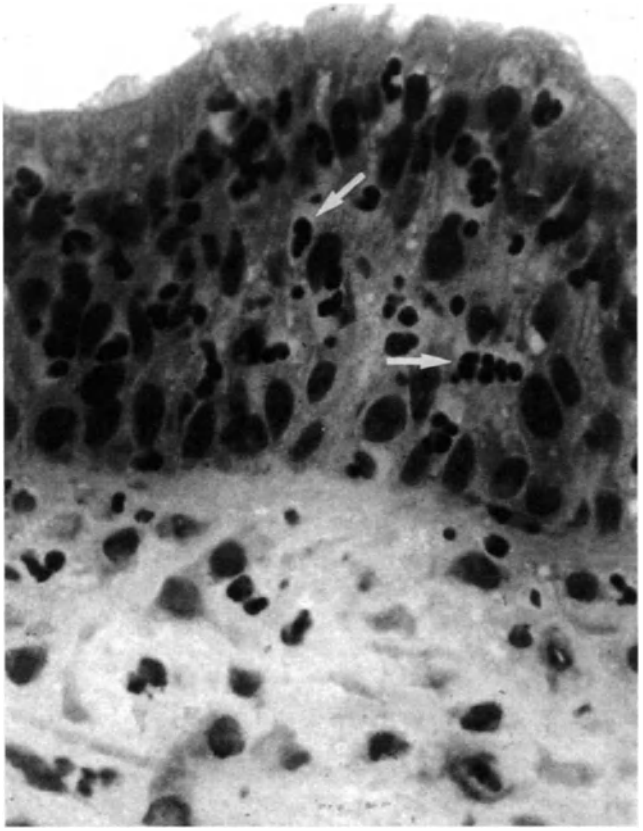


Figure 2. Inflammatory infiltrate of the airway surface epithelium in CF. Neutrophils (arrow) infiltrate the surface epithelium (×4000).

not systematically reported [70]. Moreover, the location of these glands, generally composed of mucous rather than serous cells, is more frequently identified close to the airway surface epithelium (personal data). The collecting ducts are generally distended and filled with abundant and apparently thick mucus containing inflammatory cells and exfoliated ciliated cells (Figure 3). Bronchiectasis is invariably observed in CF patients after 6 months [76, 77]; the severity of bronchial dilatations generally increases with the evolution of the disease and predominates in the upper lung lobes. Small airways also exhibit a severe inflammation with areas of stenosis

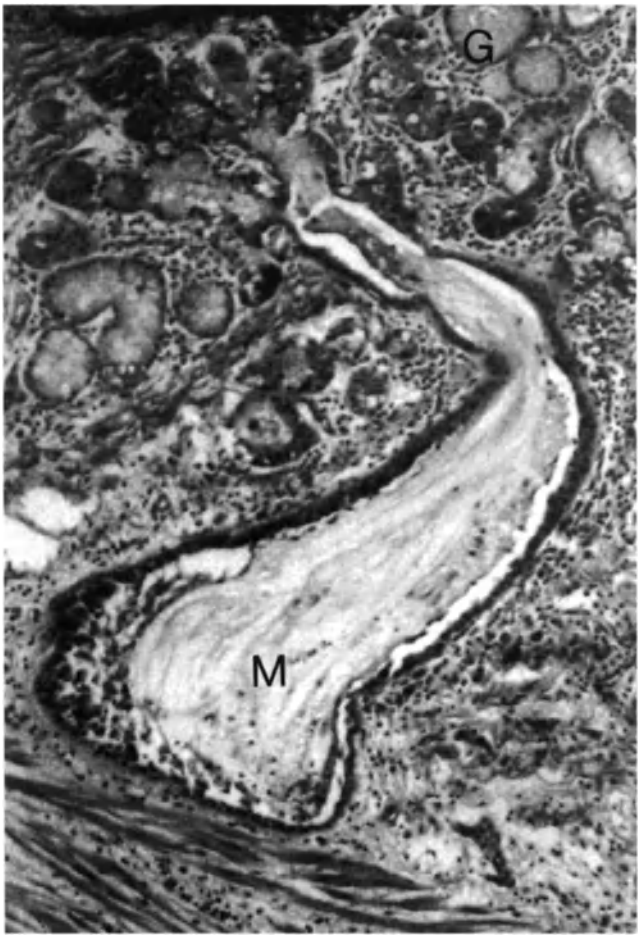


Figure 3. Inflamed CF airway mucosa. The collecting duct is distended and completely filled with mucus (M) containing desquamated epithelial and inflammatory cells. The lining epithelium of the duct is remodeled with sites of squamous metaplasia, basal hyperplasia or even complete exfoliation. Gland (G) hyperplasia is observed in the lamina propria (×100).

associated with pneumonia and alveolar emphysema. One interesting feature is the marked plasticity of the airway epithelium observed at the bronchiolar level. The Clara cells, which in normal airways represent the main bronchiolar cell phenotypes, are less frequently identified and are generally replaced by columnar ciliated and secretory cells (personal data). It is well known that cellular differentiation regulates expression of Cl^- transport and CFTR mRNA in epithelial cells [78].

The remodeling of the airway epithelium associated with severe inflammation may induce a critical role in post-transcriptional and/or post-translational regulation of CFTR protein expression in CF as well as in non-CF airways. The environmental factors and particularly the histological and inflammatory state of the surface epithelium are able to influence the variability of CFTR protein expression in non-CF and ΔF 508 homozygous nasal polyps [79]. The nasal polyp is a paradigm to mimic airway inflammatory diseases, in as much as it consists essentially of an edematous stroma with numerous inflammatory cells, including neutrophils and most commonly eosinophils, lymphocytes and macrophages covered by a respiratory-type epithelium with large areas of basal and mucous cell hyperplasia or even squamous metaplasia [30]. Although the degree of remodeling and inflammation was more severe in CF nasal polyps, the only marked difference between non-CF and CF inflamed nasal polyp tissue was the large number of neutrophils infiltrating the CF surface epithelium. The nasal polyp is therefore an ideal model for analyzing whether inflammation and remodeling of the surface epithelium can induce changes in CFTR expression at the mRNA and protein levels.

A huge variability in CFTR mRNA transcript level and CFTR protein expression and distribution was observed among the different tissue samples, but no relationship was found between the level of CFTR mRNA transcripts and CFTR protein expression, either in non-CF or CF samples. The histological analysis of non-CF and CF nasal polyp tissue indicated that the variations in expression and distribution of CFTR protein were associated with variations in the degree of surface epithelium remodeling and inflammation in the lamina propria. A surface epithelium, showing a slight basal cell hyperplasia phenotype associated with diffuse inflammation, was mainly characterized by CFTR protein distribution at the apex of ciliated cells in both non-CF and CF specimens. In contrast, in a remodeled surface epithelium associated with severe inflammation, CFTR protein presented either a diffuse distribution in the cytoplasm of ciliated cells or was absent. These results suggest that abnormal expression and distribution of CFTR protein in CF airways is not only caused by CFTR mutations and that airway surface epithelium remodeling and inflammation can modulate CFTR protein expression in non-CF and CF airways.

4.3. Relationship between P. aeruginosa Colonization and CF Airway Epithelium Remodeling

The most important question with regard to CF airway epithelium remains the bacterial colonization by *P. aeruginosa*. These bacteria are very frequently identified in the sputum of CF patients, although they are rarely encountered in other chronic pulmonary diseases like chronic bronchitis or ciliary dyskinesia syndrome, where most of the histological alterations

described in CF have been identified. According to the data reported in the literature [80], most of the *P. aeruginosa* are identified in the lumen associated with mucus plugging. An extensive microscopic immunolocalization study of *P. aeruginosa* distribution in lungs from patients with CF has been carried out by Baltimore [74]. These authors have clearly reported that *P. aeruginosa* was generally endobronchiolar and associated with bronchiolar obliterative changes, particularly in small (< 1 mm) airways. The presence of *P. aeruginosa* was mainly identified where damage was most severe. In the conducting airways, they were visualized as masses of bacteria organized in a colony-like fashion. Bacteria could also be observed more diffusely dispersed and adherent to airway walls without epithelium. The authors pointed out that, in airways or alveoli not associated with an inflammatory process, *P. aeruginosa* were never encountered. This clearly demonstrates that in CF, *P. aeruginosa* is associated with a marked remodeling of the epithelium. No invasion of *P. aeruginosa* through intact epithelium or within the inflammatory cells beneath denuded epithelium was seen. In our own experience (personal communication), a high number of *P. aeruginosa* could be visualized invading the exfoliated cells present in the intraluminal plugs, suggesting that these exfoliated airways cells may represent important reservoirs for *P. aeruginosa.*

The role of *P. aeruginosa* infection on airway mucosal integrity has been well documented in CF patients infected by *P. aeruginosa*. It is well known that during chronic infection, either in CF or other diseases, *P. aeruginosa* produces numerous substances that can damage airway epithelium, including exotoxin A, proteases, elastases, metalloproteinases, leukocidin, phospholipase C, exoenzyme S and several hemolysins [81–86]. *In vitro* studies by Stutts et al. [87] have demonstrated that *P. aeruginosa* infection increases the airway mucosal permeability in canine as well as in human bronchial epithelium, suggesting that all the toxic substances released from the bacteria may alter the tight junction barrier which normally protects the airway epithelial cells. By measuring the radiolabeled albumin in sputum from patients suffering from chronic bronchitis with or without chronic *P. aeruginosa* infection, Ishihara et al. [88] have shown that the ratio of sputum to serum radioactive albumin was significantly higher in patients infected with *P. aeruginosa*. Furthermore that the ratio at 2 and 4 h after ^{131}I albumin significantly correlated with sputum volume per day, whereas it did not correlate with any other factor (age and duration of the disease), suggesting that chronic *P. aeruginosa* infection induces an increase in airway mucosal permeability. All these data suggest that, apart from the specific genetic abnormality in CFTR protein, the infection associated with severe inflammation may induce severe alterations in CF as well as in non-CF airways.

4.4. What Could Be the Chronological Steps and Relationships between the Primary CFTR Defect and Airway Bacterial Colonization?

Although the specific chronological steps of bacterial colonization are still unknown, recent results focusing on primary airway inflammation in CF indicate the directions of research that we should follow in the future to target the primary dysfunctions possibly at the origin of bacterial adherence and colonization of CF airway cells.

A tentative description of the chronological steps of mucus and airway mucosa bacterial infection in CF is given in Figure 4. In the normal respiratory tract, the airway epithelial surface is protected from pathogenic bacterial colonization by mucociliary clearance. The mucins present in gel mucus layer exhibit a high diversity of carbohydrate receptors which specifically recognize bacteria further eliminated by the ciliary activity. A decrease in mucus transport associated with inflammation first located to the airway lamina propria may represent the first step of airway cellular dysfunction, possibly related to a local cytokine dysregulation (step I). The resulting increase in the gel mucus layer favours mucus stagnation and associated decreased mucociliary transport and the onset of polymorphonuclear neutrophil and lymphocyte migration through the airway surface epithelium (step II). Following this, the bacteria trapped in the hyperviscous (due to DNA release) and thick gel mucus may release virulence factors which will damage the epithelial integrity and induce partial or complete epithelial shedding, exposing neoreceptors of the remaining epithelial cells and unmasked extracellular matrix components of the basal lamina to bacterial adhesion (step III). Following airway injury, the surface epithelium repairs. This also represents a critical step for bacterial colonization (step IV), because the regenerating undifferentiated cells exhibit apical receptors such as asialylated gangliosides which exhibit high affinity for *P. aeruginosa*. This speculative schema underlines that, among the strategies to prevent bacterial colonization, the first one could be to prevent local inflammation in order to interrupt the circle of inflammation/-infection and airway obstruction.

Up to now, no clear explanation could relate the CFTR primary defect to *P. aeruginosa* airway chronic colonization in CF disease. Changes in ionic environment due to CFTR defect have been described to induce either upregulation of *P. aeruginosa* virulence factors [89] or a decrease of bacterial phagocytosis by alveolar macrophages [45]. More recently, Smith et al. [90] reported how CF epithelium failed to kill bacteria due to abnormal ionic content of extracellular fluid, in particular a high NaCl content, possibily related to a decrease in the bactericidal activity of a defensin-like molecule. Another recent study [91] related *P. aeruginosa* airway colonization in CF to a genetic defective internalization of *P. aeruginosa* by CFTR mutant negative airway cells.

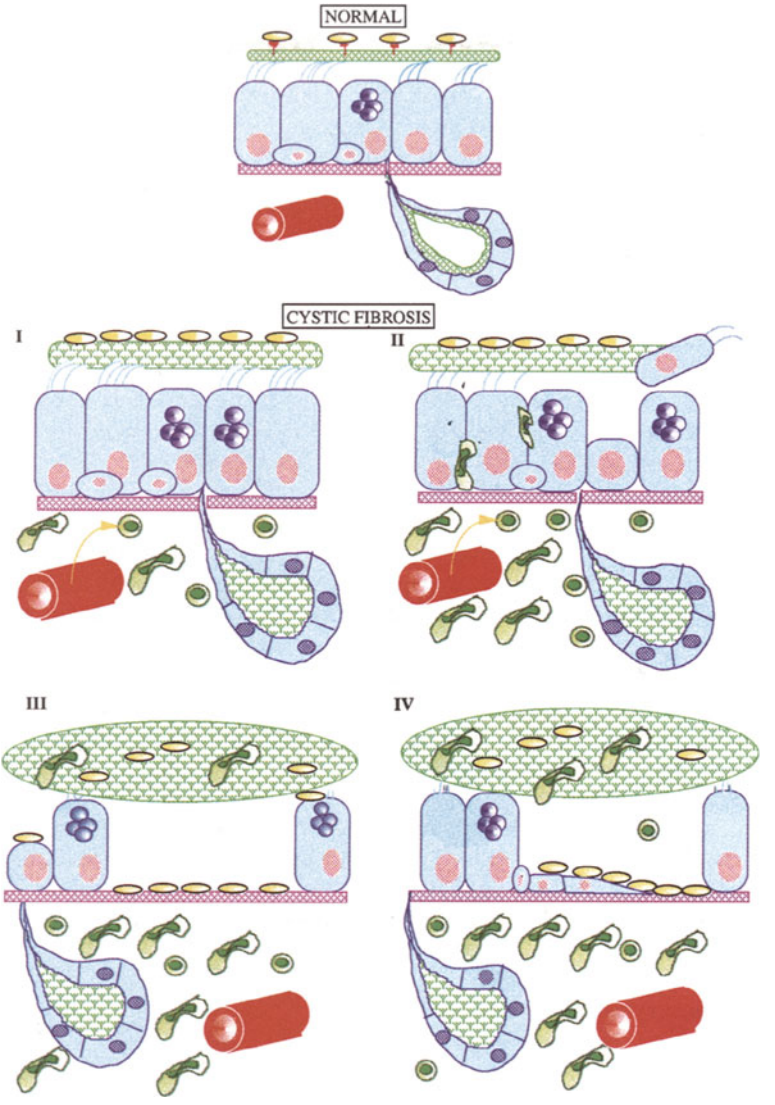


Figure 4. Tentative schematic for development of airway bacterial infection. In normal conditions, bacteria recognize carbohydrate receptors present in the mucus and are then eliminated from the airways by the mucociliary clearance mechanism. In CF, an early and moderate inflammation may be identified in the lamina propria (step I). In a following step (step II) neutrophil and lymphocyte migration through the surface airway epithelium induced an epithelial shedding. Due to mucus hypersecretion and stagnation, bacteria multiply and adhere to unmasked receptors of denuded cells and basement membrane (step III). Following cell detachment, the surface epithelium repairs by spreading and migrating cell processes. These repairing, poorly differentiated cells exhibit receptors which have a high affinity for *P. aeruginosa* (step IV).

Although altered carbohydrate composition of CF salivary mucins has been reported to increase *P. aeruginosa* adhesion in CF, there is still controversy about which specific bronchial mucin composition is responsible for increased adhesion of *P. aeruginosa* in CF airways [92–95]. All these studies report the high number of *P. aeruginosa* within CF airway lumen, which in association with decrease in mucociliary clearance and chronic airway inflammation is likely responsible for the epithelial damage. In such epithelial damage, *P. aeruginosa* has been described to find sites of adherence in the denuded basement membrane. Laminin, one of the extracellular matrix components, has recently been described to bind to *P. aeruginosa* [96], as has collagen I, which on further degradation of the basement membrane could be exposed within the connective tissue [97]. Interactions of *P. aeruginosa* with airway epithelial cells have also been studied in relation to the CFTR defect [98]. Alterations in glycosylation of epithelial components in CF and in particular of glycolipids were described as responsible for asialo GM_1 overexpression in CF epithelial cells and for increased *P. aeruginosa* adherence to CF epithelial cells [99–100]. However, de Bentzmann et al. underlined the role of asialo GM_1 as a receptor for *P. aeruginosa* adherence in undifferentiated non-CF airway epithelial cells [101]. Interestingly, *P. aeruginosa* adherence to epithelial cells has always been observed in dramatic phenotypic alterations following injury, particularly during repair. Airway epithelial cells engaged in the restoration of epithelial integrity exhibited a great adherence for *P. aeruginosa* independent of the CFTR defect. Asialylated glycolipids could serve as *P. aeruginosa* receptors in both CF and non-CF airway epithelial cells specifically expressed during epithelial repair. These results suggest that epithelial receptors for *P. aeruginosa* adherence are available in CF, secondary to intense epithelial remodeling rather than simply and directly due to a primitive CFTR genetic defect. Nevertheless, the CFTR defect may create and maintain environmental conditions favouring *P. aeruginosa* adherence to CF epithelium.

5. Summary and Concluding Remarks

Although much has been published on the abnormalities of airway mucus in CF, the fundamental question of whether defective CFTR functions are responsible for the specific alterations of the biochemical and physical properties of CF airway mucus is incompletely resolved. A defect in the sulphation and sialylation pattern of mucins appears as a possible primary defect in CF mucus, although a marked variability suggests that unknown mutant alleles may counterbalance the abnormal intracellular function of the mutated CFTR gene product. In fact, many lines of evidence suggest that most of the abnormalities of proteins, lipids and glycoproteins other than mucins identified in CF mucus result from severe airway inflamma-

tion associated with the chronic bacterial infection characteristic of CF. Recent reports show that the inflammation is found very early and could precede the infection. Airway inflammation could therefore represent the initial pathologic change responsible for the presence of mucopurulent plugs in the airways, observed in the first months of life. In advanced CF, infected sputum contains high levels of neutrophil elastases and collagenases which induce a dramatic remodeling of the airway mucosa. DNA released from bacteria, leukocytes and desquamated epithelial cells binds to proteins, mucins and phospholipids, further contribute to abnormalities in the rheological (hyperviscosity) and physical (increased work of adhesion) properties of the CF mucus. The dysregulation of the ion and water composition of the airway surface fluid, including the sol and gel layers of mucus, likely represents a key determinant not only in the dysfunction of mucociliary clearance but also in decreased lung defense against endogenous and exogenous insults.

In CF, the decreased hydration and modifications of the osmolality of the airway mucus directly affect the surface properties of mucus and impair mucus transport capacity, either by ciliary activity or cough.

Histopathologic changes in the lung associated with inflammation and infection represent crucial factors in the progression of the CF disease. Secondary to the release of *P. aeruginosa* virulence factors and of inflammatory products, the remodeling of the epithelium exposes extracellular molecule components and epithelial neoreceptors which represent new sites of bacterial adherence.

Future research should yield more precise details on the specific and chronological causes involved in the dysregulation of airway secretory functions and changes in the biochemical and physical properties of CF airway mucus. Such understanding will in turn help the development of new strategies for the early correction of CF mucus abnormalities.

Acknowledgments

The authors wish to thank Anne Quiqueret for excellent secretarial support. This work was supported by INSERM and in part by grants from MESR (Grant no. 94.C.0137) and by AFLM (Association Française de Lutte contre la Mucoviscidose).

References

1. Engelhardt JF, Yankaskas JR, Emst SA, Yang Y, Marion CR, Boucher RC, Cohn JA, Wilson JM (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genet* 2: 240–248.
2. Davis PB (1993) Pathophysiology of the lung disease in cystic fibrosis. In: Davis PB (ed.) *Lung biology in health and disease.* New York: Marcel Dekker, 193–218.
3. Boucher RC, Stutts MJ, Knowles MR, Cantley L, Gatzy JT (1986) Na^+ transport in cystic fibrosis respiratory epithelial: Abnormal basal rate and response to adenylate cyclase activation. *J Clin Invest* 78: 1245–1252.

4. Boucher RC, Cotton CU, Gatzy JT, Knowles MR, Yankaskas JR (1988) Evidence for reduced Cl^- and increased Na^+ permeability in cystic fibrosis human primary cell cultures. *J Physiol*; 405: 77–103.
5. Boucher RC (1994) Human airway ion transport: State of the Part. Part 1. *Am J Respir Crit Care Med* 150: 271–281.
6. Boucher RC (1994) Human airway ion transport: State of the Art. Part 2. *Am J Respir Crit Care Med* 150: 581–593.
7. Boat TF, Cheng PW, Leigh MW (1994) Biochemistry of mucus. In: Takishima T, Shimura S (eds) *Airway secretion.* Lung Biology in Health and Disease. New York: Marcel Dekker, 72217–72282.
8. Puchelle E, de Bentzmann S, Zahm JM (1995) Physical and functional properties of airway secretions in cystic fibrosis: Therapeutic approaches. *Respiration* 62 (Suppl 1): 2–12.
9. McPherson MA, Dormer RL, Bradbury NA, Dodge JA, Goodchild MC (1986) Defective β-adrenergic secretory responses in submandibular acini cells from cystic fibrosis patients. *Lancet* 2: 1007–1008.
10. Rogers DF, Alton EWFW, Dewar A, Lethem MI, Barnes PM (1993) Impaired stimulus-evoked mucus secretion in cystic fibrosis bronchi. *Exp Lung Res* 19: 1927–1953.
11. Merten MD, Figarella C (1993) Constitutive hypersecretion and insensitivity to neurotransmitters by cystic fibrosis tracheal gland cells. *Am J Physiol* 264: L93–99.
12. Engelhardt JF, Smith SS, Allen E, Yankaskas JR, Dawson DC, Wilson JM (1994) Coupled secretion of chloride and mucus in skin of *Xenopus laevis:* Possible role for CFTR. *Am J Physiol* 267: C491–500.
13. Kuver RN, Ramesh, Lau S, Savard SP Lee, Osborne RA (1994) Constitutive mucin secretion linked to CFTR expression. *Biochem Biophys Res Commun* 203: 1457–1462.
14. Mergey M, Lennaonar M, Veissiere D, Perricaudet M, Gruenert DC, Picard J, Capeau J, Brahimi-Horn MC, Paul A (1995) CFTR gene transfer corrects defective glycoconjugate secretion in human CF epithelial tracheal cells. *Am J Physiol* (Lung Cell Mol Physiol 13) 269: L855–864.
15. Boat TF, Cheng PW (1980) Biochemistry of airway mucus secretion. *Fed Proc* 39: 3067–3074.
16. Boat TF, Cheng PW, Lyer RN, Carlson DM, Polong I (1976) Human respiratory tract Secretions: Mucous glycoproteins of non-purulent tracheobronchial secretions and sputum from patients with bronchitis and cystic fibrosis. *Arch Biochem Bioph* 177: 95–104.
17. Lo-Guidice JM, Wieruszeski JM, Lemoine J, Verbert A, Roussel P, Lamblin G (1994) Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. *J Biol Chem* 269: 18794–18813.
18. Chace KV, Leahy DS, Martin R, Carbelli R, Flux M, Sachdev GP (1983) Respiratory mucous secretions in patients with cystic fibrosis: Relationship between levels of highly sulfated mucin component and severity of the disease. *Clin Chim Acta* 132: 143–155.
19. Thornton DJ, Sheehan JK, Carlstedt I (1991) Heterogeneity of mucus glycoproteins from cystic fibrotic sputum: Are there different families of mucins? *Biochem J* 276: 677–682.
20. Barasch J, Kiss B, Prince A, Saiman L, Gurenert D, Al-Awqati Q (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature* 252: 70–73.
21. Zhang X, Doranz B, Yankaskas JR, Engelhardt JF 81995) Genotypic analysis of respiratory mucous sulfation defects in cystic fibrosis. *J Clin Invest* 96: 2997–3004.
22. Jacquot J, Hayem A, Galabert C (1992) Functions of proteins and lipids in airway secretions. *Eur Respir J* 5: 343–358.
23. Suter S, Shaad UB, Tegner H, Ohlsson K, Desgrandchamps D, Waldvogel FA (1986) Levels of free granulocyte elastase in bronchial secretions from patients with cystic fibrosis: Effect of antimicrobial treatment against *Pseudomonas aeruginosa*-induced damage to a human pulmonary epithelial cell line. *J Infect Dis* 153: 92–909.
24. Birrer P, McElvaney NG, Rüdeberg A, Wirz-Sommer C, Liechti-Gallati S, Kraemer R, Hubbard R, Crystal RG (1994) Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *Am J Respir Crit Care Med* 150: 207–213.
25. Konstan MW, Hilliard KA, Norvell TM, Berger M (1994) Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am J Respir Crit Care Med* 150: 448–454.

26. Kahn TZ, Wagner JS, Bost AT, Martinez J, Accurso FJ, Riches DW (1954) Early pulmonary inflammation in infants with cystic fibrosis. *Am J Respir Crit Care Med* 151: 1075–1082.
27. Power C, O'Connor CM, Macfarlane D, O'Mahoney S, Gaffney K, Hayes J, Fitgerald MX (1994) Neutrophil collagenase in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 150: 818–822.
28. Picot R, Das I, Reid L (1978) Pus, deoxyribonucleic and sputum viscosity. *Thorax* 33: 235–242.
29. Lethem MI, James SL, Marriott C, Burke JF (1990) The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. *Eur Respir J* 3: 19–23.
30. Lieberman J (1979) The appropriate use of mucolytic agents. *Am J Med* 49: 1–4.
31. Girod S, Galabert C, Lecuire A, Zahm JM, Puchelle E (1992) Phospholipid composition and surface active properties of tracheobronchial secretions from patients with cystic fibrosis and chronic obstructive pulmonary diseases. *Pediatr Pulmonal* 13: 22–27.
32. Girod S, Zahm JM, Plotkowski C, Beck G, Puchelle E (1992) Role of the physicochemical properties of mucus in the protection of the respiratory epithelium. *Eur Respir J* 5: 477–487.
33. Slomiany A, Murly VLN, Aono M, Snyder CE, Herp A, Slomiany BL (1982) Lipid composition of tracheobronchial secretions from normal individuals and patients with cystic fibrosis. *Biochim Biophys Acta* 710: 106–111.
34. Gilljam H, Andersson O, Ellin A, Robertson B, Standvik B (1988) Composition and surface properties of the bronchial lipids in adult patients with cystic fibrosis. *Clin Chim Acta* 176: 29–38.
35. Quinton PM (1990) Cystic fibrosis: A disease in electrolyte transport. *FASEB J* 4: 2709–2717.
36. Matthews LW, Spector S, Lemm J, Potter JL (1963) Studies on pulmonary secretions. I. The overall chemical composition of pulmonary secretions from patients with cystic fibrosis, bronchiectasis and laryngectomy. *Am Rev Respir Dis* 88: 199–204.
37. Potter JL, Matthews LW, Spector S, Lemm J (1967) Studies on pulmonary secretions. II. Osmolality and the ionic environment of pulmonary secretions from patients with cystic fibrosis, bronchiectasis and laryngectomy. *Am Rev Respir Dis* 96: 83–87.
38. App EM, King M, Helfesrieder R, Köhler D, Matthys H (1990) Acute and long-term amiloride inhalation in cystic fibrosis lung disease. *Am Rev Respir Dis* 141: 605–612.
39. Tomkewicz RP, App EM, Zayas JG, Ramirez O, Church N, Boucher RC, Knowles MR, King M (1993) Amiloride inhalation therapy in cystic fibrosis: Influence on ion content, hydration and rheology of sputum. *Am Rev Respir Dis* 148: 1002–1007.
40. Joris L, Dab I, Quinton P (1993) Elemental composition of human airway surface fluid in healthy and diseased airways. *Am Rev Respir Dis* 148: 1633–1637.
41. Benali R, Pierrot D, Zahm JM, de Bentzmann S, Puchelle E (1994) Effect of extracellular ATP and UTP on fluid transport by human nasal epithelial cells in culture. *Am J Respir Cell Mol Biol* 10: 363–368.
42. Widdicombe JH, Widdicombe JG (1995) Regulation of human airway surface liquid. *Respiration Physiol* 99: 3–12.
43. Jiang C, Finkbeiner WE, Widdicombe JH, McCray PB, Miller SS (1993) Altered fluid transport across airway epithelium in cystic fibrosis. *Science* 262: 424–427.
44. Pillai RS, Chandra T, Miller IF, Lloyd-Still J, Yeates DB (1992) Work of adhesion of respiratory tract mucus. *J Appl Physiol* 72: 1604–1610.
45. Mizgerd JP, Kobzik L, Warner AE, Brain JD (1995) Effect of sodium concentration of human neutrophil bactericidal functions. *Am J Physiol* 269: L388–393.
46. Yeates D (1991) *Mucus rheology*. In: Crystal RG, West JB (eds) *The lung: scientific foundations*. Editors in chief Crystal RG, West JB; associate editors, Barnes PS, Cherniack NS, Weibel ER. New York: Raven Press, chap. 3.1.5, 197–203.
47. King M (1981) Is cystic fibrosis mucus abnormal? *Pediatr Res* 15: 120–122.
48. Puchelle E, Jacquot J, Beck G, Zahm JM, Galabert C (1985) Rheological and transport properties of airway secretions in cystic fibrosis: Relationship with the degree of infection and severity of the disease. *Eur J Clin Invest* 15: 389–394.
49. Chernick WS, Barbero GJ (1959) Composition of tracheobronchial secretions in cystic fibrosis of the pancreas and bronchectasis. *Pediatrics* 24: 739–745.

50. Zahm JM, Girod de Bentzmann S, Deneuville E, Perrot-Minnot C, Dabadie A, Pennaforte F, Roussey M, Shak S, Puchelle E (1995) Dose-dependent *in vitro* effect of recombinant human DNase on rheological and transport properties of cystic fibrosis respiratory mucus. *Eur Respir J* 8: 381–386.
51. Vasconcellos CA, Allen PG, Whol ME, Drazen JM, Janmey PA, Stossel TP (1994) Reduction in viscosity of cystic fibrosis sputum *in vitro* by gelsolin. *Science* 263: 969–971.
52. Galabert C, Jacquot J, Zahm JM, Puchella E (1987) Relationships between the lipid content and the rheological properties of airway secretions in cystic fibrosis. *Clin Chim Acta* 164: 139–149.
53. Houdret N, Perini JM, Galabert C, Scharfman A, Humbert P, Lamblin G, Roussel P (1986) The high lipid content of respiratory mucins in cystic fibrosis is related to infection. *Biochim Biophys Acta* 880: 54–61.
54. Verdugo P (1990) Goblet cells secretion and mucogenesis. *Annu Rev Physiol* 52: 157–176.
55. Girod S, Galabert C, Pierrot D, Boissonade MM, Zahm JM, Baszkin A, Puchelle E (1991) Role of phospholipid lining on respiratory mucus clearance by cough. *J Appl Physiol* 71: 2262–2266.
56. Girod de Bentzmann S, Pierrot D, Fuchey C, Zahm JM, Morançais JL, Puchelle E (1993) Distearoyl phosphatidylglycerol liposomes improve surface and transport properties of CF mucus. *Eur Respir J* 6: 1156–1161.
57. King M, Brock G, Lundelle C (1985) Clearance of mucus by simulated cough. *J Appl Physiol* 58: 1776–1782.
58. King M, Zahm JM, Pierrot D, Vaquez-Girod S, Puchelle E (1989) The role of mucus gel viscosity, spinnability and adhesive properties in clearance by simulated cough. *Biorheology* 26: 737–745.
59. Zahm JM, Pierrot D, Vaquez-Girod S, Duvivier C, King M, Puchelle E (1989) The role of mucus sol phase in clearance by simulated cough. *Biorheology* 26: 747–752.
60. Deneuville E, Perrot-Minnot C, Pennaforte F, Roussey M, Zahm JM, Clavel C, Puchelle E, de Bentzmann S (1997) *Am J Respir Crit Care Med* 156: 1–7.
61. Zahm JM, Gaillard D, Dupuit F, Hinnrasky J, Porteous D, Dorin JR, Puchelle E (1997) Early alterations in airway mucociliary clearance and inflammation of the lamina propria in CF mice. *Am J Physiol* 172: C853–859.
62. Dorin JR, Dickinson P, Alton EWFW, Smith SN, Geddes DM, Stevenson BJ, Kimber WL, Fleming S, Clark AR, Hooper ML (1992) Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature* 359: 211–215.
63. Standiford TL, Kunkel SL, Basha MA, Chensue SW, Lynch JP, Toews GB, Westwick J, Strieter RM (1990) Interleukin-8 gene expression by a pulmonary epithelial cell line: A model for cytokine networks in the lung. *J Clin Invest* 86: 1945–1953.
64. Nakamura H, Yoshimura K, McElvarey NG, Crystal RG (1992) Neutrophil elastase in respiratory epithelial lung fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest* 89: 1478–1484.
65. Ruef C, Jefferson DM, Schlegel-Haueter, Suter S (1993) Participation of cystic fibrosis airway epithelium in local inflammation: Regulation of the secretion of interleukin-6 and interleukin-8. *Eur Respir J* 6: 1429–1436.
66. Berger M, Bonfield T, Panuska J, Konstan M (1996) Cytokines in cystic fibrosis lung diseases. *Mediators of Inflammation* 5: 136–137.
67. Bonfield TL, Konstan MW, Burfeind P, Panuska JR, Hilliard JB, Berger M (1995) Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am J Respir Cell Mol Biol* 13: 257–261.
68. Davidson DJ, Dorin JR, McLachlan G, Ranaldi V, Lamb D, Doherty C, Govan J, Porteous DJ (1995) Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. *Nature Genet* 9: 351–357.
69. Copenhaver SC, Khan TZ, Wagene JS, Johnson Z, Vasil AI, Caroll NH, Accurso JF, Vasil ML (1994) Airway inflammation in the absence of detectable *Pseudomons aeruginosa* by culture and PCR in infants with cystic fibrosis. *Pediatr Pulmonol* S10, 283.
70. Chow CW, Landau LI, Taussig LM (1982) Bronchial mucous glands in the newborn with cystic fibrosis. *Eur J Pediatr* 139: 240–243.

71. Bredossian CWM, Greenberg SD, Singer DB, Hansen JJ, Rosenberg HS (1976) The lung in cystic fibrosis: A quantitative study including prevalence of pathologic finding among different age groups. *Hum Pathol* 7: 195–204.
72. Sturgess J, Imrie J (1982) Quantitative evaluation of the development of tracheal submucosal glands in infants with cystic fibrosis and control infants. *Am J Pathol* 106: 303–311.
73. Simel DL, Mastin JP, Pratt PC, Wisseman CL, Shelburne JD, Spock A, Ingram P (1984) Scanning electron microscopic study of the airways in normal children and in patients with cystic fibrosis and other lung diseases. *Pediatric Pathology* 2: 47–64.
74. Baltimore RS, Christie CDC, Smith GJ (1989) Immunohistopathologic localization of *Pseudomonas aeruginosa* in lungs from patients with cystic fibrosis. *Am Rev Respir Dis* 140: 1650–1661.
75. Carson JL, Collier AM, Gambling TM, Knowles MR, Boucher RC (1990) Ultrastructure of airway epithelial cell membrane among patients with cystic fibrosis. *Human Pathol* 21: 640–647.
76. Oppenheimer EH, Esterly JF (1978) Pathology of cystic fibrosis: Review of the literature and comparison with 146 autopsied cases. *Perspect Pediatr Pathol* 2: 241–278.
77. Sobonya RE, Taussig LM (1986) Quantitative aspects of lung pathology in cystic fibrosis. *Am Rev Respir Dis* 134: 290–295.
78. Montrose-Rafizadeh C, Guggino WB, Montrose MH (1991) Cellular differentiation regulates expression of Cl^- transport and CFTR mRNA in human intestinal cells. *J Biol Chem* 266: 4495–4499.
79. Dupuit F, Kälin N, Brezillon S, Hinnrasky J, Tümmler B, Puchelle E (1995) CFTR and differentiation markers expression in non-CF and ΔF 508 homozygous CF nasal epithelium. *J Clin Invest* 96: 1601–1611.
80. Jeffery PK, Brain APR (1988) Surface morphology of human airway mucosa: Normal, carcinoma or cystic fibrosis. *Scanning Microscopy* 2: 553–560.
81. Doring G, Goldstein W, Roll A, Schiotz PO, Horby N, Botzenhart K (1985) Role of Pseudomonas exoenzymes in lung infections of patients with cystic fibrosis. *Infect Immun* 49: 557–562.
82. Dolg P, Smith NR, Todd T, Irvin RT (1987) Characterization of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. *Infect Immun* 55: 1517–1522.
83. Olick J, Garber N, Shohet D (1987) Surface agglutinating activity of *Pseudomonas aeruginosa*. *Microbios* 50: 69–80.
84. Bajolet-Laudinat O, Girod de Bentzmann S, Tournier JM, Madoulet C, Chippaux C, Puchelle E (1994) Cytoxocity of *Pseudomonas aeruginosa* internal lectin PA-I in respiratory epithelial cells in primary culture. *Infect Immun* 62: 4481–4487.
85. Baker NR, Minor Y (1991) The role of exoenzyme S in adherence of *Pseudomonas aeruginosa*. Ped Pulmon (Suppl): 136–137.
86. Lingwood CA, Cheng M, Krivan HC, Woods D (1991) Glycolipid receptor binding specificity of exoenzyme S from *Pseudomonas aeruginosa*. *Biochem Biophys Res Commun* 175: 1976–1081.
87. Stutts MJ, Sckwab JH, Chen MG, Knowles MR, Boucher RC (1986) Effects of *Pseudomonas aeruginosa* on bronchial epithelial ion transport. *Am Rev Respir Dis* 134: 17–21.
88. Ishihara H, Houda I, Shimura S, Sasake H, Takishima T (1991) Role of chronic *Pseudomonas aeruginosa* infection in airway mucosal permeability. *Chest* 100: 1607–1613.
89. Cacalano G, Kays M, Saiman L, Prince A (1989) Production of the *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. *J Clin Invest* 89: 1866–1874.
90. Smith JJ, Travis SM, Greenberg EP, Welsh MJ (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85: 229–236.
91. Pier G, Groud M, Zaidi TS, Olsen JC, Johnson LG, Yankaskas JR, Goldberg JB (1996) Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science* 27: 64–67.
92. Carnoy C, Ramphal R, Scharfman A, Lo-Guidice JM, Houdret N, Klein A, Galabert C, Lamblin G, Roussel P (1993) Altered carbohydrate composition of salivary mucins from patients with cystic fibrosis and the adhesion of *Pseudomonas aeruginosa*. *Am J Respir Cell Mol Biol* 9: 323–334.

93. Devaraj N, Sheykhnazari M, Warren WS, Bhavarandon VP (1994) Differential binding of *Pseudomonas aeruginosa* to normal and cystic fibrosis tracheobronchial mucins. *Glycobiology* 4: 307–316.
94. Ramphal RN, Houdret L, Koo L, Lamblin G, Roussel P (1989) Differences in adhesion of *Pseudomonas aeruginosa* to mucin glycopeptides from sputa of patients with cystic fibrosis and chronic bronchitis. *Infect Immun* 57: 3066–3071.
95. Sajjan U, Reisman J, Doig P, Irwin RT, Forstner G, Forstner J (1992) Binding of non-mucoid *Pseudomonas aeruginosa* to normal human intestinal mucin and respiratory mucin from patients with cystic fibrosis. *J Clin Invest* 89: 657–665.
96. Plotkowski MC, Tournier JM, Puchelle E (1996) *Pseudomonas aeruginosa* strains posess specific adhesins for laminin. *Infect Immun* 64: 600–605.
97. de Bentzmann S, Plotkowski C, Puchelle E (1996) Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. *Am J Resp Crit Care Med.* 154: S155–162.
98. Zar H, Saiman L, Quittel L, Prince A (1995) Binding of *Pseudomonas aeruginosa* to respiratory epithelial cells from patients with various mutations in the cystic fibrosis transmembrane conductance regulator. *J Pediat* 126: 230–233.
99. Saiman L, Prince A (1993) *Pseudomonas aeruginosa* pili bind to asiola GM_1 which is increased on the surface of cystic fibrosis epithelial cells. *J Clin Invest* 92: 1875–1880.
100. Imundo L, Barasch J, Prince A, Al-Awqati Q (1995) Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. *Proc Natl Acad Sci USA.* 92: 3019–3023.
101. de Bentzmann S, Roger P, Dupuit F, Bajolet-Laudinat O, Fuchey C, Plotkowski MC, Puchelle E (1996) Asialo GM_1 is a receptor for *Pseudomonas aeruginosa* adherence to regenerating respiratory epithelial cells. *Infect Immun* 64: 1582–1588.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D. F. Rogers and M. I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 13
Drug–Mucus Interactions

Bayan Abdul-Haq[#], Gary P. Martin and Christopher Marriott*

Pharmacy Department, King's College London, London, UK

1. Introduction

Drug delivery via the lungs to obtain systemic effects is currently receiving considerable attention. One stimulus to this research activity has been the realisation that the inhaled route may circumvent many of the difficulties associated with the systemic delivery of biotechnologically-derived drugs such as peptides, polypeptides, antisense DNA and genes. Such difficulties include first-pass metabolism and hydrolytic and enzymatic degradation which can occur within the gastrointestinal tract. As a result, a lower dosage can be administered to achieve similar efficacy, which helps to minimize unwanted side effects [1, 2]. Furthermore, reasonable absorption values can be anticipated due to the lung's relatively large surface area of approximately 75 m^2 [3]. In addition to systemic drug delivery, aerosol inhalation as a method of drug delivery for local effects in the respiratory tract has become well established. It must be noted, however, that unlike the gastrointestinal tract, the respiratory tract is not designed to transport various materials across its epithelium. On the contrary, it is provided with protective mechanisms designed specifically to prevent materials in the inspired air from coming into contact with the tissues of the airway. Mucus is the central feature of respiratory defence, and it fulfils its protective role by

* Author for correspondence.
[#] Present address: Faculty of Pharmacy, University of Jordan, Amman, Jordan.

various mechanisms, including its involvement in mucociliary clearance, the binding and inactivation of exogeneously-applied compounds, and also by acting as a diffusion barrier to such agents [4, 5]. These protective mechanisms of mucus can limit the accessibility of a drug to its target sites, since inhaled drugs must traverse these barriers before reaching their site of action.

The viscoelastic properties of mucus are the essential features enabling mucus to perform its protective functions, especially in relation to mucociliary clearance and retardation of drug diffusion [6, 7]. Alterations in the rheological properties of the mucus gel can change the efficiency with which mucus fulfils its protective function. This means that diseases such as cystic fibrosis (CF), bacterial and viral infection, and physiological conditions associated with alterations in the quality (mainly rheological properties) of mucus, due to impaired secretion and/or changes in its composition, can influence the interaction of mucus with inhaled materials. In addition, quantitative alterations in airway mucus, as a result of enhanced secretion or accumulation, is a significant factor in determining mucus–drug interactions. The effect of altered mucus is particularly relevant when lung delivery is intended to treat "local" diseases such as chronic bronchitis or CF which are associated with alterations in the physicochemical properties and quantity of airway mucus.

The effect of mucus–drug interactions on the success of inhalation therapy has not been clearly identified. Various studies have suggested that mucus–drug interactions could be a major hindrance to drug delivery. This issue needs further investigation due to its potential influence on the success of inhaled therapy.

Prior to examination of the interactions between mucus and inhaled drug molecules and their possible effects on inhaled drug efficiency, the composition and viscoelastic properties of mucus will be briefly outlined in sufficient detail to enable understanding of the basic mechanisms underlying mucus–drug interactions.

2. Respiratory Mucus

The respiratory epithelium, like many other epithelia in direct contact with the external environment, is covered by a mucus gel. Respiratory secretions comprise a complex and variable mixture of glycoproteins, proteins, lipids and electrolytes. Mucus gel turnover is determined by the rates of mucus synthesis, secretion and degradation, and also by mucociliary clearance. The mucus glycoprotein is thought to be released into the airway from two principal sources: the mucous cells of the submucosal glands and the goblet cells of the surface epithelium [4]. The organic constituents usually account for approximately 3–5% of the wet weight, the remaining 95% being water and electrolytes [8]. The ionic composition and water content of the respiratory secretion are tightly controlled by the epithelial cells lining the

airway through the balance between chloride secretion and sodium absorption [9].

The viscoelastic properties of mucus are a reflection of the interactions between mucin molecules in the mucus gel and also with other non-mucin constituents present in the mucus layer. Based on the observation that a reduction in mucin results in collapse of the gel structure, the mucus gel network is thought to be stabilised by intermolecular disulphide bonds [10]. Mucus gels are further stabilised by non-covalent interactions between mucin molecules, thought to occur by interpenetration of the molecular domains at high glycoprotein concentrations. The effect of disulphide reduction is due to shortening of the mucin polymers, which results in destabilisation of the gel network [11]. The effect of non-covalent interactions of mucus gel stability is supported experimentally by the observation that factors which affect mucus hydration, such as the macromolecular composition and ionic content of the mucus, alter the rheological properties of mucin gels [12, 13].

3. Mucus–Drug Interactions

3.1. Mucus Effects on Drug Deposition

Effective administration of aerosolised drugs depends on maximising the deposition of drug in the airway, preferably in the region of the airway containing the site of action. The pattern of deposition of an inhaled aerosol depends on several factors, including the size and shape of the inhaled particles, the composition of the aerosol, the status of the respiratory tract and the breathing pattern of the patient [14]. Several studies have suggested that excess mucus is a significant factor in the deposition pattern of aerosols [15–17]. Severe airways obstruction has been shown to increase deposition at the site of obstruction, with little aerosol deposition occurring distal to this point [18]. In addition, severe obstruction has been suggested to deflect air into less obstructed airways, potentially enhancing penetration of aerosol into lung areas where is it less needed [19]. Severe plugging of airways, however, is not necessary for alteration of aerosol deposition. Several studies have indicated that even a moderate increase in luminal mucus can increase the deposition of particles in central airways [20, 21]. There are two main hypotheses to explain this effect. One suggests that an increase in mucus lining the airways reduces airway diameter and results in an increase in air velocity for a given flow rate. Such an increase in velocity will increase the impaction of particles and favour deposition in the central airways [22, 23]. The other hypothesis suggests that the flow of air through the airways induces a random wave motion in the surface of the mucus, which in turn leads to turbulence and deposition of particles in the area immediately behind the wave peaks [17, 24]. The presence of an elastic com-

ponent in the mucus appears to promote wave formation [24], whilst viscosity seems to be the main determinant in increased deposition [17, 24].

3.2. Mucociliary Clearance

Mucociliary clearance is central to protection of the airway tissue from inhaled irritants. In this process, inhaled foreign material is trapped in the mucus covering the airway surface and is transported toward the glottis and out of the airway by the action of cilia. The cilia beat in a coordinated manner within the periciliary fluid beneath a layer of viscoelastic mucus, the whole comprising the mucociliary apparatus. Efficient mucociliary clearance is dependent on the successful interaction of all components of the mucociliary apparatus and may be altered by any factor capable of affecting the number of cilia or their morphology, ciliary activity, ciliary coordination as well as mucus composition, secretion volume or viscoelasticity [25].

The most important implication of mucociliary clearance for respiratory drug delivery is that it reduces the amount of time a formulation is in contact with the absorbing membrane, with consequent reduction in bioavailability of the delivered drug [26]. Many drugs and their carrier formulations (excipients) have been shown to influence mucociliary clearance, which in turn might affect dosing precision. For example, diluents such as glucose or lactose are included as excipients in dry powder inhaler formulations [27]. As such, sugars hydrate by withdrawing water from the mucus gel, causing localised changes in mucociliary clearance to occur as a result of alterations in the rheological properties of the gel. Ideally, however, substances included in a formulation should be innocuous to the mucociliary clearance apparatus. This is especially relevant if use of the respiratory tract is intended for chronic administration of drugs for systemic therapy.

Reducing mucociliary clearance may be envisaged as a possible strategy to increase drug bioavailability by prolonging the contact time between the inhaled formulation and the respiratory mucosa. For example, it has been suggested that the reversible toxic effects of some pharmaceutical excipients (e.g. hydroxybenzoates) on cilia could be exploited to increase the retention time of co-administered therapeutic agents within the airways, thereby increasing drug absorption [28, 29]. However, in using such an approach, care must be taken not to permanently compromise mucociliary clearance, since this would have adverse effects on airway homeostasis and defence.

3.3. Direct Mucus–Drug Interactions

The effects of direct interactions of mucin with inhaled drugs have been largely overlooked. The most important of these are mucin–drug binding

and retardation of diffusion by the mucus layer. Data relevant to such interactions are scarce at present. However, direct mucin–drug interactions in other systems, particularly the gastrointestinal tract, have been more widely investigated, presumably because the gastrointestinal tract remains the main and most popular route for drug delivery. Therefore, much of the available evidence on mucin–drug interactions pertains to the gastrointestinal tract.

Mucin–drug interactions have been studied mainly using *in vitro* techniques. Various experimental methods have been used in studies of the effect of mucus on drug diffusion, including the use of diffusion cells [30, 31], capillary tubes [32] and epifluorescence microscopy combined with image analysis [33]. The diffusion cell method is the most frequently used method. Mucus is held between two membrane filters which are clamped between two compartments of the diffusion cell [30, 31]. Diffusion cell techniques have also been used to investigate specific mucus–drug binding interactions [34], as have gel filtration chromatography [35] and equilibrium dialysis [35–37]. Mucus samples used in the *in vitro* studies are obtained from animals, mainly the cow, pig and rat. In general, two kinds of samples are usually used in the *in vitro* studies: native mucus and purified mucin. The most commonly used method of obtaining naturally occurring mucus is by scraping the mucosa, which will inevitably lead to damage to the mucosa. Therefore, mucus samples prepared in this way will be contaminated with intracellular contents and even connective tissue matrix [30, 38, 39]. To avoid this problem, purified mucin fractions are used. A purified mucin fraction can be produced from native mucus by fractionating on a gel exclusion column followed by ultrafiltration to produce a gel [40]. However, using purified mucin to investigate mucus–drug interactions is an oversimplification of the *in vivo* situation since, in addition to mucin glycoproteins, the mucus gel contains other substances, including DNA, lipids, antibodies and enzymes, which may have profound effects on mucus–drug interactions. Thus, the data available on mucin–drug interactions must be interpreted with some caution, taking into account the type of mucin preparation employed. It must be remembered also that since variations of mucin structure and composition exist between different species and different locations, further caution must be exercised in interpreting results when using mucus obtained from different species and from different organs.

3.3.1. Mucus as a diffusion barrier: The protective role of airway mucus is believed to be largely achieved by its ability to act as a physical barrier. Respiratory mucus has been reported to cause a significant retardation in the diffusion of water and larger molecules, including a range of β-lactam antibiotics commonly used in the treatment of respiratory infection [41, 42]. However, the bulk of evidence on the role of mucus as a diffusion barrier has been obtained using gastrointestinal mucins. This is presumably

due to the ease of isolating mucus from this source, and a result of oral delivery being the preferred and the most commonly used route of drug administration. Mucin is believed to act as a molecular filter which will selectively exclude macromolecules. Large molecular mass proteins, for example myoglobin (17 kDa) or pepsin, do not significantly penetrate 1–2 mm-thick layers of gastric mucus over 24 h [43]. The diffusion coefficient for such globular proteins is of the order of 1×10^{-6} cm^2 s^{-1}, equivalent to diffusion through an unstirred layer of solution of 36 μm h^{-1}. Proteins, therefore, will take hours to diffuse freely through the unstirred layer of gastric mucus gel, the thickness of which is estimated as 180 μm in humans. In addition, even further retardation of their mobility is likely as a result of steric hindrance. It has been estimated that adherent gastroduodenal mucus is permeable to proteins, in a dose-dependent manner, up to 100 kDa [44]. Mucus has been visualised as a mesh of chain-like molecules which allow penetration by small molecules between the mucin chains [45]. However, a reduction in diffusion coefficient has also been reported for a large number of compounds, including water, urea, benzoic acid, antipyrine, aminopyrine, L-phenylalanine, hydrogen ions [46, 30] and warfarin [47]. It must be noted that there is wide variation between the reported degree of retardation conferred by mucus obtained from different parts of the gastrointestinal tract. Gastric mucus was found to cause a 4-fold retardation of hydrogen ion diffusion [46, 48] compared with a retardation of 14-fold by small intestinal mucus [30]. These results should caution against indiscriminate extrapolation of results obtained in one system to another.

CF sputum and purified CF mucus glycoprotein gels have been shown to act as more effective barriers to diffusion than comparable gels from chronic bronchitic sputa, presumably due to their state of dehydration and increased viscoelasticity. Also, non-mucin components found in mucus have been shown to influence the diffusion barrier properties of airway mucus. For example, alginate polysaccharide, which is produced by *Pseudomonas aeruginosa* in the lungs of many CF patients, can act as a diffusion barrier to β-lactam antibiotics [49, 50]. In addition, the presence of DNA at concentrations relevant to CF sputum greatly reduces the diffusion rate of antibiotics through mucin gels [51]. This could be of clinical importance, since aerosolised antibiotics are used widely in the treatment of respiratory infections associated with CF.

Some therapeutic agents have been reported to alter the viscoelasticity of mucus. For example, oxytetracycline increased the viscoelasticity of airway mucus, which means that the bioavailability of drugs co-administered with this drug may be reduced [52].

3.3.2. Binding of drugs to mucus: Drug binding to mucus accounts for the limited diffusion of many drugs. Mucus glycoproteins are complex macromolecules containing many potential sites for drug binding: the sialic acid

and acid sulphate esters in mucus glycoproteins have low p*Ka* values and are ionised under most physiological conditions. In fact, mucus glycoproteins can be thought of as negatively charged polyelectrolytes. Hence, positively charged substances are likely to bind mucin molecules via electrostatic forces. The abundance of hydroxyl groups on the sugar moiety, together with other O- and N-containing groups on the protein backbone, provide excellent sites for the formation of hydrogen bonds. Furthermore, although glycoproteins are highly soluble and extensively hydrated molecules, it was suggested that part of their structure contains a globular protein region [53], which makes binding with drugs through hydrophobic interaction a possibility [36]. Gastrointestinal mucus has been shown to bind extensively with tetracycline. Maximal binding is at pH 3, where the tetracycline is positively charged (+1 arising from ionisation of the basic dimethylamino group), and mucin is negatively charged. Both electrostatic and hydrophobic interactions were suggested to explain the pH-binding profile [36, 54]. Evidence provided by nuclear magnetic resonance (NMR) studies showed that tetracycline also binds mucin via the 10,11,12-hydroxy ketone moeity, suggesting that hydrogen bonding may also be involved in binding [37]. Cephaloridine and gentamycin have been reported to bind intestinal mucin in a pH- and NaCl-dependent manner, suggesting ionic interactions [35].

Drug binding to respiratory mucus has also been reported. Gentamycin has been shown to bind to sputum [55], and tobramycin binds to both mucin-rich sputum and DNA-rich sputum [51]. The significance of the these results is that binding to DNA could markedly affect antibiotic diffusion through the mucus, especially under some pathological conditions which are treated locally with antibiotics, such as CF, where mucus is contaminated with large quantities of serum transudate.

The ability of mucus to bind various substances can be exploited in the design of drug delivery systems. Mucoadhesive drug delivery systems (MADDS) have been designed to adhere to the mucus lining of the gastrointestinal tract. They should then be able to exert a positive influence on drug absorption by different mechanisms which involve increasing the residence time of the delivery system at the site of absorption and by intensifying the contact of the drug with the absorbing biological membranes [56]. The success of MADDS is likely to be limited by the turnover rate of mucus [57].

3.4. The Effect of Mucus on Drug Bioavailability

Despite the relative wealth of information on mucus–drug interactions, particularly involving gastrointestinal mucus, the effect of these interactions on drug bioavailability has not to date been unambiguously determined. The importance of the mucus barrier depends largely on the character-

istics of the drugs themselves, and on the extent of retardation in the process of drug absorption in comparison with other barriers such as the mucosal membrane itself. In other words, the mucus layer would be expected to be a more significant barrier for drugs that can readily cross the mucosal membrane. The mucus barrier, however, can reduce the efficiency of inhaled drug therapy regardless of whether respiratory delivery is intended for its local or systemic effects, since in both cases it has to be overcome before the drug reaches its site of action. Various approaches have been used to investigate the effect of mucus on drug transport. The most commonly used approach is to attempt to breach the mucus barrier. The assumption is that if the mucus layer presents a barrier to absorption, then degradation of the mucus structure should result in increased efficiency of drug therapy. Degradation of the mucus layer has been achieved using mucolytic agents including sulphydryl reagents, such as *N*-acetylcysteine or erdosteine, and mucolytic enzymes. Sulphydryl reagents are capable of reducing disulphide bonds in mucus through the process of sulphydryl group exchange. The collapse in mucus structure is suggested to be a result of degradation of intermolecular disulphide bonds which stabilise the mucus structure [10] and of shortening of mucin polymers [11].

N-acetylcysteine treatment resulted in a faster transport rate of testosterone across monolayers of HT29-H cells, a mucin-secreting cell line derived from colon carcinoma [58], and of human growth hormone across a rat nasal mucosal preparation [59]. A considerable increase in the absorption rate of ergot peptide alkaloids by an intestinal *in situ* perfusion model resulted from the addition of mucolytic enzymes [60]. Erdosteine increased the level of amoxicillin in sputum, but not in serum, when administered orally to chronic bronchitis patients for 7 days [61]. However, sulphydryl mucolytics can reduce a wide range of proteins in the mucosal membrane which may thus alter its permeability. Therefore, it is possible that the altered permeability observed after mucolytic treatment may be, at least in part, due to altered permeability of the apical membrane itself rather than being a consequence of breaching the mucus barrier. Reducing the barrier function of the mucus layer has been implicated in the absorption-promoting effects of some penetration enhancers. For example, although sodium salicylate reduced the depth of the glycocalyx in the rectum, it was also suggested that it reduced the barrier function of the mucus layer [62]. It has been suggested that absorption enhancement by bile salts may be brought about by effects on the mucus layer and on paracellular absorption routes. Bile salts have been reported to affect the structure of the intestinal glycocalyx [63], deplete gastric and intestinal mucus [64, 65] and reduce respiratory [66] and gastric [67] mucus structure. However, most of these penetration-enhancing compounds have been shown to have toxic effects on the epithelial membrane, making it difficult to determine how much of the enhanced absorption is due to membrane damage or to breaching of the mucus barrier.

Mucin-secreting intestinal cell lines [68, 69] have also been used to investigate the effect of mucus on drug absorption. The transport rate of testosterone, a highly lipophilic drug, across a mucin-secreting co-culture of HT29GlucH cells 3 : 1 with Caco-2 cells was reported to be significantly lower than across a non-mucin-secreting culture of HT29GlucE cells 3 : 1 with Caco-2 cells, which indicated that the mucin secreted by the former co-culture reduced testosterone permeability [70]. Testosterone has been shown to bind to pig gastric and small intestinal mucus [71]. Therefore, binding to mucin might be a contributory factor in reduced permeability. In addition, one of the ways by which mucus can alter drug permeability in the gastrointestinal tract is by promoting the unstirred water-layer effect. This is particularly relevant for the transport of highly lipophilic drugs whose diffusion across the unstirred layer may be the rate-limiting step to absorption. Therefore, the reduced permeability of testosterone may also be attributed to an enhanced unstirred water effect. Surprisingly, however, under the same experimental conditions, the transport of propranolol across the non-mucin-secreting cell line was lower than that across the mucin-secreting culture. There is a possibility that the altered permeability observed across the two co-cultures may be due to different permeabilities of the two subclones, HT29GlucH and HT29GlucE.

The addition of mucin solution was shown to retard oleic acid-induced injury to a Caco-2 cell monolayer, which was suggested to be a result of retarding oleic acid diffusion through the aqueous medium [72]. Based on these results it was suggested that mucin hypersecretion in the gastrointestinal tract is part of the cytoprotective mechanism to limit damage to the underlying epithelium from luminal irritants [72]. Mucin hypersecretion was suggested to be responsible for altering the responsiveness to aerosolised cholinergic agonists in dogs with experimentally induced chronic bronchitis as a result of restricting access to the airway mucosa [73, 74]. An altered pattern of drug deposition was eliminated as the cause of the observed decrease in responsiveness, since the increase in deposition in the conducting airway associated with hypersecretion would increase rather than decrease airway responsiveness. Similar to the gastrointestinal tract, enhanced mucin output in the upper respiratory tract was proposed to be a protective mechanism in response to inhaled irritants or toxic materials [75].

The effect of mucus on respiratory drug absorption remains to be investigated. Tracheal pouches have been reported to be a suitable *in vivo* model to investigate the possible effect of mucolytics and mucospissic agents on drug absorption [76]. In addition, mucin-secreting tracheal primary cultures [77] and cell lines which have the potential to differentiate into both secretory and squamous cell phenotypes [78] may provide suitable *in vitro* models to investigate the effect of mucus on drug absorption.

4. Conclusions

Although it is presently well documented that the efficiency of inhaled drug therapy is largely influenced by mucus through its central role in mucociliary clearance and drug deposition in the airways, only sparse data are available at present concerning direct airway mucus–drug interactions such as retardation of drug diffusion by mucin or mucin–drug binding. However, information on gastrointestinal mucin–drug interactions is currently available which suggests that the mucus barrier may be a significant hindrance to drug delivery, presumably by altering the access of drug to its site of action. Therefore, it is not unreasonable to expect similar results with respiratory tract mucins.

It must be noted, however, that mucin glycoprotein structure, in particular its degree of sulphation and sialylation, varies considerably among mucins obtained from different anatomical sites. This can significantly alter the extent of mucus–drug interactions, since both groups are heavily involved in mucus–drug binding. Furthermore, mucus at different sites is in close contact with various endogenous or exogenous agents which may alter the characteristics of mucus interactions and cautions against outright extrapolation of the results obtained in one system to another.

Consequently, more extensive investigation of mucus–drug interactions is urgently required, especially in view of the potential effect such interactions might exert on the efficiency and success of inhaled drug therapy. It is worth noting, however, that assessment of the effect of mucin–drug interactions on drug bioavailability is equally important since, despite some reports suggesting that the mucus layer can alter drug delivery, there is no conclusive evidence yet which shows such an effect. Identification of mucus–drug interactions and quantification of their effects on drug bioavailability will almost certainly help in the optimization of inhaled drug therapy.

References

1. Byron PR, Patton JS (1994) Drug delivery via the respiratory tract. *J Aerosol Medicine* 7: 49–74.
2. Martin GP, Onyechi JO, Marriott C (1994) Future prospects for pulmonary delivery of drugs. *Pharma Sciences* 4: 5–10.
3. Hollinger MA (1985) Respiratory pharmacology and toxicology. Philadelphia: WB Saunders, 1–20.
4. Lethem MI (1993) The role of tracheobronchial mucus in drug administration to the airways. *Adv Drug Delivery Rev* 11: 271–298.
5. Cross CE, Halliwell B, Allen A (1984) Antioxidant protection: A function of tracheobronchial and gastrointestinal mucus. *Lancet* 1: 1328–1329.
6. Gelman RA, Meyer FA (1979) Mucociliary transference rate and mucus viscoelasticity: Dependence on dynamic storage and loss modulus. *Am Rev Respir Dis* 120: 553–557.
7. King M, Gilboa A, Meyer FA, Silberberg A (1974) On the transport of mucus and its rheologic simulants in ciliated systems. *Am Rev Respir Dis* 110: 740–745.
8. Creeth JM (1978) Constituents of mucus and their separation. *Br Med Bull* 34: 17–24.

9. Welsh MJ (1987) Electrolyte transport by airway epithelia. *Physiol Rev* 97: 1143–1184.
10. Roberts GP (1976) The role of disulphide bonds in maintaining the gel structure of bronchial mucus. *Arch Biochem Biophys* 173: 528–537.
11. Verdugo P (1990) Goblet cell secretion and mucogenesis. *Ann Rev Physiol* 52: 157–176.
12. Mariott C, Beeson MF, Brown DT (1982) Biopolymer-induced changes in mucus viscoelasticity. In: Chantler EN, Elder JB, Elstein M (eds.) *Mucus in health and diesease.* New York: Plenum Press, 89–92.
13. Lethem MI, James SL, Marriott C (1990) The role of mucus glycoproteins in the rheologic properties of cystic fibrosis sputum. *Am Rev Respir Dis* 142: 1053–1058.
14. Newman SP (1984) Therapeutic aerosols. In: Clarke SW, Pavia D (eds) *Aerosols and the lung: Clinical and experimental aspects.* London: Butterworths, 197–224.
15. Goldberg IS, Lourenco RV (1973) Deposition of aerosols in pulmonary disease. *Arch Intern Med* 131: 88–91.
16. Kim CS, Eldridge MA, Wanner A (1988) Airway responsiveness to inhaled and intravenous carbachol in sheep: Effect of airway mucus. *J Appl Physiol* 65: 2744–2751.
17. Kim CS, Eldrige MA (1985) Aerosol deposition in the airway model with excessive mucus secretions. *J Appl Physiol* 59: 1766–1772.
18. Itoh H, Ishii Y, Maeda H, Todo G, Torizuka K, Smaldone GC (1981) Clinical observations of aerosol deposition in patients with airways obstruction. *Chest* 80 (Suppl): 837–840.
19. Kim CS, Brown LK, Lewars GG, Sackner MA (1983) Deposition of aerosol particles and flow resistance in mathematical and experimental airway models. *J Appl Physiol* 55: 154–163.
20. Dolovich MB, Sanchis J, Rossman C, Newhouse MT (1976) Aerosol penetrance: A sensitive index of peripheral airway obstruction. *J Appl Physiol* 40: 468–471.
21. Taplin GV, Tashkin DP, Chopra SK, Anselmi OE, Elam D, Calvarese B, Coulson A, Detels R, Rokaw SN (1977) Early detection of chronic obstructive pulmonary disease using radionuclide lung-imaging procedures. *Chest* 71: 567–575.
22. Agnew JE (1984) Physical properties and mechanisms of deposition of aerosols. In: Clarke SW, Pavia D (eds) *Aerosols and the lung.* London: Butterworths 49–70.
23. Isawa T, Teshima T, Hirano T, Ebina A, Anazawa Y, Konno K (1987) Effect of bronchodilation on the deposition and clearance of radioaerosol in bronchial asthma in remission. *J Nucl Med* 28: 1901–1906.
24. Kim CS, Abraham WM, Chapman GA, Sackner MA (1985) Influence of two-phase gas-liquid interaction on aerosol deposition in airways. *Am Rev Respir Dis* 131: 618–623.
25. Satir P, Sleigh MA (1990) The physiology of cilia and mucociliary interactions. *Ann Rev Physiol* 527: 137–155.
26. Lansley AB (1993) Mucociliary clearance and drug delivery via the respiratory tract. *Adv Drug Delivery Rev* 11: 299–327.
27. Timsina MP, Martin GP, Marriott C, Ganderton D, Yianneskis M (1994) Drug delivery to the respiratory tract using dry powder inhalers. *Int J Pharmaceutics* 101: 1–13.
28. Batts AH, Marriott C, Martin GP, Bond SW (1989) The effect of some preservatives used in nasal preparation on mucociliary clearance. *J Pharm Pharmacol* 41: 156–159.
29. Van der Donk HJM, Muller-Plantema IP, Zuidema J, Merkus FWHM (1989) The effects of preservatives on the ciliary beat frequency of chicken embryo tracheas. *Rhinology* 18: 119–133.
30. Winne D, Verheyen W (1990) Diffusion coefficient in native mucus gel of rat small intestine. *J Pharm Pharmacol* 42: 517–519.
31. Desai MA, Vadgama PM (1991) Estimation of effective diffusion coefficients of model solutes through gastric mucus: Assessment of a diffusion chamber technique based on spectrophotometric analysis. *Analyst* 116: 1113–1116.
32. MacAdam A (1993) The effect of gastrointestinal mucus on drug absorption. *Adv Drug Delivery Rev* 11: 201–220.
33. Henry BT, Adler J, Hibberd S, Cheema MS, Davis SS, Rogers TG (1992) Epi-fluorescence microscopy and image analysis used to measure diffusion coefficients in gel systems. *J Pharm Pharmacol* 44: 543–549.
34. Bhat PG, Flanagan DR, Donovan MD (1995) The limiting role of mucus in drug absorption: Drug permeation through mucus solution. *Int J Pharmaceutics* 126: 179–187.
35. Niibuchi J-J, Aramaki Y, Tsuchiya S (1986) Binding of antibiotics to rat intestinal mucin. *Int J Pharmaceutics* 30: 181–187.

36. Kearney P, Marriott C (1987) The effects of mucus glycoproteins on the bioavailability of tetracycline. II. Binding. *Int J Pharmaceutics* 35: 211–217.
37. Brown DT, Marriott C, Beeson MF (1983) Antibiotic binding to purified mucus glycoproteins. *J Pharm Pharmacol* 35: 80P.
38. Mantle M, Mantle D, Allen A (1981) Polymeric structure of pig small intestinal mucus glycoprotein. *Biochem J* 195: 277–285.
39. Marshall T, Allen A (1978) Isolation and characterisation of the high molecular glycoprotein from pig colonic mucus. *Biochem J* 173: 569–578.
40. Kearney P, Marriott C (1986) The effects of mucus glycoproteins on the bioavailability of tetracycline. I. Dissolution rate. *Int J Pharmaceutics* 28: 33–40.
41. Marriott C, Cheema MS (1987) Diffusion of water and antibiotics through mucus from normal and disease states. *Eur J Respir Dis* 71 (Suppl 153): 277–278.
42. Brown DT, Marriott C, Beeson MF, Barrett-Bee K (1981) Isolation and partial characterization of a rheologically active glycoprotein fraction from pooled human sputum. *Am Rev Respir Dis* 124: 285–291.
43. Hosein VK, Thakker D, Allen A, Pearson JP (1991) Barrier properties of the adherent gastric mucus gel to pepsin. *Gut* 32: A1246.
44. Bell AE, Allen A, Morris ER, Ross-Murphy SB (1984) Functional interactions of gastric mucus glycoprotein. *Int J Biol Macromolec* 6: 309–325.
45. Edwards PAW (1978) Is mucus a selective barrier to macromolecules? *Brit Med Bull* 34: 55–56.
46. Turner NC, Martin GP, Marriott C (1985) The influence of native porcine gastric mucus gel on hydrogen ion diffusion: The effect of potentially ulcerogenic agents. *J Pharm Pharmacol* 37: 776–780.
47. Allen JD, Martin GP, Marriott C, Hassan I, Williamson I (1991) Drug transport across a novel mucin secreting cell model: Comparison with Caco-2 cell system. *J Pharm Pharmacol* 43: 63P.
48. Williams SE, Turnberg LA (1980) Retardation of acid diffusion by pig gastric mucus: A potential role in mucosal protection. *Gastroenterology* 79: 299–304.
49. Bollister N, Basker M, Hodges NA, Marriott C (1991) The diffusion of beta-lactam antibiotics through mixed gels of cystic fibrosis-derived mucin and *Pseudomonas aeruginosa* alginate. *J Antimicrob Chemother* 27: 285–293.
50. Gordon CA, Hodges NA, Marriott C (1988) Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 22: 667–674.
51. Ramphal R, Lhermitte M, Filliat M, Roussel P (1988) The binding of anti-pseudomonal antibiotics to macromolecules from cystic fibrosis sputum. *J Antimicrob Chemother* 22: 483–490.
52. Martin GP, Loveday BE, Marriott C (1993) Bromohexine plus oxytetracycline: The effect of combined administration upon the rheological properties of mucus from the mini-pig. *J Pharm Pharmacol* 45: 126–130.
53. Pearson JP, Allen A, Parry S (1981) A 70000 molecular weight protein isolated from purified pig gastric mucus glycoprotein by reduction of disulphide bridges and its implication in the polymeric structure. *Biochem J* 197: 155–162.
54. Braybrooks MP, Barry BW, Abbs ET (1975) The effect of mucin on the bioavailability of tetracycline from the gastrointestinal tract: *in vivo, in vitro* correlations. *J Pharm Pharmacol* 27: 507–515.
55. Levy J (1986) Antibiotic activity in sputum. *J Pediatr* 108: 841–846.
56. Helliwell M (1993) The use of bioadhesives in targeted delivery within the gastrointestinal tract. *Adv Drug Delivery Rev* 11: 221–251.
57. Lehr C-M, Pelma FGJ, Junginger HE, Tukker JJ (1991) An estimate of turnover time intestinal mucus gel layer in the rat *in situ* loop. *Int J Pharmaceutics* 70: 235–240.
58. Wikman A, Karlsson J, Carlstedt I, Arturrsson P (1993) A drug absorption model based on the mucus layer producing human intestinal goblet cell line HT29-H. *Pharm Res* 10: 843–852.
59. O'Hagan DT, Critchley H, Farraj N, Fisher AN, Johansen BR, Davis SS, Illum L (1990) Nasal absorption enhancers for biosynthetic human growth hormone in rats. *Pharm Res* 7: 772–776.

60. Franz JM, Vonderscher JP, Voges R (1980) Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes. *Int J Pharm* 7: 19–28.
61. Ricevuti G, Mazzone A, Uccelli E, Gazzani G, Fregnan GB (1988) Influence of erdosteine, a mucolytic agent, on amoxycillin penetration into sputum in patients with an effective exacerbation of chronic bronchitis. *Thorax* 43: 585–590.
62. Sithigorngul P, Burton P, Nishihata T, Caldwell L (1983) Effects of sodium salicylate on epithelial cells of the rectal mucosae of the rat: A light and electron microscopic study. *Life Sci* 33: 1025–1032.
63. Guarini S, Fano RA, Rompianesi E, Martinelli AM, Ferrari W (1986) The effect of sodium deoxycholate given by gavage with heparin on the histology of the intestinal mucosa of the rat. *J Pharm Pharmacol* 38: 922–924.
64. Slomiany BL, Aono M, Murty VLN, Piasek A, Slomiany A (1984) Effect of bile acids on the glycoprotein constituent of gastric mucus. *J Appl Biochem* 6: 308–313.
65. Whitmore DA, Brooks LG, Wheeler KP (1979) Relative effects of different surfactants on intestinal absorption and the release of proteins and phospholipids from the tissue. *J Pharm Pharmacol* 31: 277–283.
66. Martin GP, Marriott C, Kellaway IW (1978) Direct effect of bile salts and phospholipids on the physical properties of mucus. *Gut* 19: 103–107.
67. Heer JS, Roberts CJ, Davies MC, Martin GP, Marriott C (1995) The effect of bile salts on the structure of mucus. *J Pharm Pharmacol* 47: 1125P.
68. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 47: 323–330.
69. Huet C, Sahuquillo-Merino C, Coudrier E, Louvard D (1987) Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT29) provide new models for cell polarity and terminal differentiation. *J Cell Biol* 105: 345–357.
70. Allen J (1992) *A mucus secreting cell co-culture model for drug absorption studies*. PhD Thesis, Brighton Polytechnic, CNAA, Brighton, UK.
71. Hughes DRL (1988) The influence of intestinal mucus on drug absorption. PhD Thesis, Brighton Polytechnic, CNAA, Brighton, UK.
72. Cepinskas G, Specian RD, Kvietys PR (1993) Adaptive cytoprotection in the small intestine: Role of mucus. *Am J Physiol* 264: G921–G927.
73. Drazen JM, O'Cain CF, Ingram RH (1982) Experimental induction of chronic bronchitis in dogs: Effects on airway obstruction and responsiveness. *Am Rev Respir Dis* 126: 75–79.
74. Shore SA, Kariya ST, Anderson K, Skornik W, Feldman HA, Pennington J, Godleski J, Drazen JM (1987) Sulphur-dioxide-induced bronchitis in dogs: Effects on airway responsiveness to inhaled and intravenously administered methacholine. *Am Rev Respir Dis* 135: 840–847.
75. Davis CW, Dowell ML, Lethem MI, Van Scott M (1992) Goblet cell degranulation in isolated canine tracheal epithelium: Response to exogenous ATP, ADP and adenosine. *Am J Physiol* 262: C1313–C1323.
76. Martin GP, Loveday BE, Marriott C (1990) The effect of bromohexine hydrochloride on the viscoelastic properties of mucus from the mini-pig. *Eur Respir J* 3: 392–396.
77. Kim KC, Lee BC (1991) P2 purinoceptor regulation of mucin release by airway goblet cells in primary culture. *Br J Pharmacol* 103: 1053–1056.
78. Doherty MM, Liu J, Randell SH, Carter C, Davis W, Nettesheim P, Ferriola P (1995) Phenotype and differentiation potential of a novel rat tracheal epithelial cell line. *Am J Respir Cell Mol Biol* 12: 385–395.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D.F. Rogers and M.I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 14
Therapeutic Approaches to the Lung Problems in Cystic Fibrosis

Myra Stern* and Eric W. Alton

Ion Transport Unit, National Heart and Lung Institute, London, UK

1. Introduction

Pulmonary disease is almost universal in adults with cystic fibrosis (CF), accounting for 75% of hospital admissions and virtually all the mortality [1]. Clinically the presentation is one of thick mucus retention in the small airways which causes airway obstruction and infection leading to bronchiectasis and ultimately to respiratory failure. Although the lungs of CF babies may be structurally normal at birth, the disease process commences very soon after, and mucus impaction with subsequent infection induces bronchiolitis, cyst formation and bronchiectasis which has been observed at autopsy in patients as young as 1 month old [2]. Pathogens associated

* Author for correspondence.

with the recurrent infections of CF are not generally found in non-CF subjects: *Staphylococcus aureus* is often acquired in the first few years of childhood, and subsequent colonisation of the lungs with non-mucoid strains of *Pseudomonas aeruginosa* is then followed by mucoid strains of this organism. Increased infection and the consequent inflammatory responses lead to airway fibrosis, distal hyperinflation and areas of collapse. There is also goblet cell metaplasia with mucus hypersecretion. Thus, with increasing disease, ventilation-perfusion imbalances lead to hypoxia, pulmonary hypertension and later in the disease, cor pulmonale.

While much is now known about the basic molecular and biochemical defects in CF (see below), the pathogenesis of the pulmonary manifestations is not yet fully elucidated. One possibility is that reduced or absent cyclic adenosine monophosphate (cAMP)-mediated chloride transport in the airway epithelium results in suboptimal airway hydration. Consequently, there is an increase in mucus viscosity detrimental to mucociliary function and which may lead to increased bacterial adherence and colonisation. A further possible explanation relates to the recent observation that lung epithelial cells produce antibiotic-like molecules named defensins [3]. An important characteristic of these agents is dependence of their function on total salt concentration. Thus, although CF cells are able to generate normal levels of these molecules, their function is impaired because of the abnormal salt concentration found in their airway lining fluid. Finally, CF cells show increase numbers of the asialoGM_1 binding sites for *P. aeruginosa* attachment [4]. Thus any one of these, or some combination, may be responsible for the pathogenesis of the disease.

Standard therapy is directed towards physical clearance of sputum and aggressive antibiotic therapy of the lung pathogens. This, combined with pancreatic enzyme supplementation and intensive dietary support, has improved the longevity of CF patients from about 1 year in the 1930s to about 30 years at present. New and experimental approaches to therapy are directed more towards correction of basic, known pathogenic mechanisms and, as described below, include the development of new mucolytic agents, treatment of the ion transport defects associated with CF, suppression of the chronic inflammatory pathways which arise in response to bacterial colonisation and finally gene therapy.

2. Conventional Therapy of Lung Disease in CF

Clearance of pulmonary secretions is achieved by daily physiotherapy undertaken by the parents of children or by adult patients themselves. Specific techniques have been developed to assist clearance, including postural drainage, mechanical percussion, active cycle of breathing techniques, periodic continuous airway pressure an intermittent positive pressure breathing [5]. The short-term benefits of these methods on lung function in

CF have been well documented; longer-term outcomes have not been well studied, but some studies have shown positive results [6].

The objectives of antibiotic therapy against colonising organisms is to decrease bacterial load and thus reduce the inflammatory cycles that are initiated in response to infection. Oral and nebulised antibiotics against *S. aureus* and *P. aeruginosa* are generally used, and there is evidence that this lessens the decline in lung function [7] and reduces the chronic colonisation with *P. aeruginosa* [8]. Aggressive treatment of acute infective exacerbations with a combination of at least two intravenous antipseudomonal agents is thought to have been responsible for improving morbidity and mortality of CF [9].

Bronchodilators may be beneficial in patients who, after administration, demonstrate objective improvement on lung function testing [10, 11]. They may be particularly useful prior to physiotherapy. The use of mucolytic therapy with agents like *N*-acetylcysteine [12, 13] and MESNA (sodium 2-mercaptoethane sulphonate) [14] however, is largely historical.

3. New and Experimental Modalities of Treatment

3.1. Mucolytic Agents

3.1.1. Aerosolised dornase alfa (rhDNase): The development of the mucolytic agent rhDNase for the treatment of CF lung disease has been rapid and represents an important addition to available therapies. It had been known for many years that the increased viscoelasticity associated with CF sputum is, at least in part, due to the release of extracellular DNA by necrotic neutrophils. DNA, itself a highly viscous material, is found in high concentrations (3–14 mg/ml) in purulent but not in non-purulent CF secretions [15, 16]. The use of bovine pancreatic deoxyribonuclease I to cleave neutrophil-derived DNA and thus reduce the viscosity of lung secretions was first tested both *in vitro* and *in vivo* more than 30 years ago [17–22]. Following reports of adverse pulmonary reactions to the enzyme, however [23], it fell into disuse.

The reapplication of the idea of DNA cleavage to the problem of sputum viscosity followed the cloning of the gene for a human DNase I from a pancreatic complementary DNA (cDNA) library using oligonucleotide probes based on the amino acid sequence of bovine pancreatic DNase I [24]. A full-length clone encoding a 260-amino acid protein was isolated and the plasmids transfected into Chinese hamster ovary cells. From the supernatants of these cells, a 37-kDa protein which efficiently cut high molecular weight DNA was purified and shown to have an amino acid sequence identical to the naturally occurring enzyme found in the blood, in pancreatic secretions and in saliva. Initial *in vitro* studies using both a subjective "pourability" assay, and more objective quantification using a

viscometer, confirmed that the enzyme, human recombinant DNase 1 (hrDNase), reduced the viscosity of CF sputum. Briefly, addition of rhDNase (50 μg/ml) but not saline dramatically increased the pourability of CF sputum and similarly reduced its viscoelasticity in a concentration-dependent fashion. Additional comprehensive *in vitro* studies using other viscometric techniques confirmed these effects and showed that rhDNase reduces the adhesiveness and increases mucociliary transportability of CF sputum on the surface of a frog palate [25–27].

The *in vitro* studies were followed by extensive *in vivo* clinical studies in CF patients. Two short-term phase I and two short-term phase II studies in which rhDNase was administered using a simple jet air-driven nebuliser were performed in normal adults and in adults and children with CF [28–31]. In phase I, doses of up to 40 mg/day were examined primarily to assess safety. No acute adverse events were detected, and a significant improvement in spirometry (FEV_1 and FVC) was observed following 6 days of treatment. The phase II studies were randomised double-blind placebo-controlled clinical studies evaluating the efficacy and safety of short-term (10-day) administration of rhDNase. One was carried out in the UK [30] and one in the US [31]. CF patients with mild to moderate respiratory disease (FVC >40% predicted) were enrolled. Treatment groups, randomised in the UK study to receive either placebo or rhDNase (2.5 mg twice daily) and in the US study to receive either placebo or rhDNase (0.6 mg, 2.5 mg or 10 mg), were compared with respect to mean performance in pulmonary function test scores at intervals during and after treatment through to day 42. FEV_1 was the main outcome measure. Secondary outcome measures included the effects of treatment on various quality-of-life measures, including a dyspnoea score, general well-being and CF-related symptoms. Both studies again confirmed safety. Improvement in pulmonary function was evident within 3 days of rhDNase treatment, with FEV_1 increasing significantly in both studies but declining towards baseline after withdrawal of rhDNase. A long-term (24-week), multicentre phase IIb study thus went on the assess the long-term efficacy of intermittent treatment with rhDNase. The data showed that each time therapy was interrupted, pulmonary function returned to baseline, suggesting the importance of regular administration of the enzyme to maintain efficacy.

The primary clinical study in support of rhDNase was a long-term phase III, multicentre, randomised, double-blind, placebo-controlled trial with a parallel group design and open-label extension of 24 weeks [32]. It was conducted at 51 CF centres in the US, and evaluated 943 patients randomised to three treatment groups: rhDNase 2.5 mg daily, rhDNase 2.5 mg twice daily or placebo. The objectives of the study were broadened to include not only confirmation of the spirometric improvement demonstrated in the phase I and II studies, but also the effects of rhDNase in reducing respiratory tract infective exacerbations requiring parenteral antibiotics, as

well as on quality-of-life measures and safety. The study population was examined in subgroups of patients defined by age, gender, baseline FEV_1, body mass index and concurrent use of physiotherapy, bronchodilators and prophylactic antibiotics. This study confirmed that rhDNase significantly improved FEV_1 from baseline as compared with placebo, with a mean improvement over 24 weeks of approximately 5.7 ($p < 0.001$). It further demonstrated that rhDNase reduces the incidence of respiratory tract infective exacerbations requiring parenteral antibiotics. Compared with placebo the risk was reduced in the once-daily and twice-daily dosing groups by 28% ($p = 0.04$) and 37% ($p < 0.01$), respectively. The rate of hospitalisations, the number of days missed from school or work, and the frequency of CF-related symptoms were also reduced regardless of patient age, gender or baseline lung function. No major adverse events were documented during the 24-week study period, and in particular, there was no anaphylaxis. Mild, transient adverse effects of rhDNase were limited to upper airway irritation (voice alteration, pharyngitis, laryngitis), rash, chest pain and conjunctivitis. A small number of patients (2–4%) developed serum antibodies to rhDNase, but this was not associated with decreased clinical efficacy of the treatment.

All patients who completed the double-blind study were eligible to go on to participate in a 24-week open-label extension, receiving rhDNase, 2.5 mg twice daily. This confirmed the original findings, showing that the improvements in pulmonary function and the reduction in infective exacerbations was maintained for at least the 48 weeks of treatment studied.

The studies described above involved patients with mild or moderate disease ($FEV_1 \geq 40\%$ predicted) and for patients ≥ 5 years. Although to date there have been no further studies of paediatric patients with CF under 5 years, a short-term (14-day) randomised placebo-controlled study of 70 patients with severe pulmonary disease ($FEV_1 < 40\%$) has been completed. The initial 2-week study confirmed only safety in this population of patients, while significant improvements in pulmonary function compared with placebo were not demonstrated. However, 64 patients continued to participate in a 24-week open extension to the trial on a twice-daily dose of rhDNase. A mean improvement in FEV_1 and FVC of 11.4 and 22.8%, respectively, was documented in this phase of the study, suggesting that sicker patients may need longer periods of treatment to show significant benefit.

In summary, extensive clinical trial data have established the efficacy and safety of nebulised rhDNase, and it has become widely incorporated into standard therapy for CF. A dose of 2.5 mg daily is recommended for most patients, although some (>21 years, <85% FVC) benefit from a twice-daily regime. The treatment needs to be administered regularly to maintain clinical benefit, and in patients with more severe disease, longer initial periods of treatment may be required to establish clinical improvement.

3.1.2. Recombinant bacterial alginate lyase (rbAlginase): The opportunistic pathogen *P. aeruginosa* is commonly isolated from the airways of patients with cystic fibrosis. Typically the airways are initially colonised with non-mucoid strains [33, 34], and after a variable period, usually 1–2 years, mucoid strains emerge that produce large quantities of the large molecular weight encapsulating exopolysaccharide, alginate. Alginate is resistant to human polysaccharide metabolism, and it accumulates in CF sputum, reaching levels of up to 200 μg of alginate per ml of sputum [35, 36]. Compared with non-mucoid strains, infection with mucoid strains is associated with poorer clinical state and lower pulmonary function [37, 38] and intractable infections which are almost impossible to eradicate [39]. Alginate has been implicated as a virulence factor in this colonisation, facilitating bacterial adherence to epithelial cells of the lungs [40, 41], limiting antibiotic diffusion [42], acting as a free-radical scavenger [43] and displaying both immunosuppressive and antiphagocytic properties [44–49], seemingly to evade host defences. Alginate is also thought to produce an increase in the viscoelasticity of CF sputum which, as described for neutrophil-derived DNA, has been suggested to contribute both to the establishment and persistence of pulmonary infections in CF.

Alginate-degrading enzymes have been isolated from many sources, including marine algae and molluscs and micro-organisms. The latter have proved to be a prolific source of alginases, with enzyme activity detectable *in vitro* in some species of alginate-producing bacteria, including *P. aeruginosa* [50]. However, it has not been clearly demonstrated that the enzyme is expressed or functional in the environment of purulent CF airway secretions. Many other non-alginate-producing bacteria produce inducible alginase which in many cases has been purified, for example from *Vibrio harveyi* and *Vibrio alginolyticus* [51] and from *Klebsiella aerogenes* and *Haliotis* [52]. Furthermore, the gene for the alginase produced by *Klebsiella pneumonia* has been cloned [53] and overexpressed in *Escherichia coli* under the control of the *lac* promoter [54]. Both the purified and recombinant forms of the enzyme have been shown *in vitro* specifically to degrade alginates, which has raised the possibility of using exogenous alginase degrade alginate in CF *Pseudomonas*-infected sputum – theoretically a powerful strategy for therapeutic intervention. Data from a number of *in vitro* studies support this idea. Purified alginase has been shown to inhibit the adherence capacity of mucoid but not non-mucoid strains of *P. aeruginosa*, to act synergistically with antibiotics against the bacteria [55] and to enhance phagocytosis of mucoid strains of *P. aeruginosa* [56]. However, in a study, which specifically investigated the use of exogenous alginase to disrupt alginate and effect a change in the rheological properties of CF sputum *in vitro*, only limited success was achieved [36]. Addition of the enzyme purified from a mucoid strain of *P. aeruginosa* to CF sputum led to disruption of alginate and a change in the viscoelastic properties in

only a small percentage of the samples studied, and the effects, where positive, were limited. The data indicated that the ability of the enzyme to disrupt sputum alginate may be substantially reduced by the presence of high levels of Zn^{2+} and/or Ca^{2+} present in the secretions, and that cations "bound" to the complex gel matrix may further impede the actions of alginase. In a separate study which investigated the effects of alginase on CF sputum-mediated inhibition of gene transfer efficiency *in vitro*, addition of the enzyme to sputum-covered epithelial cell monolayers prior to transfection was not shown to improve gene transfer [57].

The results of the two latter studies seem to indicate that, in practice, the potential for application of alginase either as a therapy for CF or as adjunctive therapy for gene transfer in CF is limited. To date there have been no further reported *in vitro* studies to support the instigation of clinical trials of recombinant bacterial alginase for CF patients.

3.1.3. Gelsolin: Filamentous actin derived from degenerating leukocytes in CF sputum is thought to be another contributor to its increased viscosity. Actin comprises 10% of total leukocyte protein [58] and forms long protease-resistant filaments that are highly viscoelastic [59]. Sputum samples from CF patients have been shown to contain filamentous actin, and the addition to the sputum of gelsolin, a plasma-derived protein that rapidly severs non-covalent bonds within actin filaments [60], rapidly decreases the viscosity of the sputum *in vitro* [61]. Plasma gelsolin is a normal constituent of extracellular fluids and in concert with Gc-globulin is thought to scavenge actin released from cells during inflammation. Because inflamed airways contain plasma proteins, it is likely that plasma gelsolin is naturally present in CF airways, but in insufficient quantities to break down the actin released from the very high accumulation of necrotic leukocytes. The use of exogenous gelsolin as a potential mucolytic agent for treatment of CF has thus been raised.

DNase 1 and Gc-globulin, both monomer-binding proteins, can also shorten actin filaments by preventing monomers from adding back onto filaments after they dissociate. This action may even in part contribute to the ability of rhDNase to reduce the viscosity of CF sputum, but compared with the severing of actin filaments by gelsolin, the mechanism is very inefficient. Furthermore, purified actin has been shown *in vitro* to inhibit the DNA-hydrolysing activity of DNase 1 [62]. These observations nevertheless raise the possibility of a doubly useful role for gelsolin as a mucolytic agent in CF, first, by virtue of its direct proteolytic action on actin and, second, by its ability to enhance DNase activity by facilitating clearance of actin. Synergism between gelsolin and hrDNase in reducing CF sputum viscosity has been documented in two separate studies [61, 63].

To date, no data on the use of gelsolin *in vivo*, have been reported, nor have there been reports of any clinical trials.

3.2. Treatment of Ion Transport Defects

As previously described, the thickened airway secretions characteristic of CF have been attributed to the abnormal ion transport functions which reflect mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-mediated chloride channel in the apical membrane of airway and intestinal epithelial cells [64–66]. These cells exhibit excessive sodium (and thus water) absorption [67, 68] as well as limited chloride (and thus water) secretion via CFTR in response to cAMP-mediated stimuli [64, 69, 70]. However, secretion of chloride via a population of calcium-mediated chloride channel remains intact [70]. Although the link between the ion transport defects and the clinical manifestations of CF remains essentially speculative, it has been reasonably hypothesised that the defects result in suboptimal hydration of the airway mucosal surface, thus contributing to abnormal rheology of airway secretions and abnormal mucociliary clearance [71]. Pharmacological agents which target and modulate these defects *in vitro* are therefore being tested in clinical trials with a view to developing novel therapies that might be particularly useful in children with CF prior to the onset of irreversible lung disease. The agents under investigation may be divided into those which modulate the increased sodium absorption and those which modulate defective chloride secretion.

3.2.1. Modulation of increased sodium absorption: Amiloride: Amiloride has been shown both *in vivo* and *in vitro* to inhibit sodium transport across normal epithelial cells [72] as well the hyperabsorption of sodium characteristic of CF [67, 68]. These observations underlie the hypothesis that inhalation of aerosolised amiloride *in vivo* might inhibit excessive sodium and water absorption in CF airway epithelia and thus improve the rheology and clearance of airway secretions. Initial *in vivo* studies in sheep confirmed that it was possible to deliver sufficient quantities of aerosolised amiloride directly to the airways to inhibit sodium and water absorption [73], and the earliest human clinical studies of inhaled amiloride confirmed safety [74]. Further studies demonstrated improved pulmonary mucociliary clearance following administration of inhaled amiloride, both acutely [71] and over a 3-week period [75]. A 24-week double-blind crossover study of nebulised amiloride in a small number of CF patients followed [76]. The data which emerged suggested that amiloride improved the rheological and clearance properties of the sputum and lessened the decline in lung function seen when CF patients were taken off all other treatments. However, these encouraging results were not sustained in a number of further clinical trials unable to document additional beneficial effects of amiloride when added to standard current therapy [77–79]. Clinical trial data on the use of amiloride in paediatric patients with CF remains limited, although safety has been confirmed in two reported studies [80, 81]. The outcome of

a Glaxo Pharmaceuticals-sponsored multicentre, placebo-controlled clinical study of the safety and efficacy of amiloride in CF patients down to the age of 10 years is still pending.

3.2.2. Modulation of impaired chloride secretion: ATP and UTP: In a view of emerging clinical trial data which suggest that inhaled amiloride has limited efficiency in arresting decline of lung function, strategies aimed at targeting the CF chloride secretion defect together with the sodium absorption defect are now being investigated. To this end, agents which stimulate chloride secretion through calcium-mediated pathways, which as mentioned above remain intact in CF, are being exploited. The triphosphate nucleotides [adenosine triphosphate (ATP) and uridine triphosphate (UTP)] are two such agents which induce chloride secretion across human airway epithelia *in vitro* and *in vivo* [82–84]. After pretreatment with amiloride, addition of ATP or UTP to the nasal epithelium *in vivo* stimulates chloride secretion in both normal and CF subjects via an alternative (non-CFTR) population of chloride channels that are calcium-activated [82, 85]. The duration of action is relatively short *in vivo* (approximately 60% of maximal effect for 6 min [85]). In addition to their effects on chloride secretion, both agents also stimulate ciliary beat frequency and goblet cell degeneration in canine and human airway epithelium [86]. These cellular effects suggest a further role for their use as therapeutic agents in CF.

Because both ATP and adenosine, the nucleoside metabolite of ATP, induce bronchoconstriction in asthmatic patients, ongoing studies of inhaled nucleotides in human clinical trials have focused on UTP. Neither UTP nor its nucleoside metabolite uridine are associated with this problem. Initial studies have demonstrated that aerosolised UTP is safe after short-term and long-term dosing in animals [87], and short-term dosing in normal and CF human subjects [88]. Further clinical trials have demonstrated that aerosolised UTP, with or without amiloride, stimulates mucociliary clearance in both normal and CF subjects [89, 90]. In a more recent study, the effects of aerosolised UTP and amiloride, alone and in combination, on the mucociliary clearance of radiolabelled particles in adult patients with CF were documented [91]. There were no clinically significant adverse effects from therapeutic doses of UTP/amiloride, while mucociliary clearance rates from the peripheral airways of the patients increased to near-normal values during the 40-min period studied. Further studies are in progress to determine the pharmokinetics, safety and efficacy of UTP/amiloride on airway surfaces of both adults and children with CF.

3.3. Novel Anti-inflammatory Agents

Evidence suggests that persistent infection with organisms such as *S. aureus* and *P. aeruginosa* evokes a continuous host inflammatory response that not

only fails to eradicate the organisms but also produces a cycle of immune hyper-responsiveness as well as neutrophil- and cytokine-mediated tissue damage in the lungs (reviewed in [92]). A number of therapeutic approaches based on interruption of these processes are being investigated. These include studies on the use of generalised anti-inflammatory agents like corticosteroids and the non-steroidal anti-inflammatory drug ibuprofen. The theoretical application of more specific agents targeted against neutrophil elastase or inflammatory cytokines like tumor necrosis factor are also under investigation.

3.3.1. Corticosteroids: Prednisolone has been used for many years with benefit in CF patients with associated allergic bronchopulmonary aspergillosis, evidence of immune-mediated arthritis and with resistant bronchospasm. It has not, however, been used routinely in the management of CF. One trial has suggested that at a dose of 2 mg/kg given on alternate days, prednisolone produced a reduction in respiratory problems and improved lung function [93]. A follow-up study, however, has demonstrated that side-effects of this treatment outweigh benefits [94]. However, as the potent anti-inflammatory action of corticosteroids remains a good rationale for therapy and the inhaled route is likely to cause fewer side-effects, the benefits of using inhaled steroids on a long-term basis are currently being investigated. Large, randomised placebo-controlled trials of inhaled fluticasone and budesonide have been undertaken in London and Copenhagen, respectively, and the results are pending.

3.3.2. Ibuprofen: Because of the side-effects associated with corticosteroids, non-steroidal anti-inflammatory agents have been considered as an alternative. Ibuprofen in high doses has been shown to inhibit migration, adherence and aggregation of neutrophils as well as the release of lysosomal enzymes [95–99]. Furthermore, in a rat model that mimics CF, high-dose ibuprofen significantly reduced lung inflammation without increasing the burden of *P. aeruginosa* [100].

The first double-blind human clinical trial of ibuprofen for CF has recently been reported [101]. 85 CF patients with mild lung disease (FEV_1 > 60% predicted) were randomised to receive high-dose ibuprofen or placebo orally twice daily for 4 years. FEV_1 was selected as the primary outcome measure, although percentage of ideal body weight, a chest X-ray score and the frequency of hospital admissions were also assessed. The data suggested that ibuprofen, taken consistently for 4 years, was safe and that it significantly slowed progression of lung disease in CF patients over 5 years of age in whom disease was mild from the outset. Of note, however, was that this effect was particularly evident in patients who completed the treatment and who were initially less than 13 years old. This group consisted of a very small sample of only 36 patients. Thus, whilst the results are encouraging, further studies are required to confirm the benefits of this agent.

3.3.3. α1-Proteinase inhibitor: Sputum from CF patients contains large numbers of neutrophils and high concentrations of neutrophil-derived proteinases [102]. Important amongst these is elastase, which is released during neutrophil activation, phagocytosis and necrosis [103]. Although healthy subjects have sufficient antiproteinases such as α1-proteinase inhibitor (α1-PI) and secretory leukoprotease inhibitor [104], the anti-elastase defence in CF airways is overwhelmed, resulting in unopposed elastase activity [105]. Through unihibited proteolysis and elastolysis, elastase is associated with progressive lung destruction [106] and, in addition, is an extremely potent secretagogue for airway submucosal glands [107]. Thus, CF sputum has been shown to induce a considerable secretory response from cultured tracheal gland serous cells.

A recently reported *in vitro* study which quantitatively investigated the interaction of CF sputum with exogenous α1-PI demonstrated a dose-related inhibition of neutrophil elastase activity by α1-PI, which also inhibited purified elastase- or CF sputum-induced secretion by porcine tracheal glands [108]. These results suggest that administration of exogenous antiproteinases like α1-PI might be a reasonable approach to therapy for CF. To date, however, there have been no reported *in vivo* studies to confirm this hypothesis.

3.3.4. Tumour necrosis factor-α and leukotrienes: Tumour necrosis factor (TNF) is a monocyte/macrophage-derived cytokine that is secreted in response to a variety of infectious and inflammatory stimuli and plays a central role in the orchestration and amplification of the ongoing inflammatory cycle. It is strongly chemotactic for neutrophils and can induce their degranulation [109, 110]. It also upregulates and primes human-5-lipoxygenase *ex vivo* [111]. Leukotrienes, which are arachidonic acid products of the 5-lipoxygenase pathway, are present in CF sputum at concentrations capable of causing mucosal inflammation and bronchial hyperresponsiveness [112]. It has subsequently been reported that TNF-α is itself elevated in CF sputum, even at times of apparent clinical stability, and that there is a significant inverse correlation between the levels of this cytokine and the lung function of the CF patients from whom the sputa were obtained [113]. These findings support a hypothesis that the use of 5-lipoxygenase inhibitors, specific leukotriene receptor antagonists or TNF-α antagonists might constitute a novel approach to CF therapy. Again, this possibility remains theoretical, with no reported *in vivo* studies to confirm its validity.

3.4. Gene Therapy

The cloning of the CF gene and the characterisation of the protein for which it codes (CFTR) [114–116] has raised the possibility of *in vivo* gene

therapy. Theoretically, early delivery of sufficient normal *CFTR*-cDNA to numbers of affected cells should result in the production of enough normal protein to prevent the major clinical manifestations of the disease. In practice, many important issues need to be resolved before gene therapy can be used to manage CF. What is the normal pattern of CFTR, and must a similar pattern be produced to achieve clinical benefit? Which organs or cells should be targeted, and what proportion of these cells need to express the protein for clinical benefit? What is the safest and most efficient way of transferring *CFTR*-cDNA to somatic cells, and how best may the gene be delivered to patients? How often will administration need to be repeated to sustain clinical benefit, and would over-expression of CFTR be deleterious in any way?

3.4.1. Specific cell targeting and expression: The use of *in situ* hybridisation to detect mRNA and the use of antibodies to detect protein have provided extensive data on the normal pattern of expression of CFTR in humans. These studies have focused particularly in the lung as the site of major morbidity in CF and, therefore, to be the obvious organ to target for gene therapy.

CFTR is expressed in the fetal lung from the first trimester onwards, but varies in amount and localisation and decreases after birth. In the adult lung, the highest level of expression occurs in the submucosal glands [117]. Expression in the surface epithelium is much less, apart from a subpopulation of both ciliated and non-ciliated cells in the peripheral airways, although the precise cell types are as yet unidentified [118]. Of interest is the observation that, overall, expression of CFTR in the lung is very low.

The elucidation of these patterns of expression has a number of implications for cell targeting in gene therapy for CF. First, despite the high level of expression of CFTR in the airway epithelium of normal foetal lungs *in utero,* lung development, as judged by light microscopy and pulmonary function, is essentially normal in CF newborns [119]. Thus, prenatal expression may not be necessary. Second, although expression of CFTR is low in the epithelium of the distal airways, the site of these cells corresponds to the development and progression of pathology in CF lungs. This observation has led investigators to target the epithelium of the distal airways for gene therapy. It is an assumption which may, however, prove to be incorrect. Although submucosal glands lie relatively distant from the site of CF pathology, they have the highest expression of CFTR and might well prove to be the appropriate site to target. Third, the fact that, overall, expression of CFTR is very low may suggest that achieving expression in only a small number of cells will be required for clinical benefit. The observation that heterozygotes have no lung pathology supports this optimistic hypothesis, as do some further studies. *In vitro* experiments with cultured CF airway epithelial cells studied before and after transfection with normal CFTR indicate that expression in only 6–10% of cells in a monolayer

corrects the chloride defect to the same level as expression in 100% of the cells [120]. A further study in which different transgenic CF mice were interbred both with each other and with wild-type mice produced animals with *CFTR*-mRNA levels ranging from 0% in the null mice of 100% in wild types [121]. Correlation between the phenotypic features of the mice and their level of normal CFTR revealed large increases in both chloride transport as well as in survival of the animals with very small increments in levels of normal *CFTR*-mRNA. This study further supports the possibility that low levels of successful gene transfer to correctly targeted cells may be required to achieve clinical benefit in CF. Nevertheless, the exact number or type of airway cells requiring transfection remains unclear and much debated.

Targeting other organs involved in CF pathology, for example the pancreas and intestinal tract, remains of secondary importance, since these are at present neither the site of life-threatening pathology nor without reasonably effective therapy.

3.4.2. Gene delivery systems: The two main approaches currently used are based on adenoviruses and cationic liposomes. Adeno-associated viruses [122], retroviral vectors [123], receptor-mediated gene transfer [124] and mammalian artificial chromosomes [125] also remain under investigation.

3.4.2.1. Adenovirus vectors. This family of viruses, which normally cause infections of the upper respiratory tract, can be modified for gene therapy by replacing or inactivating the genes for viral replication. The adenovirus genome is linear and double-stranded, and can accommodate foreign genes of up to 7.5 kb in length. The gene of interest may be incorporated into the viral genome to allow its introduction and expression into the relevant cells. For use as gene therapy vectors, the E1 and E3 gene regions are deleted, and the vectors are packaged by growth in the human embryonic kidney helper cell line, which expresses the E1 (replicative) function constitutively [126]. The advantages of adenoviruses include their natural tropism for epithelial cells and a high efficiency of gene transfer both *in vitro* [127] and *in vivo* [128]. There are no known associations of human malignancies with adenoviral infection.

There are, however, some disadvantages associated with this system, including their transient nature of expression, since the adenovirus does not integrate into the cell's genome and will be lost as the epithelial cells undergo regular replacement. This would imply that even successful gene transfer would need to be routinely repeated to sustain clinical benefit, and repeated application might prove difficult or inefficient in the face of risks of viral recombination to a replication-competent virus, the induction of cellular immunity [129, 130] or the production of neutralising antibodies [131]. It is now also clear that inflammation developes in a dose-related manner in response to increasing adenoviral titre administered [128]. This

may relate to the antigenicity of viral coat proteins and the cytotoxic T-cell response.

3.4.2.2. Cationic liposomes. Liposome-mediated gene transfer has emerged as another promising approach to gene delivery. Cationic liposomes spontaneously bind DNA and fuse to cell membranes [132]. They have been used to transfer several genes into cells both *in vitro* and *in vivo* [133–135]. Liposomes, although cytotoxic at high doses *in vitro*, are generally thought to be less toxic and therefore safer than viral vectors, but early concerns about their use related to variable and less efficient gene transfer efficiency. This concern has not altogether been borne out. Studies using one such lipid composed of 3β[*N*-(*N*,N^1-dimethylaminoethanecarbamoyl)] cholesterol dioleoyl phosphatidylethanolamine (DC-Chol/DOPE) shows high transfection efficiency and low cytotoxicity [136], and no autoimmune reaction or transfer of DNA into gonadal cells was observed after repeated injection of plasmid DNA-DC-Chol/DOPE in mice [137]. In studies in mice and rats using either nebulisation [133] or tracheal instillation [134] of liposome-DNA complexes, the airway epithelium was shown to be the major target of gene transfer, and expression of the reporter gene could be detected 3–4 weeks after administration without any apparent toxicity. A study of the effects of DC-Chol/DOPE on the nasal epithelium of normal volunteers showed no evidence of cytotoxicity [138], and in a double-blind randomised trial of DC-Chol/DOPE-mediated gene transfer to the nasal epithelium of CF patients [139], there were no adverse systemic effects nor histological evidence of local damage.

3.4.3. Administration of the gene delivery system: Delivery of the gene complex directly to the airways offers the opportunity to reach the respiratory epithelium in a highly targeted way. Although many current trials still use instillation into the airway, nebulisation is likely to offer a less invasive and more acceptable way of achieving direct airway delivery, especially if application needs to be repeated routinely. Nebulisation, which is already widely used for delivery of antibiotics, bronchodilators and DNase in CF, has the advantage of widespread deposition, and is likely to reach distal airway epithelial cells. The disadvantage of this approach is that it may well be ineffective at reaching submucosal glands, should these be important targets. Concerns that the nebulisation process itself might damage the functional integrity of the gene complex has not been borne out by an *in vitro* study using the reporter gene β-galactosidase complexed with DC-Chol/DOPE with nebulisation of the complexes onto normal and CF airway cell lines [140]. Concerns about the inhibitory effects of thick CF airway secretions on the efficiency of topical gene transfer to the airways are justified, and the use of DNase as adjunctive treatment prior to nebulised gene transfer has been shown to be beneficial *in vitro* [140].

3.4.4. Detecting the effects of gene transfer: It is clearly important to be able to monitor the efficacy of gene transfer. Detection of *CFTR*-mRNA and CFTR protein provide evidence of successful gene transfer but no indication of whether the protein has remained functional. Measurement of potential difference (PD), which reflects the ion transport functions of CFTR and reliably distinguishes CF from non-CF cells, provides an index of functional correction in the nasal epithelium following gene transfer [141, 142]. Lower airway measurements of PD are technically more difficult, but current studies are underway to establish this technique [143]. A number of other techniques, for example Ussing chambers [144], patch clamping [145] and epifluorescence using the halide-sensitive fluorophore SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium) [146] also allow functional analysis of cells obtained from either nasal or bronchial epithelium by brushing. A recent study [147] has demonstrated that nasal and bronchial cells isolated by brushings can be used within 1 h after removal from the subject for SPQ studies. The cells demonstrate the characteristically reduced cAMP-mediated chloride secretion but preserved calcium secretion of CF. Furthermore, liposome-mediated transfer of normal copies of *CFTR*-cDNA *in vitro* was shown to correct the defect towards normal. Thus, this technique might prove a convenient assay for gene therapy assessment, particularly in studies involving the lower airways.

Finally, measurements that reflect clinical benefits of gene transfer might include end points relating to susceptibility to infection by organisms such as *P. aeruginosa*, which is thought to adhere preferentially to CF cells [148, 149]. Assays of bacterial adherence before and after gene transfer are currently being developed [150]. The effects of gene transfer on mucus rheology and mucociliary clearance have not to date been studied, but might prove an important clinical outcome measure following gene therapy.

3.4.5. Progress to date:

3.4.5.1. Studies in cell lines and in animals. Since the earliest reports of successful transfer of *CFTR*-cDNA to epithelial cells *in vitro* [151, 152], further studies using different gene delivery systems both *in vitro* and *in vivo* have confirmed proof of principle. Thus, transfer of a normal copy of the transgene corrects the chloride defect. With respect to *in vivo* studies, Crystal et al., using both adenovirus [153] and liposome-mediated [134] gene transfer, established that the CF gene could be expressed in the airways of mice *in vivo*. Instillation of the transgene into the lungs was followed 24 h later by detection of *CFTR*-mRNA, which could still be detected up to 4 weeks later. Adenoviral-mediated gene transfer was also successfully demonstrated in the epithelium of bronchial xenografts in immune-deficient mice [154]. Further, transgenic CF mice generated in a number of separate laboratories have been used to demonstrate that resto-

ration of the cAMP-mediated chloride secretion can be achieved following direct instillation of a liposome-DNA complex into the trachea [155] or nebulisation of a liposome-DNA complex into mouse lungs [156]. A number of studies using non-human primates have also reported positive results, using both reporter genes and *CFTR*-cDNA [128, 157, 158] with transgene expression, albeit patchy, seen throughout the airways, including the alveoli.

3.4.5.2. Studies in humans. The first reported study of gene therapy in CF patients [159] used adenovirus to transfer *CFTR*-cDNA to the nasal epithelium of three CF volunteers. The results were encouraging in that *CFTR*-mRNA was detected in two of the three subjects, and a small degree of functional correction of the bioelectric abnormality associated with the defective chloride transport was documented over a 10-day period following treatment. A degree of localised inflammation around the site of application was thought to be secondary to the method of delivery. Nevertheless, it is possible that this could itself have been responsible for the bioelectric changes that were documented.

The second reported study [160] assessed both upper- and lower-airway application of adenoviral-mediated *CFTR*-cDNA transfer to four CF subjects. No measures of CFTR function were included, but *CFTR*-mRNA was present in one of the four nasal specimens, and CFTR protein was detected in one of the nasal and one of the bronchial specimens. However, the patient who received the highest dose developed fever, hypotension and an inflammatory reaction in the lungs. This resolved over the course of a month and was thought to be due to an increase in interleukin-6. This trial thus demonstrated some of the difficulties relating to gene therapy, particularly to the lower airways: safety, efficiency and relevant end points to assess.

A double-blind, randomised, placebo-controlled study of liposome-mediated *CFTR*-cDNA transfer to the nasal epithelium of 15 ΔF508 homozygote CF patients has also been completed [139]. No safety problems were encountered, assessed either clinically or by blinded analysis of nasal biopsies. Both plasmid DNA and *CFTR*-mRNA were detected from the nasal biopsy specimens of five of the eight treated subjects. Functionally, the chloride secretion defect showed a significant improvement of 20% towards the normal values and in two of the treated patients reached values within the normal range. These changes could be detected for approximately 7 days.

Finally, the most recently published study of 12 patients who received escalating doses of adenoviral-CF gene complexes to the nose [161] demonstrated no toxicity at low doses but also no evidence of successful gene transfer at low doses. At high doses, the transgene could be detected in about 1% of cells, while at the highest dose there was evidence of mucosal inflammation and no evidence of functional correction.

Thus, it would seem that the data to date reflect both proof of the gene transfer principle but also illustrate the many difficulties that remain to be resolved and the challenges which lie ahead. A further 11 clinical trials are either planned or under way. These efforts and the associated developments in technology reflect a great impetus towards the search for a definitive treatment for CF lung disease, and it is likely that some clinical benefit will ensue, particularly if it becomes possible to institute therapy prior to the onset of lung damage.

Acknowledgements

This work was supported by the Cystic Fibrosis Trust, UK. E.A. is a Wellcome Trust Senior Clinical Fellow.

References

1. Penketh ARL, Wise A, Mearns MB, Hodson ME, Batten JC (1987) Cystic fibrosis in adolescents and adults. *Thorax* 42: 526–532.
2. Bedrosian CW, Greenberg SD, Singer DB, Hansen JJ, Rosenberg HS (1976) The lung in cystic fibrosis: A quantitative study including prevalence of pathologic findings among different age groups. *Hum Pathol* 7: 195–196.
3. Smith JJ, Travis SM, Greenberg EP, Welsh MJ (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85: 229–236.
4. Saiman L, Prince A (1993) *Pseudomonas aeruginosa* pili bind to asialoGM_1 which is increased on the surface of cystic fibrosis epithelial cells. *J Clin Invest* 92: 1875–1880.
5. Pryor JA, Webber BA (1992) Physiotherapy in cystic fibrosis: Which technique? *Physiotherapy* 78: 105–108.
6. Reisman JJ, Rivington-Law B, Corey M (1988) Role of conventional physiotherapy in cystic fibrosis. *J Padiatr* 113: 632–636.
7. Hodson ME, Penketh ARL, Batten JC (1981) Aerosolised carbenicillin and gentamicin treatment of pseudomonas aeruginosa infection in patients with cystic fibrosis. *Lancet* ii: 1137–1139.
8. Littlewood JM, Miller MG, Ghoneim AT, Ramsden CH (1985) Nebulised colomycin for early pseudomonas colonisation in cystic fibrosis. *Lancet* i: 865–869.
9. Littlewood JM, Smye SW, Cunliffe H (1993) Aerosol antibiotic treatment in cystic fibrosis. *Arch Dis Child* 68: 788–792.
10. Ormerod LP, Thompson RA, Anderson CM, Stableforth DE (1980) Reversible airway obstruction in cystic fibrosis. *Thorax* 35: 768–772.
11. Avital A, Sanchez I, Chernick V (1992) Efficacy of salbutamol and ipratropium bromide in decreasing bronchial hyperreactivity in children with cystic fibrosis. *Pediatr Pulmonol* 13: 34–37.
12. Lorin MI, Denning CR (1978) Cystic fibrosis. In: Scarpellil EM, Auld PAM, Goldman HS (eds.) *Pulmonary disease of the fetus, newborn and child.* Philadelphia: Lea & Febiger, 306.
13. Mearns M (1979) Aerosol therapy in cystic fibrosis. *Paedatr Fortbildk Praxis* 48: 76–93.
14. Weller PH, Ingram D, Preece MA, Matthew DJ (1980) Controlled trial of intermittent aerosol therapy with sodium-2-mercaptoethanesulphonate in cystic fibrosis. *Thorax* 35: 32–36.
15. Chernick WS, Barbero GJ (1959) Composition of tracheobronchial secretions in cystic fibrosis of the pancreas and bronchiectasis. *Pediatrics* 24: 739–745.
16. Potter J, Mathews LW, Lemm J, Spector JS (1960) Composition of pulmonary secretions from patients with and without cystic fibrosis. *Am J Dis Child* 199: 493–495.

17. Armstrong JB, White JC (1950) Liquification of viscous purulent exudates by deoxyribonuclease. *Lancet* 2: 739–742.
18. Chernick WS, Barbero GJ, Eichel JH (1961) *In vitro* evaluation of effect of enzymes on tracheobronchial secretions from patients with cystic fibrosis. *Pediatrics* 27: 589–596.
19. Elmes PC, White JC (1953) Deoxyribonuclease in the treatment of purulent bronchitis. *Thorax* 8: 295–300.
20. Salomon A, Herschfus JA, Segal MD (1954) Aerosols of pancreatic dornase in bronchopulmonary disease. *Ann Allergy* 12: 71–79.
21. Spier R, Witebsky F, Paine JR (1961) Aerosolised pancreatic dornase and antibiotics in pulmonary infections. *JAMA* 178: 878–886.
22. Lieberman J (1968) Dornase aerosol effect on sputum viscosity in cases of cystic fibrosis. *JAMA* 205: 303–313.
23. Raskin P (1968) Bronchospasm after inhalation of pancreatic dornase. *Am Rev Respir Dis* 98: 597–598.
24. Sahk S, Capron DJ, Hellniss R, Masters SA, Baker CL (1990) Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc Natl Acad Sci USA* 87: 9188–9192.
25. Rubin BK, Ramirez OE, Baharav AL (1993) The physical and transport properties of CF sputum after treatment with rhDNase. *Pediatr Pulmonol* 16(Suppl 9): 251.
26. Tomkiewicz RP, Shak S, King M (1993) Effects of rhDNase on cystic fibrosis sputum viscosity *in vitro*. *Pediatr Pulmonol* 9(Suppl): A251.
27. Sha PL, Ingham S, Marriott C, Scott SF, Hodson ME (1994) The *in vitro* effects of two novel drugs on the rheology of cystic fibrosis and bronchiectasis sputum. *Eur Resp J* 7 (Suppl 18): 12S.
28. Aitken ML, Burke W, McDonald G, Villalom M, Shak S, Montgomery B, Smith AL (1992) Effect of inhaled recombinant human DNase on pulmonary function in normal and cystic fibrosis patients: Phase I study. *JAMA* 267: 1947–1951.
29. Hubbard RC, McElvaney NF, Birrer P, Shak S, Robinson WWK, Jolley C, Wu M, Chernick S, Crystal RG (1992) A preliminary study of aerosolised recombinant human deoxyribnuclease 1 in the sputum of cystic fibrosis. *N Engl J Med* 326: 8125–8132.
30. Ranasinha C, Assoufi B, Shak S, Christiansen D, Fuchs H, Empey D, Geddes D, Hodson M (1993) Efficacy and safety of short-term administration of aerosolised recombinant human DNase 1 in adults with stable stage cystic fibrosis. *Lancet* 342: 199–202.
31. Ramsey B, Astley SJ, Aitken ML, Burke W, Colin AA, Dorkin HL, Eisenberg JD, Gibson RL, Harwood IR, Schidlow DV et al. (1993) Efficacy and safety of short-term administration of aerosolised recombinant human deoxyribonuclease in patients with cystic fibrosis. *Am Rev Respir Dis* 148: 145–151.
32. Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, Rosenstein BJ, Smith AL, Wohl ME (1994) Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. *N Engl J Med* 331: 636–642.
33. Doggett RG, Harrison GM, Stillwell RN, Wallis ES (1966) An atypical *Pseudomonas aeruginosa* with cystic fibrosis of the pancreas. *J Pediatr* 68: 215–221.
34. Hoiby N (1974) *Pseudomonas aeruginosa* infection in cystic fibrosis: Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. *Acta Pathol Microbiol Sand* [B] 82: 551–558.
35. Baltimore RS, Mitchell M (1980) Immunologic comparison of mucoid strains of *Pseudomonas aeruginosa*: Comparison of susceptibility to opsonic body in mucoid and nonmucoid strains. *J Infect Dis* 141: 238–247.
36. Mrsny RJ, Lazazzera BA, Daugherty AL, Schiller NL, Patapoff ATW (1994) Addition of a bacterial alginate lyase to purulent CF sputum *in vitro* can result in the disruption of alginate and modification of sputum viscosity. *Pulm Pharmacol* 7: 357–366.
37. Henry DL, Dorman DC, Brown J, Mellis C (1982) Mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Austr Paediatr J* 18: 43–45.
38. Pederson SS, Hoiby N, Espersen N, Koch C (1994) Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 47: 6–13.
39. Doggett RG, Harrison GM (1969) Significance of the pulmonary flora associated with chronic pulmonary disease in cystic fibrosis. In: Lawson D (ed.) *Fifth International Cystic Fibrosis Conference*. Churchill College, Cambridge.

40. Marcus H, Baker NR (1985) Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. *Infect Immun* 47: 723–729.
41. Ramphal R, Pier GB (1985) Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in the adherence to tracheal cells. *Infect Immun* 47: 1–4.
42. Bayer AS, Park S, Ramos MC, Nast CC, Eftekhar F, Schiller NL (1992) Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa. Infect Immun* 60: 3979–3985.
43. Learn DB, Brestel EP, Seetharama S (1987) Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. *Infect Immun* 55: 1813–1818.
44. Meshulam T, Obedeanu N, Morzbach D, Sobel JD (1984) Phagocytosis of mucoid and non-mucoid strains of *Pseudomonas aeruginosa. Clin Immunol Immunopathol* 32: 151–165.
45. Oliver AM, Weir DM (1982) Inhibition of bacterial binding to mouse macrophages by *Pseudomonas* Alginate. *J Clin Lab Immunol* 10: 221–224.
46. Ruben RW, Holt PG, Papadimitrou JM (1980) Antiphagocytic effect of *Pseudomonas aeruginosa* exopolysaccharide. *J Clin Pathol* 33: 1221–1222.
47. Simpson JA, Smith SE, Dean RT (1988) Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. *J Gen Microbiol* 134: 29–36.
48. Krieg DP, Helmke RG, German VF, Mangos JA (1988) Resistance of mucoid *Pseudomonas* to nonopsonic phagocystosis by alveolar macrophages *in vitro. Infect Immun* 56: 3172–3179.
49. Laharrangue PF, Corberand JX, Fillola G, Gleizes BJ. Fontanilles AM, Gyrard E (1964) *In vitro* effect of the slime of *Pseudomonas aeruginosa* on the function of human polymorphonuclear neutrophils. *Infect Immun* 44: 760–762.
50. Dunne WM, Buckmire FLA (1985) Partial purification and characterisation of polymannuronic acid depolymerase produced by a mucoid strain of *Pseudomonas aeruginosa* isolated from a patient with cystic fibrosis. *Appl Environ Microbiol* 50: 562–567.
51. Kitamikado M, Yamaguchi K, Tseng C-H, Okabe B (1990) Method designed to detect alginate-degrading bacteria. *Appl Environ Microbiol* 2939–2940.
52. Haugen F, Kortner F, Larson B (1990) Kinetics and specificity of alginate lyases. Part I. A case study. *Carbohydr Res* 198: 101–109.
53. Caswell RC, Gacesa P, Lutrell KE, Weightman AJ (1989) Molecular cloning and heterologous expression of a *Klebsiella pneumoniae* gene encoding alginate lyase. *Gene* 75: 127–134.
54. Gacesa P, Caswell RC (1990) Control and heterologous expression in *Escherichia coli* of the *Klebsiella pneumoniae* gene encoding alginate lyase. *Hydrobilogia* 204/205: 661–666.
55. Mai GT, McCormack JG, Seow WK, Pier GB, Jackson LA, Thong YH (1993) Inhibition of adherence of mucoid *Pseudomonas aeruginosa* by alginase, specific monoclonal antibodies and antibiotics. *Infect Immun* 61: 4338–4343.
56. Efekhar F, Speert DP (1988) Alginase treatment of mucoid *Pseudomonas aeruginosa* enhances phagocytosis by human monocytoe-derived macrophages. *Infect Immun* 56: 2788–2793.
57. Stern M, Caplen NJ, Sorgi F, Huang L, Gruenert DC, Geddes DM, Alton EWFW (1995) effect of mucolytic agents on liposome-mediated gene transfer across a CF sputum barrier *in vitro. Pediatr. Pulmonol* Suppl 12: 222.
58. Stossel TP, In: Gallin JI, Goldstein I, Snyder P (eds.) *Inflammation, basic principles and clinical correlates.* New York: Raven, 459–475.
59. Jammey PA, Hvidt S, Kas J, Lerche D, Maggs A, Sackmann E, Schliwa M, Stossel TP (1994) The mechanical properties of actin gels: Elastic modules and filament motions. *J Biol Chem* 269: 32502–32513.
60. Yin HL, Stossel TP (1979) Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature* 281: 583–586.
61. Vasconcellos CA, Allen PG, Wohl ME, Drazen JM, Janmey PA Stossel TP (1994) Reduction in viscosity of cystic fibrosis sputum *in vitro* by gelsolin. *Science* 263: 969–971.
62. Lazarides E, Lindberg U (1974) Actin is the naturally occurring inhibitor of deoxyribonuclease 1. *Proc Natl Acad Sci USA* 71: 4742–4746.

63. Tomkiewicz RP, De Sanctis GT, Fisher R, Rubin BK, Drazen JM, King M (1994) Effects of the actin-severing protein gelsolin on cystic fibrosis sputum viscoelasticity *in vitro*. *Pediatr Pulmonol* Suppl 10: 242.
64. Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ (1991) Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253: 202–205.
65. Anderson MP, Rich DP, Gregory RJ, Smith AE, Welsh MJ (1991) Generation of cAMP-activated chloride currents by expression of CFTR. *Science* 251: 679–682.
66. Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR (1992) Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell* 68: 809–818.
67. Boucher RC, Stutts MJ, Knowles MR, Cantley L, Gatzy JT (1989) Na^+ transport in cystic fibrosis respiratory epithelia: Abnormal basal rate and response to adenylate cyclase activation. *J Clin Invest* 78: 1245–1252.
68. Knowles MR, Gatzy J, Roucher RC (1981) Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *N Engl J Med* 305: 1489–1495.
69. Quinton PM (1990) Cystic fibrosis. A disease in electrolyte transport. *FASEB J* 4: 2709–2717.
70. Boucher RC, Cheng EHC, Paradiso AM, Stutts MJ, Knowles MR, Earp HS (1989) Chloride secretory response of cystic fibrosis human airway epithelia: Preservation of calcium but not protein kinase C- and A-dependent mechanism. *J Clin Invest* 84: 1424–1431.
71. Kohler D, App E, Schmitz-Schumann M, Weurtemberger G, Matthys H (1986) Inhalation of amiloride improves the mucociliary and the cough clearance in patients with cystic fibrosis. *Eur J Resp Dis* (Suppl 146) 69: 319–326.
72. Knowles M, Murray G, Shallal J, Askin F, Ranga V, Gatzy J, Boucher R (1984) Bioelectric properties and ion flow across excised human bronchi. *J Appl Physiol* 56: 868–977.
73. Mentz WM, Brown JB, Friedman M, Stutts MJ, Gatzy JT, Boucher RC (1986) Deposition, clearance and effects of aerosolized amiloride in sheep airways. *Am Rev Respir Dis* 134: 938–943.
74. Knowles MR, Church NL, Waltner WE, Gatzy JT, Boucher RC (1992) Amiloride in cystic fibrosis: Safety, pharmacokinetics and efficacy in the treatment of pulmonary disease. In: Cragoe EJ, Kleyman TR, Simchowitz (eds.) *Amiloride and its analogues: Unique cation transport inhibitors.* New York: VCH Publishers, 301–306.
75. App EM, King M, Helfesrieder R, Kohler D, Matthys H (1990) Acute and long-term amiloride inhalation in cystic fibrosis lung disease. *Am Rev Respir Dis* 141: 605–612.
76. Knowles MR, Church NL, Waltner WE, Yankaskas JR, Gilligan PH, King M, Edwards LJ, Helms W, Boucher R (1990) A pilot study of aerosolised amiloride for the treatment of cystic fibrosis lung disease. *N Engl J Med* 332: 1189–1194.
77. Graham A, Hasani A, Alton EWFW, Martin GP, Marriott C, Hodson ME, Clark SW, Geddes DM (1993) No added benefit from nebulised amiloride in patients with cystic fibrosis. *Eur Resp J* 6: 1243–1248.
78. Robinson M, Donnelly PM, Donnelly J, Torzillo P, Bye PTP (1995) Effect of long-term inhalation of amiloride on lung function and exercise capacity in adults with cystic fibrosis. *Am Rev Respir Dis* Suppl 12: A20.
79. Bowler JM, Kelman E, Worthington D, Littlewood JM, Watson A, Conway SP et al. (1995) A double-blind placebo-controlled trial of nebulised amiloride added to the standard therapy of a respiratory exacerbation in cystic fibrosis. *Pediatr Pulmonol* Suppl 12: A20.
80. Anderson WR, Church NL, Wisniewski ME, Hsyu PH (1995) Effects of aerosolised amiloride on pulmonary function in children with cystic fibrosis. *Resp Crit Care Med* 151: A19.
81. Church NL, Hsyu PH, Wisniewski ME, Anderson WH (1995) Safety trial of nebulised amiloride in children with cystic fibrosis. *Resp Crit Care Med* 151: A19.
82. Mason SJ, Paradiso AM, Boucher RC (1991) Regulation of transepithelial ion tranport and intracellular calcium by extracellular adenosine triphosphate in human normal and cystic fibrosis airway epithelium. *Br J Pharmacol* 103: 1649–1656.
83. Knowles MR, Clark LL, Boucher RC (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N Engl J Med* 325: 533–538.

84. Browon HA, Lazarowski ER, Boucher RC, Harden KT (1991) Evidence that UTP and ATP regulate phosholipase C through a common extracellular 5′-nucleotide receptor in human airway epithelial cells. *Mol Pharmacol* 40: 648–655.
85. Knowles MR, Clark LL, Boucher RC (1992) Extracellular ATP and UTP induce chloride secretion in nasal epithelia of CF patients and normal subjects *in vivo*. *Chest* 101 (Suppl): 60S–63S.
86. Davis CW, Dowell ML, Lethem MI, Van Scott M (1992) Goblet cell degranulation in isolated canine tracheal epithelium: Response to exogenous, ATP, ADP and adenosine. *Am J Physiol* 262: C1313–C1323.
87. Mason SJ, Oliver KN, Bellinger D, Meuten DJ, Pare PD, Knowles MR, Boucher RC (1993) Studies of absorption and acute and chronic effects of aerosolized and parenteral uridine 5′-triphosphate (UTP) in animals. *Am Rev Respir Dis* 147: A27.
88. Oliver KN, Hohneker KH, Noone PG, Boucher RC, Knowles MR (1993) Aerosolized uridine 5′-triphosphate (UTP) as a potential new therapy for cystic fibrosis (CF) lung disease: Acute safety studies in humans. *Am Rev Respir Dis* 147: A28.
89. Oliver K, Hohneker K, Noone PG, Boucher RC, Knowles MR (1994) Acute safety studies of aerosolized uridine 5′-triphosphate (UTP) in normals and cystic fibrosis (CF) patients. *Pediatr Pulmonol* Suppl 9: 248.
90. Bennet W, Oliver K, Zemen K, Hohneker KW, Boucher RC, Knowles MR (1994) Acute effects of aerosolized uridine 5′-triphosphate (UTP) plus amiloride on mucociliary clearance in cystic fibosis. *Am J Resp Crit Care Med.* 149: A670.
91. Bennet WD, Oliver KN, Zeman KL, Hohneker KW, Boucher RC, Knowles MR (1996) Effect of uridine 5′-triphosphate plus amiloride on mucociliary clearance in adult cystic fibrosis. *Am J Resp Crit Care Med.* 153: 1796–1801.
92. Warner JO (1992) Immunology of cystic fibrosis. *British Medical Bulletin* 48: 893–911.
93. Auerbach HS,Williams M, Kilpatrick JA (1985) Alternate day prednisolone reduces morbidity and improves pulmonary function in cystic fibrosis. *Lancet* 686–688.
94. Rosenstein BJ, Eigen H (1991) Risks of alternate day prednisolone in patients with CF. *Pediatrics* 87: 245–246.
95. Brown KA, Collins AJ (1977) Action of nonsteroidal, anti-inflammatory drugs on human and rat peripheral leukocyte migration *in vitro*. *Ann Rheum Dis* 36: 239–243.
96. Higgs CA, Eakins KE, Mugridge KJ, Moneada S, Vane JR (1980) The effects of non-steroidal anti-inflammatory drugs on leukocyte migration in carrageenin-induced inflammation. *Eur J Pharmacol* 66: 81–86.
97. Shimanuki T, Nakamura RM, Dizerga GS (1985) Modulation of leukotaxis by ibuprofen: A quantitative determination *in vivo*. *Inflammation* 9: 285–295.
98. Venezio FR, Divencenzo C, Pearlman F, Phair JF (1985) Effects of the newer nonsteroidal anti-inflammatory ibuprofen, fenprofen and sulindac on neutrophil adherence. *J Infect Dis* 152: 690–694.
99. Kaplen HB, Edelson HS, Korchak KM, Given WP, Abramson S, Weissmann G (1984) Effects of non-steroidal anti-inflammatory agents on human neutrophil function *in vitro* and *in vivo*. *Biochem Pharmacol* 33: 371–378.
100. Konstan MW, Vargo KM, Davis PB (1990) Ibuprofen attenuates the inflammatory response to *Pseudomonas aeruginosa* in a rat model of chronic pulmonary infection: Implications for anti-inflammatory therapy in cystic fibrosis. *Am Rev Respir Dis* 141: 186–191.
101. Konstan MW, Byard PJ, Hoppel CL, Davis PB (1995) Effect of high dose ibuprofen in patients with cystic fibrosis. *N Engl J Med* 332: 848–854.
102. Goldstein W, Döring G (1986) Lysosomal enzymes from polymorphonuclear leucocytes and proteinase inhibitors in patients with cystic fibrosis. *Am Rev Respir Dis* 134: 49–56.
103. Weisman G, Zurier RB, Hoffstein S (1972) Leucocyte proteases and the immunological release of lysosomal enzymes. *Am J Pathol* 68: 539–563.
104. Travis J, Fritz H (1991) Potential problems in designing elastase inhibitors for therapy. *Am Rev Respir Dis* 143: 1412–1415.
105. Suter S, Schaad UB, Tegner H, Ohlsson K, Desgrandchamps D, Waldvogel FA (1986) Levels of free granulocyte elastase in bronchial secretions from patients with cystic fibrosis: Effect of antimicrobial treatment against *Pseudomonas aeruginosa*. *J Infect Dis* 153: 902–909.

106. Bruce MC, Poncz L, Klinger JD, Stern RC, Tomashefski JR, Dearborn DG (1983) Biochemical and pathological evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. *Am Rev Respir Dis* 132: 529–535.
107. Schuster A, Ueki I, Nadel JA (1992) Neutrophil elastase stimulates tracheal submucosal gland secretion that is inhibited by ICI 200, 355. *Am J Physiol* 262: L86–L91.
108. Hansen G, Schuster A, Zubrod C, Wahn V (1995) Alpha1-Proteinase inhibitor abrogates proteolytic and secretogogue activity of cystic fibrosis sputum. *Respiration* 62: 117–124.
109. Ming WJ, Bersani L, Mantovani A (1987) Tumor necrosis factor I schemotactic for monocytes and polymorphonuclear leukocytes. *J Immunol* 87: 1469–1474.
110. Klebanoff SJ, Vadas MA, Harlan JM, Sparks LH, Gamble JR, Agosti JM, Waltersdorph AM (1986) Stimulation of neutrophils by tumor necrosis factor. *J Immunol* 136: 4220–4225.
111. Toubin R, Elsas PP, Fiers W, Dessain AJ (1987) Recombinant human TNFα enhances leukotriene synthesis in neutrophils and eosinophils stimulated by Ca^{++} ionophore A23187. *Clin Exp Immunol* 70: 484–490.
112. Sampson AP, Spencer DA, Green CP, Piper PJ, Price JF (1990) Leokotrienes in the sputum and urine of cystic fibrosis children. *Br J Clin Pharmacol* 30: 861–869.
113. Greally P, Hussein MJ, Cook AJ, Sampson AP, Piper PJ, Price JF (1993) Sputum tumor necrosis factor-α and leukotriene concentrations in cystic fibrosis. *Arch Dis Child* 68: 389–392.
114. Rommens JM, Ianuzzi MC, Kerem B-S, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N et al. (1989) Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science* 245: 1059–1065.
115. Riordan JR, Rommens JM, Kerem B-S, Alon N, Rozmahel R, Grzelczak Z et al. (1989) Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science* 245: 1066–1973.
116. Kerem B-S, Rommens JM, Buchanan JA, Markiewicz D, Cox T, Chakravarti A, Buchwald M, Tsui L-C (1989) Identification of the cystic fibrosis gene: Genetic analysis. *Science* 245: 1073–1080.
117. Engelhardt JF, Yankaskas JR, Ernst SA, Yang Y, Marino CR, Boucher RC, Cohn JA, Wilson JM (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genet* 2: 240–248.
118. Engelhardt JF, Smith SS, Allen E, Yankaskas JR, Dawson DC, Wilsom JM (1994) Coupled secretion of chloride and mucus in the skin of *Xenopus laevis:* Possible role for CFTR. *Am J Physiol* 267: C491–C500.
119. Oppenheimer EH, Esterly JR (1975) Pathology of cystic fibrosis: Review of the literature and comparison with 146 autopsied cases. *Perspect Pediatr Pathol* 2: 241–278.
120. Johnson LG, Olsen JC, Sarkadi B, Moore KI, Swanstrom R, Boucher RC (1992) Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. *Nature Genet* 2: 21–25.
121. Dorin JR, Farley R, Webb S, Smith SN, Farini E, Delaney SJ, Wainwright BJ, Alton EWFW (1996) A demonstration using mouse models that successful gene therapy for cystic fibrosis requires only partial gene correction. *Gene Therapy* 3: 797–801.
122. Carter B, Flotte T, Zeitlin P, Solow R (1992) Adeno-associated virus vectors and complementation of cystic fibrosis. *Pediatr Pulmonol* 10 (Suppl 6): 187–188.
123. Bayle JY, Johnson LG, St George JA, Boucher RC, Olsen JC (1993) High efficiency gene transfer to primary monkey airway epithelial cells with retrovirus vectors using the gibbon ape leukemia virus receptor. *Hum Gene Ther* 4: 161–170.
124. Harris CE, Agarwal S, Hu P, Wagner E, Curiel DT (1993) Receptor-mediated gene transfer to airway epithelial cells in primary culture. *Am J Resp Cell Mol Biol* 9: 441–447.
125. Huxley C (1994) Mammalian artificial chromosomes: A new tool for gene therapy. *Gene Ther* 1: 7–12.
126. Graham FL, Smiley J, Russel WC, Nairn R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36: 59–72.
127. Rosenfeld MA, Chu CS, Seth P, Danel C, Banks T, Yoneyama K, Yoshimura K, Crystal RG (1994) Gene transfer to freshly isolated human respiratory epithelial cells *in vitro* using a replication-deficient adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA. *Hum Gene Ther* 5: 331–342.

128. Zabner J, Peterson DM, Puga AP, Graham SM, Couture LA, Keyes LD, Lukason MJ, St George JA, Gregory RJ, Smith AE et al. (1994) Safety and efficacy of repetitive adenovirus-mediated transfer of CFTR-cDNA to airway epithelia of primates and cotton rats. *Hum Gene Ther* 6: 75–83.
129. Trapnell B (1993) Adenoviral vectors for gene transfer. *Adv Drug Deliv Rev* 12: 185–199.
130. Simon RH, Engelhardt JF, Yang Y, Zepeda M, Weber-Pendleton S, Grossman M et al. (1993) Adenovirus-mediated transfer of the CFTR gene to lungs of non-human primates: toxicity study. *Hum Gene Ther* 4: 771–780.
131. Yei S, Mittereder N, Tang K, O'Sullivan C, Trapnell B (1994) Adenovirus-mediated gene transfer for cystic fibrosis: Quantitative evaluation of repeated *in vivo* vector administration to the lung. *Gene Ther* 1: 192–200.
132. Hug P, Sleight RG (1991) Liposomes for the transformation of eukaryotic cells. *Biochim Biophys Acta* 1097: 1–17.
133. Stribling R, Brunette E, Liggit D, Gaensler K, Debs R (1992) Aerosol gene delivery *in vivo. Proc Natl Acad Sci USA* 89: 11277–11281.
134. Yoshimura K, Rosenfeld MA, Nakamura H, Scherer EM, Parvarani A, Lecocq J-P, Crystal RG (1993) Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after *in vivo* intratracheal plasmid mediated gene transfer. *Nucleic Acids Res* 20: 3233–3240.
135. Canonica AE, Conary JT, Meyrick BO, Brigham KL (1994) Aerosol and intravenous transfection of human alpha 1-antitrypsin gene to lungs of rabbits. *Am J Resp Cell Mol Biol* 10: 24–29.
136. Stewart MJ, Plautz GE, Del Buono L, Yang ZY, Gao X. Huang L, Nabel EG, Nabel GJ (1992) Gene transfer *in vivo* with DNA-liposome complexes: Safety and acute toxicity in mice. *Hum Gene Ther* 3: 267–275.
137. Nabel EG, Gordon D, Yang ZY, Xu L, San H, Plautz GE, Wu B-Y, Gao X, Huang L, Nabel GJ (1992) Gene transfer *in vivo* with DNA-liposome complexes: Lack of autoimmunity and gonadal localization. *Hum Gene Ther* 3: 649–656.
138. Middleton PG, Caplen NJ, Gao X, Huang L, Gaya H, Geddes DM, Alton EWFW (1994) Nasal application of the cationic liposome DC-Chol/-DOPE does not alter ion transport, lung function or bacterial growth. *Eur Respir J* 7: 442–445.
139. Caplen NJ, Alton EWFW, Middleton PG, Dorin JR, Stevenson BJ, Gao X, Durham SR, Jeffery PK, Hodson ME, Contelle C (1995) Liposome-mediated CFTR-gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med* 1: 39–46.
140. Stern M, Caplen NJ, Sorgi F, Huang L, Gruenert DC, Geddes DM, Alton EWFW (1995) The effect of mucolytic agents on liposome-mediated gene transfer across a CF sputum barrier *in vitro. Pediatr Pulmonol* Suppl 12: 152.
141. Middleton PG, Geddes DM, Alton EWFW (1994) Protocols for *in vivo* measurement of the ion transport defects in cystic fibrosis nasal epithelium. *Eur Respir J* 7: 2050–2056.
142. Knowles MR, Paradiso AM, Boucher RC (1995) *In vivo* nasal potential difference: Techniques and protocols for assessing efficacy of gene transfer in cystic fibrosis. *Hum Gene Ther* 6: 445–455.
143. Alton EWFW, Chadwick SL, Smith SN, Pastorini U, Scallen M, Geddes DM (1996) Lower airway potential difference measurements in non-CF and CF subjects. *Pediatr Pulmonol* Suppl 13: 276.
144. Tsang VT, Alton EWFW, Hodson ME, Yacoub M (1993) *In vitro* biolectric properties of bronchial epithelium from transplanted lungs in recipients with cystic fibrosis. *Thorax* 48: 1006–1011.
145. Grygorczyk R, Bridges MA (1992) Whole cell chloride conductances in cultured brushed human nasal epithelial cells. *Can J Physiol Pharmacol* 70: 1134–1141.
146. Verkman A (1990) Development and biological applications of chloride-sensitive fluorescent indicators. *Am J Physiol* 259: C375–C388.
147. Stern M, Munkonge F, Caplen NJ, Sorgi F, Huang L, Geddes DM, Alton EWFW (1995) Quantitative fluorescence measurements of chloride secretion in native airway epithelium from CF and non-CF subjects. *Gene Ther* 2: 766–774.
148. Saiman L, Prince A (1993) *Pseudomonas aeruginosa* pili bind asialo-GM1 which is increased on the surface of cystic fibrosis epithelial cells. *J Clin Invest* 92: 1875–1880.

149. Schwab UB, Wold AE, Carson JL, Leigh MW, Cheng PW, Gilligan PH, Boat TF (1993) In creased adherence of *Staphylococcus aureus* from cystic fibrosis lungs to airway epithelial cells. *Am Rev Respir Dis* 148: 365–369.
150. Davies J, Stern M, Dewar A, Caplen NJ, Munkonge FW, Pitt T, Sorgi F, Huang L, Bush A, Geddes DM et al. (1995) Adherence of *Pseudomonas aeruginosa* to cystic fibrosis respiratory epithelium is reduced by liposome-mediated CFTR gene transfer. *Thorax* 50(Suppl 2): A48.
151. Drumm ML, Pope HA, Cliff WH, Rommens JM, Marvin SA, Tsui LC, Collins FS, Frizzell RA, Wilson JM (1990) Correction of the cystic fibrosis defect *in vitro* by retrovirus-mediated gene transfer. *Cell* 62: 1227–1233.
152. Rich DP, Anderson MP, Gregory RJ, Cheng SH, Paul S, Jefferson DM, McCann JD, Klinger KW, Smith AE, Welsh MJ (1990) Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride regulation in cystic fibrosis. *Nature* 347: 358–363.
153. Rosenfeld MA, Yoshimura K, Trapnell BC, Yoneyama K, Rosenthal ER, Dalemans W, Fukayama M, Bargon J, Stier LE, Stratford-Perricaudet L (1992) *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* 68: 143–155.
154. Engelhardt JF, Yang Y, Stratford-Perricaudet LD, Allen ED, Kozarsky K, Perricaudet M, Yankaskas JR, Wilson JM (1993) Direct gene transfer of human CFTR in human bronchial epithelial of xenographs with E1 deleted adenoviruses. *Nature Genet* 4: 27–34.
155. Hyde SC, Gill DR, Higgins CF, Trezise AE, MacVinish LJ, Cuthbert AW, Ratcliff R, Evans MJ, College WH (1993) Correction of ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature* 362: 250–255.
156. Alton EW, Middleton PG, Caplen NJ, Smith SN, Steel DM, Munkonge FM, Jeffrey PK, Geddes DM, Hart SL, Williamson R (1993) Non-invasive liposome mediated gene delivery can correct ion transport defect in cystic fibrosis mutant mice. *Nature Genet* 5: 135–142.
157. Bout A, Perricaudet M, Baskin G, Imler JL, Scholte BJ, Pavirani A, Valerio D (1994) Lung gene therapy: *In vivo* adenovirus-mediated gene transfer to rhesus monkey airway epithelium. *Hum Gene Ther* 5: 3–10.
158. Engelhardt JF, Simon RH, Yang Y, Zepeda M, Weber-Pendleton S, Doranz B, Grossman M, Wilson JM (1993) Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: Biological efficiency study. *Hum Gene Ther* 4: 759–769.
159. Zabner J, Couture LA, Gregory RJ, Graham SM, Smith AE, Welsh MJ (1993) Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with CF. *Cell* 75: 207–216.
160. Crystal RG, McElvaney NG, Rosenfeld MA, Chu CS, Mastrageli A, Hay JG, Brody SL, Jaffe HA, Eissa NT, Danel C (1994) Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nature Genet.* 8: 42–51.
161. Knowles MR, Hohneker KW, Zhou Z, Olsen JC, Noaj TL, Hu PC, Leigh MW, Engelhardt JF, Edwards LJ, Jones KR et al. (1995) A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. *N Engl J Med* 333: 823–831.

Airway Mucus: Basic Mechanisms
and Clinical Perspectives
ed. by D.F. Rogers and M.I. Lethem
© 1997 Birkhäuser Verlag Basel/Switzerland

CHAPTER 15
Therapeutic Approaches to Airway Mucous Hypersecretion

Atsushi Yuta and James N. Baraniuk

Division of Rheumatology, Immunology and Allergy, Georgetown University, Washington, DC, USA

1. Introduction

Airway mucus is the intraluminal product of glandular and goblet cell exocytosis, vascular permeability, cellular infiltration and desquamation. Factors promoting each of these processes may contribute to a state of

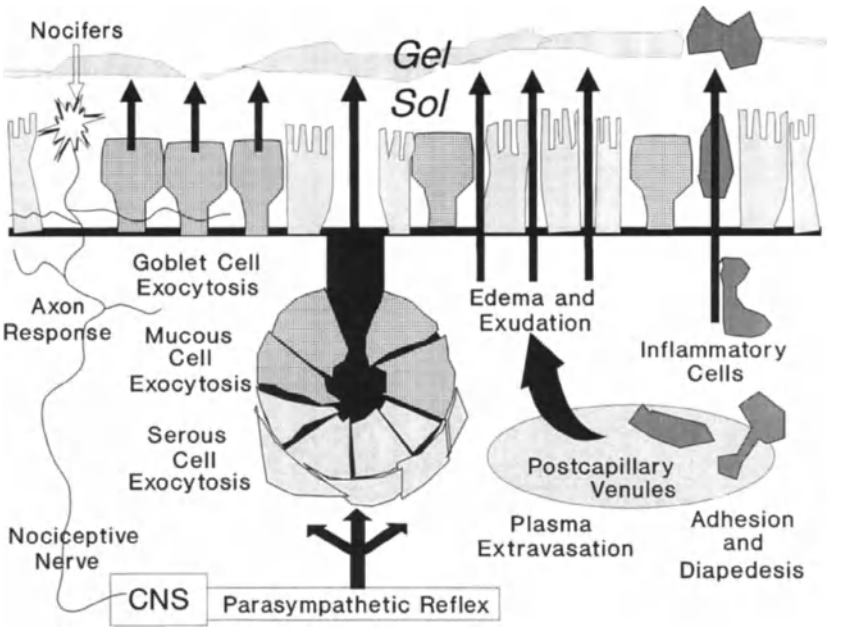


Figure 1. "Mucus" refers to the fluid lining the mucosal epithelium, and is composed of secreted macromolecules derived from glandular and epithelial goblet cell exocytosis and plasma components that exudate across post-capillary venule and epithelial barriers. There is a constant influx of inflammatory cells, and these contribute significantly to mucus during inflammatory conditions. Regulation of these secretory processes by neural reflexes and inflammatory mediator release is complex.

"mucous hypersecretion" (Figure 1). The challenges of quantifying these processes *in vivo* have made it difficult to determine the relative importance of serous, mucous and goblet cell exocytosis, leak, neural reflexes and inflammatory cell infiltration in various bronchial nasal, sinus and middle ear diseases, and the effects of individual drugs on these processes.

Submucosal glands contain two populations of cells based upon their Alcian Blue-periodic acid Schiff base (PAS) staining characteristics [1, 2]. Essentially all submucosal gland cells in the bronchial and nasal mucosa are PAS-positive, indicating the presence of a high concentration of carbohydrate residues that probably represent mucins [1–3]. Alcian Blue-positive cells are defined as "mucous", and contain acidic, sulfated carbohydrate polymers ("acid mucin") [1]. Alcian Blue-negative, PAS-positive cells are thought to contain "neutral mucin", and have been termed "seromucous" or "serous" [1]. Serous cells of the nasal and bronchial mucosa contain many proteins including lysozyme, lactoferrin, secretory leukocyte protease inhibitor (SLPI) and secretory component. Secretory component

is the transport protein for locally synthesized dimeric immunoglobulin A (IgA) that is secreted as secretory (sIgA) from serous cells [4]. Regulation of intracellular glycosyl transferases that construct the carbohydrate side chains of mucins and the molecular mechanisms of glandular exocytosis are beyond the scope of current therapeutics, but are targets for future drug development.

The complexity of mucus, multiplicity of mechanisms affecting mucus production and diversity of diseases associated with mucous hypersecretion, combined with the lack of effective therapies and understanding of mechanisms of drug action, have severely hindered the rational scientific development of effective therapies for mucous hypersecretion [5]. We can gain a better understanding of current therapeutic modalities by appreciating the history of mucous hypersecretion.

2. A History of Mucous Hypersecretion and Its Therapy

Mucous hypersecretion is a component of many disease processes that have afflicted *Homo sapiens* for millennia [6]. The search for effective therapies surely extends to paleolithic times that predate written records. Shen Nung summarized the Chinese experience to 3500 BC when codifying the contemporary Chinese Pharmacopeia in the initial Chinese "Book of Herbs" (Pen Ts'ao, "Legendary Red Emperor") [7]. Ma Huang, the source of ephedrine, a sympathomimetic catecholamine, and numerous other herbal preparations were described.

Systematized drug therapy for cough and mucous hypersecretion was introduced by the Sumerians of Mesopotamia in approximately 3000 BC [8]. The objective of therapy was to drive evil from the body by inducing nausea, emesis, diarrhea and coughing. This was associated with incantations and ceremonial procedures. The drugs included excreta, *Ammi* (may contain chromones), mandrake (anticholinergic properties), terpenes, aloes, belladonna, castor oil and mint. Although most of these agents were not subjected to rigorous double-blind placebo-controlled investigation, many of the components and their derivatives are still used as expectorant elixirs and prescribed along with the familiar incantations and machinations of the physician garbed in a white cloak and ceremonial stethoscope necklace.

The most favoured compounds of Mesopotamian culture had a profound influence on the development of Egyptian and Greek medical therapies. The School of Hippocrates (460–370 BC) applied scientific rationalization to their use, and appreciated their general lack of efficacy. Dioscorides (AD 78) recommended a number of expectorants, including cinnamon, radish, garlic, honey, flax, comfrey, mandrake, peppers, honey and pine extract. Celsus (AD 1) and Galen (AD 2) recommended thymol, storax, turpentine and other agents that persist as expectorants in current pharmacopeias.

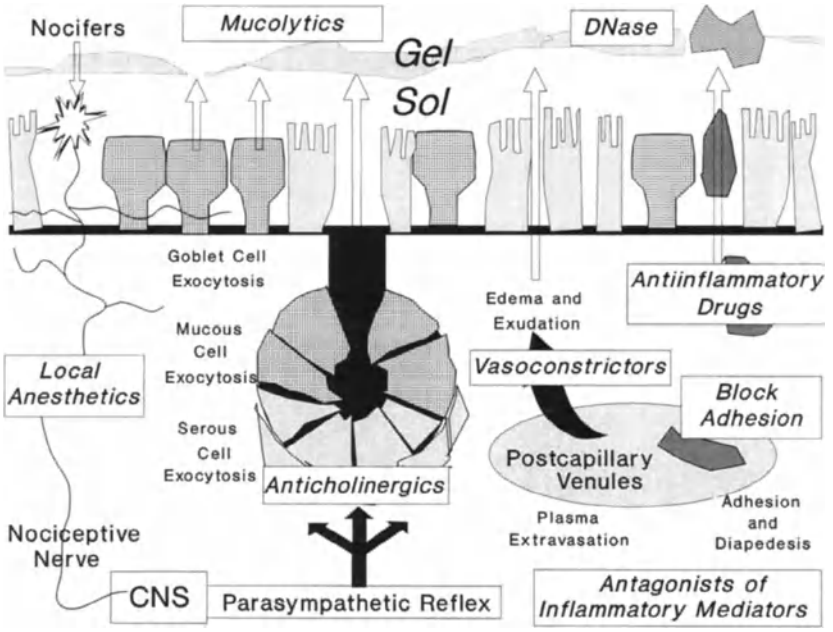


Figure 2. Therapeutic strategies attempt to chemically reduce disulfide bonds in secreted mucoglycoconjugates, degrade mucus DNA, reduce cholinergically mediated glandular exocytosis and interfere with post-capillary venule plasma extravasation and cellular infiltration. Antagonists of specific inflammatory mediators and glucocorticoids are effective in inflammatory conditions.

Major advances for the treatment of mucous hypersecretion were made by Anglo Saxon herbalists in the first half of the 10th century with the introduction of leeches. Maimonides in his famous 12th-century "Treatise on Asthma" recommended chicken soup flavoured with ginger, cloves, coriander and spikenard for coughing up phlegm.

Importation of Aztec and South American medical practices to Europe introduced the use of *Datura*, guaiac, tobacco, sarsaparilla, ipecacuanha, quinine and chile peppers as mucokinetic agents. Guaiac wood imported from the north coast of South America was initially used for the treatment of syphilis, but late in the 19th century became one of the most popular oral agents for treatment of cough and cold in the United States.

The lengthy list of traditional herbal preparations from these diverse cultures has received relatively little intensive scientific scrutiny despite forming the basis for many current mucotherapies. Stramonium was used in ancient Hindu medicine and became the first important European bronchodilator. Cigarettes made from the Malabar nut tree, *Adhatoda vesica*, led to the development of bromhexine and ambroxol. Cromolyn is derived from *Ammi*. Radish seedlings produce *S*-carboxymethylcysteine. Deriva-

tives of the ginkgo biloba tree are potent inhibitors of platelet activating factor [9].

3. Mucolytic Agents: Thiols with Free Sulfhydryl Group

Once it was determined that disulfide bonds contributed to mucus viscosity and gel formation, the search was on for mucolytic drugs with free sulfhydryl groups that could dissolve these bonds *in vivo* (Table 1).

3.1. Cysteine

L-Cysteine and its derivatives with free sulfhydryl groups [10–13] have mucolytic activity and reduce the viscosity of sputum *in vitro*. Cysteine is also a precursor for glutathione, so could promote antioxidant effects.

Table 1. Mucoactive drugs

Mucolytic thiol drugs with a free sulfhydryl group
Cysteine and derivatives
Dithiothreitol
N-Acetylcysteine
Thiopronine
Sodium 2-mercaptoethane sulfonate (MESNA)
Mucokinetic drugs with a blocked thiol group
Carbocysteine
Letosteine
Stepronine
Expectorant drugs that may increase mucus secretion
Sobrerol
Bromhexine
Ambroxol
Inorganic and organic iodides
Domiodol
Guaiacol and derivatives
Guaifenesin
Ipecacuanha
Volatile inhalants and balsams
Proteolytic enzymes
Trypsin
Gelsolin
Others
Anticholinergic agents
Water
Hypertonic solutions
DNase
Glucocorticoids
Erythromycin

3.2. Dithiothreitol

Dithiothreitol is the most potent mucolytic thiol but is too irritating for clinical use. It is commonly used to homogenize sputum specimens in laboratory investigations [10, 11].

3.3. N-Acetylcysteine

N-acetylcysteine (NAC) reduces the specific viscosity of porcine gastric mucin extract with the maximal effect at 100 mM [12].

The mechanism of action of NAC may depend on its route of administration. Aerosolized inhaled NAC may dissociate disulfide bonds of mucoglycoproteins to reduce viscosity. Topically inhaled, oral and intravenous NAC may increase intracellular glutathione and protect against free radical damage. NAC (200 mg three times a day orally for 8 weeks in healthy smokers) may reduce superoxide radical generation by alveolar macrophages [13].

Nebulized NAC increases sputum volume and decreases sputum viscosity (10 min inhalations of 20% solution). An early treatment effect is noted within 2 weeks, consisting of an increase in volume of more dilute and less viscous sputum, followed by a late effect with progressive attenuation of bronchial hypersecretion and reduced incidence of acute exacerbations of bronchial infection over 6 months [14]. NAC may improve sputum clearance and loosen mucus plugs, but does not appear to change FEV_1. However, beneficial effects have been difficult to demonstrate and reproduce in double-blind placebo-controlled studies [15–17]. Aerosolized NAC is no different from placebo in chronic bronchitis or asthma.

Side effects of aerosolized NAC include nausea and stomatitis. Hyperresponsive asthmatics can develop bronchospasm. Oral dosing is associated with dyspepsia, nausea and diarrhea.

3.4. Thiopronine

In addition to mucolytic properties, thiopronine protects guinea pigs against histamine-induced bronchoconstriction [18]. The effect was equivalent to theophylline.

3.5. Sodium 2-Mercaptoethane Sulfonate

Sodium 2-mercaptoethane sulfonate (MESNA) has a free thiol group, and has no irritating or bronchodilating activity. In placebo-controlled studies, MESNA nasal spray was similar to NAC, and more effective than bromhexine [19]. Sputum weights were significantly lower on MESNA in

a crossover design placebo-controlled study. Mild gastrointestinal discomfort was present in 4% of subjects.

4. Mucokinetic Agents: Thiols with a Blocked Sulfhydryl Group

Some thiol drugs do not have a free sulfhydryl group. They do not appear to break mucin disulfide bonds, but may act via alternative mechanisms.

4.1. Carbocysteine

Carbocysteine (*S*-carboxymethylcysteine) is a cysteine derivative with a blocked thiol group that does not reduce disulfide bonds [20]. Carbocysteine protected against the macroscopic and microscopic alterations induced by SO_2 inhalation in rats [21]. Carbocysteine (500 mg/kg/day orally) increased production of sialic acid-labeled mucin by 73% and reduced fucose-labeled mucins by 29% in 50 chronic bronchitis patients [22]. Mucociliary clearance was reduced, while viscosity was unaffected [23–25]. Changes in FEV_1 have been inconsistent. Sputum IgA content may be increased, and albumin concentrations (product of vascular permeability) reduced. One study has reported a decrease in goblet cell hyperplasia compared to placebo treatment [22]. Carbocysteine may be useful in the treatment of otitis media with effusion [26].

4.2. Letosteine

Letosteine, a cyclic derivative of cysteine, appears to have properties similar to carbocysteine, and may favour synthesis of sialomucins [27].

4.3. Stepronine

Stepronine [2-(α-thenoylthio)propionylglycine (lysine salt)] is metabolized in the intestine to remove the thenoyl moiety and release thiopropionylglycine, which has a free sulfhydryl group. This compound can reduce mucin disulfide bonds, and may also activate sialytransferase and increase sialomucin production.

5. Expectorants: Drugs That May Increase Mucus Secretion

5.1. Sobrerol

Sobrerol is a cyclic terpene derivative [28]. Sobrerol may increase mucus production and volume but reduce overall viscosity. Several open clinical

studies suggest clinical benefit on expectoration with an increase in FEV_1 from 75% of predicted to 80% compared with no change in a placebo-controlled group.

5.2. *Bromhexine*

Bromhexine (*N*-methyl-*N*-cyclohexane-3,5-dibromo-2-aminobenzylamine hydrochloride) is derived from vasicine, an alkaloid from *Adhatoda vasica nees* [29]. Bromhexine has been promoted for chronic bronchitis, bronchiectasis, mild asthma, sinusitis and otitis media. Bromhexine may increase mucus secretion, reduce sputum viscosity and facilitate expectoration, but has minimal effects on pulmonary function indices and rates of resolution of sinus disease. The molecular mechanism of action is unclear.

5.3. *Ambroxol*

Ambroxol is related to bromhexine and appears to stimulate mucus secretion, yet promotes a normalization of mucus viscosity in viscid secretions. In studies of bronchitis, bronchiectasis and chronic cough in smokers and emphysema subjects, ambroxol has shown a significant reduction in cough frequency and intensity. A morphological study indicated that ambroxol normalized the epithelial histology compared with a placebo group. Significant improvements have also been claimed for FEV_1 and other pulmonary function measures, frequency of infective exacerbations and days lost from work due to illness. Ambroxol may be marginally superior to *N*-acetylcysteine, bromhexine, thiopronine and letosteine. The most common complications have been nausea, vomiting, diarrhea, rash, vertigo and sleeplessness.

5.4. *Inorganic and Organic Iodides*

Although iodides have long been used as expectorants clinical efficacy has not been demonstrated [30]. Iodides should not be used clinically, because of their indisputable toxicity for induction of thyroid disease.

SSKI (saturated solution of potassium iodide) is a common preparation [30]. Major reported benefits have been improvement of cough, decreased breathlessness and reduction in sputum viscosity. Dyspepsia occurs in 11% on small doses and 40% on higher doses. Parotid gland swelling appears to be due to excessive stimulation of salivary production. Hypersensitivity reactions include acne, other skin rashes and adenopathy. Pregnant women should not take iodides because neonatal thyroid suppression, cretinism and goiter may result.

Iodinated glycerol has been used since 1915 for chronic asthma, cough, bronchospasm and expectoration. Limited studies in chronic bronchitis subjects suggests that the subgroup with the most copious sputum production have a significant decrease in mucus production. Nebulized iodine preparations cannot be advocated.

5.5. Domiodol

Domiodol (4-hydroxymethyl-2-iodomethyl-1,3,-dioxolane) is an iodinated organic compound [31]. In a placebo-controlled crossover study, domiodol significantly increased the volume of secretions in chronic bronchitis subjects [32]. This was deemed beneficial because patients were able to most easily cough up the mucus. Other controlled studies have compared domiodol to sobrerol and *S*-carboxymethylcysteine, but without placebos, making it difficult to compare efficacy. The end points used to compare efficacy contribute to the confusion regarding mucoactive drugs. It is not clear if there were changes in the physical properties of the mucus, or concentrations of constituent macromolecules. The use of increased mucus production as an end point is of interest, since it underscores our ambiguous attitude toward mucous hypersecretion. On the one hand, it is of interest to stop mucus production, but in some cases it is the aim to generate a less viscous mucus that may be expectorated more easily. This ambivalence highlights our poor understanding of mucous hypersecretion and the actions of mucokinetic and other mucoactive drugs.

5.6. Guaiacol and Derivatives

Handelich identified a resin from guaiac wood that yielded creosote, guaiacol and other phenolic resins [33]. Guaiacol derivatives have been widely marketed, including Thiokol which helped launch the success of the Hoffmann-La Roche pharmaceutical company [33].

5.7. Guaifenesin

Guaifenesin (glyceryl guaiacolate) has been successfully marketed as guaifenesin, Resyl, Robitussin, Fenisin and other products [33]. Guaifenesin has no mucolytic action but may decrease the surface tension of bronchial sputum. There is no evidence to suggest antiseptic or antitussive properties. It may have a mild anesthetic effect, since one guaiacol derivative led to the synthesis of benzocaine. Guaifenesin's principal benefit appears to be as an expectorant (but not antitussive) for the symptomatic treatment of

coughs productive of scanty amounts of thick viscous secretions. Doses of 100–200 mg four times per day are recommended, although up to 2400 mg per day may be required.

5.8. Ipecacuanha

Ipecacuanha is derived from the Brazilian *Cephaelic ipecacuanha* plant, and has long been advocated for asthma, bronchitis and related diseases [33]. Its more common use is as an emetic, an effect that may be mediated by direct stimulation of central nervous system (CNS) receptors in the medulla, and "gastric irritant" effects. Ipecacuanha activates gastric nociceptive vagal afferents that recruit CNS efferent vagal reflexes. These promote gastric secretion and include a "mucokinetic" pulmonary component ("gastropulmonary vagal mucokinetic reflex") [33]. While ipecacuanha can augment the outflow of respiratory tract fluids, there have been no clinical evaluations of this mechanism in humans. However, Ziment [33] has noted that the emetic effect of ipecacuanha and similar drugs can be reduced by milk, presumably by adsorption of the drug to bovine proteins. This reduces ipecacuanha's ability to stimulate gastric irritant receptors and may reduce the beneficial pulmonary reflex effects. Milk has a reputation for impairment of mucokinesis, but does not appear to increase mucus viscosity or production.

5.9. Volatile Inhalants and Balsams

A large number of volatile oils have been used in cough, cold and mucokinetic preparations [34]. Camphor, a component of Vicks products, has a traditional reputation as a mild expectorant. Despite widespread use, few of this class of compounds have been subjected to scientific investigation.

5.10. Expectorants

Ziment proposes that many expectorants act as nonspecific irritants of vagal afferent nerves in the gastric mucosa that provoke central parasympathetic reflexes that promote gastric and bronchial secretion via parasympathetic cholinergic reflexes ("gastropulmonary mucokinetic reflexes") [33]. Hence, a common property of many of these "expectorants" may be their ability to activate irritant neurons and recruit parasympathetic glandular secretory reflexes. In the bronchi, these reflexes may stimulate serous cell transport of secretory IgA and other serous cell products to produce a thin "serous" secretion with decreased viscosity. The lack of clear efficacy

of these irritant drugs suggests that this mechanism is unlikely to affect long-term outcome in bronchorrhea. Iodinated glycerol offers a case in point, since it may reduce chest discomfort and coughing in some patients with chronic bronchitis without affecting dyspnea or lung function [35].

6. Anticholinergic Agents

Cholinergic parasympathetic nerve activity is the greatest tonically active stimulus for glandular exocytosis and mucus secretion in human airways. Muscarinic M_3 receptors on submucosal glands mediate the secretory response. Ipratropium offers a rational approach to block these cholinergic secretory reflexes and does reduce sputum volume, but does not alter sputum viscosity [36]. While ipratropium has generally been employed as a bronchodilator, it can alter mucus composition in chronic bronchitis and increase mucociliary clearance [37]. Glycopyrolate, a quaternary ammonium derivative of atropine, has been reported to have antisecretory activities without the side effects of atropine [38].

Anticholinergic agents such as atropine and ipratropium reduce rhinorrhea in patients with allergic rhinitis [39]. The effects are dose-dependent between 40 and 20 µg [40]. Ipratropium bromide decreased rhinorrhea significantly, but had no effect on sneezing or nasal blockage [41]. In a long-term open study (10 months) of nonallergic perennial rhinitis subjects, ipratropium bromide significantly decreased nasal discharge, and reduced the secretory response to methacholine provocation from 0.35 ml to 0.12 ml ($p = 0.05$) [42]. The pharmacokinetics of ipratropium bromide nasal spray were studied in normal volunteers (0.03%, 0.06%, 0.12%), patients with perennial rhinitis (0.03% and 0.06%) and the common cold (0.06%). Approximately 10% of the active drug was absorbed systemically after intranasal adminstration. There were no significant pharmacokinetic differences between the normal volunteers and patients with perennial rhinitis or common cold. Plasma ipratropium concentrations were always undetectable after use of 0.03% solution, with infrequent, low, transient concentrations detectable after 0.06%. Measurements of pupil diameter and vital signs showed no significant changes compared with placebo [43].

In an 8-week double-blind study of ipratropium bromide nasal spray (0.03%, three times per day, 42 µg of active drug) in 233 patients with perennial rhinitis, there was a significant 30% decrease in nasal secretion weights. Minor infrequent side effects such as nasal dryness and epistaxis did not limit treatment [44]. In a 1-year open phase III trial of 0.03% ipratropium bromide nasal spray (42 µg per nostril three times per day) for perennial rhinitis, only 17 of 285 patients (6%) were considered treatment failures [45]. The higher dose of 0.06% ipratropium bromide was also effective [46]. Ipratropium bromide was also effective for the common cold

[47]. In a dose–response study, 0.06% (84 μg) was more efficacious than 0.03% (42 μg) solution, and only marginally less efficacious than 0.12% (168 μg) solution [48]. Adverse events were dose-related.

Rhinovirus 39 is one of the major causes in the common cold. Human *in vivo* studies demonstrate that the most important component of nasal hypersecretion in rhinovirus infections is vascular permeability [49]. The effects of ipratropium bromide nasal spray were studied by Gaffey [50]. Normal subjects were inoculated by rhinovirus 39, and 73% of the ipratropium bromide-treated group (80 μg three times daily) and 79% of the no-treatment group became infected. Only 50% of the ipratropium group had clinical colds, compared with 76% of the no-treatment group ($p < 0.05$) [50]. In contrast, 125 μg three times daily of atropine methonitrate had no effect on symptoms or signs, while 250 μg four times per day induced only a reduction of nasal secretion weights [51]. Atropine methonitrate was not as effective as ipratropium bromide.

Atropine and ipratropium also have different effects on mucociliary transport. The transport rate of ^{99}mTc-labeled resin particles that move in the gel layer was decreased by atropine. However, the clearance of ^{99}mTc-labeled saline, which moves in both the gel and sol (periciliary) layers, was not changed, suggesting that atropine's effect on mucociliary function was due to alteration of the gel layer [52]. In contrast, ipratropium bromide did not alter mucociliary transport [53].

7. Water

Water has long been touted as a valuable mucokinetic. However, scientific data to support these claims are lacking. Drinking 200 ml of cold water had no effect on nasal mucus velocity, whereas hot water and chicken soup increased velocity, a fact attributed to heat and aroma rather than fluid intake [54]. Aerosolized water has been advocated, but appears to be a good agent for inducing cough and bronchospasm in asthma. As noted by Ziment [55], therapeutic humidifiers including ultrasonic and microsonic mists have no rational value in treating lung disease and must be condemned. The only value of aerosolized water is to add moisture to dry oxygen or medical gas mixtures.

8. Hypertonic Solutions

Hypertonic solutions of saline, urea and ascorbic acid were at one time thought to promote ciliary motility, proteolysis and mucus liquefaction [55]. This was due to interference with intra- and intermolecular binding and osmotic hydration of luminal fluid. However, these solutions have been shown to induce coughing and bronchospasm, which clearly limits their use.

9. Proteolytic Enzymes

9.1. Trypsin

Nebulized trypsin degrades mucoproteins and fibrin in sputum [33]. Uncontrolled studies reported improvement in clinical symptoms, but it was unclear if this was related to digestion of sputum. The recommended dose was 25000 to 200000 units aerosolized one to six times per day for several days. Concerns regarding clinical harm due to α_1-antitrypsin deficiency, allergic reactions, hemoptysis, mucosal metaplasia and emphysematous degradation of alveolar walls have led to abandonment of trypsin in therapy. Similarly, serratopeptidase, fericase, onoprose, neuraminidase, chymotrypsin, papain, bromelain, ficin, helicidin, leucine amino peptidase, elastase and ribonuclease have been considered for use to degrade mucus but have not been considered for clinical development [56].

9.2. Gelsolin

Globular and filamentous actin is released from desquamated cells in airways, and contributes to the viscosity of sputum. Gelsolin is a human plasma protein that cleaves filamentous actin and reduces viscosity. Preliminary studies suggest efficacy in cystic fibrosis [57] and other disorders where neutrophils and other cells are present in airways and actin is released.

10. DNase

Purulent nasal and lung secretions contain large amounts of DNA from dead neutrophils. DNA contributes to mucus viscosity. Human DNase was cloned and sequenced from a pancreatic complementary DNA (cDNA) library [58], and was shown to code for a 260-amino acid protein. Inhalation of recombinant human DNase decreases the size and concentration of DNA in sputum from cystic fibrosis subjects [59]. DNA concentration was reduced from 0.6 mg/ml to 0.3 mg/ml ($p < 0.05$), while the size of DNA by gel electrophoresis was decreased from 1.3 kbp to 0.4 kbp ($p < 10^{-9}$). These reductions decrease mucus viscosity and improve mucus transport capacity [60]

DNase has become an important treatment in cystic fibrosis, and is discussed at length elsewhere in this book. Phase I and phase II placebo-controlled studies in cystic fibrosis patients indicate that rhDNase can induce significant improvements in forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) [61–63]. In a randomized double-blind placebo-controlled phase III study, 968 adult and children with cystic

fibrosis were treated with one- or twice-daily rhDNase inhaler (2.5 mg) for 24 weeks [64, 65]. rhDNase administered once daily and twice daily reduced the age-adjusted risk of respiratory exacerbations by 28% ($p = 0.04$) and 37% ($p < 0.01$), respectively. FEV_1 was improved by $5.8 \pm 0.7\%$ and $5.6 \pm 0.7\%$, respectively. There were more frequent voice alteration and laryngitis in rhDNase group, but there were no instances of anaphylaxis. Two years' treatment was safe [66].

11. Glucocorticoids

Since glucocorticoids are potent anti-inflammatory agents, it would be reasonable to expect they would be beneficial in mucous hypersecretion. In human nasal mucosal explants, 1 μM dexamethasone did not alter expression of serous cell lysozyme or lactoferrin, mucoglycoprotein or mucous cell Alcian Blue staining material. Prednisone had a similar lack of effect on secretion of mucin-like glycoprotein into sputum in asthmatic subjects [67]. In contrast, 10 μM dexamethasone decreased spontaneous release of ^{3}H-glucosamine-labeled mucoglycoconjugates from tracheal explants [68, 69]. This effect was present only in the first 8 and 16 h using human trachea [69], but lasted several days using feline trachea [68]. Glucocorticoids may increase the intensity of Alcian Blue staining in human bronchial glands, but the molecular mechanism accounting for this finding is unclear [70]. It is conceivable that the major effects of glucocorticoids in inflammatory airway diseases are on inflammatory cells, and that changes in glandular exocytosis are the results of reductions in release of secretagogue mediators such as leukotrienes, histamine, neutrophil elastase and cholinergic reflexes. This hypothesis requires confirmation, but does suggest specific roles for antagonists of these mediators in specific situations where the mediators are present and active as secretagogues.

12. Erythromycin

Erythromycin is a macrolide antibiotic but appears to also inhibit immune function and mucus production. Erythromycin inhibits respiratory glycoconjugate secretion from human airways [71] and Cl^- ion transport [72]. Clinically, erythromycin suppresses mucus production in severe bronchorrhea [73], diffuse panbronchitis [74, 75], sinobronchial syndrome [76] and otitis media with effusion [77]. In sinusitis, erythromycin was a useful adjunctive therapy in subjects with lymphocytic infiltrates, but less so in those with eosinophilic (allergic) infiltrates [78].

The molecular mechanisms of erythromycin's effects remain to be determined, but may involve actions on host cell function in addition to antimicrobial effects. Erythromycin reduces neutrophil chemotaxis in

bronchoalveolar lavage fluid [79–81] and inflammatory responses of neutrophils, lymphocytes and macrophages [82]. Erythromycin also modulates airway smooth muscle [83] and neural tone [84].

13. Conclusions

Despite intensive interest in mucus hypersecretion, it is essential to define the specific components of mucosal secretions that are "hypersecreted". This requires determining the origin of exocytosed macromolecules and relative contributions of submucosal gland mucous and serous cells and epithelial goblet and other secretory cells, degree of vascular permeability and cellular phenotype of inflammatory infiltrates. If allergic inflammation is present, as in allergic rhinitis and asthma, then glucocorticoids would reduce eosinophil, T-lymphocyte and mast cell infiltration and in turn reduce secretion of mucus. In chronic bronchitis, a neutrophilic infiltrate is present, but glucocorticoids may be less effective. Since parasympathetic reflexes play a role, anticholinergic agents such as ipratropium are useful to decrease glandular mucoglycoconjugate exocytosis. Vasoconstrictors such as α-adrenergic agonists are of value in hyperpermeability states such as common cold. DNase appears to be appropriate for decreasing the viscosity of sputum in cystic fibrosis. Despite their widespread use, the clinical utility of the mucolytic and expectorant drugs remains in doubt. Thus, there is a great need to better define the nature of mucus, and to develop better ways to modulate the production of its various components in diseases marked by bronchorrhea and rhinorrhea.

As the biology of mucins and the effects of proinflammatory mediators on mucus products are delineated, it is anticipated that exciting, efficacious and innovative new therapies will emerge that replace the currently available derivatives of paleolithic empiricism. Reduction of expression of specific mucin or glycosyl transferase genes, improved degradation of intraluminal mucus components, and agents that promote the differentiation of "normal" epithelial cell populations offer great potential benefits for those patients who gurgle in their own respiratory secretions.

References

1. Reid L (1977) Secretory cells. *Fed Proc* 36: 2703–2707.
2. Lamb D, Reid L (1970) Histochemical and autoradiographic investigation of serous cells of the human bronchial glands. *J Pathol* 100: 127–138.
3. Verma M, Davidson EA (1994) Mucin gene: Structure, expression, and regulation. *Glycoconjugate J* 11: 172–179.
4. Brandtzaeg C (1984) Immune functions of human nasal mucosa and tonsils in health and disease. In: Bienenstock J (ed.) *Immunology of the lung and upper respiratory tract.* New York: McGraw-Hill, 28–95.

5. Braga PC (1989) Mucoactive drugs: Guidelines for proper experimental and clinical pharmacological studies: In: Braga PC, Allegra L (eds) *Drugs in bronchial mucology.* New York: Raven Press, 35–58.
6. Ziment I (1989) Historic overview of mucoactive drugs. In: Braga PC, Allegra L (eds) *Drugs in bronchial mucology.* New York: Raven Press, 1–34.
7. Hsu HY, Peacher WG, Chen H (1977) History of Chinese medical science. *Bulletin of the Oriental Arts Institute* 2: 6.
8. Majno G (1975) The healing hand, man and wound in the ancient world. Cambridge: Harvard University Press.
9. Chung KF, Dent G, McCusker M, Guinot P, Page CP, Barnes PJ (1987) Effect of a ginkgolide mixture, BN 52063, in antagonizing skin and platelet response to platelet activating factor. *Lancet* 8527: 248–251.
10. Lee HS, Majima Y, Sakakura Y, Kim BW (1991) A technique for quantitative cytology of nasal secretions. *Eur Arch Otorhinolaryngol* 248: 406–408.
11. Lee HS, Majima Y, Sakakura Y, Shinogi J, Kawaguchi S, Kim BW (1983) Quantitative cytology of nasal secretions under various conditions. *Laryngoscope* 103: 533–537.
12. Richardson P (1987) Oral *N*-acetyl-cystine: How does it act? *Eur J Resp Dis* 70: 71–72.
13. Ziment I (1989) Cystein and its derivatives. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press, 71–75.
14. Stephan U, Böwing B, Goering U, Wiesemann HG, Reinhardt M, Hirche H, Brandt H (1980) Acetylcysteine in chronic treatment of cystic fibrosis. *Eur J Respir Dis* 61 (Suppl 111): 127–131.
15. Boman G, Backer U, Larsson S, Melander B, Wahlander L (1983) Oral acetyl cystine reduces exacerbation rate in chronic bronchitis, report of a trial organized by the Swedish Society for Pulmonary Disease. *Eur J Resp Dis* 64: 405–415.
16. Grassi C, Morandini GC (1976) A controlled trial of intermittent oral acetyl-cystine in the long-term treatment of chronic bronchitis. *Eur J Clin Pharmacol* 9: 393–396.
17. Multicenter Study Group (1980) Long-term oral acetyl cystine in chronic bronchitis: a double-blind controlled study. *Eur J Respir Dis* 61 (Suppl 111): 93–108.
18. Colombo F, Borella F, Rampoldi C, Zheng Y (1989) Thiopronine. In: Braga PC, Allegra L (eds) *Drugs in bronchial mucology.* New York: Raven Press, 103–118.
19. Steen SN, Ziment I, Freeman D, Thomas JS (1974) Evaluation a new mucolytic drug. *Clin Pharmacol Ther* 16: 58–62.
20. Guffanti EE, Rosseti S, Scaccabarozzi S (1989) Carbocysteine. In: Braga PC, Allegra L (eds) *Drugs in bronchial mucology.* New York: Raven Press, 147–170.
21. Quevauviller A, Huyen VN, Garcet S, Lakah L (1967) Activité des mucolytiques sur la retention experimentale du mucus intra-bronchique chez le rat. *Therap* 22: 485–493.
22. Miskovits G, Szule P, Appel J, Meszaros M (1984) La mucoregolation element important dans le traitement du syndrome bronchitique. *Med Klin* 79: 88–92.
23. Majima Y, Hirata K, Takeuchi K, Hattori M, Sakakura Y (1990) Effects of orally administered drugs on dynamic viscoelasticity of human nasal mucus. *Am Rev Respir Dis* 141: 79–83.
24. Majima Y, Inagaki M, Hirata K, Takeuchi K, Morishita A, Sakakura Y (1988) The effect of an orally administered proteolytic enzyme on the elasticity and viscosity of nasal mucus. *Arch Otolaryngol* 244: 355–359.
25. Sakakura Y, Majima Y, Saida S, Ukai K, Miyoshi Y (1985) Reversibility of reduced mucociliary clearance in chronic sinusitis. *Clin Otolaryngol* 10: 79–83.
26. Majima Y, Takeuchi K, Sakakura Y (1990) Effects of myringotomy and orally administered drugs on viscosity and elasticity of middle ear effusions from children with otitis media with effusion. *Acta Otolaryngol Suppl* (Stockh) 471: 66–72.
27. Candiani C, Savio GC (1989) Letosteine. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press, 171–188.
28. Romandini S (1989) Sobrerol. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press, 203–220.
29. Zuliani G, Marenco G, Daniotti S (1989) Bromhexine. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press 221–238.
30. Ziment I (1989) Inorganic and organic iodides. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press, 251–260.

31. De Rosa G, Donati C, Hodel CM (1989) Domiodol. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press, 239–249.
32. Ferrari S, Donati C, Legnani W (1981) Studio clinico controllato dell'attivata terapeutica del domiodolo. *Riv Patol Clin* 36: 139–154.
33. Ziment I (1988) *Respiratory pharmacology and therapeutics.* Philadelphia: WB Saunders.
34. Ziment I (1989) Volatile inhalants and balsams. In: Braga PC, Allegra L (eds) Drugs in bronchial mucology. New York: Raven Press. 323–334.
35. Petty TL (1990) The national mucolytic study: Results of a randomized, double-blind, placebo-controlled study of iodinated glycerol in chronic obstructive bronchitis. *Chest* 98: 1309–1310.
36. Ghafouri MA, Patil KD, Kass I (1984) Sputum changes associated with the use of ipratropium bromide. *Chest* 86: 387–393.
37. Cugell W (1986) Clinical pharmacology and toxicology of ipratropium bromide. *Am J Med* 81 (Suppl 5A): 18–23.
38. Gal TJ, Suratt PM, Lu J (1984) Glycopyrrolate and atropine inhalation: Comparative effects on normal airway function. *Am Rev Respir Dis* 129: 871–873.
39. Jackson RT, Teichgraeber J (1981) Low-dose topical atropine for rhinitis. *Arch Otolaryngol* 107: 288–289.
40. Sjögren I, Jonsson L, Köling A, Jansson C, Osterman K, Håkansson B (1988) The effect of ipratropium bromide on nasal hypersecretion induced by methacholine in patients with vasomotor rhinitis. *Acta Otolaryngol* (Stockh) 106: 453–459.
41. Meltzer EO (1992) Intranasal anticholinergic therapy of rhinorrhea. *J Allergy Clin Immunol* 90: 1055–1064.
42. Borum P, Mygind N, Larsen FS (1983) Ipratropium treatment for rhinorrhea in patients with perennial rhinitis: An open follow-up study of efficacy and safety: *Clin Otolaryngol* 8: 267–272.
43. Wood CC, Fireman P, Grossman J, Wecker M, MacGregor T (1995) Product characteristics and pharmacokinetics of intranasal ipratropium bromide. *J Allergy Clin Immunol* 95: 1111–1116.
44. Bronsky EA, Druce H, Findlay SR, Hampel FC, Kaiser H, Ratner P, Valentine MD, Wood CC (1995) A clinical trial of ipratropium bromide nasal spray in patients with perennial nonallergic rhinitis. *J Allergy Clin Immunol* 95: 1117–1122.
45. Grossman J, Banov C, Boggs P, Bronsky EA, Dockhorn RJ, Druce H, Findlay SR, Georgitis JW, Hampel FC, Kaiser H, et al. (1995) Use of ipratropium bromide nasal spray in chronic treatment of nonallergic perennial rhinitis, alone and in combination with other perennial rhinitis medications. *J Allergy Clin Immunol* 95: 1123–1127.
46. Kaiser HB, Findlay SR, Georgitis JW, Grossman J, Ratner PH, Tinkelman DG, Roszko P, Zegarelli E, Wood CC (1995) Long-term treatment of perennial allergic rhinitis with ipratropium bromide nasal spray 0.06%. *J Allergy Clin Immunol* 95: 1128–1132.
47. Borum P, Olsen L, Winter B, Mygind N (1981) Ipratropium nasal spray: A new treatment for rhinorrhea in the common cold. *Am Rev Respir Dis* 123: 418–420.
48. Diamond L, Dockhorn RJ, Grossman J, Kisicki JC, Posner M, Zinny MA, Koker P, Korts D, Wecker MT (1995) A dose-response study of the efficacy and safety of ipratropium Abromide nasal spray in the treatment of the common cold. *J Allergy Clin Immunol* 95: 1139–1146.
49. Yuta A, Van Deusen M, Gaumond E, Ali M, Baraniuk JN, Skoner D, Doyle W, Cohen S (1996) Vascular permeability and mucous hypersecretion after rhinovirus infection. *Am J Resp Cri Care Dis* 153: A865 (abstract).
50. Gaffey MJ, Hayden FC, Boyd JC, Gwaltney JM Jr (1988) Ipratropium bromide treatment of experimental rhinovirus infection. *Antimicrob Agents Chemother* 32: 1644–1647.
51. Gaffey MJ, Gwaltney JM Jr, Dressler WE, Sorrentino JV (1987) Intranasally administered atropine methonitrate treatment of experimental rhinovirus colds. *Am Rev Respir Dis* 135: 241–244.
52. Takeuchi K, Suzumura E, Majima Y, Sakakura Y (1990) Effect of atropine on nasal mucociliary clearance. *Acta Otolaryngol* (Stockh) 110: 120–123.
53. Ohi M, Sakakura Y, Murai S, Miyoshi Y (1984) Effect of ipratropium bromide on nasal mucociliary transport. *Rhinology* 22: 241–246.

54. Saketkhoo K, Januskiewicz A, Sackner MA (1978) Effects of drinking hot water, cold water and chicken soup on nasal mucus, velocity and nasal airflow resistance. *Chest* 74: 408–410.
55. Ziment I (1989) Drugs modifying the sol layer and the hydration of mucus. In: Braga PC, Allegra L (eds) *Drugs in bronchial mucology.* New York: Raven Press 293–322.
56. Ziment I (1989) Enzymes. In: Braga PC, Allegra L (eds) *Drugs in bronchial mucology.* New York: Raven Press 129–135.
57. Vasconcellos CA, Allen PG, Wohl ME, Stossel TP (1994) Reduction in viscosity of cystic fibrosis sputum *in vitro* by gelsolin. *Science* 263: 969–971.
58. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL (1990) Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc Natl Acad Sci USA* 87: 9188–9192.
59. Brandt T, Breitenstein S, Hardt HV, Tümmler B (1995) DNA concentration and length in sputum of patients with cystic fibrosis during inhalation with recombinant human DNase. *Thorax* 50: 880–882.
60. Zahm JM, Girod de Bentzman S, Deneuville E, Perrot-Minnot C, Dabadie A, Pennaforte F, Roussey M, Shak S, Puchelle E (1995) Dose-dependent *in vitro* effect of recombinant human DNase on rheological and transport properties of cystic fibrosis respiratory mucus. *Eur Respir J* 8: 381–386.
61. Aitken ML, Burke W, McDonald G (1992) Recombinant human DNase inhalation in normal subjects and patients with cystic fibrosis: A phase I study. *JAMA* 267: 1947–1951.
62. Hubbard RC, McElvaney NG, Birrer P, Shak S, Robinson WW, Jolley C, Wu M, Chernick MS, Crystal RG (1992) A preliminary study of aerosolized recombinant human deoxyribonuclease I in the treatment of cystic fibrosis. *N Engl J Med* 326: 812–815.
63. Ranasinha C, Assoufi B, Shak S, Christiansen D, Fuchs HJ, Empey D, Geddes D, Hodson M (1993) Efficacy and safety of short-term administration of aerosolized recombinant human deoxyribunuclease in patients with cystic fibrosis. *Lancet* 342: 199–202.
64. Ramsey BW, Astley SJ, Aitken MI, Burke W, Colin AA, Dorkin HL, Eisenberg JD, Gibson RL, Harwood IR, Schidlow DV, et al. (1993) Efficacy and safety of short-term administration of aerosolized recombinant human deoxyrobonuclease I in patients with cystic fibrosis. *Am Rev Respir Dis* 148: 145–151.
65. Fuchs HJ, Borowitz DS, Christiansen DH., Morris EM, Nash ML, Ramsey BW, Rosenstein BJ, Smith AL, Wohl ME (1994) Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function with cystic fibrosis. *N Engl J Med* 331: 637–642.
66. Shah P, Scott SF, Geddes DM, Hodson ME (1995) Two years experience with recombinant human DNase I in the treatment of pulmonary disease in cystic fibrosis. *Respir Med* 89: 499–502.
67. Claman DM, Bousher HA, Lui J, Wong H, Fahy JV (1994) Analysis of induced sputum to examine the effects of prednisone on airway inflammation in asthmatic subjects. *J Allergy Clin Immunol* 94: 861–869.
68. Lundgren JD, Hirata F, Marom Z, Logun C, Kaliner M, Shelhamer J et al. (1988) Dexamethasone inhibits respiratory glycoconjugate secretion from feline airways *in vitro* by the induction of lipocortin (lipomodulin) synthesis. *Am Rev Respir Dis* 137: 353–357.
69. Marom Z, Shelhamer J, Alling D, Kaliner M (1984) The effects of corticosteroids on mucous glycoprotein secretion from human airways *in vitro. Am Rev Respir Dis* 129: 62–65.
70. Sturgess J, Reid L (1972) An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland. *Clin Sci* 43: 533–543.
71. Goswami SK, Kivity S, Marom Z (1990) Erythromycin inhibits respiratory glycoconjugate secretion from human airway *in vivo*. *Am Rev Respir Dis* 141: 72–78.
72. Tamaoki J, Isono K, Sakai N, Kanemura T, Konno K (1992) Erythromycin inhibits Cl secretion across canine tracheal epithelial cells. *Eur Respir J* 5: 234–238.
73. Marom ZM, Goswami SK (1991) Respiratory mucus hypersecretion (bronchorrhea): A case discussion-possible mechanism(s) and treatment. *J Allergy Clin Immunol* 87: 1050–1055.
74. Ohno S, Sugiyama Y, Kitamura S (1993) Clinical effects of low-dose and long-term erythromycin in diffuse panbronchitis with chronic respiratory failure. *Jpn J Thrac Dis* 31: 1251–1256.

75. Yamamoto M, Kondo A, Tamura A, Izumi T, Ina Y, Noda M (1990) Long-term therapeutic effects of erythromycin and new quinolone antibioterial agents on diffuse panbronchitis. *Jpn J Thrac Dis* 28: 1305–1313.
76. Nishi K, Myou S, Ooka T, Fujimura M, Matsuda T (1993) Effect of erythromycin on mucocilliary transport and clinical symptoms in patients with sinobronchial syndrome. *Jpn J Thrac Dis* 31: 1367–1376.
77. Iino Y, Sugita K, Toriyama M, Kudo K (1993) Erythromycin therapy for otitis media with effusion in sinobronchial syndrome. *Arch Otolarnygol Head Neck Surg* 119: 648–651.
78. Iino Y, Okura S, Shiga J, Toriyama M, Kudo K (1994) Histopathological studies on paranasal mucosa from patients treated with erythromycin. *J Otolaryngol Jpn* 97: 1070–1078.
79. Kadota J, Sakito O, Kohno S, Sawa H, Mukae H, Oda H, Kawakami K, Fukushima K, Hiratani K, Hara K (1993) A mechanism of erythromycin treatment in patients with diffuse panbronchitis. *Am Rev Respir Dis* 147: 153–159.
80. Tamaoki J, Tagaya E, Yamawaki I, Sakai N, Nagai A, Konno K (1995) Effect of erythromycin on endotoxin microvascular leakage in the rat trachea and lungs. *Am J Respir Crit Care Med* 151: 1582–1588.
81. Oda H, Kadota, J, Kohno S, Hara K (1994) Erythromycin inhibits neutrophil chemotaxis in bronchoalveoli of diffuse panbronchitis. *Chest* 106: 1116–1123.
82. Roche Y, Gougerot-Pocidalo MA, Fay M, Forest N, Pocidalo JJ (1986) Macrolides and immunity: Effects of erythromycin and spiramycin on human mononuclear cell proliferation. *J Antimicrob Chemother* 17: 195–203.
83. Shachter EN, Marom Z (1991) Erythromycin reduces airway responsiveness to methacholine. *Am Rev Respir Dis* 143: A428.
84. Tagaya E, Tamaoki J, Konno K (1994) Erythromycin inhibits cholinergic neuro-effector transmission in canine airway smooth muscle. *Res Com Mol Path Pharm* 85: 181–192.

Index