Novartis Foundation Symposium 248

MUCUS HYPERSECRETION IN RESPIRATORY DISEASE

2002

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MUCUS HYPERSECRETION IN RESPIRATORY DISEASE

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Symposium on Mucus hypersecretion in respiratory disease, held at the Novartis Foundation, London, 26–28 February 2002

Editors: Derek J. Chadwick (Organizer) and Jamie A. Goode

This symposium is based on a proposal made by William Davis and Alan Jackson

Participants

Peter J. Barnes Head of Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, UK

Carol Basbaum (*Chair*) Department of Anatomy, University of California, Box 0452, HSW 1326, 505 Parnassus Avenue, San Francisco, CA 94143, USA

Ingemar Carlstedt Department of Cell and Molecular Biology, Mucosal Biology Group, Lund University, BMC, C13, SE-22184 Lund, Sweden

Lauren Cohn Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, USA

Henry Danahay Novartis Horsham Research Centre, Wimblehurst Road, Horsham, West Sussex RH12 5AB, UK

William Davis Cystic Fibrosis/Pulmonary Research and Treatment Center, The University of North Carolina at Chapel Hill, 6009 Thurston-Bowles, CB 7248, Chapel Hill, NC 27599-7248, USA

Bernd Disse Department of Therapeutic Area Respiratory Diseases, Boehringer Ingelheim Pharma KG, Binger Strasse 173, D-55216 Ingelheim am Rhein, Germany

John F. Engelhardt Department of Anatomy and Cell Biology, University of Iowa, Room 1-111 Bowen Science Building, 51 Newton Road, Iowa City, IO 52242-1109, USA

John V. Fahy Division of Pulmonary and Critical Care Medicine, and the Cardiovascular Research Institute, University of California, San Francisco, 505 Parnassus Avenue, San Francisco, CA 94143-0111, USA

Jack R. Harkema Department of Pathology & Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA

Stephen T. Holgate Mail Point 810, RCMB Division, IIR, Level D, Centre Block, Southampton General Hospital, School of Medicine, Tremona Road, Southampton SO16 6YD, UK

Alan D. Jackson Novartis Respiratory Research Centre, Wimblehurst Road, Horsham, West Sussex RH12 4HE, UK

Peter K. Jeffery Lung Pathology Unit, Department of Gene Therapy, Imperial College School of Medicine, The Royal Brompton Hospital, Sydney Street, London SW3 6NP, UK

Roy C. Levitt Genaera Corporation, 5110 Campus Drive, Plymouth Meeting, PA 19462, USA

Yuehua Li North Carolina State University, Pylon I Research Laboratories, 617 Hutton Street, Raleigh, NC 27606, USA

Jay A. Nadel Cardiovascular Research Institute, Department of Medicine and Physiology, University of California, San Francisco, CA 94143-0130, USA

Paul Nettesheim Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, P O Box 12233, 111 T W Alexander Drive, Research Triangle Park, NC 27709, USA

Charles G. Plopper Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, 1321 Haring Hall, University of California-Davis, Davis, CA 95616-8732, USA

Chris Poll Novartis Horsham Research Centre, Wimblehurst Road, Horsham, West Sussex RH12 5AB, UK

Scott H. Randell The University of North Carolina at Chapel Hill, Cystic Fibrosis/Pulmonary Research and Treatment Center, CB 7248, Room 4011, Thurston-Bowles, Chapel Hill, NC 27599-7248, USA

Duncan F. Rogers Department of Thoracic Medicine, National Heart & Lung Institute, Imperial College, Dovehouse Street, London SW3 6LY, UK

Mary C. Rose Research Centre for Genetic Medicine, Children's Research Institute, Children's National Medical Center, George Washington University, Washington, DC 20010, USA

Bruce K. Rubin Department of Pediatrics, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1081, USA

Matthias Salathe Division of Pulmonary and Critical Care Medicine, University of Miami School of Medicine, R-47, 1600 N W 10th Ave, RMSB 7063a, Miami, FL 33136, USA

John K. Sheehan Cystic Fibrosis Centre, The University of North Carolina at Chapel Hill, 6009 Thurston-Bowles, CB 7248, Chapel Hill, NC 27599-7248, USA

Yohannes Tesfaigzi Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive, SE, Albuquerque, NM 87108, USA

B. Boris Vargaftig Unité de Pharmacologie cellulaire, Unité Associée Institut Pasteur-INSERM U485, Institut Pasteur, 25 Rue du Dr Roux, 75015 Paris, France

Pedro Verdugo Department of Bioengineering, University of Washington, Box 357962, Seattle, WA 98195, USA

Jørgen Vestbo Department of Respiratory Medicine 223, Hvidovre University Hospital, Kettegaard Alle 30, Hvidovre, DK-2650, Denmark

Chair's introduction

Carol Basbaum

Department of Anatomy, University of California San Francisco, Box 0452, HSW 1326, 505 Parnassus Avenue, San Francisco, CA 94143, USA

I'll begin by comparing the healthy and diseased airway, with respect to mucus production. When I'm referring to the diseased airway, this could just as well be any of the major airway diseases — cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), or asthma. In all three we tend to see the same types of structural changes. These are an increase in the proportion of surface-lining cells that make mucus and a growth of submucosal glands (first described by Lynne Reid with her famous gland:wall ratio work in the 1960s). In the gland itself there is also a greater proportion of mucous cells than in glands in the normal, healthy airway. The gland serous cell persists in the diseased airway, but at lower concentrations. The other salient point is that each of the three diseases is launched or triggered by certain environmental stimuli. CF is of course a genetic disease, but it is the onset of bacterial infection that precipitates a downward spiral resulting in the death of the patient. Overall we might say that in going from a healthy airway to a diseased airway, there has to be an environmental perturbation of one sort or another. This could be tobacco smoke (linked to COPD), allergens (linked to asthma) or pathogens (linked to infectious conditions, including CF). These environmental perturbations recruit a population of inflammatory cells, usually a combination of neutrophils and Th2 lymphocytes. Clearly these cells contribute to mucus overproduction, but specific cause–effect mechanisms remain incompletely understood. Indeed, when goblet cell metaplasia and gland enlargement occur, we have to identify the sequence of molecular events mediating these phenomena and determine whether they are precipitated directly by the intrusive environmental stimulus, or alternatively by the associated inflammation.

There are several outstanding issues and questions I think we should address at this meeting. I have broken these down into two categories, asthma and COPD. During this meeting we may add to this list, but hopefully we will at least come up with answers for some of these initial questions.

In asthma, mouse models indicate that Th2 lymphocytes are responsible for releasing mediators that result in goblet cell metaplasia. The mediators that have been implicated are interleukin (IL)-4, IL-5, IL-9 and IL-13. But it is not clear what

the hierarchical relationship among these cytokines is. Which of them find receptors on the host epithelial cells and stimulate mucin production and secretion? In addition, two groups have recently described a chloride channel that is closely correlated — and potentially causally related — to the development of mucus production in asthma (Nakanishi et al 2001, Zhou et al 2001). What roles do these channels play in mucin induction? The CLCA1 channel, in particular, may turn out to be an important target for the modulation of mucus production. In COPD a key issue is whether overproduction of mucus is a direct effect of smoke on the epithelial cells, or rather a secondary response to the influx of inflammatory cells that occurs in the smoker's lung. The mucus gland hypertrophy is something that is seen in all three of the diseases — asthma, CF and COPD. It may account for the longevity and irreversibility of mucus overproduction in these diseases. Even in people who stop smoking, there is a persistence of mucus overproduction. This may be due to the architectural change in the airway with the enlargement of mucous glands. Finally, it would be good to know exactly which inflammatory cells are involved in each disease. It may well be rational to target each of the diseases differently. There is no point in giving an asthmatic a drug against a cytokine that is present in COPD but not in asthma. At this point, let's move on to the first paper.

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Epidemiological studies in mucus hypersecretion

Jørgen Vestbo

Department of Respiratory Medicine, Hvidovre University Hospital, Kettegaard Alle 30, Hvidore, DK-2650, Denmark

Abstract. Respiratory mucus in epidemiology has mainly been studied using standardized questionnaires including questions on cough and phlegm. In chronic obstructive pulmonary disease (COPD) much controversy exists regarding the importance of mucus hypersecretion. From being the key element in the 'British hypothesis' it was reduced to being an innocent disorder in the 1980s but is now again recognized as a potential risk factor for an accelerated loss of lung function. Whereas early studies in mainly occupational cohorts showed no effect of chronic mucus hypersecretion on decline in lung function, such an effect has been shown in subsequent studies on general population samples. Chronic mucus hypersecretion also increases risk of hospital admission which may be due to an increased risk of lower respiratory tract infection. In severe COPD this may explain the increased mortality associated with the presence of mucus. In asthma recent findings suggest that in epidemiology chronic mucus hypersecretion may indicate lack of control which leads to an accelerated loss of lung function and increased mortality in subjects with self-reported asthma.

2002 Mucus hypersecretion in respiratory disease. Wiley, Chichester (Novartis Foundation Symposium 248) p 3–19

Phlegm or symptomatic mucus hypersecretion is a respiratory symptom which is known by all as a frequent mild complication to the common cold and most other upper airway infections. Chronic phlegm is generally believed to be due to chronic mucus hypersecretion (CMH) which is less frequent varying roughly from 2% in female never-smokers to 30% in male heavy smokers. Increased prevalence of CMH is associated with increasing age, male gender, tobacco smoking, childhood respiratory infections, frequent lower respiratory tract infections, occupational exposures, and presence of asthma. It is, however, especially in chronic obstructive pulmonary disease (COPD) that CMH has been studied, and CMH is generally synonymous with chronic bronchitis as defined at the Ciba Guest Symposium in 1959 (Ciba Foundation 1959). CMH can easily be assessed in both clinical practice and in the epidemiological setting and questions on mucus hypersecretion from the British Medical Research Council Questionnaire

(Medical Research Council 1960, 1966) has been a part of virtually every survey in respiratory epidemiology since 1970.

Initial studies

The rationale for looking at CMH in respiratory epidemiology is closely linked to infections and the 'British hypothesis' linking recurrent lower respiratory tract infections (LRTIs) with development of COPD (Pride & Burrows 1995). Whereas lower respiratory tract infections (LRTIs) undoubtedly become more frequent with advancing COPD the role of recurrent LRTIs in the pathogenesis of COPD — in the early stages as well as later — was unclear. The British hypothesis was the basis for the seminal study of male post workers in London by Fletcher et al (1976) in which they showed that CMH was only weakly associated with decline in lung function (i.e. forced expiratory volume in 1 second; FEV_1) (Fletcher et al 1976, Fletcher & Peto 1977). In their book they state 'The hypersecretory disorder is also caused, in susceptible subjects, by smoking and consists of chronic excessive secretion of bronchial mucus sufficient to cause expectoration. It encourages recurrent clinical bronchial infections which are thus a common feature of the disorder. These cause only temporary increases in expectoration. The disorder is not usually progressive and usually remits on stopping smoking. Susceptibility to it correlates with, but is distinct from, susceptibility to the obstructive disorder.' (Fletcher et al 1976.)

A lack of association between CMH and FEV_1 decline was subsequently reported in other surveys, such as the study in Paris area workers (Kauffmann et al 1979), showing lack of association between CMH and FEV_1 decline and the Tecumseh study showing lack of association between CMH and subsequent development of COPD (Higgins et al 1982). Interest in mucus hypersecretion was further diminished after publication of the pooled mortality study by Peto et al (1983) in which CMH was of little relevance as a prognostic marker of mortality from COPD after taking FEV_1 into account (Peto et al 1983). These findings have subsequently been reproduced in British civil servants (Ebi-Kryston 1988, 1989) and South African gold miners (Wiles & Hnizdo 1991). It is probably worthwhile mentioning that all of these studies have been done in men only.

More recent studies

However, within the last 15 years several studies have shown CMH to be a less-innocent disorder. Three mortality studies have all shown an association between CMH and mortality. In a mortality study of an occupational cohort of 1061 men, Annesi & Kauffmann (1986) found CMH to be a significant predictor of overall

mortality after adjustment for FEV_1. Although the overall risk ratio for phlegm production was only 1.59 there was no tendency towards a weakened relationship in subgroups with severely decreased FEV_1. Cause-specific mortality was not available for analysis. Annesi & Kauffmann stressed that although the risk ratio was not large the findings had important implications because of the high prevalence of phlegm; in the Paris study the prevalence in working men was approximately 40%. Vollmer et al (1989) analysed mortality in a population of 698 subjects and found that CMH was associated with mortality but only in subjects with low lung function; information on cause-specific mortality was available but number of deaths was too small for significant conclusions to be drawn. Two large population studies have found CMH to predict mortality from COPD. In the large Six Cities study in the USA, Speizer et al (1989) examined mortality in 8427 subjects followed for 9–12 years and found CMH to be associated with COPD mortality. Using data from the Copenhagen City Heart Study in which 12 557 men and women were followed Lange et al (1990) showed that CMH was a significant predictor of mortality from COPD and asthma — with COPD accounting for the majority of deaths — also after adjusting for lung function. There was a significant interaction between CMH and FEV_1 leading to a much worse prognosis for subjects with CMH *and* decreased lung function as shown in Fig. 1. An association between CMH and COPD mortality does not prove that CMH has a distinct role in the pathogenesis of COPD. By increasing the risk of LRTIs, CMH could increase the risk of mortality as patients with severe COPD will have an increased risk of an unfavourable outcome of an LRTI. That this seems relevant is supported by supplementary findings from the Copenhagen City Heart Study where the excess risk of mortality associated with CMH was due to an increased risk of death associated with infection whereas no association was found between CMH and death without infection (Prescott et al 1995), as shown in Fig. 2.

Regarding an association between CMH and development of COPD, fewer studies have confirmed an association between CMH and FEV_1. Sherman et al (1992) reported an excess FEV_1 decline associated with CMH in 3948 subjects in the US Six Cities study. The association was, however, only found in men and the excess annual decline was only 4.5 ml/year. CMH was in this study found in 15% of men and 9% of women (Sherman et al 1992). In the Copenhagen City Heart Study a clearer effect of CMH on decline in lung function was found and the size of the effect made it clinically relevant (Vestbo et al 1996). In this large population-based study comprising 5354 women and 4081 men aged 30–79 years with spirometry at two surveys five years apart, CMH was significantly associated with FEV_1 decline. The effect was most prominent among men, where CMH at both surveys was associated with an excess FEV_1 decline of 22.8 ml/year (95% confidence interval 8.2–37.4) compared to men without mucus hypersecretion,

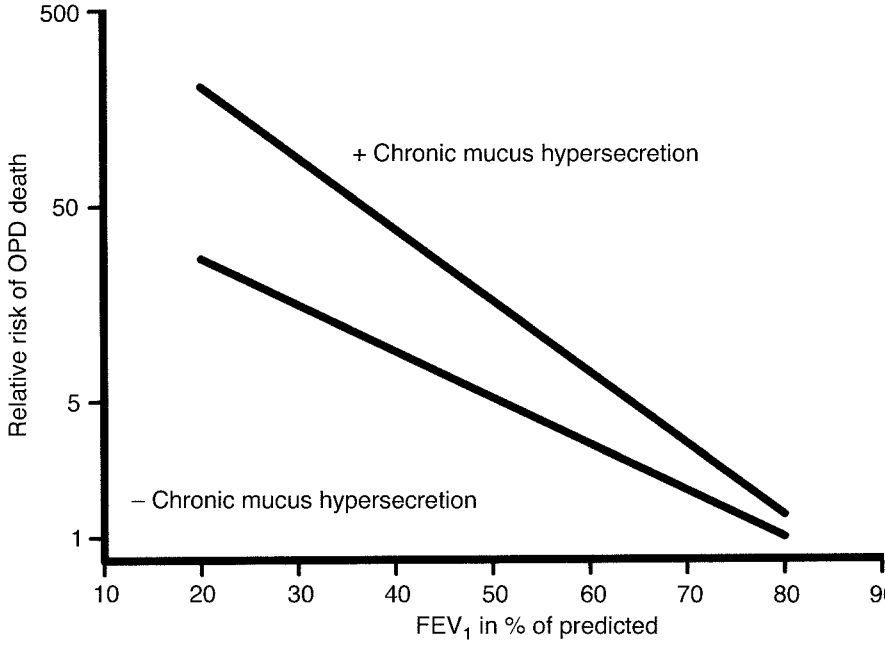

FIG. 1. Mortality from obstructive pulmonary disease in the Copenhagen City Heart Study according to FEV_1 in % predicted and presence or absence of chronic mucus hypersecretion. (Modified from Lange et al 1990.)

after adjusting for age, height, weight change and smoking. In women, the excess decline was 12.6 ml/year (0.7–24.6). Using both *any* mucus secretion at any of the surveys and CMH at any of the surveys made it possible to look for an association between degree of mucus secretion and excess FEV_1 decline. After adjusting for age, height, weight change and smoking, the effect of any mucus at any survey was 7.76 ml/year (– 2.3–17.7) in men and 4.56 ml/year (– 3.0–12.0) in women. CMH at any survey lead to an increase in FEV_1 decline of 20.76 ml/year (11.8–29.6) in men and 7.66 ml/year (0.7–14.5) in women. These associations are shown in Fig. 3.

In the Copenhagen City Heart Study CMH was also a significant predictor of subsequent hospital admission for COPD. Using data from the nationwide Danish Hospital Register the risk of hospitalization associated with CMH was 5.3 (95% confidence interval 2.9–9.6) for men and 5.1 (2.5–10.3) for women (Vestbo et al 1996). When using any mucus secretion at either survey and CMH at either survey as descriptors of mucus, an increasing risk of hospitalization was found with increasing degree of mucus secretion. As shown in Fig. 4, this was found in both sexes and both before and after including FEV_1 at second survey in the statistical model. An association between CMH and hospitalization due to

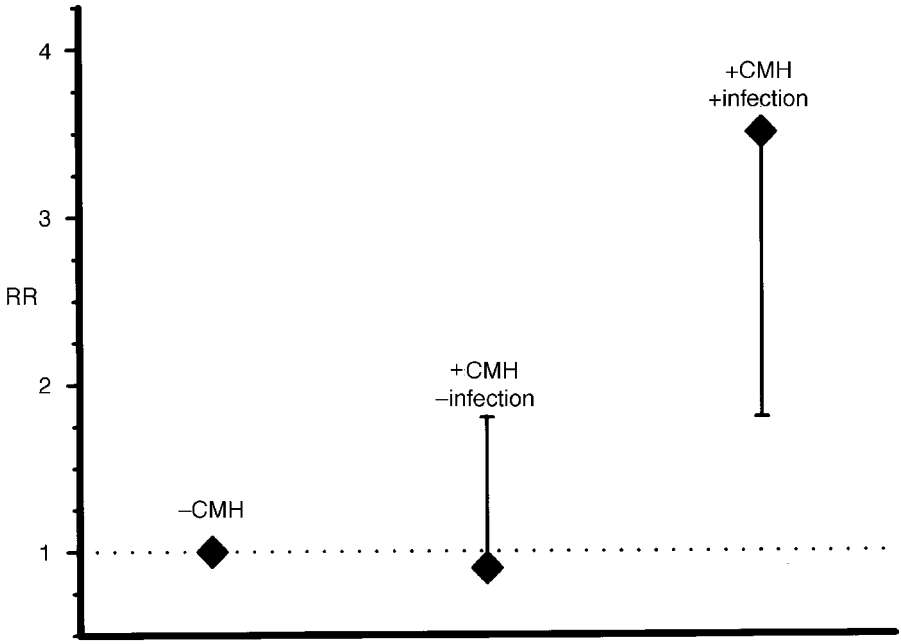

FIG. 2. Relative risk of COPD death associated with chronic mucus hypersecretion in the Copenhagen City Heart Study. Risk for subjects without CMH is 1 (left), for subjects dying without sign of terminal infection CMH did not increase the relative risk (middle) whereas for subjects dying from infection CMH increased the relative risk significantly (right). (Data from Prescott et al 1995.)

COPD had been demonstrated previously in a smaller Danish male population sample (Vestbo et al 1989). In this random sample of 876 men CMH was also associated with an increased risk of medication with drugs used for treating COPD at an 11 year follow-up (Vestbo & Rasmussen 1989).

CMH in asthma

CMH is frequent in asthma. It is generally believed to be a marker of poor asthma control although a study in the clinical setting failed to show any association between CMH and lability in peak expiratory flow in 130 asthma patients (Openshaw & Turner-Warwick 1989). In respiratory epidemiology the diagnosis of asthma is often self-reported and most often little detailed information is available for proper characterization and evaluation of asthma control. In the Copenhagen City Heart Study subjects with self-reported asthma had a higher mortality than subjects without asthma, the hazards ratio (~ relative risk) was 1.6 (Lange et al

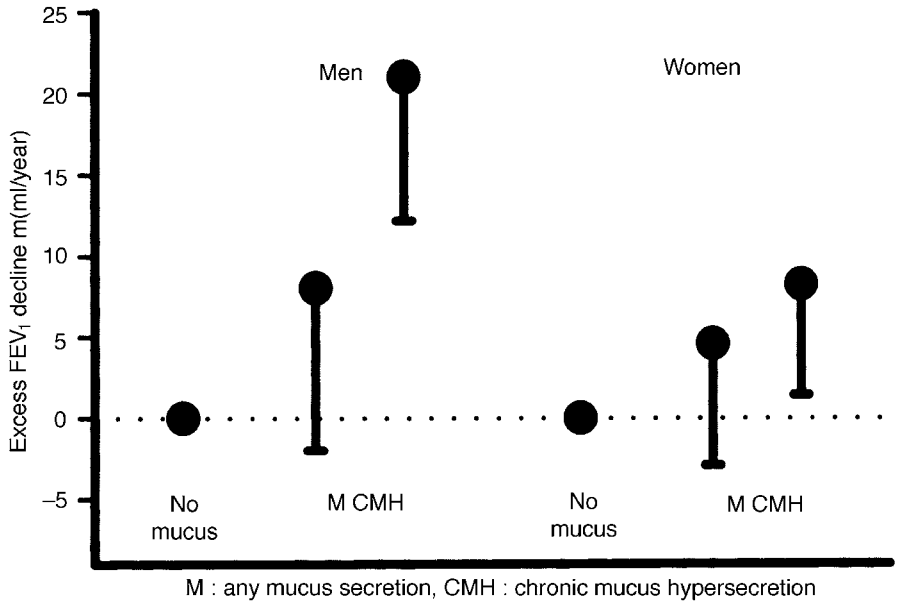

FIG. 3. FEV_1 decline in relation to absence of mucus, presence of mucus, and presence of chronic mucus hypersecretion for both men and women. The graph shows results from multiple linear regression analyses. (Modified from Vestbo et al 1996.)

1996). The strongest risk factor was decreased lung function but from the analyses it seemed that at least some of the excess risk was mediated through the presence of CMH. In support of CMH playing an important role in asthma, data from the same population showed that asthma patients experience a rapid decline in FEV_1 and that CMH is a significant predictor of an excess decline (Lange et al 1998).

Discussion

CMH is a frequent finding in respiratory epidemiology and whereas early studies ruled out any significant rule of CMH in the pathogenesis of COPD, findings from more recent studies have questioned such a clear rejection. This naturally leads to the inevitable question: do these studies differ in a degree sufficient to explain the contradictory findings?

The question is not easily answered. In this short review only epidemiological studies with sufficiently well described methodology and methods have been included and in none of them obvious bias seems present. Two features, however, differ in 'negative' and 'positive' studies. The most obvious is the timing and the other is the source or study base from which the study

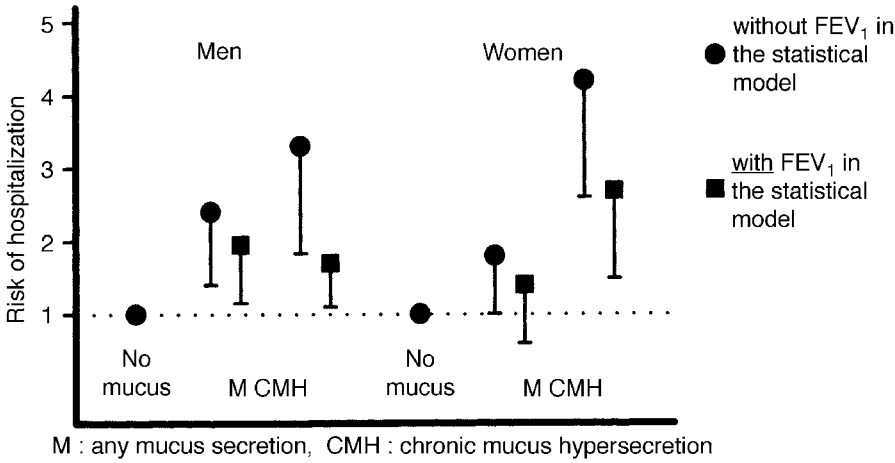

FIG. 4. Risk of hospital admission in relation to absence of mucus, presence of mucus, and presence of chronic mucus hypersecretion for both men and women. The graph shows results from Cox regression analyses with and without FEV_1 in the survival model. (Modified from Vestbo et al 1996.)

populations were sampled. Early occupational cohorts were bound to be much more heavily exposed to particulate air pollution than subsequent general population samples. This is clearly reflected in the prevalences of CMH. In the occupational cohorts prevalence of CMH varies from approximately 20% to > 40% whereas it was 12% in the Copenhagen City Heart Study, of similar size in the US Six Cities Study and even lower in a smaller study of randomly sampled Danish men. It could be hypothesized that CMH in epidemiology can reflect different mechanisms of mucus production and that CMH in response to outdoor air pollution is of another nature than the less frequent CMH reported by subjects in less polluted surroundings.

Much focus has been on FEV_1 decline as FEV_1 is considered the hallmark of COPD. However, the period of time in life combined with the level of lung function from which the decline takes place probably matters. Presumably the association between CMH and FEV_1 decline found in the Copenhagen City Heart Study is not contradictory to the findings of Fletcher et al (1976). Although there was no statistical interaction between level of FEV_1 and the association it is likely that most of the association demonstrated is due to an association among subjects who already have some degree of airflow limitation. In contrast, Fletcher et al examined the association in an occupational cohort of men who were without obvious COPD. In fact, Fletcher et al clearly state that 'In the *preclinical* stages of these disorders, which we have studied, we find no causal relationship between them, for neither mucus hypersecretion nor clinical

chest illnesses cause accelerated loss of FEV'. Recently, global guidelines for COPD have been published and in these guidelines COPD Stage 0 has been introduced, denoting subjects without airflow obstruction but with chronic respiratory symptoms, CMH (Pauwels et al 2001). In order to test if this Stage 0 was useful we examined risk of developing COPD defined as airflow obstruction in the Copenhagen City Heart Study. We compared Stage 0 with asymptomatic smokers and in this population study — where we had previously demonstrated an association between CMH and FEV_1 decline — we were unable to demonstrate that Stage 0 conferred a higher risk of developing COPD than absence of symptoms (Vestbo & Lange 2002).

Perhaps there has been too much focus on FEV_1 and mortality as a result of the seminal papers by Fletcher et al (1976) and Peto et al (1983). By looking at FEV_1 at one end and the extreme consequence of FEV_1 decline, mortality, at the other end of the spectrum of COPD, it could be argued that more intermediate aspects of the impact of CMH on COPD morbidity are lacking. Since COPD patients are often hospitalized during the course of disease, hospitalization is a valuable index of COPD morbidity and could prove a more 'sensitive' measurement than mortality in epidemiological studies on COPD. In studies looking at hospitalization, CMH has been a significant predictor of COPD admission, in the studies mentioned above as well as in a recent Spanish study showing CMH to be a predictor of readmission in COPD (Miravitlles et al 2000).

Was the British hypothesis then true after all? Probably not. CMH facilitates infection especially when severe airflow limitation is present but so far no evidence exists which proves that this is the mechanism through which the harmful effect of CMH is mediated. That this area is fully open to further research is probably most clearly demonstrated by recent analyses from the large Lung Health Study, a North American controlled trial of an inhaled anticholinergic and intense smoking cessation advice in patients with mild COPD. In a longitudinal analysis on 5887 subjects Kanner et al (2001) found the well-known association between CMH and an increased frequency of lower respiratory illnesses. They then studied the effect of lower respiratory illnesses on FEV_1 decline and found that among smokers and intermittent quitters lower respiratory illnesses led to a significantly increased decline in FEV_1; this was not the case in sustained quitters. In smokers and intermittent quitters each lower respiratory illness led to an excess decline of 7.1 and 7.3 ml, respectively. More studies are clearly needed in spite of the already now appearing feeling of *déjà vu*!

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DISCUSSION

Rubin: One important epidemiological question is that of *causation* versus *association*. This is particularly important, as many of us are interested in specific therapy directed at preventing mucin gene expression, and this may be an important physiological response rather than a problem. There is also a problem with defining chronic mucus hypersecretion. Most often, this is measured by the volume of sputum expectorated and the frequency of expectoration, as reported by patients. Studies have shown that the volume of mucus expectorated does not correlate with the response to mucolytic therapy, even for effective therapies such as DNAse for cystic fibrosis. It is not possible to predict 'responders' by the volume that they are expectorating, nor do we see a change in volume expectorated as a response to therapy. The first gauntlet that I would like to throw down is that we need a better way to define chronic mucus hypersecretion than just expectorated volume. Perhaps a non-invasive radiographic technique for quantifying total airway mucus burden might help us get our hands round some of these problems relating to causation/association and potential therapies.

Vestbo: Most epidemiologists just use these simple questions and rely heavily on the validation procedures. Before Fletcher and co-workers did their study in London, they did many initial studies looking at how the questionnaire could be validated. In these studies, they did not just rely on questionnaire data; they had people bring in their sputum for examination. They found that this extra information added very little compared with the questionnaire. This is why the rest of us just smile and use the questions. When we are examining 14 000 people, it is much easier to ask questions than get them to bring in mucus for examination. You are quite right, though: it is a very crude measure. Perhaps there is nothing more to be gained from a crude measure like this. The question is then how to find something else you can apply to 14 000 people.

Basbaum: Might it matter where the mucus is located? A radiographic study might show where it is located in the airway, and this could be clinically significant.

Rubin: Because of the different viscoelastic characteristics of secretions relative to airway tissue, it may be possible to use magnetic resonance imaging (MRI) techniques to identify one versus the other. What is expectorated comes from the nose, saliva and lower respiratory tract. We subtract out what is swallowed. Perhaps because of all these variables, the crude measurement of expectoration frequency and volume relates very poorly to other clinical outcomes.

Disse: I fully agree with your position that collecting mucus from patients is a nightmare. If it's going to be done correctly it needs to be done over 24 h in the clinic with appropriate advice. If the patient collects at home the quality isn't that good. It needs to go to a laboratory as soon as possible. This makes it very complicated. I'm not aware of any larger study in which this has been successfully completed.

Nadel: Anatomically, cough and sputum production is coming from the large airways. The cough receptors are at bifurcations of the major airways, and the gland ducts are also found there. The presumption is that most of the secretions that patients bring up and express are coming at least in part from glands. In the peripheral airways, if peripheral plugging occurs are there any symptoms at all?

Vestbo: Our idea was that in the measurement of mucus hypersecretion in the early epidemiological studies there was a dissociation between mucus in the peripheral airways and mucus in central airways. We thought that the mucus in the central airways was more of an irritant mucus type in the older studies. In an occupational cohort in a highly polluted area, this could mean quite a lot. There are prevalences of mucus secretion in the order of 40–50% in those older studies. Now, we would tend to think that there was a better correlation. We are not saying that phlegm measured by questionnaire today is coming from peripheral airways, but perhaps there is a better association in individuals between bringing up phlegm and having inflammation and increased numbers of goblet cells in peripheral airways. So now we have a better signal. Although it is just as wrong as it was 20 years ago, there is better association between signal and what may be going on more peripherally.

Nadel: Do you think that major airway mucus hypersecretion and aspiration could be an important mechanism? During sleep there is no cough, so if people are producing a lot of secretions in their major airways, isn't it possible that some of the pathophysiology has to do with factors such as infection, which could be seeded by aspiration?

Vestbo: It could be. In our study we wanted to dissociate mucus from infection. From the American Lung Health Study it seems that this could be a mechanism.

Barnes: Could you discuss the relationship between cigarette smoking and chronic mucus hypersecretion? Do all cigarette smokers get chronic mucus

hypersecretion? A cigarette dose–response could be measured in epidemiological studies. Is there a difference between people in susceptibility (probably genetic) to chronic mucus hypersecretion in relation to cigarette dose? Is this associated with the risk of developing COPD?

Vestbo: We have found that there is a relationship between smoking and chronic mucus hypersecretion. The more you smoke the more likely you are to have chronic mucus hypersecretion. But even in those who smoke > 30 cigarettes/day, only 45–50% will report chronic mucus hypersecretion. This is what epidemiologists term the 'healthy smoker' effect. These are the ones who have no big problems arising from their smoking. There is a dose–response relationship, but we do not have the power in our studies to look at specific interactions in smoking groups. It is interesting that those who stopped smoking and continued to have mucus hypersecretion seemed to have the same accelerated rate of decline of lung function as those who continued smoking.

Barnes: So some people stop having mucus hypersecretion on smoking cessation, and some don't?

Vestbo: Yes. Most quitters stop having mucus hypersecretion, and it seems that those who do not stop coughing phlegm have gained very little from smoking cessation.

Rogers: It seemed that in your populations where there were quitters, people who continued to smoke, and intermittent quitters, the intermittent quitters actually fared worse than the people who continued to smoke. Do you have an explanation for this?

Vestbo: This was from the Lung Health Study. But the differences between these two groups, the continuing smokers and intermittent quitters, was not statistically significant. However, there is a study from the Tuscon group (Sherrill et al 1996), in which they looked at people who stopped smoking and restarted, and this group had a poorer prognosis than smokers who had never stopped. They suggested that one of the dangerous things was the process of starting smoking, which somehow did some extra damage. In some ways this is similar to the process of dieting, where studies have shown that if you go on several diets and then gain weight after each, the changes that take place during this post-diet weight gain are perhaps more harmful than what you gain from losing weight. The process of restarting smoking could be worse than just continuing to smoke.

Rogers: Perhaps quitters lose their protective mucus layer.

Basbaum: That's an interesting idea. Is chronic bronchitis protective against lung cancer in smokers?

Vestbo: No. On the contrary, it seems that there is a positive association between chronic mucus hypersecretion and lung cancer. This is very much biased by the correlation between mucus and low lung function, because low lung function is a strong predictor of mortality from lung cancer. I'm not sure that this can be

completely separated. However, I don't think there are any studies that have shown a protective effect of mucus.

Basbaum: It is just the simplistic idea that there are carcinogens in smoke that might be prevented from reaching the cells by the mucus barrier.

Vestbo: This is probably outweighed by the decreased mucociliary clearance in the smokers.

Barnes: We need to know what happens to mucus hypersecretion when people stop smoking. If it is true that in some people it resolves and in others it persists, this could be an important difference that may give us insight into the persistence of disease.

Tesfaigzi: You mentioned that some 40% of smokers get CMH, and that some of these recover from this when they quit smoking whereas others don't. Are there data from this 40% showing how they recover?

Vestbo: Not that I know of, although this could be looked at in the Lung Health Study. They have the most precise data on smoking cessation and its consequences, both for lung function and symptoms. When we tried to look at this in the general population, in Denmark we found that there aren't very many heavy smokers who stop smoking, and not enough for us to address this properly. Perhaps this is a question that is not best solved by epidemiology. It may be better to take a clinical sample.

Tesfaigzi: We are looking at sputum from COPD patients. The interest is that somehow some of these regulators of apoptosis that we see increased in our rat model might not be well regulated in certain subjects with COPD. Our hypothesis is that there is some polymorphism that doesn't allow the down-regulation of these inhibitors of apoptosis. Therefore these people are not able to reduce the numbers of mucus cells in their lung.

Basbaum: For those unfamiliar with Yohannes Tesfaigzi's work in animals, let me try to explain. He has given a variety of irritating stimuli, such as lipopolysaccharide (LPS) and ozone. He has shown that during the induction of this mucous hyperplasia, some of the new mucous cells up-regulate a gene called *Bcl-2*, which is a survival gene. Perhaps what nature intends is that acute injury causes a transient increase in mucous cells to form a barrier, and then once the danger is passed the lesion is resolved through apoptosis of the newly made mucous cells. However, the presence of *Bcl-2* in certain cells makes it impossible for those cells to undergo apoptosis. All you would need in a subpopulation of human beings would be a mutation that interferes with the normal regulation of *Bcl-2* to cause survival of mucous cells where they are not supposed to be.

Barnes: It could be persistence of the inflammation. There is considerable evidence that people with COPD who stop smoking have persistent inflammation, measured in bronchial biopsies, bronchoalveolar lavage and non-invasive markers of inflammation. Our hypothesis is that all cigarette smokers

get inflammation, but on quitting smoking, normal smokers have resolution of this inflammation, but those with COPD have persistent inflammation.

Jeffery: Marina Saetta studied a population of chronic bronchitics, some of whom gave up smoking. When she subsequently biopsied the airways of the subjects who continued to hypersecrete, they continued to have inflammation at similar levels to those seen previously. Also, we have been concerned in many of the biopsy studies we have done that we recruit both ex-smokers and also current smokers into a similar grouping. We have investigated and separated ex- and current smokers to see whether they are different in terms of their baseline inflammation. They are not; they are exactly the same. The ex-smokers continue to have the same level of inflammation as the current smokers.

Basbaum: This is a subpopulation of ex-smokers, presumably.

Jeffery: It is the sub-population of ex-smokers that we select for our clinical trials because they continue to have symptoms of chronic sputum. To come back to what Peter Barnes was saying, we also need to study the other group — the group of ex-smokers that get better (i.e. the ones who cease to produce sputum).

Barnes: They are the group of people that are difficult to find as they do not see doctors.

Engelhardt: Couldn't this finding, that patients who quit continue to decline in lung function, just be that they are the population that has the worst problems to begin with, and this is why they decided to quit? If you are having bad lung problems it might prompt you to quit. The point about beginning to translate this to animals is a good one, because there are selection biases in patient studies.

Sheehan: I want to emphasize the point that it is wrong to think of mucus as being a homogeneous substance. We are just publishing a study in which we looked at about 30 different sputum samples from patients with cystic fibrosis and COPD (Kirkham et al 2002). The amount of mucin present in these samples was variable. There was a clear relationship between the amount of MUC5B in relation to infection. The nature and quality of the sputum and the presence of specific mucin gene products in different sputum samples were quite clear when the burden of infection was high. It could be that some mucuses are more difficult to clear on the basis of where they come from, the nature of the mucins and the kind of gel they make. This may fit in with what you are saying in relationship to infection. It would be interesting to start to parse this out by looking at more sputum samples to see whether we could dissect away some of these factors. It appears that the burden of MUC5B is coming up from the glands, and most of the MUC5AC under normal conditions would be a surface goblet cell product.

Disse: Dr Vestbo had an interesting hypothesis. The older epidemiological studies didn't show a relationship between CMH and FEV_1 decline, whereas the newer studies seem to. You said that in earlier studies the mucus was more irritative and protective, whereas in later studies the mucus is inflammatory. How would

you see the Lung Health Study? This study recruited during 1986–1989 and followed through to 1994. The original publication from 2000 suggested there is no relationship between FEV_1 decline and respiratory symptoms including cough and expectoration (Scanlon et al 2000).

Vestbo: If we go back to the Fletcher study, one of the problems is that they wanted to look at recurring infections and the decline in lung function. They actually used mucus as a proxy variable, because there was this clear relationship between mucus and infections. It was easier to measure the mucus. They also measured infections though, and had a large amount of data on recurrent infections. But although they thought that mucus would be a good indicator, at that time it probably was not, whereas it is now. Now there is a clearer relationship, but the Lung Health Study shows that we do not have to use a proxy variable. We can ask people about lower respiratory illnesses and get this data more directly. The problem with epidemiology is distinguishing whether we are looking at the result of recurrent respiratory illnesses resulting in mucus or whether we are looking at mucus as a signal of something going on in the airways that happens to lead to more infections.

Verdugo: I wonder whether early childhood infection could imprint the future reactivity of the mucosa. If this were to be true, the extreme situation would be in cystic fibrosis, where the infections start very early and continue when the over-production of mucus is present. Is it possible that the non-reactive smoker might have been a child with few airway infections, while the reactive smoker might have been a child with early airway infections?

Vestbo: Our problem is that we only have our data on childhood infection from our last panel study. There are other population studies that have better information and there are cohort studies taking place which cover this spectrum. We could also speculate that those who had recurrent infections in childhood had a poor growth of lung function, so that their maximally attained lung function would be lower than those without infection. Currently, we have no way of disentangling these things. There are those who promote the idea that the maximally attained lung function is the most important risk factor for COPD.

Basbaum: Let me try to rearticulate Pedro Verdugo's hypothesis. A child who is exposed to infection frequently may develop gland enlargement and goblet cell proliferation. If the individual then smokes when he is an adolescent, there is a lot more mucus to be released. Pathogens come in and it becomes almost like a cystic fibrosis situation in that the bacteria may not be cleared as adequately as they would be in an airway with lower (more optimal) amounts of mucus. In such an individual, smoke may have more severe clinical consequences than in someone with relatively low mucus production.

Verdugo: I was thinking more in terms of an increasing pattern of reactivities that could be induced by early infection.

Holgate: Dr Shaheen has worked with David Barker for some time on this idea of initial fetal imprinting, and then moving forward to the first three years of life with frequent virus infections. There's quite convincing evidence on retrospective look-backs on appropriate data sets that have been collected. One of the most powerful influences was maternal and family cigarette smoking in relation to the child that was brought up in that atmosphere. Perhaps the early life programming was a pollutant that was active in the airways during the time they were developing in the first few years of life.

Basbaum: Projecting that out, what does it mean? Is it going too far to say that this child growing up in a passive smoking environment will have larger glands and more goblet cells?

Holgate: The data show that looking back the 'decliners in lung function', as opposed to 'mucus producers', seem to have these environmental risk factors more evidently expressed in the first 3–5 years of life. Whether early life events in humans are able to alter airway morphogenesis towards mucus production requires further work.

Rubin: Paradoxically, the hygiene hypothesis states the opposite effect. The more siblings you have and the more infections you get in childhood, the less likely you are to have asthma. The hygiene hypothesis postulates that the development of both asthma and allergy in children is inversely related to the frequency of infections in early life. This has since been expanded to include exposure to farm animals and pets, older children in the home and day care attendance (Cookson & Moffatt 1997, Von Mutius 2001, Martinez 2001, 2002, Ball et al 2000). The common theme relates to the total burden of antimicrobial and endotoxin exposure in early life 'priming' the immune system for a Th1 response rather than an allergic Th2 response.

Holgate: Most of the evidence around this hypothesis orientates around atopy, rather than the development of chronic non-allergic lung disease. It is very important that we disaggregate these two things, or else one gets into a debate about early life factors that can influence the allergic immune response, as opposed to the tissue response to altered immunity and of Th2-type inflammation. Most of the early-life virus observations and the concept of early-life programming of the immune system on the Th1/Th2 system relate to atopy, i.e. the ability of a human to generate IgE directed to specific environmental allergens. This is, of course, an important risk factor for asthma severity but not for COPD.

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Post-secretory fate of host defence components in mucus

Matthias Salathe, Rosanna Forteza and Gregory E. Conner

Division of Pulmonary and Critical Care Medicine, University of Miami School of Medicine, R-47, 1600 NW 10th Ave, RMSB 7063a, Miami, FL 33136, USA

Abstract. Airway mucus is a complex mixture of secretory products that provide a multifaceted defence against infection. Among many antimicrobial substances, mucus contains a peroxidase identical to milk lactoperoxidase (LPO) that is produced by goblet cells and submucosal glands. Airway secretions contain the substrates for LPO, namely thiocyanate and hydrogen peroxide, at concentrations sufficient for production of the biocidal compound hypothiocyanite, a fact confirmed by us *in vitro*. *In vivo*, inhibition of airway LPO in sheep significantly inhibits bacterial clearance, suggesting that the LPO system is a major contributor to host defences. Since secretory products including LPO are believed to be steadily removed by mucociliary clearance, their amount and availability on the surface is thought to be controlled solely by secretion. In contrast to this paradigm, new data suggest that LPO and other substances are retained at the ciliary border of the airway epithelium by binding to surface-associated hyaluronan, thereby providing an apical, fully active enzyme pool. Thus, hyaluronan, secreted from submucosal gland cells, plays a previously unrecognized pivotal role in mucosal host defence by retaining LPO and possibly other substances important for first line host defence at the apical surface 'ready for use' and protected from ciliary clearance.

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The airways are naturally exposed to a large burden of both organic and inorganic matter. Thus, the airway mucosa has developed sophisticated and complex defence systems against inhaled toxins and particles, including infectious disease agents (for review see Wanner et al 1996). These include a physical barrier (epithelial cells and secreted mucus) and mechanical clearance (cilia and cough). To protect against biological pathogens, airway surface liquid contains a variety of defence substances including immunoglobulins, lactoferrin, lysozyme and defensins (Wanner et al 1996, Travis et al 2001). We have recently shown that goblet cells and submucosal gland cells also secrete lactoperoxidase (LPO) that uses hydrogen peroxide (H_2O_2) to oxidize thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$) (Salathe et al 1997, Gerson et al 2000). Among the

known airway epithelial defence systems, the LPO system is unique because it is also active against viruses and fungi in addition to its bacteriostatic activity (Yamaguchi et al 1993, Popper & Knorr 1997). This brief report will summarize our data on the LPO system and on the fate of LPO after secretion into the airways.

The LPO system in airways

When we started to analyse the H_2O_2 scavenging activity of sheep tracheal mucus, we found it to be dependent on mucus concentration, independent of the presence of lipids, and not due to Fe^{2+} catalysed formation of OH^- by the Fenton reaction (Salathe et al 1995). The activity was heat and protease sensitive (100 °C) as well as non-dialysable (< 10 kDa). Azide inhibited the scavenging activity at lower concentrations than those required for myeloperoxidase (MPO), suggesting that the activity was enzymatic, not due to MPO or glutathione peroxidase (resistant to azide). These data strongly suggested that the H_2O_2 scavenging activity in mucus was due to the secretion of a peroxidase (Salathe et al 1995).

The presence of an endogenous peroxidase in the airway mucosa had already been well established. Several groups detected endogenous peroxidase activity by cytochemistry in goblet cells, airway submucosal glands and also in nasal glands (Christensen et al 1981, Christensen & Hayes 1982, Watanabe & Harada 1990). We then demonstrated peroxidase activity in mucus by oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB), 4-aminoantipyrene, *o*-phenylenediamine and by the triiodide formation assay developed for LPO (Salathe et al 1997). Using this latter assay, ovine airway mucus contained approximately 0.7 units of LPO-like activity per mg of dialysed and lyophilized mucus, equivalent to approximately 10 μg of enzyme. Thus, we estimated that airway peroxidase constitutes about 1% of all secreted macromolecules in the sheep trachea.

Airway LPO purification from ovine tracheal lavage was achieved by a two-column procedure on S-Sepharose and lentil lectin Sepharose (Salathe et al 1997). This purification procedure confirmed that the peroxidase was responsible for 1–2% of the total secreted protein. The absorption spectra of the purified enzyme showed a major peak at 412 nm consistent with LPO and inconsistent with MPO (absorbs at 430 nm) and glutathione peroxidase (absorbs only in the UV range). The purified enzyme had an apparent molecular weight of 83 kDa, nearly identical in size to sheep milk LPO and contained peroxidase. To determine its molecular nature, purified ovine airway LPO was sent for amino acid determination (Gerson et al 2000). The sequence of the N-terminus (although partially blocked) and of a CNBr peptide was identical to bovine LPO and to the deduced protein sequence of the cDNA isolated from an ovine tracheal mucosa

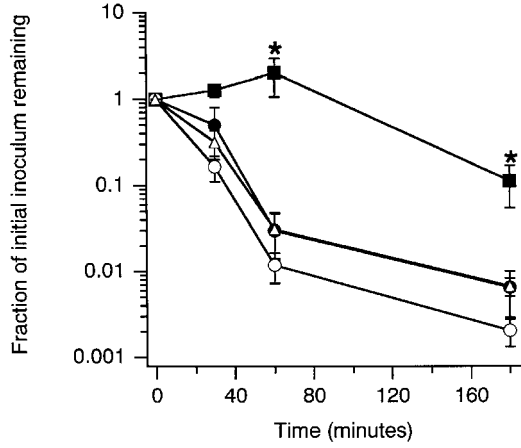

FIG. 1. Experimental bacterial challenge of sheep airways. Sheep were either pretreated with dapsone in PBS (*closed squares*) or with PBS alone (*closed circles*). Control sheep (n= 6) and pretreated animals (n= 4) were challenged with *P. haemolytica* (ATCC no. 29698) in 3 ml of PBS. Immediately after, 30 min, 1 h and 3 h after challenge, samples of tracheal surface fluid were collected and quantitative bacterial cultures used to determine CFU. Values, normalized to the initial value after challenge, are plotted as means ± SE. Control sheep showed rapid clearance of inhaled bacteria. Dapsone treatment significantly inhibited bacterial clearance at 60 and 180 min (*P< 0.05). Treatment with 5 mg bovine milk LPO reversed the impaired clearance in dapsone-treated animals (*open triangles*). As an additional control, treatment with 5 mg of LPO alone did not significantly improve clearance of bacteria (*open circles*). Reproduced with permission from Gerson et al (2000).

mRNA library. *Invitro*, airway LPO was capable of catalysing the formation of the biocidal compound HOSCN/$OSCN^-$ (Gerson et al 2000).

These data suggested that airway LPO could be part of the airway mucosal defence against infection. But in order to function as a protective mechanism against infection in the airway, substrates for LPO (H_2O_2 and SCN^-) must also be present in the lumen. Since H_2O_2 and superoxide have been detected in airways by others (e.g. Adler et al 1992, Kinnula et al 1992, Liberman et al 1995), we measured SCN^- in airway secretions from intubated sheep. We found a concentration of 0.16 mM, high enough to serve as a substrate for LPO (Pruitt et al 1988) and considered bacteriostatic (Reiter & Perraudin 1991). The demonstration of SCN^- in secretions, together with the detection of H_2O_2 and LPO, shows that all components of the LPO biocidal system are present in the airway lumen.

The hypothesis that the LPO system functions *invivo* to maintain airway sterility was examined using experimental bacterial challenge of the sheep respiratory tract (Gerson et al 2000). Sheep were pretreated by aerosol with dapsone, an inhibitor of peroxidases including airway LPO, or treated with PBS as a control (Fig. 1). The

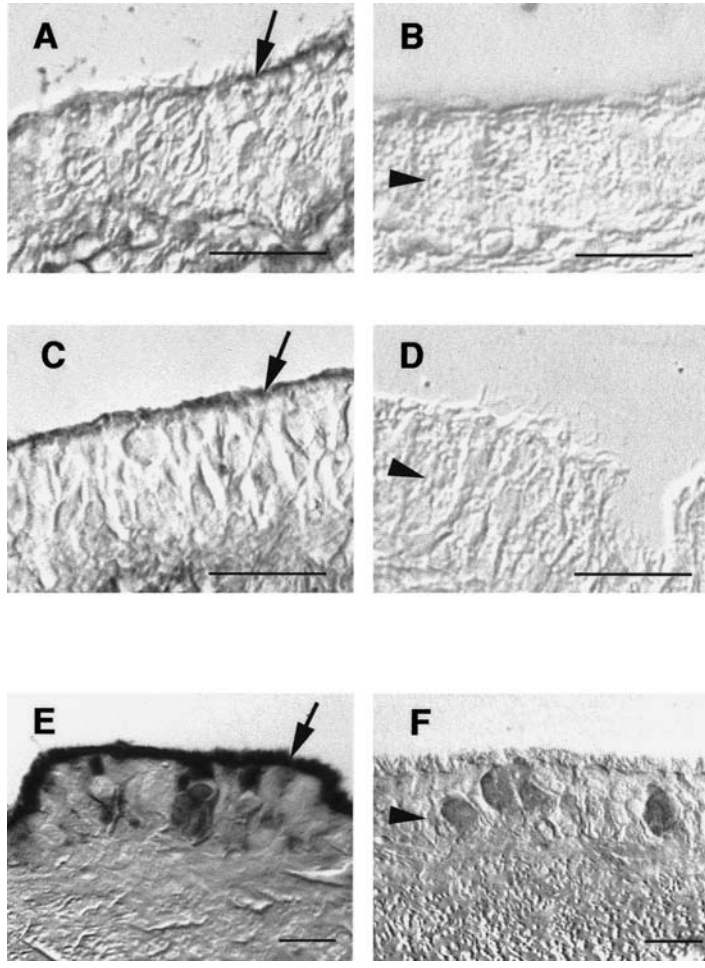

FIG. 2. Staining for hyaluronan and LPO in airway epithelial cells. Paraffin sections of ovine trachea were stained with a biotinylated hyaluronan-binding protein and avidin-alkaline phosphatase (A–D) showing that hyaluronan is localized to the ciliary border of the epithelium in addition to its known localization in the submucosal interstitium (A). Incubation with hyaluronidase (37 °C overnight) removed specific staining for hyaluronan (B), whereas incubation with chondroitinase ABC at pH 7.5 did not change the staining pattern for hyaluronan (C). When chondroitinase ABC was used at pH 5.6, where it has hyaluronidase activity, hyaluronan staining was also removed from the sections (D). Labelling with anti-LPO antibodies and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (E) also revealed specific staining along the ciliary border of the airway epithelium. Incubation with hyaluronidase removed surface-bound LPO (F). All bars are 10 μM. Arrows depict the ciliary border of the epithelium. Arrowheads point to the epithelial cell layer. Reproduced with permission from Forteza et al (2001).

animals were challenged by aerosol with *Pasteurella haemolytica*, a natural pathogen in sheep. Immediately following the challenge and sequentially thereafter, a sample of airway fluid was collected by cytology brush and quantitative culture of these samples performed. Sheep pretreated with dapsone had significantly slower clearance compared to control animals at 60 and 180 min after the challenge. To show that the result of the dapsone treatment was due primarily to inhibition of peroxidase activity, dapsone-pretreated animals were administered bovine milk LPO by aerosol immediately following the bacterial challenge. In dapsone-treated animals, bovine LPO post-treatment reversed the effects of dapsone, supporting the interpretation that dapsone was primarily inhibiting endogenous peroxidase (Fig. 1). This was not due to unspecific LPO effects, as LPO treatment alone did not significantly improve clearance of the PBS-pretreated control animals. Thus, sheep whose airway LPO was inhibited by dapsone were compromised in their ability to clear viable bacteria from their airway suggesting that peroxidase was a significant contributor to bacterial clearance from the airways.

Postsecretory fate of LPO

As indicated, LPO is secreted into the airway lumen. Secretions are believed to be constantly cleared by mucociliary action. Consequently, secretion has been postulated to be the main determinant of enzyme availability and activity on mucosal surfaces. Since many aspects of innate mucosal defence depend on the continued presence of secreted proteins and enzymes on the mucosa, rapid removal requires continued secretion to maintain the surface presence of secreted components important for host defence. However, we found that this paradigm does not necessarily apply in the airways (Forteza et al 2001).

Immunohistochemistry of ovine tracheal sections revealed specific staining for LPO not only in submucosal gland cells and in goblet cells, but also along the ciliated border of the airway epithelium (Fig. 2). Direct visualization of LPOs activity in tissue sections using H_2O_2 and diaminobenzidine confirmed the results obtained by immunostaining, ruling out non-specific adherence of antibodies to the ciliary border. Since we have shown that tissue kallikrein obtained from airway secretions was bound to hyaluronan (Forteza et al 1999), we wondered whether hyaluronan immobilizes LPO at the apex of epithelial cells. Histochemistry of tracheal sections for hyaluronan using a biotinylated hyaluronan-binding protein also labelled the ciliated border of the epithelium. Digestion with hyaluronidase eliminated the apical staining for both hyaluronan and LPO. But hyaluronidase did not remove all glycoconjugates from the apical border of the epithelium as evidenced by the retention of Alcian blue and PAS positive material. In addition, chondroitinase ABC did not eliminate staining for

hyaluronan and LPO at pH 7.5 where it has little hyaluronidase activity. When chondroitinase ABC was used at pH 5.6, however, where it has high hyaluronidase activity, apical staining for hyaluronan was also eliminated (Fig. 2). Thus, these data suggested that cell membrane-bound hyaluronan was retaining LPO at the airway epithelial surface. Using non-denaturing agarose gel electrophoresis, we confirmed that bovine milk LPO actually binds to hyaluronan. The binding could be reversed by hyaluronan digestion with hyaluronidase. Since LPO has an alkaline p*I* but no specific hyaluronan binding sites, non-specific electrostatic interactions could account for its association with this glycosaminoglycan.

In contrast to tissue kallikrein which is inhibited by hyaluronan binding (Forteza et al 1999), TMB assays of LPO activity revealed that its binding to hyaluronan did not inhibit its activity. This makes sense since airway LPO contributes to host defence against inhaled bacteria (Gerson et al 2000). Thus, hyaluronan serves as an anchor for LPO and possibly other enzymes at the airway surface.

To examine whether hyaluronan actually protected LPO from removal by mucociliary clearance, we used fluorescently labelled LPO and albumin (Forteza et al 2001). Labelled LPO and albumin were applied onto the same region of the surface of a freshly isolated trachea and the migration of the fluorescence was measured over a 30 min period. LPO was not transported after application whereas albumin moved forward over the whole 30 min period. The immobilization of the enzyme was in fact due to hyaluronan since LPO was not immobilized when the tracheal epithelial surface was pretreated with hyaluronidase, moving at the rate of albumin over the 30 min observation period. These data show that airway LPO is bound to the airway epithelial surface by hyaluronan and not transported away by mucociliary clearance.

Summary

The data presented here suggest that the LPO system plays an important role in innate airway host defence. They also contradict the paradigm that secreted substances are rapidly cleared by mucociliary action and provide evidence that at least some enzymes are retained at the surface by binding to hyaluronan. In the case of LPO, the binding seems to occur through electrostatic interactions and in this sense it is remarkable that many antimicrobial substances in nasal secretions (but likely also in airway secretions) are cationic in nature (Cole et al 1999) and could bind to hyaluronan as well. Thus, the example of the LPO antimicrobial system shows that hyaluronan serves a previously unrecognized role in mucosal host defence by immobilizing enzymes at the surface, thereby providing an apical enzyme pool ready for use that is protected from mucociliary clearance.

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DISCUSSION

Vargaftig: Tissue kallikrein is obviously a very potent releaser of bradykinin. Augmentation of bradykinin content is one of the explanations for cough but not for asthma.

Salathe: If tissue kallikrein is activated it will produce bradykinins in the airways and cause, in addition to cough, bronchoconstriction and airway hyperreactivity in asthmatics but not normal subjects.

Vargaftig: Do you know whether the amount of kallikrein released from the surface is enough to produce sufficient of bradykinin to cause bronchoconstriction?

Salathe: We haven't measured the exact amount released from the hyaluronan-bound pool at the surface. In general, the majority of tissue kallikrein is bound to hyaluronan in the airways and therefore inhibited; only a small amount is active before hyaluronan is degraded.

Harkema: Could you comment on the motility of cilia during these events?

Salathe: This is something we are working on intensively. We looked for one of the extracellularly expressed receptors for hyaluronan, namely RHAMM (receptor for hyaluronan-mediated motility), because this receptor has been described to be present in sperm tails. Sperm tails have a requirement for hyaluronan for their flagellum to be fully active. It has been shown that blocking RHAMM reduces sperm motility. If hyaluronan is put on airway epithelial cell cultures, ciliary beat frequency increases. We know that this effect of hyaluronan is mediated through RHAMM because a functionally blocking antibody against RHAMM prevents this increase in motility. The problem is that RHAMM is an unusual receptor. It has no transmembrane domain and, while it is known that it signals in multiple cell types, it is unclear how it does it. There is some evidence that RHAMM, with hyaluronan attached, can be internalized into the cell and RHAMM can activate ERK. How this relates to ciliary beating, however, is not clear. Receptors associated with ERK stimulation are not the receptors we usually associate with altering ciliary beat frequency.

Harkema: Are these receptors specific for ciliated cells?

Salathe: They are on ciliated but not goblet cells. They are probably on cilia themselves, and on the apical membrane. But there is also a large pool of intracellular RHAMM in the apical portion of ciliated cells.

Jeffery: Have you undertaken electron microscopic immunocytochemistry to localize whether RHAMM is really associated with the cilia, or on that very typical microvillus border of ciliated cells? There is a glycocalyx there; it is a very glycoconjugate-rich layer. I would predict that it is on the microvillus border and not the cilia *per se*. Often with immunostaining one sees an area that is stained much greater than that expressing the antigen.

Salathe: We haven't done these experiments, so we don't know.

Sheehan: Out of all the glycosaminoglycans you might choose, hyaluronan has the lowest charge density. If you want to invoke a simple electrostatic basis for the interaction with peroxidase, it would not be the candidate that one would expect to bind, especially when there is evidence for sulfated glycans in the epithelial surface layer which have a much higher charge density and would be much more avid ligands.

Basbaum: Perhaps we don't want something that is too avid.

Sheehan: If we want to invoke a non-specific electrostatic interaction, we would have to let the charge density be the chooser. I am saying that there are many other charged species that would have a more avid affinity on the basis of electrostatics. This raises two questions. If it is bound to hyaluronan, what is the mechanism? I would suggest that it would have to have a protein specificity that relates it to the hyaluronan. An alternative hypothesis would be that there are other molecules that do bind to hyaluronan, such as sulfated proteoglycans that could bind peroxidase electrostatically.

Salathe: In vitro you can make LPO bind to hyaluronan. However, we can't rule out the possibility that it binds to something else in the airway and that this complex binds to hyaluronan.

Sheehan: I can see from your experiments that you couldn't have distinguished between a fellow traveller with the hyaluronan network and direct hyaluronan binding.

Basbaum: You mentioned an experiment with chondroitinase at a certain pH that was supposed to degrade chondroitin sulfate and not hyaluronan. Does that address this issue in any way?

Salathe: The data only tell us that when we use chondroitinase at a low pH it cleaves hyaluronan and we lose staining at the surface, while chondroitinase at pH 7.5 does not cleave hyaluronan and we do not lose hyaluronan staining at the surface. But whatever the interaction of LPO with hyaluronan is, we are going to lose it once you start breaking down hyaluronan; even if the LPO was bound to a secondary molecule, and not to hyaluronan directly, you would get the same kind of result.

Sheehan: There are many examples of hyaluronan–protein complexes, and many of them are dissociable by hyaluronan oligosaccharides. Have you tried these on your cells?

Salathe: We know that tissue kallikrein needs to bind to a 10-mer hyaluronan oligosaccharide to be inactivated. We have not investigated other specific interactions. An interesting question is what size of hyaluronan activates RHAMM and how this relates to biological functions such as ciliary beating. We are starting to look at these questions, but have no answers yet.

Basbaum: The underlying significance seems to extend beyond LPO and tissue kallikrein. There are a lot of positively charged bactericidal molecules in the mucus,

including lactoferrin, lysozyme and defensins. It is not an accident that they are positively charged, because this helps them to bind to or intercalate into the negatively charged bacterial membrane. Might the macromolecular structure we are talking about here serve that purpose of immobilizing (at least transiently) a large number of these different cationic molecules? If this is the case, are they most adaptive and beneficial when they are mobilized, or do we want them to be released under certain conditions? If so, under what conditions?

Salathe: We do not know. However, there is still the possibility of secretion, no matter what you do. The coat is already there before the necessity for secretion and could provide a first line of defence.

Basbaum: That's what I mean by 'immobilized'. If the bacterium comes along it may be sufficient just to have these molecules present. But perhaps under other conditions you would want them to be cleaved and released. It may be like an ion exchange system, where they are released by an electrostatic interaction.

Sheehan: There is a literature building up in the matrix field about the way these kinds of molecules are commonly involved in an activation phenomenon involving protein binding. For example, heparan sulfate is a cofactor in the binding to receptors for various growth factors. It is a speculation that something like this could be happening here.

Barnes: There's a marked increase in the production of hydrogen peroxide in COPD and severe asthma, particularly during exacerbations. Is there any reduction in LPO activity in these conditions?

Salathe: I don't know in COPD. In asthma, LPO activity goes up.

Basbaum: So are oxygen radicals coming from the inflammatory cells?

Salathe: Large proportions of the oxygen radicals probably are, but the airway epithelium itself is capable of making at least some. Bill Abraham has data looking at allergen challenge in sheep (Liberman et al 1995). He detected superoxide radicals being produced in the airway epithelium. The problem with this is that we really don't know what enzyme system is responsible for its production in airway epithelia. Superoxide can be produced by multiple mechanisms, but in inflammatory cells it is mainly made by NADPH oxidase. But not all components of NADPH oxidase have been found in airway epithelial cells.

Basbaum: Peter Barnes, you brought up the fact that there is an increased oxidant load in the asthmatic airway. Where does this come from?

Barnes: I presume ROS are mainly derived from inflammatory cells, such as eosinophils, neutrophils and macrophages in severe asthma and chronic obstructive pulmonary disease (COPD). But epithelial cells may also contribute. We are interested in increased oxidative stress in severe airway disease because hydrogen peroxide markedly reduces the anti-inflammatory effects of steroids through an inhibitory effect on histone deacetylases. The mechanism you describe could be important, because if defective, and therefore not able to

remove hydrogen peroxide, this could lead to the steroid insensitivity which is characteristic of COPD and severe asthma.

Engelhgardt: Did you say that the predominant site of LPO production was in the mucous glands, or do the goblet cells in the surface airway epithelium also make it?

Salathe: It is produced in goblet cells but most appears to be made in submucosal gland cells.

Engelhardt: In cystic fibrosis where the glands may have problems secreting, I wonder whether LPO concentrations in the mucous secretions may be reduced. This might be a factor perpetuating neutrophil damage and not enough clearance of the hydrogen peroxide.

Salathe: The activity of LPO itself is very difficult to measure in secretions from patients with cystic fibrosis. Because there is so much myeloperoxidase from the inflammation, it is impossible to distinguish these two activities enzymatically. Also, antibodies are usually not good enough to separate the different peroxidases for use in activity assays.

Nadel: If LPO is an important molecule, is it co-secreted with mucin?

Salathe: Since it is present in secretory granules, it is co-secreted with mucins in goblet cells. Using electron microscopy, we have shown that it is detectable at all levels of the secretory pathway. The gene seems to be the same LPO gene known from the mammary gland, but the promoter must be different since LPO is induced in the mammary gland but constitutively expressed in the airway.

Basbaum: You don't think it is induced by bacteria?

Salathe: We haven't done these studies.

Rose: It is interesting that LPO is biosynthesized in the goblet cells, because most of the host defence molecules are synthesized in the serous cells. Are there other examples of defence molecules synthesized in the goblet cells?

Salathe: Not that I know of.

Verdugo: Have you compared the enzymatic activity of this LPO when it is bound with its activity when it is free?

Salathe: Yes. When it is bound to hyaluronan, the activity does not change when tested with tetramethylbenzidine, which is a substrate for LPO.

Verdugo: So this is acting as an immobilized enzyme. It doesn't matter whether the enzyme is free or if it is bound. This is interesting.

Holgate: I am particularly taken by the study you did showing that the average molecular size of hyaluronic acid decreases in the airways after allergen challenge. The results appear quite dramatic. In Southampton, in collaboration with researchers at Bayer, Germany, we have been looking at a split product of hyaluronan in the circulation as a biomarker of activity in asthma. It really does look to be quite good, since very high circulating levels occur in proportion to asthma severity. Have you explored this at all in any other clinical situation?

Salathe: I am not sure whether the hyaluronan split products that appear in the circulation stem from the airway lumen (as the one we showed in our study does). What happens with hyaluronan that has been secreted into the airways? Rather than being removed through the circulation, I would have predicted that it is transported out of the airways by mucociliary clearance. However, there is also hyaluronan in the subepithelial layer. Perhaps what is appearing in the circulation is from this pool that is not actually in the airway lumen itself. Such split products may just be a general feature of how it is broken down. If you create enough radicals you may be able to break it down at any location. I'm not positive that this is therefore specific for asthma.

Holgate: In chronic severe asthma we see up to 20-fold increases in this soluble hyaluronan fragment. It seems to be quite a good biomarker of disease activity, which is obviously reflecting different processes than classic eosinophil products. I do think it is a biomarker worth pursuing further.

Davis: Matthias Salathe, is there enough hyaluronic acid in the gel, by mass, for it to be so important that we shouldn't be talking about a different form of secretion? I assume it is *not* coming from secretory cells. If so, where is it coming from?

Salathe: It is likely coming from submucosal gland cells.

Davis: Would you expect to find it in secretory granules?

Salathe: This is a really interesting question, because how it is produced there is unknown. We know that hyaluronic acid is produced by hyaluronic acid synthases, which are usually plasma membrane enzymes. So it may not be in secretory granules. But we don't even know which hyaluronic acid synthases are expressed in the airways.

Davis: Do you have any idea what fraction of the mucus consists of hyaluronic acid?

Salathe: I don't know.

Basbaum: Have you succeeded in staining the gland granules with anti-hyaluronan?

Salathe: Not with anti-hyaluronan. But we have tried to stain with the binding protein. We don't see much in secretory granules. What we see is that tissue kallikrein seems to already be bound to hyaluronan when it is detectable in the airway lumen.

Sheehan: There is a growing literature on intracellular hyaluronan. It has been implicated in several cellular processes. We don't know how it gets there, i.e. we don't know whether there are synthases that work inside cells. Thus, the source of the hyaluronan that is to be found in this layer with the cilia may not be from goblet cells. In other words, it could be being synthesized into that environment by a synthase that is located in the plasma membrane. The cell copy number could be only two or three and they could make quite enough hyaluronan to perform the function that Matthias is discussing, thus we need to be careful in the interpretation of the histology.

Salathe: On cancer cells it is possible to picture hyaluronic acid synthase. On almost all other cells we can't find them because of the low copy number. Some of the intracellular hyaluronan is believed to be secreted first and then taken up again, although this is also quite controversial.

Nettesheim: In looking at therapeutic options, are we sure that we really want to reduce mucus hypersecretion? Here we are talking about defensive molecules. If their production is co-regulated with mucus secretion, how desirable is it to suppress antibacterial and antioxidant molecules?

Basbaum: That's quite a profound question. Underlying our discussion at this meeting will be questions about whether we understand how mucins are overproduced, and if we have a step by step molecular understanding of how it occurs. There will be questions regarding which step in the signalling cascade is the most beneficial one to interrupt. In general, steps that are most specific to the molecule of interest are those we want to target. If mucin induction is almost uniformly dependent on NF-jB, for example, does this mean that we should use NF-jB as a therapeutic target?

Nettesheim: This also connects to Jay Nadel's comment on how much we have to know about the specificity of regulation of these various molecules in order to understand where we want the therapeutic target to be. If the target is too broad we may be doing as much damage as good.

Jeffery: Is it a question of the quality of components as opposed to the quantity? We have been talking about *hyper*secretion. Jørgen Vestbo mentioned chronic mucus hypersecretion, and he related it to morbidity and mortality of COPD. With regard to chronic mucus hypersecretion, one either has it or not, by the definition of chronic bronchitis. All the studies that we have discussed had to do with patients who were being defined as chronic bronchitic. There is a large group of subjects who are recurrent hypersecretors and who don't quite make it to the MRC definition of chronic bronchitis. Perhaps, in these individuals mucus production has increased to the correct level to act as a host defence. We have set an artificial definition above which we define hypersecretion. Normal respiratory defences are then overwhelmed, such as mucociliary clearance. The system cannot cope with the excessive quantity of mucus. This then becomes 'disease', as opposed to a protective mechanism. Perhaps we should be targeting the entire process: we should be merely limiting quantity in our therapy, and maintaining the quality as it is.

Disse: This is probably not an all-or-nothing situation. We might well find a bell shaped dose–response in clinical trials. This would mean we have a narrow therapeutic range.

Rogers: John Engelhardt's finding in a variety of patients was that MUC5B seemed to be an important molecule associated with infection. Getting back to Peter Barnes' point about the quality of the mucus, we probably need to know a

lot more about the make-up of normal mucus versus pathophysiological mucus. This is a combination of volume and quantity, and MUC5B.

Basbaum: There are insoluble and soluble mucins. Ingemar Carlstedt will address this. For example, should we be looking at insoluble mucins and hoping to reduce them, and letting the soluble ones flow?

Carsltedt: I don't think we're yet in a position where we can answer this question. When I talk about 'soluble' and 'insoluble' mucins, this is under very specific conditions — i.e. during extraction with guanidinium chloride. Obviously, the oligomeric mucins are 'insoluble' in a physiological sense, because they are forming a gel. When we take a respiratory secretion and spin it hard, most of them come down with the gel. It is only in severe cystic fibrosis patients that we see a lot of mucin in the sol phase. The properties soluble/insoluble imply that there is something different in their macromolecular architecture, but we don't know how this relates to their function, for example, how they are transported by the cilia. There are two major sources of mucus: the goblet cells and the submucosal glands, and it should in principle be possible to down-regulate one and not the other. But again, how this affects function is not known. When we started to use antibodies, we believed that it was a simple situation with MC5AC produced by the goblet cells and MUC5B by the glands. However, in irritated airways we see goblet cells that produce MUC5B and also some MUC2. MUC2 is highly 'insoluble' and structurally different from MUC5B. However, the functional implications of this are unknown. Does this upset the cilia, and is this something we want to down-regulate? Finally, we know that the tissue produces several mucin 'glycoforms' but we do not know how this affects the properties of the secretion.

Basbaum: Earlier on we were talking about Alex Silberberg's work. I remember Alex used frog palate models. He put mucus or synthetic viscous solutions on the frog palate and studied how it was cleared under various experimental conditions. Has anyone done this type of experiment using a MUC2 polymer versus a MUC5AC polymer on a frog palate? Would this be interesting to do? It is possible that when cystic fibrosis patients become infected with *Pseudomonas* or *Staphylococcus* and start making MUC2 mucin, the integration of the MUC2 monomers into the gel will make it significantly more difficult for the cilia to move the mucus. This should be testable in a frog palate model.

Nadel: I think this is an interesting question. Mucins have a unique property called thixotropy. When a thixotropic material is stirred, its viscosity decreases. When a cilium moves the mucus forward, as it stirs the mucin it decreases the viscosity and allows it to go forward. Then, when there is a relaxation behind, there is an increase in viscosity and the material behind snaps forward. This is what Silberberg's major description was. The question is, what is the relationship between thixotropy and the structures of mucins that Ingemar Carlstedt is discussing? As far as I understand it, there may be no way of imitating mucins, in

terms of clearance. Is there a relationship between these structural determinants and thixotropic properties?

Verdugo: This is a terribly complex gel we are talking about. It is a semi-ordered system, which we demonstrated in a paper several years ago was a liquid crystalline order (Viney et al 1993). It is not a completely random network. Nonetheless, there is no question that the degree of hydration determines the viscoelastic properties of the gel. In gels such as mucus, which contain a tangled polymer network, the number of tangles per unit volume of gel primarily determines the rheological properties. As the gel swells the tangle density decreases and the gel becomes more fluid. In gels that are made of very long polymers, the disentangling ability produced by hydration is slow as it depends on the square of the polymer length. If you increase the amount of longer mucins in the gel, this will change the kinetics of hydration of the mucus in a drastic manner. In order to relax, the chains need to shear, one across the others. In this respect, for a collection of mucins where the charge density is the same but the length differs, the mucosa could regulate the properties of the resulting gel that is coming out by expressing mucins of different length. To what extent does the expression of mucins of different length effect the properties of the mucus? And to what extent is there an imprinting of expression of the particular types of mucin that don't lend themselves to good hydration in COPD? These are interesting questions.

Sheehan: We have recently got agarose gel electrophoresis experiments to work at the level where we can see oligomerization of the MUC5AC gene product (Sheehan et al 2000). We think this is a major gene product from the surface epithelium in the lung. We have studied a variety of sputa from a range of individuals, and in general, in healthy individuals we find a very highly oligomerized product. We are talking in excess of 12–15 monomers, which is as high as we can go with this method, and it could be much higher. This is a molecule in excess of 30–40 million Da, and longer than 10 lm if it was to be thought of as a single strand. Yet in a number of individuals we see monomers and dimers of that molecule. In controlled experiments using induction in different individuals we can get a huge range in that oligomerization. In other studies we have been doing it is clear that one cannot equate mucus with mucins. There are many other proteins in mucus, and they have a profound effect on the rheology. We have just done some rather simple studies in saliva where we tried to recreate the properties of saliva using what clearly were salivary mucins, and we weren't able to do that without thinking of the incorporation of other proteins into the system. We are now starting to study the morphology of the mucins, looking at them with the electron microscope. We are using gentler and gentler extraction methods. Our first studies were really focused on describing the phenotype. But now that we know all of that we are much more interested in whether there is a residual organization to these macromolecules that reflects how they are made and how they are in the goblet

cells, and what they then look like when they leave and are hydrated. Then we are telling a lot of very different stories about how those mucins look. They can appear as very tightly coiled up bundles, or they can be long, linear and flexible. It is clear that there are different complexes of different mucins coming from different cells. We haven't parsed all of that back into the quality of the mucus gel, but preliminary indications are that mucin 'morphology' will affect mucus quality.

Basbaum: So is the consensus among the biochemists that we can't at present assign any physical properties of mucus to specific MUC family members?

Carlstedt: I don't think we can, and I want to point out that we are discussing the oligomeric mucins (MUC2, MUC5AC, MUC5B and MUC6). I also want to emphasize that there are many post-translational 'complications', such as the degree of oligomerization, glycosylation and proteolytic cleavage events. All these modifications of the primary gene product add properties to the final mucus gel.

Basbaum: Can't one assume that the addition of MUC2 to the airway mucus polymer will increase its viscosity?

Carlstedt: Not necessarily, but it may turn out to be that simple.

Basbaum: Are all the mucins interchangeable?

Sheehan: I would say not. In our hands the glycotype of the different protein phenotypes is very different. It is also very well maintained. We have looked at MUC5AC from very highly inflammatory situations through to normal. It maintains its glyco-phenotype strongly, even through CF. Even though we can see that it has been proteolytically cleaved, it seems to have a recognizable glycotype.

Basbaum: Effectively, you are saying why would nature go to the trouble of systematically parsing the mucins out organ by organ, if there weren't some functional significance to this.

Sheehan: We just haven't been able to decode them. Are these rheological effects? And how are these effects affected by other proteins that bind and modulate the properties of these molecules? We see very strongly conserved phenotypes and glycotypes at the tissue level, but we can't say much more than this at the present.

Jackson: I have a very basic question. Do they mix? Does MUC5AC actually mix with MUC5B, for example?

Sheehan: That's a good question. It has worried me for a long time. We always think of them mixing, but I don't think they do.

Jackson: At a meeting last year Samuel Ho showed some images where MUC5AC and MUC6 were clearly not mixed on the surface of the epithelium (Ho et al 2000).

Carlstedt: We must distinguish between mucus and mucins here. Mucins would certainly mix if in solution. The question is, if you pour out a secretion of MUC6 on top of a secretion of MUC5AC, do they anneal?

Rogers: The probes that are used for the inspection and elucidation of the genes that produce mucins are way ahead of the antibodies that we have to look at the actual gene products. At the moment we are concentrating on MUC5AC, MUC5B and MUC2. Are there mucins in the secretions that are products of genes that have yet to be discovered because we lack the appropriate antibodies? Have you got good probes for MUC3 or MUC4?

Carlstedt: We have been struggling with MUC4. We have tried some 30 different antibodies but so far we have not been able to identify MUC4 as a biochemical entity. However, some of the antibodies work quite well on immunohistochemistry.

Fahy: On this topic of the difference between mucin and mucus, the main components of mucus are mucin glycoproteins, plasma proteins and the products of cell death (such as actins and DNA). Clearly, there is a huge difference between airway mucus in the allergic airway environment, and a suppurative airway environment. The relationship between mucin glycoproteins and these partners in the mucus could have a tremendous effect on the physical properties of mucus. We really can't use the terms 'mucus' and 'mucin' interchangeably, and we can't go from asthma to cystic fibrosis and think of them as just involving different mucin gene expression. We compared asthmatic sputum with cystic fibrosis sputum. One of the characteristics of asthma sputum is relatively large amounts of albumin from microvascular leakage. In cystic fibrosis, large numbers of neutrophils result in high concentrations of DNA and actin. Earlier we discussed using mucus production as a symptom in epidemiological studies. This was well defended as something we have to do, and has value, but we will have to be more sophisticated about how we look at outcomes of mucus-directed therapy. I think we will have to look at the structure of the airway and the components of the mucus (e.g. mucin glycoproteins, glycosylation, antibacterial molecules). If we are going to predict the outcome of an intervention over the long-term, we will need very careful short-term studies about what the intervention is doing to the components of the mucus.

Verdugo: We need to remember that we are dealing with the matrix of a secretory granule. This matrix is tailored for the active products that it carries. For example, in the mast cell there is a matrix of heparin that is carrying a particular product, histamine. And in the chromaffin cell the matrix is chromagranin that is carrying catecholamine. What are the products that are stored in these granules, and how are the matrices adapted to the particular products they release? This is something that might be modulating the type of matrix that is needed. We think we are dealing with a gel, but in fact the goblet cell is a secretory cell and it is producing not only the gel (which is a matrix) but also a bunch of other active products that are stored in the granules. The variations of what goes into the post-translational modifications and the packing of the granule, might be tailored to whatever the

cell is going to be releasing and what is being sequestered from outside that needs to be exported by the cell.

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Mechanisms of submucosal gland morphogenesis in the airway

Mohammed Filali, Xiaoming Liu, Ningli Cheng, Duane Abbott, Vladimir Leontiev and John F. Engelhardt[1]

Department of Anatomy and Cell Biology, and Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA

Abstract. Submucosal glands (SMGs) are thought to play an important role in the pathogenesis of a number of hypersecretory lung diseases including cystic fibrosis, asthma, and chronic bronchitis. In such diseases, severe SMG hypertrophy and hyperplasia is characteristic of disease progression. Our laboratory has focused efforts on defining both the mechanism of SMG morphogenesis and the identification of SMG stem cells. To this end, we have identified a transcription factor (LEF1) that is temporally and spatially uniquely regulated in SMG progenitors during the initial stages of gland development. LEF1 expression is absolutely required for SMG development in mouse and ferret tracheas, but is insufficient to induce *de novo* gland development in the absence of other unknown co-factors. In an effort to delineate the transcriptional cascades responsible for inducing LEF1 expression and subsequent SMG development in the airway, we have begun to dissect the regulation of the LEF1 promoter using cell line and transgenic mouse models. Current efforts are focused on defining the *cis*-acting elements and transcriptional binding factors responsible for Wnt induction of the LEF1 promoter and determining whether the Wnt/β catenin cascade plays a role in submucosal gland development *in vivo*.

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Submucosal glands (SMGs) in the airway secrete fluid, mucous and bacteriocidal proteins, which are important in maintaining normal lung function. In the normal human airway, SMGs are restricted to the cartilaginous airways, the trachea and bronchi. Anatomically, SMGs are composed of a series of interconnecting tubules and ducts localized in the interstitium beneath the surface airway epithelium. The most distal regions of the network are comprised of serous acini and tubules. Secretory products move vectorially from the distal serous tubules through

[1]This chapter was presented at the symposium by John Engelhardt, to whom correspondence should be addressed.

mucous tubules and accumulate in collecting ducts. At the proximal end of submucosal glands, collecting and ciliated ducts connect the glandular tubules to the airway lumen (Meyrick et al 1969). Each of these spatially distinct regions of the SMG has specific cell types controlling the content and viscosity of secretory products, as well as timing the expulsion of secretions in response to airway irritation and infection (Nadel & Davis 1980, Tom-Moy et al 1983).

SMGs are also thought to play an important role in the pathogenesis of a number of hypersecretory lung diseases such as cystic fibrosis (CF), chronic bronchitis and asthma. In such diseases SMG hypertrophy (expanded growth of existing glands) and hyperplasia (growth of new glands) occurs. The potential involvement of SMGs in the pathogenesis of CF is suggested by several findings, including the high level of cystic fibrosis transmembrane conductance regulator (CFTR) expression in SMGs (Engelhardt et al 1992), and the severe hypertrophy and hyperplasia of SMGs characteristic of the progressing disease (Oppenheimer & Esterly 1975). The cellular processes regulating hypertrophic responses of SMGs are currently poorly understood. A better understanding of these processes may aid in the development of therapies to reduce hypersecretatory responses of glands. Additionally, knowledge regarding submucosal gland developmental processes will help to identify airway stem/progenitor cells with capacity for gland development in the airway. Identification of such progenitors may also aid gene therapy efforts targeting SMGs in genetic diseases such as cystic fibrosis.

In humans, SMG development initiates during the 15th week of gestation and continues for approximately 10 weeks. However, *de novo* gland formation may also continue, albeit at a lower level, in fully differentiated adult diseased airways (Oppenheimer & Esterly 1975). Clonal analysis in human bronchial xenografts has also revealed pluripotent progenitor cells in the surface airway epithelium, with full capacity for generating both surface epithelium and submucosal glands (Engelhardt et al 1995). Morphological studies in several species including human, rhesus monkey and ferret, have revealed five stages of SMG development in the airway. In stage 1, epithelial buds, appearing as solid cylinders, grow into the underlying mesenchymal lamina propria. By stage 2, the cylinders have formed lumens that are continuous with the airway surface. At stage 3, the tubules have bifurcated ventrally into two lateral ducts. During stages 4 and 5, further dichotomous branching of ducts occurs, followed by the differentiation of serous and mucous tubule components (Jeffrey & Reid 1977, Tos 1968). The focus of this presentation is to investigate regulatory mechanisms involved in stage 1 gland development.

Although little is currently known about the cellular factors controlling gland developmental processes in the airway, insight can be gleaned by analogy with similar systems of organogenesis. It is likely that reciprocal inductions between

epithelium and mesenchyme play a crucial role during the progressive invagination, branching, and arborization of SMG tubules and ducts. These interactions may be mediated by direct cell–cell contact, by contact with the extracellular matrix, and/or by diffusible factors, as is characteristic of tubulogenesis in kidney, liver, lung, pancreas and mammary gland (Gurdon 1992, Hay & Zuk 1995, Hogan 1999). To date, a number of signalling pathways and transcription factors have been implicated in controlling similar developmental processes, including the Wnt/wingless pathway, patched (Ptc)/sonic hedgehog (shh), bone morphogenic proteins (BMP) 2 and 4, and hepatocyte growth factor. In this group of developmental regulators is the sequence-specific HMG-box transcription factor LEF1 (lymphoid enhancer binding factor 1), which has been previously implicated in hair follicle and tooth development (Kratochwil et al 1996, van Genderen et al 1994, Zhou et al 1995).

Members of the TCF (T cell factor) family transcription factors, including LEF1, share an identical DNA recognition sequence (CCTTTGAACT) through a conserved HMG (high mobility group) domain (Travis et al 1991, Waterman & Jones 1990). Functionally, the hydrophobic arm of the HMG box recognizes irregular DNA structures, such as pyrimidine-rich cruciform or kinked DNA, and intercalates into the minor groove. Consequently, TCF/LEF1 factors bend DNA locally, bringing distantly located DNA binding proteins into juxtaposition, thereby facilitating the protein–protein interactions necessary for transcriptional activation (Grosschedl et al 1994). Unlike other members of the TCF family, LEF1 protein also contains a context-dependent activation domain (CAD) that is dependent on the co-activator ALY in regulating expression of the T cell receptor α (TCRα) (Okamura et al 1998). By virtue of LEF1's role as an 'architectural' transcription factor with context-dependent *trans*-activation characteristics, it has been suggested that LEF1 effectively coordinates multiple developmental pathways in regulating gene expression (Dassule & McMahon 1998, Hsu et al 1998, Riese et al 1997). Recently, LEF1 and other TCF family members, in association with a requisite co-factor, β catenin, have been demonstrated to mediate signalling of the Wnt pathway, which has diverse and critical roles in development (Behrens et al 1996, Capdevila et al 1998, Moon et al 1997).

LEF1 expression was originally identified in cells of the B and T lymphocyte lineages (Travis et al 1991, Waterman et al 1991). However, both LEF1 and the closely related TCF1 (T cell factor 1), were found to be expressed in distinct but overlapping distributions in non-lymphoid tissues during murine embryogenesis (Oosterwegel et al 1993). Further experimentation demonstrated that homozygous germline mutation of LEF1 in mice resulted in salient abnormalities in hair follicle position and orientation (van Genderen et al 1994). These LEF1 'knockout' mice exhibit developmental impairment of many additional organs that require mutual inductive epithelial–mesenchyme interactions, including kidney,

teeth and mammary glands. As part of an effort to identify cellular factors and developmental pathways that control SMG formation in airway, we have recently reported that that LEF1 expression is induced at a high level in SMG progenitor cells at the earliest stage of gland bud formation in ferret trachea (Duan et al 1998). Subsequently, we have shown using multiple ferret and mouse models that LEF1 expression is necessary but insufficient for gland development in the airway (Duan et al 1999).

Results and discussion

In vitro studies of LEF1 promoter regulation

To further address aspects of LEF1 regulation during submucosal gland development in the airway, we cloned a 12 kb fragment of the human LEF1 gene and promoter. The largest lambda phage clone contained the first three exons and 2.5 kb of the LEF1 promoter. Using various fragments of the LEF1 promoter we have established reporter assays based on an *in vitro* cell line and an *in vivo* transgenic mouse to study regulation of this promoter. Promoter deletional analysis in 293 cells has demonstrated four unique transcriptional start sites spanning approximately 1 kb of the promoter and first exon. Studies evaluating several Wnt genes demonstrated transcriptional induction (7–20-fold) of the full length LEF1 promoter by both Wnt3 and Wnt3A but not by Wnt7A (Filali et al 2002). Wnt induction of the LEF1 promoter reporter constructs was substantiated by findings that the endogenous LEF1 mRNA transcript and protein in 293 cells were also induced following transfection with a Wnt3A expression plasmid. Studies using dominant active and negative forms of LEF1 and/or β catenin were used to evaluate the mechanism of Wnt3A-induced LEF1 promoter activity. These studies demonstrated that the stabilized mutant of β catenin (S37A) could induce the LEF1 promoter similarly to Wnt3A expression. Furthermore, expression of both Wnt3A and β catenin (S37A) did not significantly induce LEF1 promoter activity above that seen with Wnt3A alone, suggesting that Wnt3A and β catenin may be acting in series. These findings are similar to studies recently reported by Hovanes and colleagues that demonstrated induction of the LEF1 promoter in the presence of wild type TCF1/β catenin expression (Hovanes et al 2001). However, a difference in our present study is that we demonstrated induction by β catenin alone. Given the fact that numerous TCF1/LEF1 binding consensus sequences reside within the LEF1 promoter, we investigated whether LEF1 might be responsible for autoregulation of its own promoter through β catenin association. Strikingly, the β catenin binding mutant of LEF1 [LEF1(m5)] dramatically decreased promoter activity suggesting that LEF1 may play an inhibitory role in regulating its own

promoter. Recently a dominant negative isoform of LEF1 (lacking the β catenin binding domain) has been reported to be produced from a second promoter in intron 2 of the LEF1 genomic locus (Hovanes et al 2001). These authors hypothesized that this dominant negative isoform of LEF1 may compete with TCF1/β catenin binding in the promoter to down regulate expression. Our results demonstrating inhibition of LEF1 promoter activity by LEF1(m5) substantiate this finding. However, no effects on LEF1 promoter activity were seen with a dominant active fusion of LEF1/β catenin or with wild-type LEF1, suggesting that sites of interaction with LEF1 do not likely play a role in Wnt3A induction through β catenin.

Linear deletion of the LEF1 promoter to − 768 bp demonstrated a significant loss in Wnt3A induction and a substantial rise in baseline promoter activity in the absence of Wnt3A. Internal deletion of − 884 to − 768 bp of the promoter confirmed the existence of a Wnt3A-responsive element (WRE) that appeared to act by a mechanism of de-repression following Wnt3A stimulation. However, studies placing the WRE in isolation 5′ or 3′ to a minimal SV40 promoter suggested that although this element contains all the necessary information responsible for Wnt3A induction, it does not possess repressor function out of the context of the LEF1 promoter. Furthermore, this element gave rise to inducible expression of the SV40 promoter in the presence of β catenin (S37A) expression alone, substantiating that β catenin is likely mediating Wnt-responsiveness at the WRE. Whether this effect is a direct consequence of β catenin interaction with factors binding at the WRE, or an indirect effect by which β catenin alters the abundance of WRE binding factors, remains to be determined. Nonetheless, the ability of the WRE to convey Wnt3A/β catenin responsiveness to a heterogonous promoter, in a context independent fashion, fulfils the requirement for defining the WRE as an enhancer. Repressor functions of this element within the context of the LEF1 promoter are likely due to context dependent interactions with other binding factors.

In vivo studies of LEF1 promoter regulation in transgenic mice

To establish whether fragments of the LEF1 promoter used in our *in vitro* studies contained all the sequence information necessary to reproduce the native LEF1 expression profile, we performed studies in transgenic mice. Twelve founder transgenic lines were screened for LacZ expression from two different LEF1 promoter/LacZ expression cassettes. Of these 12 founders, four lines were established which expressed LacZ in specific anatomical regions associated with LEF1 expression and inductive epithelial/mesenchymal interactions. Expression cassettes which contained either 2.5 kb of the LEF1 promoter or 2.5 kb of the LEF1 promoter plus the first two introns gave distinct patterns of expression.

Studies evaluating regulated LacZ expression in the newborn trachea of transgenic and non-transgenic littermates demonstrated patterns of X-gal staining consistent with expression of LEF1 mRNA in the invading tips of newly growing tracheal cartilage. Evaluation of LacZ expression in tracheal gland progenitors was difficult due to the infrequent abundance of glands in the mouse trachea. However, examples of regulated LacZ expression in infrequent cells of the tracheal epithelium were observed in 3 day postnatal samples (the time in which gland buds begin to form). Due to the difficulty in directly assessing gland progenitors in the mouse, the developmental pattern of LacZ expression was evaluated in other organs also known to regulate LEF1 expression as a feature of inductive epithelial morphogenesis. The most notable organs affected in LEF1 knockout mice include vibrissal (whisker) and hair follicles. In these organs, LEF1 plays an important role in initiating bud formation at the epithelial surface of the skin. Consistent with the important role of LEF1 in follicle formation, LacZ expression was observed in the budding epithelium of the dermis and at subsequent stages of the forming whisker/hair follicle. Interestingly, at early stages of whisker formation, LacZ was located in the region of the dermal papilla of the follicle bulb and at later stages in the follicular bulge which is thought to be a source of migrating follicular stem cells during hair follicle regeneration. Although these results on whisker/hair follicle are only surrogate endpoint for evaluating *cis*-acting elements in the LEF1 promoter that regulate expression during inductive epithelial morphogenesis of SMGs, they do suggest that we have successfully isolated a segment of the LEF1 promoter which contains all the necessary *cis*-acting sites required to properly regulate LEF1 gene expression *in vivo*.

Conclusions and future directions

The activation of LEF1 gene expression in SMG progenitor cells of the airway plays a pivotal role in the inductive processes that control gland development. The successful isolation of a LEF1 promoter segment capable of reconstituting the native pattern of LEF1 gene regulation seen *in vivo* will have obvious applications for studying SMG biology in the mouse. For example, we now know what regions of the LEF1 promoter are required for Wnt induction *in vitro*. The question as to whether Wnt/β catenin pathways play a role in SMG development in the airway remains an open question. We are now positioned to utilize similar LEF1 promoter/LacZ reporter transgenic mouse models with various *cis*-activating elements deleted to dissect the importance of the Wnt/β catenin pathways in activating LEF1 expression during SMG development. Such information will help to clarify the early molecular events that control SMG progenitor cell commitment in the formation of airway glands.

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DISCUSSION

Basbaum: Have you looked for LEF1 in instances of pathogenic gland enlargement in humans or animals?

Engelhardt: No.

Basbaum: Would you expect it to be there?

Engelhardt: We have mainly studied the processes that occur during the initial stages of gland development. This is the stage where epithelial cells are committing to form a gland. As these cells begin to invade, they express LEF1 at the tips of invading gland buds. This process may be very different from what happens when a gland enlarges during hypertrophy. If one believes that there is hyperplasia of glands in certain human airway diseases, I might expect that this process is more similar to what we have studied during development. This would be a disease condition that leads to the formation of new glands, rather than just expansion of existing glands. I suspect that the actual mucous cell hypertrophy or metaplasia probably doesn't involve LEF1 since this process just involves expansion of gland mass and might be different than that for initially establishing a glandular tubular network, which involves LEF1.

Basbaum: Are you implying that gland hypertrophy in disease is a bit like squeezing a toothpaste tube? The gland gets bigger and pushes the connective tissue, as opposed to a phenotypic transformation that occurs when the mesenchyme interacts with epithelium to make a gland.

Engelhardt: You could say that. I believe that gland hyperplasia is probably part of the disease process, at least in CF. There are probably new glands formed there. These regulatory processes are going to be more relevant to our studies on LEF1 at this stage.

Basbaum: So *de novo* formation of glands in an adult organism is taking place past the time when new glands wouldn't normally form.

Nadel: But similar genes may be expressed. A disease process may well involve similar mechanisms of expression to those that occur during fetal development.

Engelhardt: There could be overlap. Unfortunately, there are very few markers of specific cell phenotypes in the epithelium to study this with. But if you take morphology as a marker, these cells are not serous and they are not goblet cells when they are expressing LEF1. They are undifferentiated when these tubules are elongating; the differentiation process happens later. Perhaps they might dedifferentiate in disease, and then they could recover that phenotype and invade. But we haven't looked at this.

Plopper: Even in adults, a lot of gland growth involves the formation of these tubes. I wouldn't be surprised if this was the case. If you get twice as many mucous or serous elements than are in these tubes, you have to have more tubes. There is only one way they are going to grow.

Randell: Paul Nettesheim published a study long ago, addressing the question of gland neogenesis versus hyperplasia (Nettesheim & Martin 1970). There are rudimentary gland-like structures up and down the trachea. In the normal mouse or rat they predominate in the upper trachea. In the chronically injured rat and mouse, new glands occur further down the trachea, and existing glands in the upper trachea increase in size. If the rodent model is in any way applicable to the human, there are probably elements of both neoplasia and hypertrophy.

Jeffery: It has always been my understanding that it was an increase in gland size, rather than an increase in the number of glands, that occurs in chronic obstructive pulmonary disease (COPD). Surely, if it is true across species, here you have a tool to investigate that very issue. If you found that LEF1 was being expressed in these disease situations, it would at least give you an indication that there was an attempt to form new glands. It would help us to understand better the process of so-called gland hypertrophy. The assumption was always that it has been an increase in size not in number; this may be incorrect, and here you have a tool with which to investigate this. Duncan Rogers, I and others have been used to the rat as an experimental model, but occasionally we utilize the mouse. In the rat there are some submucosal glands, particularly in the upper third of the trachea, but they are relatively sparse. In the mouse, there are virtually none. On the other hand, if you were to go just slightly higher anatomically, above the ligature rather than below, and go into the larynx, you would see a wealth of glands I suspect. If you are using the rat or mouse as an experimental system, I would suggest that you look for glands in the larynx.

Engelhardt: Does the size of the glands in the larynx change with disease?

Jeffery: I don't know, but in terms of morphogenesis, if you want to get a handle on the development of glands I wouldn't go to the trachea in the mouse but rather to the larynx.

Harkema: I would suggest you go even further up the respiratory tract. In the nose there are numerous subepithelial glands that may increase in size after irritant exposure. Right at the junction between the olfactory epithelium and the respiratory epithelium there are submucosal glands similar to those found in the human. After irritant exposure (tobacco smoke or ozone) these glands may enlarge or proliferate.

Basbaum: So there is branching morphogenesis of existing glands?

Harkema: It looks that way.

Engelhardt: Are these in the septum?

Harkema: These are actually in the septum and on the lateral wall. This is right at the junction between the olfactory and the respiratory epithelium. An easier structure to look at initially is the Bowman's gland located in the olfactory mucosa in the narrow epithelium. These glands contain predominantly mucous goblet cells. This would be a really interesting site to explore in terms of mucin gene expression or gland development. When people suffer injury to their olfactory epithelium, this neuroepithelium can undergo what is called respiratory metaplasia. In this process, respiratory epithelium replaces olfactory epithelium. People think that the respiratory epithelium may originate from underlying Bowman's glands.

Basbaum: Are you saying there is *de novo* gland formation from the Bowman's glands, and that it is conspicuous and well localized?

Harkema: Yes.

Basbaum: And this occurs in response to what?

Harkema: The only source of mucus in the nasal airways covered by olfactory epithelium is from the underlying Bowman's glands.

Basbaum: And these Bowman's glands are minimal in a healthy animal?

Harkema: I believe these glands have the potential to enlarge or proliferate in response to chronic injury. There are other glands in the nasal airway that are more like respiratory submucosal glands. These mucus-producing glands do appear to enlarge and proliferate in response to chronic ozone exposure in laboratory rats.

Basbaum: Do they undergo branching morphogenesis?

Harkema: I would think so.

Basbaum: If there were a site where we could always find branching morphogenesis that would undergo an exuberant dynamic change as the result of a specific injury, this would enable us to look at the role of LEF1. John Engelhardt, it sounds like you are leaning towards the idea that LEF1 is mainly involved in development, and perhaps not in pathology.

Engelhardt: Because we haven't looked at this I can't really comment. My gut feeling would be that it isn't involved in pathological responses, but from what I've just heard I wouldn't rule it out.

Basbaum: What precisely is LEF1?

Engelhardt: LEF1 is an HMG box transcription factor. It associates with β catenin and binds to promoters to regulate transcription. β catenin is the transcriptional activator; LEF1 binds to DNA and then reconfigures the DNA such that it brings transcription factors juxtaposed to each other so it can regulate transcription.

Basbaum: It doesn't sound as though LEF1 should have a specific role in gland morphogenesis.

Engelhardt: LEF1 has been defined in mammary gland development. Expression profiles of LEF1 are very similar in mammary gland and submucosal gland development. LEF1 has also been defined in many other developmental processes which involve epithelial–mesenchymal interactions. Its regulatory involvement in development is not limited to gland morphogenesis.

Tesfaigzi: What genes are regulated by LEF1?

Engelhardt: It was originally identified to regulate TCR transcription (LEF stands for 'lymphoid enhancing factor'). It regulates the TCR during T cell development. There isn't a huge amount known about the precise genes that it regulates. The field is pretty descriptive at this point. We made mice in that overexpressed LEF1 specifically in the airway, and we were surprised that there was no phenotype. We knew the gene was functional, though, because when we backcrossed it onto the LEF1 knockout mice it rescued the glandular developmental phenotype. So LEF1 is required for inductive gland development but it is not the main regulator.

Nadel: What is the relationship between β catenin and LEF1 to E cadherin expression in this case? In many systems the E cadherin/β catenin complex prevents β catenin from binding to LEF1 and causing its downstream effects on the nucleus. Is that true in this system. In other words, if you up-regulate E cadherin do you inhibit this system?

Engelhardt: In experiments where we used GFP reporters driven by the LEF1 promoters, overexpression of Sonic hedgehog and Patched induced expression. If you also overexpress E cadherin, this can inhibit that induction by Sonic hedgehog and Patched. There's a lot of precedent for the involvement of E cadherin/β catenin complexes in regulating tumorigenesis and invasion. If E cadherin is part of this pathway and is regulating intracellular β catenin, then indeed E cadherin could be an important component in the regulation of transcriptional complexes such as LEF1 or TCF.

Plopper: Could you expand more on how LEF was defined as a lymphoid-enhancing factor? We are concerned about gland expansion and inflammatory

processes, and you are saying that this is actually identified as being involved with the lymphoreticular system. It is now thought that some of the factors that regulate lymphoid development also control brain development, so why wouldn't it be controlling gland development? This may be an important factor.

Engelhardt: This is an example where evolution has utilized a pathway for a completely different purpose. In development LEF1 is involved in epithelial–mesenchyme interactions. In the T cell it is well characterized and regulates expression of the TCR, through very different processes. This difference may have to do with the other co-factors that are expressed in this co-operative transcriptional complex.

Basbaum: The contrast between gland bud formation and carcinoma invasion may be relevant here. The basement membrane is breached in both of these processes. In tumour invasion it is destroyed, but what happens to it in the case of the growing gland bud? Is it destroyed, or does it just get bigger to accommodate the bud?

Engelhardt: I don't know. I made a conscious decision not to study things downstream. We haven't really looked at how LEF1 regulates these factors. Historically, if we look in other model systems, LEF1 is probably involved in regulating metalloproteinase expression that may degrade the extracellular matrix and allow room for the epithelium to invade. It may even guide where the invading tubules go on the basis of the path of least resistance.

Basbaum: Does Bernfield's work help us here? He has done some work on branching morphogenesis in salivary glands.

Engelhardt: I've followed more of the work on mammary gland development. LEF1 hasn't really been characterized in salivary glands, although we are pretty sure that it is expressed there in our LEF1 promoter/LacZ reporter mice. Ocular gland formation is controlled by fibroblast growth factor (FGF) secretion at specific spatial regions that allow for the invasion of the conjuctival epithelium. Reciprocal interactions between epithelium and mesenchyme are involved. These principles are likely to hold for many different types of glands, although the factors may change slightly.

Randell: We localized mouse tracheal epithelial stem cells by finding label-retaining cells. We performed long-term BrdU labelling of injured animals. Because stem cells revert to infrequent cycling, they become apparent as BrdU label-retaining cells. They are localized to gland ducts in the upper trachea and are also found near rudimentary glands in the lower trachea. This is a very similar distribution pattern to the LEF1 promoter-driven LacZ expression.

Basbaum: Can we put them together?

Engelhardt: That's a possibility.

Randell: Our current understanding of β catenin partners in skin development and hair folliculogenesis has taken a small army of cell biologists. John

Engelhardt needs to be commended here. He has shown that LEF1 is necessary but not sufficient. What other factors are necessary to create glands? There are going to be many events that need to be precisely regulated temporally and spatially. John is an army of one, and he is the only one studying it. Ultimately, we will need a greater research effort to understand the regulation of gland neogenesis and hyperplasia.

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Mucin-producing elements and inflammatory cells

Peter Jeffery and Jie Zhu

Lung Pathology, Department of Gene Therapy, Imperial College School of Medicine, Royal Brompton Hospital, Sydney Street, London SW3 6NP, UK

Abstract. Airway goblet cells and submucosal glands form the major sources of human respiratory mucins. In the adult, mucus-secreting glands occupy about one-third of the inner airway wall wherever there is supportive cartilage (i.e. from the larynx to small bronchi). In hypersecretory conditions such as chronic bronchitis, asthma and cystic fibrosis, glands are considered to be the major source of tracheobronchial mucus, especially that which is expectorated abnormally as sputum. In contrast, goblet cells are regularly found throughout the tracheobronchial tree. Normally sparse or absent in bronchioles (i.e. small airways of less than 1mm diameter), goblet cells appear and increase in number in airway hypersecretory conditions: their secretions likely contribute to airflow obstruction and early closure of bronchioles, especially during expiration. The increase in gland mass has been considered to be *the* histological correlate of mucus-hypersecretion in conditions such as chronic bronchitis. However, there appears to be a better association of sputum production with scores of airway wall inflammation than with gland size *per se*. Thus, while the absolute mass of mucus-secreting tissue is important, it is likely that the release of inflammatory cell secretions (e.g. neutrophil elastase, mast cell chymotryptase), mediators of inflammation (e.g. interleukin 4, 13) and products of the metabolism of arachidonic acid (such as 15-HETE) contribute more than previously realized to the hypersecretion of mucus in chronic bronchitis. New data discussed herein provide supportive evidence for this hypothesis and identify a newly reported link between plasma cells and mucus-hypersecretion by submucosal glands. These considerations demonstrate the complexity of targets that need to be considered for the treatment of mucus hypersecretion.

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Airway mucus hypersecretion is a common symptom associated with a number of distinct pulmonary conditions including chronic bronchitis, asthma and cystic fibrosis. The initiating primary cause of the hypersecretion in each of these conditions is likely to be different: for example particulate/noxious gases (cigarette smoke and atmospheric pollution), allergen or infection, respectively. In the normal lung the respiratory portion of the lung (alveoli) is protected from the damaging effects of these agents by short-lasting responses that include neural

reflexes (e.g. bronchoconstriction), cough, acute discharge of mucus, increased mucociliary clearance, and innate and acquired immune reactions (i.e. acute inflammation). However, in genetically determined susceptible individuals and as a consequence of chronic irritation (e.g. heavy smoking for many years), repeated exposure to allergen or chronic infection, there is an increase in the amounts (mass) of mucus-secreting tissue in both large and small airways, and a failure to clear mucus from the airway lumen necessitating cough in an attempt to remove the excess mucus as sputum. Chronic productive cough is the clinical hallmark of chronic bronchitis and is also a common symptom of asthma and patients with the genetic disease cystic fibrosis. In each condition secretions may occlude the conducting airways. Each is also characterized by chronic airway inflammation, albeit the predominant pattern of inflammatory cells may differ. The present chapter focuses on the interrelationships of mucus-secreting tissue and inflammatory cells in chronic bronchitis, and finally between inflammatory cell products and mucus itself, especially in asthma.

Mucus volume, gland size and inflammatory cells

The volume of mucus found in the airway lumen may increase in a number of ways. It may be a consequence of an increase in mass of the mucus-producing elements, or an increased synthesis and release due to enhanced neural drive by the nerves that supply submucosal glands, or a failure of the mucociliary system to clear the airway lumen of mucus, or the result of infiltrating inflammatory cells releasing factors that stimulate increased release of mucus.

The increase in mass includes hypertrophy of submucosal mucus-secreting glands, present deep in the airway walls of bronchi (i.e. proximal airways that have supportive cartilage plates in their walls) and a hyperplasia (i.e. increase in number) of goblet cells present in the surface epithelium lining bronchi. Moreover, goblet cells (but not glands) appear in bronchioli (i.e. airways devoid of cartilage and smaller than 2 mm in diameter), in which they are usually sparse or absent and increase in number (i.e. mucous metaplasia) (Jeffery 2001a). Removal of mucus from this distal site depends upon mucociliary clearance, as it is particularly difficult to clear by cough. In contrast cough is considered to be highly effective in clearing mucus from about the first six generations of airway branching. Effective mucociliary clearance depends upon the interaction of mucus of the correct viscosity and elasticity with the coordinated beating of cilia. As the airways become smaller the cilia become shorter and ciliated cells become less frequent. In the larger airways chronic irritation and infection damage cilia, reducing or stopping the removal of mucus by the mucociliary system: this failure results in pooling of mucus and an increase in the volume of retained lumenal secretions.

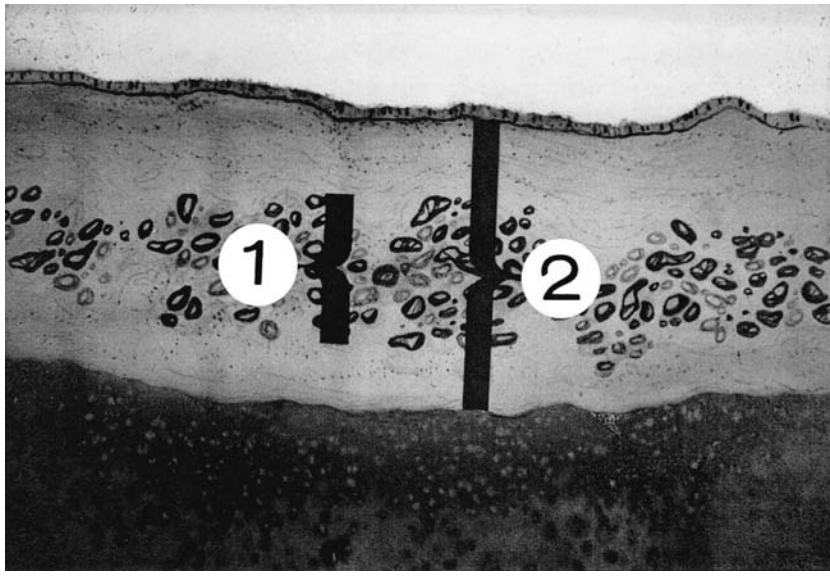


FIG. 1. Representation of the bronchial wall showing airway lumen (top) and supportive cartilage (bottom). In this example of chronic bronchitis the gland to wall ratio (referred to as the Reid Index) is approximately 1:2 instead of the normal 1:3.

Submucosal glands produce the bulk of the mucus found in bronchi. These normally occupy approximately one-third of the inner airway wall, that which lies between the inner aspect of the cartilage and the base of the epithelium (i.e. the Reid Index) (Reid 1954). In chronic bronchitis the hypertrophy of submucosal glands is reflected by an increase in the Reid Index from about 0.33 to 0.50 or greater so that the wall area occupied by gland can increase to more than half (Fig. 1). Gland hypertrophy occurs also in asthma and to a similar extent as that reported in chronic bronchitis (Dunnill et al 1969). It has been considered that the gland hypertrophy in chronic bronchitis represents the histological correlate of mucus hypersecretion (i.e. sputum production) (Reid 1954). However, a study of 101 randomly selected cases coming to autopsy, some of whom were productive of sputum and some of whom were not, demonstrated that the wall area occupied by gland showed a unimodal rather than bimodal distribution of gland area. Moreover 6 % of cases with bronchitis had a Reid Index less than 0.36 and 9% of cases without chronic bronchitis had an index above 0.55 (Thurlbeck & Angus 2002). Another study demonstrated that scores of inflammation rather than hypertrophy provided the better morphological correlate of mucus hypersecretion (Mullen et al 1985, 1987). Whilst these studies support the concept of gland hypertrophy contributing an excess of lumenal mucus they demonstrate that even glands of normal size can

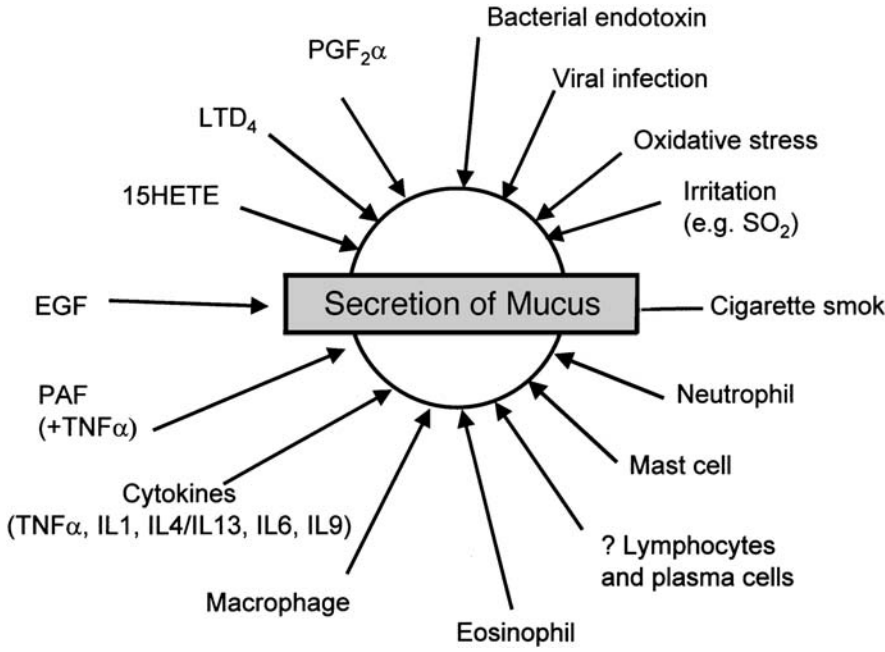


FIG. 2. A summary of the main non-neural mediators and cell products that influence secretion of respiratory tract mucus.

hypersecrete, and implicate other factors, especially infiltration of the airway wall by inflammatory cells, as a likely cause.

Apart from neural reflexes, there are numerous agents that can stimulate the production and secretion (release) of mucus. These include infection, oxidative stress (including tobacco smoke), selected products released by a variety of inflammatory cells, and several mediators of inflammation (including some of the metabolites of arachidonic acid) (see Fig. 2).

Neutrophils, infection and hypersecretion

Loss of ciliary motility combined with excessive lumenal mucus and changes in its rheological (i.e. flow) characteristics lead to failure of mucociliary clearance. This results in the appearance of a relatively thick continuous layer or 'blanket' of secretions overlying now less effective cilia. Bacteria trapped in these stagnant secretions grow *in situ* and release a range of toxic products, including pyocyanins and rhamnolipids: these damage the underlying epithelium and its cilia. Once damaged, epithelial cells slough and bacteria attach to the lateral walls of

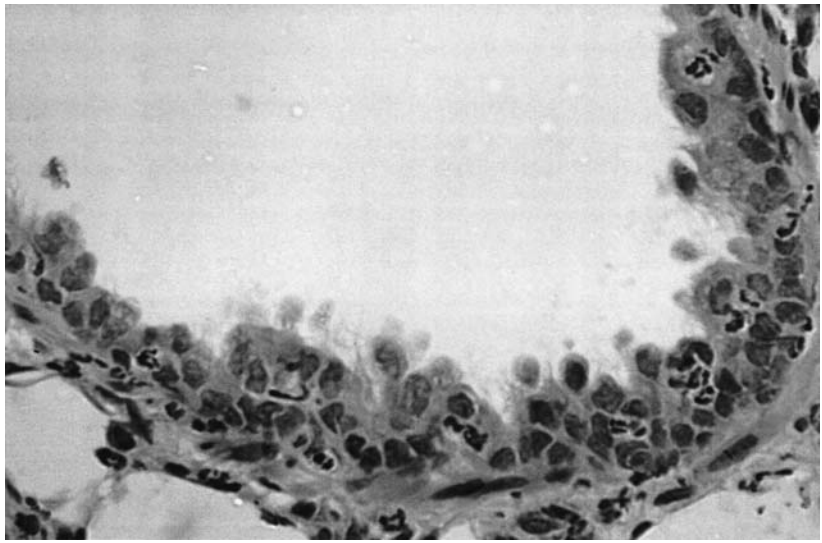
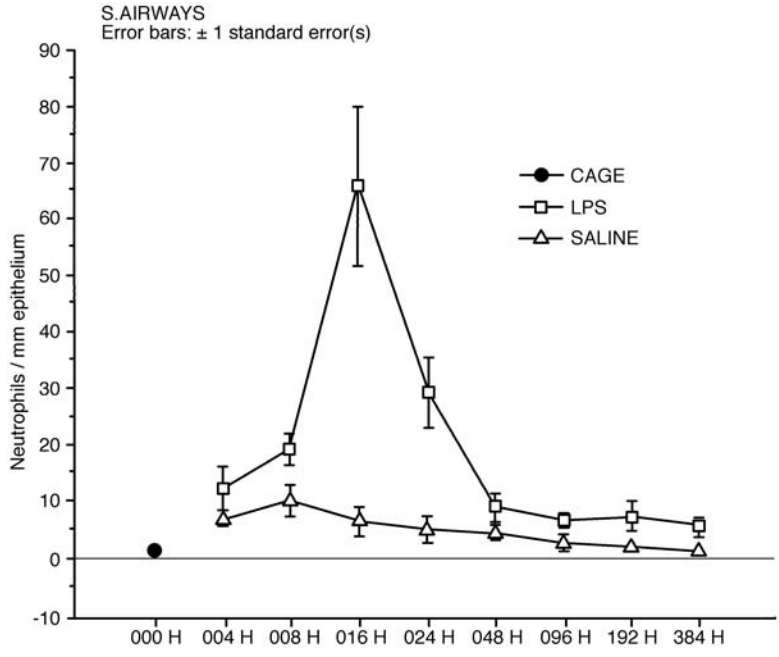


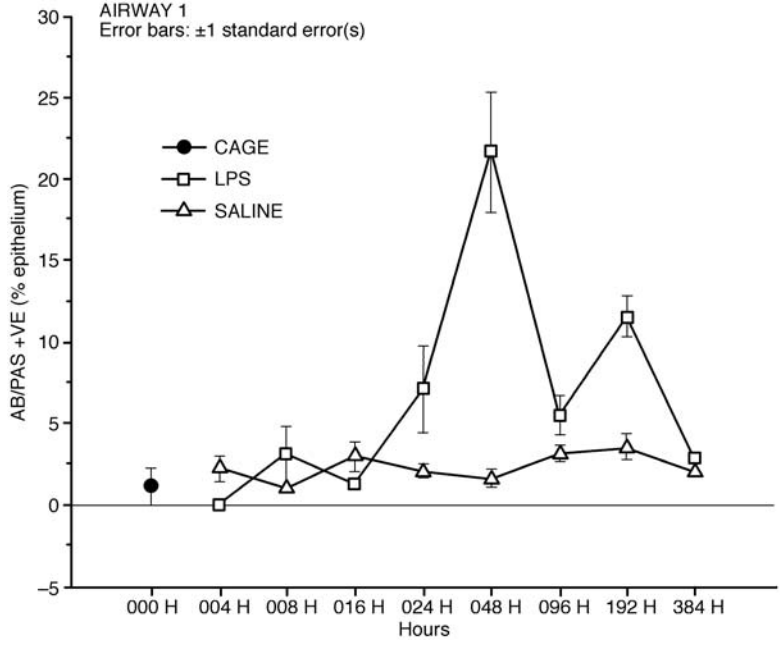
FIG. 3. Histological section of the rat airway mucosa 16 h after a single intratracheal lumenal exposure to LPS. There is marked infiltration of the airway epithelium by neutrophils. Haematoxylin and eosin stain (D. Li, D. Wang & P. K. Jeffery, unpublished results).

epithelial cells, colonizing the deeper airway mucosa and initiating an inflammatory response. In smokers this process may become self-perpetuating.

Bacterial wall components such as lipopolysaccharide (LPS) are powerful inducers of neutrophil recruitment. For example, we and others have found that a single intratracheal instillation of 100 μg LPS given experimentally to specific-pathogen-free laboratory rats results in marked neutrophil recruitment to the mucosa of all conducting airways and the lung parenchyma (Steiger et al 1995) (Fig. 3). The neutrophilia begins at 4 h post-instillation, peaks at 16 h and returns to near saline control levels by 48 h (D. Li, D. Wang & P. K. Jeffery, unpublished results) (Fig.4a). Following the initiation of tissue neutrophilia, mucins begin to be synthesized and the number of goblet cells increases, peaking at 48 h post-instillation (Fig. 4b). The goblet cell hyperplasia is seen in relatively large airways where goblet cells are normally sparse, and there is mucous cell metaplasia in the smaller (distal) airway branches where goblet cells are normally absent (Figs 5a,b). This experimental system represents a relatively good model for the changes to goblet cells seen in humans with chronic obstructive pulmonary disease (COPD). By electron microscopy there is evidence of cell transformation: transitional forms between Clara and goblet cells, serous and goblet, and even ciliated and goblet cells are observed (Fig. 6), emphasizing the extreme lability of epithelial cells.

There is evidence of increased tissue neutrophilia, associated with mucus-secreting glands in humans also (Saetta et al 1997). Submucosal glands of airway





tissue resected from smokers demonstrate increased numbers of elastase-positive neutrophils adjacent to the secretory acini (units) in individuals productive of sputum (i.e. with chronic bronchitis) (Figs 7a,b). A powerful mucous secretogogue activity for neutrophil elastase has been demonstrated previously (Nadel 1991, Sommerhoff et al 1990). Moreover, a single experimental intratracheal instillation of human neutrophil elastase given to laboratory rodents has been shown to induce progressive and irreversible goblet cell hyperplasia and metaplasia (associated with the development of emphysematous destruction of lung parenchyma) over several months (Christensen et al 1977).

T lymphocytes and regulatory cytokines

There is also a trend to increased numbers of gland-associated CD8+ cells (probably cytotoxic T lymphocytes) in smokers with chronic bronchitis such that the ratio of CD4+ :CD8+ cells falls significantly (Saetta et al 1997). This reflects the predominance of airway mucosal CD8+ cells reported originally by our group in smokers with COPD and confirmed in all airway generations and lung parenchyma to be a characteristic of COPD (Jeffery 2001b, O'Shaughnessy et al 1997). It should be noted that smoking *per se* is associated with a peripheral blood leukocytosis. There is a reversible decrease in the CD4:CD8 ratio in heavy smokers and even moderate smoking reduces the CD4:CD8 ratio in bronchoalveolar lavage (BAL) fluid of otherwise asymptomatic smokers (Costabel et al 1986, Miller et al 1982).

The CD8+ predominance in COPD contrasts with the CD4+ T cell predominance characteristic of non-smokers with asthma and the increased eosinophilia and expression of the regulatory cytokines interleukin (IL)4 and IL5. The altered pattern of T cell subsets led us to the hypothesis that IL4 and IL5 would not, therefore, be expressed in smokers with chronic bronchitis or COPD and that eosinophilia would not but rather neutrophilia would be the characteristic mucosal infiltrate in COPD. The hypothesis has not been supported by our subsequent investigation (Zhu et al 2001a). Instead, examination of bronchial biopsies in chronic bronchitis has demonstrated that IL4+ cells are frequently found in the subepithelial zone whether it is by immunostaining for the protein (Fig. 8) or *in situ* hybridization (ISH) to detect mRNA (i.e. expression of the

FIG. 4. (*Upper*) Neutrophil recruitment to the airway epithelium over time (hours) after a single intratracheal instillation of 100 μg LPS given experimentally to specific-pathogen-free laboratory rats. There is a peak at 16 h (D. Li, D. Wang & P. K. Jeffery, unpublished results). (*Lower*) Goblet cell increase after a single intratracheal instillation of 100 μg LPS showing a peaks at 48 h and 192 h post-instillation (D. Li, D. Wang & P. K. Jeffery unpublished results). Alcian blue/periodic acid–Schiff (AB/PAS) stain to demonstrate intracellular mucin.

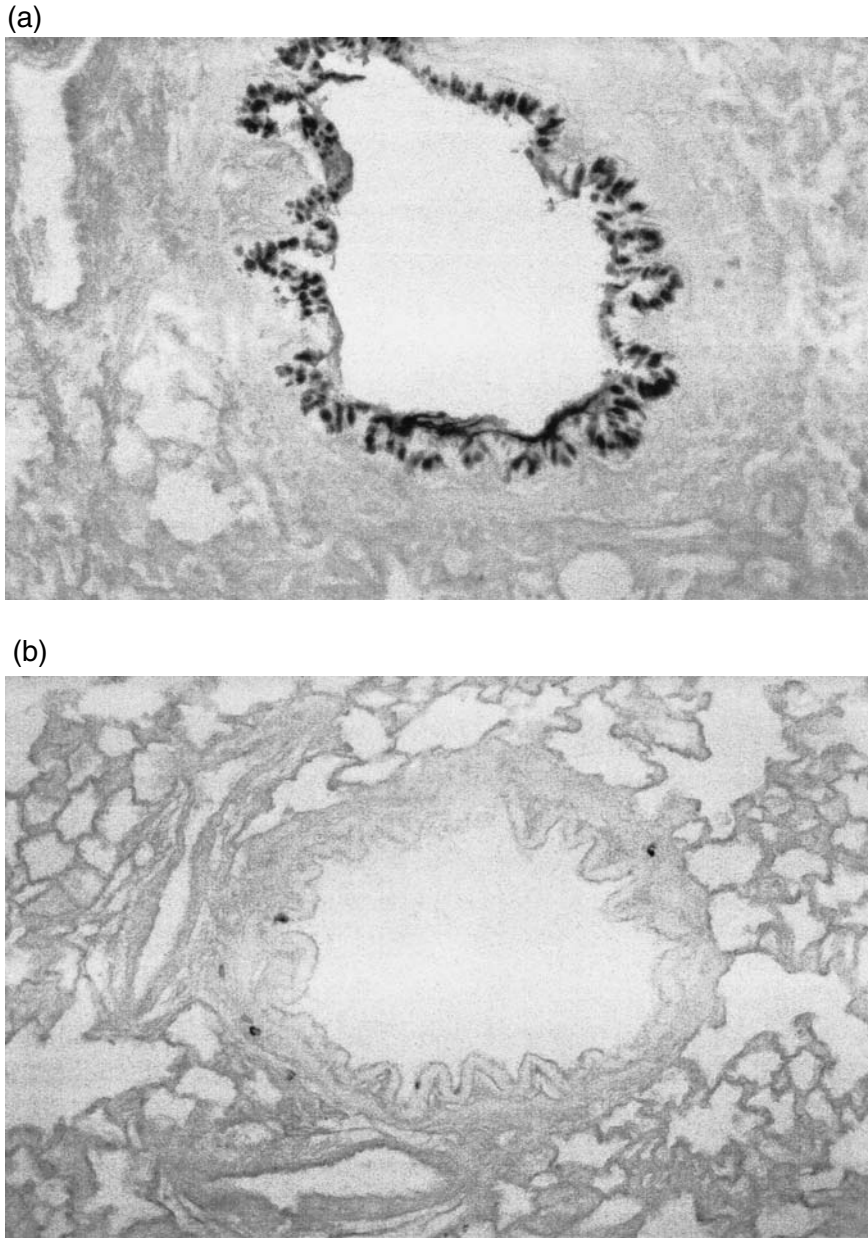


FIG. 5. AB/PAS stain demonstrating (a) mucous metaplasia in a peripheral airway of an animal administered LPS as compared with (b) the absence of intracellular mucin in a control animal given saline. In the latter only mast cells are stained.

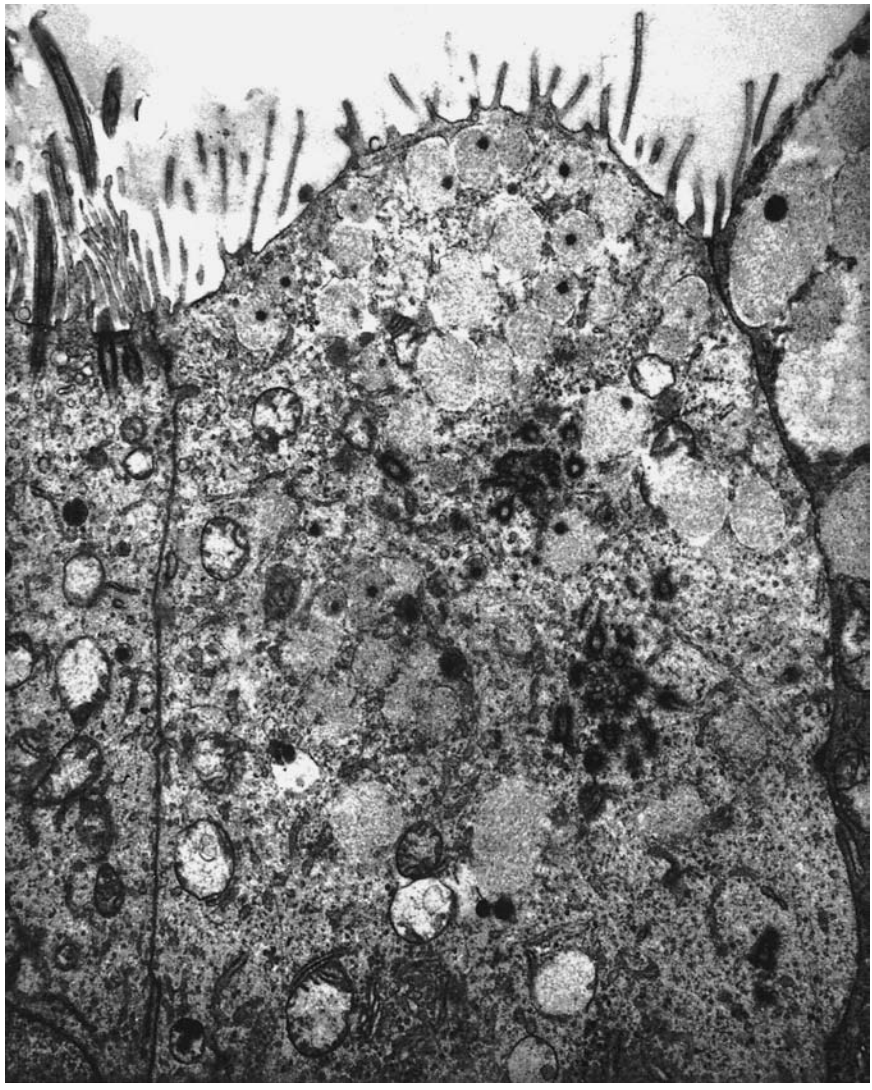


FIG. 6. Electron micrograph showing an airway epithelial cell with ultrastructural features of both ciliated and mucous cells, i.e. the electron-lucent cytoplasm, elongate microvilli and ciliary basal bodies are characteristic of ciliated cells and lie adjacent to mucous secretory granules.

gene). Application of ISH to airway tissues resected from the lungs of smokers with chronic bronchitis has shown that the *IL4* gene is strongly expressed by mononuclear cells closely associated with mucus-secreting gland acini (Fig. 9). We have shown that the number of these cells is increased in smokers when there

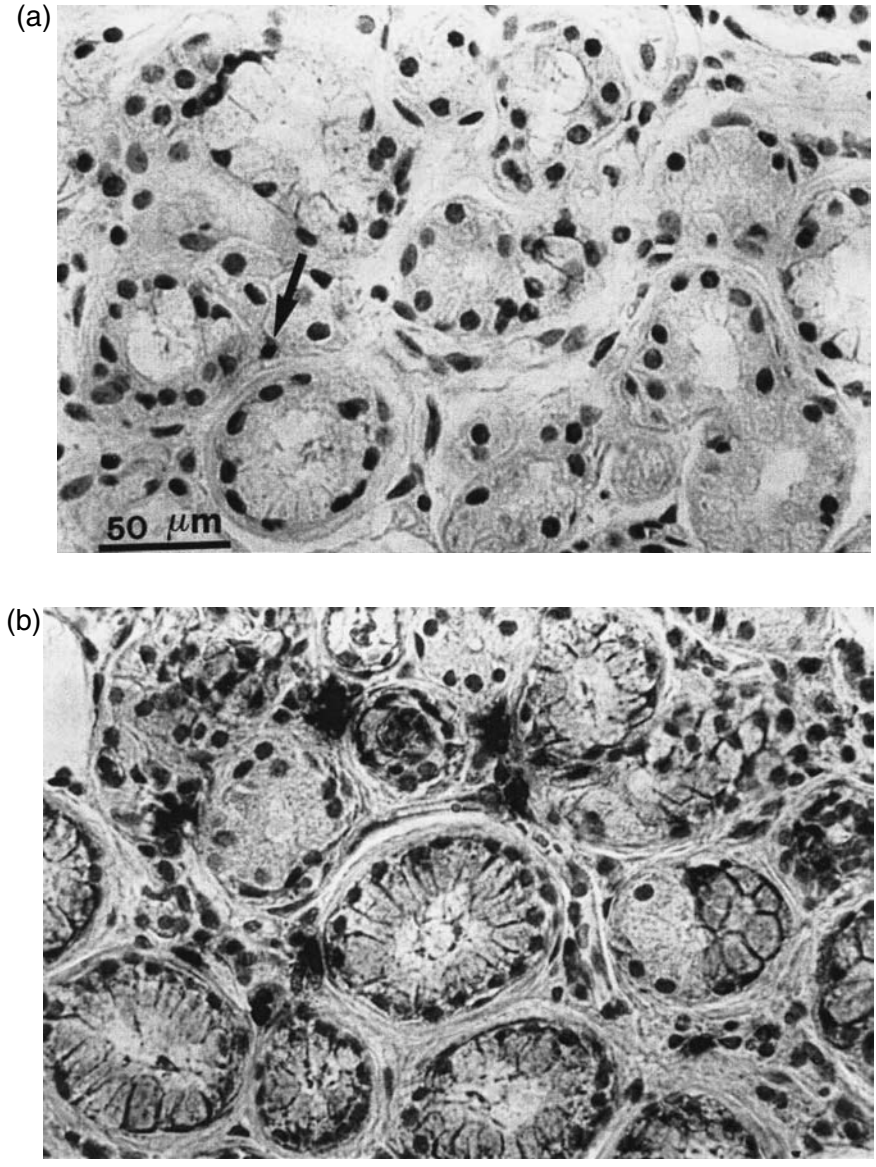


FIG. 7. Human airway submucosal glands in lungs resected from: (a) individuals who are not productive of sputum (i.e. without chronic bronchitis) when neutrophils are rarely found as compared with (b) smokers with chronic bronchitis demonstrating increased numbers of elastase-positive neutrophils adjacent to the secretory acini (by courtesy of Professor M. Saetta, Padua).

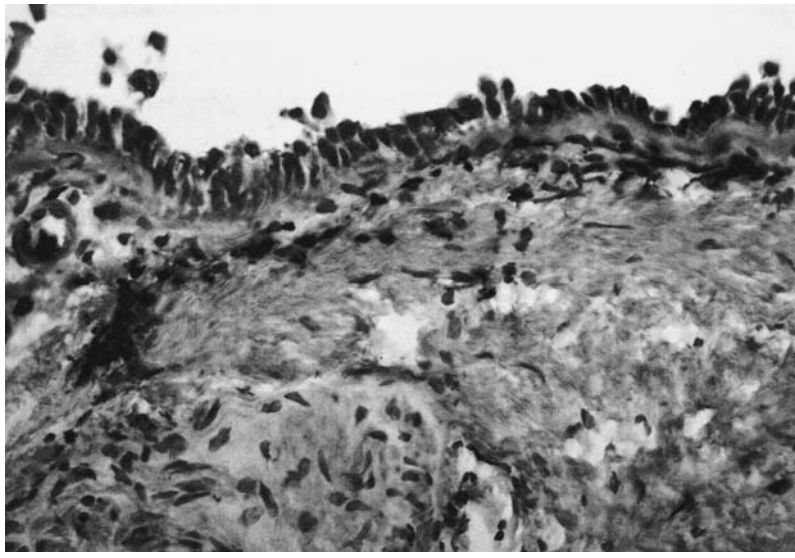


FIG. 8. Bronchial biopsy in a smoker with chronic bronchitis. Immunostaining for IL4 protein shows cells expressing IL4 are frequently present in the subepithelial zone. Alkaline phosphatase/anti-alkaline phosphatase technique.

is chronic mucus-hypersecretion (Zhu et al 2001a) (Fig. 9). In contrast the expression of *IL5* is less strong and the cells expressing it are less frequent (Zhu et al 2001a).

It is now appreciated that IL4 has a number of novel roles to play in inflammation. Apart from its complementary role with IL5 and specific chemoattractants in the recruitment of eosinophils, IL4 and the related cytokine IL13 are associated with induction of increases in mucus-secreting tissue. *IL4+ /IL4+* (i.e. transgenic) mice develop an excess of goblet cells in their airways (Temann et al 1997). Moreover, intratracheal instillation of IL4 to BALB-c mice induces an increase of *MUC5* gene and of AB/PAS+ airway goblet cells within 24 h of exposure (Dabbagh et al 1999).

What then is the source of IL4 in the airways of smokers with mucus-hypersecretion? Is it CD8+ cells, CD4+ cells, mast cells or other as yet unidentified cells? We have carried out double-labelling experiments in order to determine the answer. In these experiments gene expression by cells is marked by radioisotopically labelled mRNA whereas the phenotype of the cell is determined by its positivity with an immuno-stain: cells positive for both appear with both dense clusters of autoradiographic grains and a red chromagen. As a control procedure, the technique demonstrates that approximately 15% of CD8+ cells express the gene for tumour necrosis factor (TNF)α or 34% of

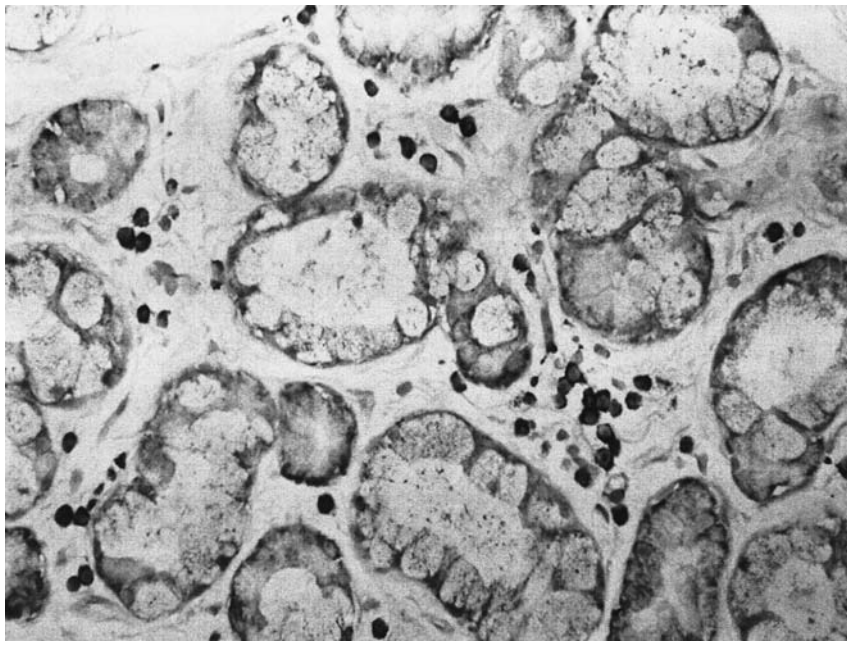


FIG. 9. The method of *in situ* hybridization applied to airway tissues resected from the lungs of smokers with chronic bronchitis demonstrates that the *IL4* gene is strongly expressed by mononuclear cells (dark black) closely associated with mucus-secreting gland acini.

TNFα-producing cells are also CD8+ . However, in our investigation of airway tissue from a case of chronic bronchitis, of over 1000 CD8+ cells counted not one expressed the gene for IL4 (Zhu et al 2001a). Clearly, it is not the CD8+ cell (neither TC1 nor the TC2 subset recently described) expressing this pro-mucus IL. Of CD4+ cells or mast cells only about 2% and 1% express the *IL4* gene, respectively. In contrast and much to our surprise we have recently demonstrated that 67% of plasma cells associated with gland acini in chronic bronchitis express the *IL4* gene (Zhu et al 2001b). This provides a novel source for IL4 in human airways hitherto not discovered: this observation requires validation and further study.

Mast cells and mucus

Several studies have demonstrated an increase in the number of mast cells in the airway mucosa of smokers with chronic bronchitis, whether or not there is associated airflow obstruction (Grashoff et al 1997, Pesci et al 1994, Zhu et al 2001b). The study of bronchial biopsies by Pesci and colleagues reported in a

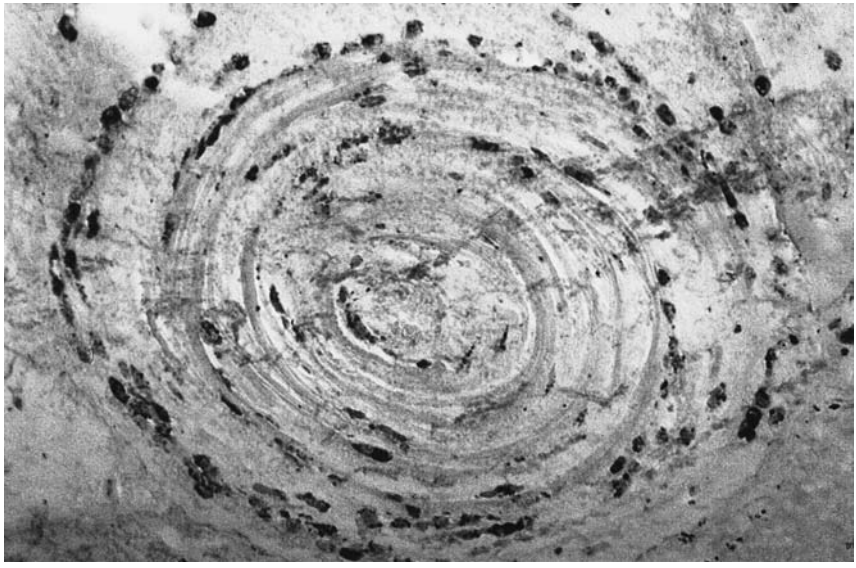


FIG. 10. Immunostaining of an airway plug in a case of fatal asthma. The antibody used is raised against the eosinophil granule 2 (EG2) component of eosinophil cationic protein (ECP). The EG2+ eosinophils are present as concentric lamellae and there is extracellular ECP mixing with mucinous secretions.

comparison with non-smokers that smokers with chronic bronchitis had greater numbers of mast cells both in the epithelium and also in association with bronchial glands: in the latter case there was evidence of degranulation (Pesci et al 1994). Whilst not all studies have supported the observation of increased mast cell numbers in the glands (Zhu et al 2001b) the observation is relevant to other experimental studies that demonstrate the secretogogue activity of mast cell chymotryptase (Nadel 1991, 1989, Sommerhoff et al 1989).

Products of metabolism of arachidonic acid

There are several distinct enzyme-dependent pathways for the metabolism of arachidonic acid. IL4 has been shown to stimulate 15 lipoxygenase (15LO) activity with resultant production of 15-HETE that has been shown by some but not all investigators to have mucus-secretagogue effects (Johnson et al 1985, Marom et al 1981). We have recently applied immuno and ISH techniques to bronchial biopsies of non-smokers and smokers and resected airway tissue of symptomatic and asymptomatic smokers to investigate protein and gene expression of 15LO. We have shown by comparison with non-smokers, that smoking up-regulates 15LO protein expression and both protein and gene are

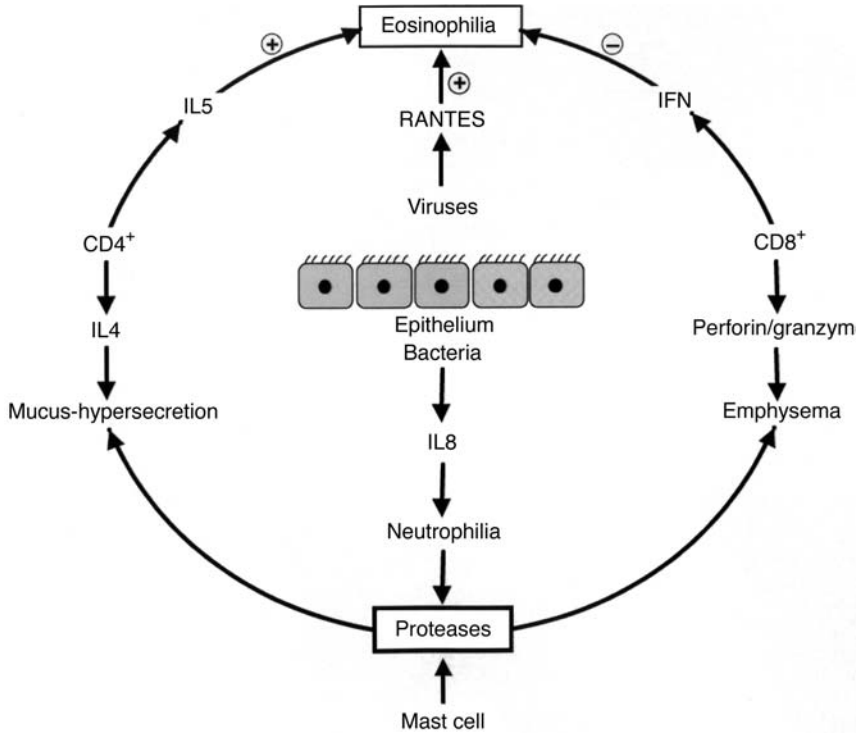


FIG. 11. A novel CD4:CD8 'balance' hypothesis to explain factors influencing the presence or absence of tissue eosinophilia in smokers with chronic bronchitis or COPD. Infection by viruses or bacteria will influence the expression of chemoattractants specific for eosinophils or neutrophils but their effects will depend on the prevailing balance of regulatory interleukins produced by CD4+ or CD8+ lymphocytes. These interleukins and the proteases produced by lymphocytes, neutrophils, macrophages and mast cells may also influence mucous metaplasia, release of mucins and contribute to the tissue destruction characteristic of emphysema associated with severe COPD.

increased significantly in smokers with chronic bronchitis (Zhu et al 2001c). While IL4 increases expression of 15LO, IL4 *per se* has the effect of attenuating mucus production *in vitro*, indicating that these are biologically independent events (Jayawickreme et al 1999).

Other cells and mediators

Other cells and mediators of inflammation have the potential to induce or modulate the secretion of mucus. A novel macrophage-derived mucus secretagogue (MMS-68) has been reported in the airway fluid of smokers with chronic

bronchitis (Sperber et al 1995). In another study eosinophil cationic protein has been shown to stimulate mucin ('glycoconjugates') in tracheal cultures of experimental animals yet, interestingly, eosinophil major basic protein appears to inhibit it (Lundgren et al 1991). Tumour necrosis factor (TNF) is a multifunctional, proinflammatory cytokine that has also been shown to stimulate mucin secretion and *MUC2* gene expression in a concentration-dependent manner in human airway organ cultures and cultures of human airway epithelial cells (Levine et al 1995). It is also known that TNF induces the expression of airway epithelial epidermal growth factor receptors whose activation leads to increased mucin gene expression and goblet cell metaplasia (Nadel & Burgel 2001). There is also evidence for mucin secretagogue activity for IL1 (Cohan et al 1991) and platelet activating factor (Larivee et al 1994).

Direct interaction of inflammation and mucins

Finally, we have immunostained the tenacious plugs that block the airways in cases of fatal asthma and demonstrated the inclusion of activated (EG-2+) eosinophils and their released eosinophil cationic protein admixed with the mucinous secretion (Fig. 10). There is support for our contention that part of the increased viscosity of these exudate-rich plugs in asthma is likely due to electrostatic interaction between positively charged secretions released from eosinophils and the negatively charged mucin molecules (due to terminal carboxyl and sulphate radicals) (List et al 1978). Thus inflammatory cells and their mediators play distinct roles in the production and release of mucus, and in addition likely increase the viscosity, adhesivity and surface tension of the secretions, thereby reducing the lumen and stability of the airways (Macklem et al 1970).

Concluding comments and hypothesis

It is likely that inflammatory cells recruited to the airway wall and their released products provide a major drive to mucus-hypersecretion. Increased numbers of neutrophils, mast cells and interestingly plasma cells probably play key roles in controlling the extent of mucus-hypersecretion in human conditions such as chronic bronchitis, asthma and cystic fibrosis. The relative roles played by these inflammatory cells in each of these conditions likely depend on the initial stimulus: cigarette smoke, allergen or bacterial and viral infection. Mediators of the inflammatory cascade including a number of products of arachidonic acid metabolism also have the potential to up-regulate or even may down-regulate production of mucus. It is now appreciated that regulatory cytokines such as the IL4/IL13 family can also switch on the *MUC* genes associated with the generation of increased mucus-producing tissue, resulting in both increased numbers of

epithelial goblet cells and proliferation of gland acinar cells leading to gland hypertrophy. Whilst it seems logical to consider that increased size of the mucus-producing factory will inevitably lead to increased production of mucus this does not necessarily follow and it appears that inflammation *per se* is probably what determines how productive is the mucus-producing factory. These considerations and results are in keeping with seminal observations made by Lord Florey, namely that at moist mucosal surfaces hypersecretion of mucus is an integral part of the inflammatory response.

It appears that the relationships (whether causal or merely associated) of the distinct patterns of regulator and inflammatory effector cells, increased mucus and remodelling in COPD and asthma are still far from fully understood. In COPD, the hypotheses that mucus hypersecretion is due to the release of neutrophil elastase and that proteolysis by neutrophil elastase leads to emphysema are attractive but there are alternatives to consider. We propose a more encompassing 'balance' hypothesis (Fig. 11) that extends our original hypothesis, set out in a previous publication in this series (Jeffery 2001b).

Our proposal illustrates an eosinophilic response dependent on the balance of CD4+ /CD8+ cells, the particular predominance resulting from genetic and environmental influences (e.g. exposure to cigarette smoke) and the associated release of IL5 (together with the chemoattractant RANTES favouring eosinophilia) or interferon (suppressing eosinophilia) respectively.

The CD8 predominance and release of TNFα in COPD would favour proteolysis or apoptosis of virally infected cells due to perforin and granzyme release, respectively, leading to emphysematous destruction. This would occur especially in a subpopoulation of smokers with relatively high starting numbers of CD8+ cells. Contrary to popular expectation, as in asthma, cells expressing IL4 are also frequent in smokers with chronic bronchitis. Plasma cells, CD4+ and mast cells appear to contribute, and IL4/IL13, together with mast cell-derived chymotryptase and neutrophil elastase, likely lead to the increased production and increased secretion of mucus in conditions that include chronic bronchitis. As always no one cell or mediator is responsible: instead the data remind us that there are multiple targets to challenge our development of a strategy to attenuate the detrimental aspects of chronic mucus hypersecretion.

Acknowledgements

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DISCUSSION

Rogers: You showed what appeared to be a selective infiltration of neutrophils into the submucosal glands. This is an interesting observation. IL4 is present, but presumably is not chemotactic for neutrophils. Do submucosal glands produce specific neutrophil chemotactic factors?

Jeffery: Dr Zhu, working in my laboratory, has shown marked up-regulation of 15LO protein and gene expression by glands in CB. If active, this would increase 15HETE and the recruitment of neutrophils. As you rightly say, IL4 would not be expected to do this — this up-regulates VCAM on vascular endothelium acting on VLA4. This would selectively recruit eosinophils, and this is not observed.

Nadel: IL4 and IL13 are very potent inducers of IL8. I was going to ask a similar question: how do you get a co-relationship between IL4, IL13 and neutrophils? I think the answer is very likely to be via the induction of IL8.

Jeffery: You could perhaps widen the possibilities and consider ENA78, which is also a neutrophil chemoattractant. In addition to IL8 we have been examining gene expression for other powerful neutrophil chemoattractants in our biopsy studies. It seems that ENA78 is hugely up-regulated in COPD, at least during exacerbations. In contrast, we have been surprised that IL8 is much less so. I agree that IL8 is a potential candidate: IL8 is probably playing a role here but there are other neutrophil chemoattractants, and I suggest that ENA78 is another worthwhile candidate.

Sheehan: I have a comment on your mucus stickiness. We did a study some years ago rather motivated by some of Lynne Reid's work suggesting that DNA and proteoglycans may be major contributors to the stickiness of asthma plugs (Sheehan et al 1999). We couldn't show this, but what was undoubtedly true was that there was a massive amount of mucin in the secretion — much higher concentrations than we'd ever found associated with ordinary sputum samples. The biochemistry would be worth following here. We only looked in one patient who died in status asthmaticus. It would be nice to know how general this was. We found one very obvious thing about this mucus. We could take it out and reduce it, and it fell apart. It was held together by disulfide bonds. It wouldn't have fallen apart had it been a proteoglycan or DNA-induced stickiness.

Jeffery: What of electrostatic interactions with inflammatory cell products, for example, cationic molecules such as eosinophil major basic protein?

Sheehan: We couldn't rule that out. The major observation we made was that there were high concentrations of very high molecular weight mucins. Indeed, there was so much that they couldn't be hydrated. There couldn't have been enough water available. We took out 3 g of this gel and we extracted it in three litres of 6 M guanidine, and it turned the whole lot into a thick, viscous goo. We were just unfolding what was essentially a compact assembly of very large molecules. It was a shocking realization for me. The amount of mucin secreted into that environment was so great it turned it into a totally intractable gel.

Basbaum: That is consistent with what pathologists say about asthmatic mucin. So would you question the view that the asthmatic mucus is thick because of inflammatory cell DNA, and instead suggest that it is just pure mucin?

Sheehan: I would suggest this, but only on the basis of just one study. But still it was very dramatic. From our data, you would definitely have something to look for. There are now some very clear indicators that would tell us immediately whether it was a mucin-based gel or not.

Basbaum: When you say it fell apart on reduction, does this imply that the monomers are linked by the von Willebrand domains?

Sheehan: They were heavily oligomerized, yes.

Basbaum: Is COPD mucus different?

Sheehan: I would simply say that it was the sheer concentration that we were faced with that gave it such remarkable physical properties. We looked for DNA and proteoglycans specifically. Our conclusion was that they were there, but they could in no way account for this dramatic property.

Nadel: John Sheehan, is your implication that the stickiness of this mucus that goes into three litres of solution is because of inadequate hydration?

Sheehan: We are pulling this rubbery stuff up from the large airway, and physically it is a remarkable substance. The implication to me, as a biophysical sort of person, is that the mucin concentration is very high and that if we allow it to hydrate further it will go on and on expanding. It was nowhere near its fully expanded random coiled state. If you allow it to seek that state, it will get there and unfold itself into its full length.

Holgate: We have been culturing epithelial cells from brushings obtained from patients with chronic severe asthma and have compared these with cells from normal and smoking-induced COPD. After they reach confluence, the cells are differentiated at an air–liquid interface. We see that the nature of the mucus in the differentiated cells and the asthmatic material appears different from what is seen either in the normal or the COPD. First of all, it is produced in larger quantities. But it may not be the quantity of mucus that we are looking at here. There is plenty of water to hydrate this material because they are at a liquid interface. It may be that what we are seeing is more hydration of the mucus. The cilia become bound up in the mucus, and as a consequence become 'paralysed'. In contrast, in the COPD cultures we see the mucus being secreted but the cilia are able to continue their beating unimpeded. Secondly, the proportion of the epithelial cells that are mucus secreting may differ. The columnar layer is greater in the asthmatic epithelium than in COPD, even in the absence of any added cytokine. It seems that because in each case the cells were cultured through three passages before maturing them at the air–liquid interface, there may be some fundamental (genetic?) differences in the maturation of the 'stem cells' in the different disease states. We clearly need more chemistry information about the nature of this mucus in the different settings to explore some of the very interesting ideas that you have just put forward.

Jeffery: There is a histological study by Aikawa that showed high-powered light micrographs of the goblet cells in asthmatic deaths (Aikawa et al 1992). The goblet cells had discharged their mucin, but although it was secreted it was still adherent to the cell apices: it remained stuck to the goblet cells. The illustrations are quite dramatic. This might be relevant to what you are describing in these cultured cells of asthmatic epithelia.

Rubin: We have measured the biophysical and surface properties of secretions from patients with fatal asthma, as well as those from non-fatal asthma. We found

that they were cohesive, but not adhesive. The product of adhesivity and cohesivity is tenacity, so the tenacity was actually rather low. But they were profoundly viscous and elastic, giving a huge complex modulus. In cystic fibrosis, conversely, the viscosity has been shown by us and others to be no greater than that in COPD and significantly less than that in asthma. But the secretions in CF are profoundly tenacious, as a result of high cohesivity and adhesivity. When treated with DNAse, there is a much greater reduction in tenacity than there is in viscosity. There are clear differences between the different diseases.

Sheehan: We did a set of serial extraction experiments on the mucus plugs I described from the fatal asthma airway, with the idea that perhaps there is a subset of the mucins important for this property. In other words, we had done studies that showed substantial MUC5AC content and different glycoforms of MUC5B present. Indeed, after extracting it sequentially, we were left with an absolutely pure mucin gel. But it was a particular glycoform of MUC5B and it had a very curious morphology that I didn't recognize as being typical of secretions we had previously studied. We speculated that either the hyperplasia had given rise to a population of mucin-secreting cells that were in some way special, or that the asthma process itself doesn't modulate and modify this population of molecules in the appropriate way. As a result, the mucus doesn't have the right physical properties. In either event, this might be quite interesting. When I hear this discussion on tenacity, I think that it will relate to a subset of distinctive molecules of a particular genotype that have a particular property. This would be worth studying in these plugs.

Nadel: Is it possible that vasoconstriction in bronchial artery or other vessels that perfuse airways could play a major role in the viscoelastic properties of mucins under these extraordinary circumstances?

Verdugo: I think the ratio of water to solid material is a critical factor. If there is not enough water on the surface epithelium you are releasing a granule that is not going to hydrate.

Nadel: It occurred to me that rather than the biochemical properties of the mucin, something else happening in the epithelial system, such as vasoconstriction, would limit blood flow under one circumstance and change the viscosity of the secretion.

Basbaum: Perhaps the presence or absence of albumin could play a role.

Sheehan: If it was albumin, you would expect our reduction experiment to make it worse. This would have just unfolded everything. Our observations fit together. There was a remarkably high mucin concentration, and breaking the disulfide bonds undoubtedly broke that mucin down. When we got the purified mucin gel, we could scission through it with reducing agents and it just fell apart.

Rubin: That is tremendously consistent. Lack of hydration itself would actually decrease cohesivity. A critical hydration is required for maximal cohesivity. Lack

of hydration would be more likely to affect adhesivity, a surface property, without affecting wettability. The changes you are suggesting both suggest a lack of hydration but also a very significant structural alteration so that this behaves very differently, not only under stress/strain conditions and traditional rheological conditions, but also when evaluating the surface interactions.

Basbaum: Could lipid play a role?

Sheehan: Possibly, from the point of view of the morphology. We couldn't tell you why the morphological characteristics were different.

Basbaum: John Fahy, I know that you have been studying goblet cells from asthmatic patients. Have you compared them head to head with goblet cells from COPD patients?

Fahy: We published a study on biopsy homogenates that we think are goblet-cell rich, which show that *MUC5AC* is the predominant mucin gene (Ordonez et al 2001). *MUC5B* expression was decreased in asthma. This is holding up in some more specific studies in laser-captured goblet cells.

Basbaum: It is important for people to realize that there are on-going studies in which it is possible to microdissect goblet cells from human asthmatics versus human bronchitics, and examine gene expression profiles.

Fahy: We are looking at broad-based gene profiling in laser-captured goblet cells and gland cells. We are doing this across multiple diseases. We have just got past some technical hurdles and are still in the sample collection phase.

Could neutrophil elastase or other non-protease products of neutrophils, including arachadonic acid metabolites, be growth factors for submucosal glands? It seems to me that we have very few clues about growth factors for submucosal cell gland enlargement. John Engelhardt mentioned LEF1 in his paper, and he wasn't even sure it was involved in enlargement of glands. The only data we have indicate that there seems to be a relationship between neutrophil airway inflammation and submucosal cell gland enlargement. Could neutrophils drive gland enlargement as distinct from secretion?

Basbaum: That's easy enough to test. Jack Harkema, I know that you have taken neutrophils from endotoxin-inoculated animals and examined the neutrophils *in vitro*. But have you ever looked at whether they contain mitogens?

Harkema: Not mitogens. Charlie Plopper has conducted a study in neonatal monkeys with allergic airway disease that were exposed to ozone. I have examined the nasal mucosa from some of these animals and often find neutrophils congregated around submucosal glands. This is similar to what Peter Jeffery was saying with the plasma cells. If this is combined with some BrdU labelling, which we are starting to look at, there may be some association between differentiating cells and submucosal gland proliferation.

Verdugo: Are any chemoattractants released by the goblet cells or gland cells? Why do these white cells come and guard the mucosa, even in conditions when

there is no need for them? That is, why are there inflammatory cells present when there is no infection?

Jeffery: I could give you an example of exacerbations of COPD and another hypothesis that our subsequent observations did not support. Prior to Marina Saetta's observations, we did not expect to see tissue eosinophilia in any biopsy samples of patients with COPD. There were occasional eosinophils, but in general it was not a characteristic of COPD. However, in exacerbations in COPD there was a profound eosinophilia, and we were interested in this. We looked at the biopsies that Dr Saetta had originally examined, and added further patients to our study. Indeed, we confirmed the previous reports of true eosinophilia. We then went on to look at the chemoattractants in exacerbation. We looked at eotaxin, MCP4 and RANTES. To our surprise, eotaxin was present in COPD, but it was not up-regulated in association with any exacerbation. Nor was MCP4. RANTES, on the other hand, was hugely up-regulated. The increase in gene expression for RANTES was highly significant and showed a good correlation with EG2 positively (Zhu et al 2001). In exacerbations, it is epithelial and also inflammatory cell up-regulation of RANTES that is drawing the eosinophils into the mucosa and encouraging their migration towards the epithelium. Probably beyond and into the lumen, because RANTES will be released into the airway lumen as much of it is released at the surface of cells rather than at the base. In the exacerbations that we have looked at recently in which there is tissue neutrophilia, again we find that IL8 is present but it is not especially up-regulated in that situation. It is ENA78 that is expressed in the epithelia and significantly up-regulated. Thus we have two molecules which seem to be importantly expressed by the epithelium, one responsible for drawing in the eosinophils (RANTES) and the other likely attracting neutrophils (ENA78).

Nettesheim: I think there is another side to the IL4 story. We have been interested in studying leukotrienes and COX2 products as well as 15LO production in normal human bronchial cells *in vitro*. We have found that IL4 is a strong inducer of 15LO and causes a drastic up-regulation of 15HETE production (Jayawickreme et al 1999). For other purposes we have done similar studies more recently and we find that mucin gene expression and mucus production is not increased; if anything, it is decreased. In contrast, we think that some of the COX2 products might be critical in up-regulating some mucins.

Cohn: We think that IL4 is a marker for Th2 cells and their activation, but doesn't function as the effector molecule. Instead, IL13 is the prime effector molecule. Along these lines, have you looked at IL13 in humans? One interesting issue is whether human and mouse are the same in this regard.

Jeffery: We haven't yet looked in our tissues for IL13 or IL9 in this system.

Cohn: I found it surprising that the plasma cells are making so much IL4. They are not known to be major producers of IL4. You said you saw both mRNA and protein.

Jeffery: Yes, and a further comment: in our judgement there are many more plasma cells than neutrophils around these glands in chronic bronchitis.

Basbaum: Is there a precedent for plasma cells making IL4?

Cohn: IL4 was defined as a B cell growth factor made by T cells. But do B cells make it? We never hear about this. Also, we haven't studied plasma cells in tissues very much.

Jeffery: It is interesting to reflect back on the observation that the predominant cell types described around the gland acini by Mullen and colleagues were lymphocytes and plasma cells (Mullen et al 1985, 1987). I think this shows the importance of careful observation even in haematoxylin and eosin stained preparations.

Vargaftig: We have been studying IL4 receptor knockouts. With the system we developed we could show some residual IL4 production in those animals, even though by definition they should not be able to produce IL4. We think the origin of this extra IL4 may be the NK cells. I was also very interested by the possibility that plasma cells can make IL4, but did you consider this alternative?

Jeffery: No, but it would be worthwhile studying this, as NK cells are reported to be increased in COPD.

Disse: Which of the established drug regimens may be active? You mentioned in conjunction with the IL4 findings that corticosteroids are active in combating mucus hypersecretion.

Jeffery: We have done a bronchial biopsy study looking at the effect of inhaled corticosteroids (ICS) in a group of patients virtually identical to those used in the ISOLDE study. Over the first three months in the ISOLDE study lung function improves, but during this time in our biopsy study ICS reduce only the numbers of mast cells (Gizybi et al 2002). I wasn't referring to IL4 there; I was speculating that the reduction in mast cells might explain the reduction in mucus, and therefore the observed short-term improvement in FEV_1 that overrides the continuing decline in lung function in these patients with COPD. As soon as the about-turn improvement is maximized, then the long-term steady decline in FEV_1, with the same slope as seen in untreated patients, reappears.

Disse: That small initial improvement in lung function is always seen, and I have often heard discussed anecdotal reports that inhaled steroids reduce hypersecretion. But if you look at published studies there is that increase in lung function, but disappointingly there is no effect on symptoms reported. If you go through other drug classes, nothing is published for leukotriene C4/D4 antagonists and little for COX inhibitors (Tamaoki et al 1992).

Jeffery: What you say about the lack of effect on mucus hypersecretion is interesting. If it is true, it would then appear that the short-term improvement in lung function following ICS does not seem to be due to their effect on mucus secretion.

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Respiratory tract mucins: structure and expression patterns

Julia R. Davies, Annkatrin Herrmann, Wayne Russell, Naila Svitacheva, Claes Wickström and Ingemar Carlstedt[1]

Mucosal Biology Group, Department of Cell and Molecular Biology, Lund University, BMC, C13, S-221 84, Lund, Sweden

Abstract. Goblet cells produce mainly MUC5AC, but also MUC5B and some MUC2 in apparently 'irritated' airways. MUC5B dominates in the submucosal glands although a little MUC5AC and MUC7 are usually present. MUC4 originates from the ciliated cells. After separation into a gel and a sol phase, lysozyme and lactoferrin are enriched in the salivary gel phase suggesting that mucus may act as a matrix for 'protective' proteins on the mucosal surface. A salivary MUC5B N-terminal fragment consistent with a cleavage event in the D′ domain was detected with antibodies against various N-terminal peptide sequences suggesting that assembly of MUC5B occurs through a mechanism similar to that of the von Willebrand factor. Identification of additional cleavage sites C-terminal to the D′ domain suggests that most of the N-terminal low-glycosylated part of MUC5B may be removed without affecting the oligomeric nature of the mucin. Possibly, the generation of mucins with different macromolecular properties through proteolytic 'processing' is one way of adapting the mucus polymer matrix to meet local physiological demands. Monomeric mucins that appear to turn over rapidly in the airway epithelium have been identified using radiolabelled mucin precursors. 'Shedding' of such mucins after microbe attachment may prevent colonization of epithelial surfaces.

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The mucosal surfaces represent the interface between the external environment and the inner tissues. From the external side, these surfaces may be exposed to bacteria and viruses, gaseous irritants and enzymes as well as fluctuations in temperature and pH. They are highly adapted to resist such challenges. On the epithelial surface, a layer of mucus usually provides the first line of defence, and underlying the mucus gel are epithelial cells covered with a luminal glycocalyx. In addition, airway epithelial cells are likely to play a key role in mucosal protection by relaying signals from the lumen — 'outside-in-signalling' — and thus mobilizing, for

[1]This paper was presented at the symposium by Ingemar Carlstedt, to whom correspondence should be addressed.

instance, elements of the immune system in the sub-epithelial connective tissues. This chapter focuses upon the respiratory tract mucosa and the mucins that provide the first line of defence against the outside world.

Respiratory mucus is a gel with well-defined physical properties

Respiratory mucus interacts with cilia on the luminal edge of the ciliated cells to trap and propel inhaled particles and microorganisms towards the larynx. In order for this mucociliary transport system to function optimally, the physical properties of the mucus gel must lie within defined limits (Silberberg 1983). The polymer matrix of mucus is a network of entangled oligomeric mucins, which are secreted from goblet cells in the surface epithelium and mucous cells in the submucosal glands. While mucins provide the 'structural' framework for mucus, the term mucus is usually used to describe the entire 'functional' gel formed through the addition of a range of other proteins to the mucin matrix. In chronic bronchitis, asthma and cystic fibrosis the quantity of airway mucus produced is increased and studies have indicated that the expression of some mucin genes may be altered or up-regulated. In addition, other factors including the presence of inflammatory cells, and bacteria and their products (e.g. DNA), as well as 'transudate' proteins may profoundly affect the properties of the mucus gel. Little is currently known about *qualitative* changes in mucus and/or how changes in mucins, such as the production of different mucin 'glycoforms' and changes in proteolytic processing of the macromolecules (see below), affect mucus in disease.

Gel–sol phase separation allows identification of mucus-associated proteins

The mucus polymer matrix formed by the oligomeric mucins provides an unstirred layer that may act as a reservoir for molecules associated with defence on the mucosal surface. Alternatively, such molecules may be retained at the mucosal surface through molecular interactions with the mucin matrix. A range of proteins including lysozyme, lactoferrin, sIgA, β defensins, salivary agglutinin (encoded by the *DMBT* gene) and trefoil factors have been identified in respiratory mucus (Diamond et al 2000) but little is known about whether or not they are associated with mucins. We use high-speed centrifugation to separate mucous secretions into a 'gel' and a 'sol' phase thus allowing identification of molecules associated with the gel-forming mucins. Such studies have revealed that, for example, lactoferrin and lysozyme are associated with the salivary mucus gel (Wickström et al 2000). It should be noted, that 'gel' phase represents the gel-forming mucins and associated molecules but that the 'sol' phase, as defined here, does not necessarily correspond to the physiological 'sol' phase in which the cilia beat.

What is a mucin?

Mucins are high-molecular-mass glycoproteins in which the apoprotein is substituted with large numbers of *O*-linked glycan chains confined to domains — mucin domains — that 'dominate' the properties of the macromolecule. The mucin (*MUC*) gene family currently contains 13 members numbered in order of their description (*MUC1–4*, *MUC5AC*, *MUC5B*, *MUC6–9* and *MUC11–13*) (for a review see Moniaux et al 2001). Although the complete cDNAs have only been sequenced for six mucins (*MUC1*, *MUC2*, *MUC4*, *MUC5B*, *MUC5AC* and *MUC7*), all members of the family appear to share the common feature of tandemly repeated nucleotide sequences which give rise to serine- and/or threonine- and proline-rich regions (STP-rich regions) in the apoprotein. The number of tandem repeats, and thus the length of the mucin domains in the mature mucin, is subject to genetic variation referred to as VNTR (variable number tandem repeat) polymorphism. According to their predicted structures, the members of the mucin family are usually divided into cell-associated and secreted species.

Cell-associated mucins

The predicted sequences of the major isoforms of MUC1, MUC3, MUC4, MUC8, MUC11 and MUC13 all contain a hydrophobic membrane-spanning domain, suggesting that these mucins are membrane-bound. However, with the exception of MUC1, the products of the genes are poorly defined biochemically. The mucin domain(s) are present within the extracellular portion of the glycoprotein. Both human MUC4 and the rat MUC4 homologue — the sialomucin complex (SMC) — are synthesized as a single apoprotein but subsequent cleavage gives rise to a membrane-spanning domain (termed MUC4β and ascites sialoglycoprotein 1 [ASGP1] in human and rat, respectively) and an extracellular mucin-like subunit (termed MUC4α and ASGP2, respectively) which exist as a heterodimeric complex (Carraway et al 2000, Moniaux et al 2001). MUC1 and MUC13 also appear to have a similar structure (Ligtenberg et al 1992, Williams et al 2001). For MUC3 and MUC4, splice variants have been described which lack the transmembrane domain and thus are expected to give rise to soluble forms of the mucins (Crawley et al 1999, Williams et al 1999, Moniaux et al 2001).

The *MUC1*, *MUC3*, *MUC4*, *MUC8*, *MUC11* and *MUC13* genes are all expressed on epithelial surfaces and, due to the extension provided by the mucin domains, the extracellular domains of, for instance, MUC1 and MUC4 are predicted to extend far above most other molecules on the luminal surface (Fig. 1). MUC3, MUC4 and MUC13 contain EGF-like domains in the extracellular part of the transmembrane subunit (Williams et al 1999, Moniaux

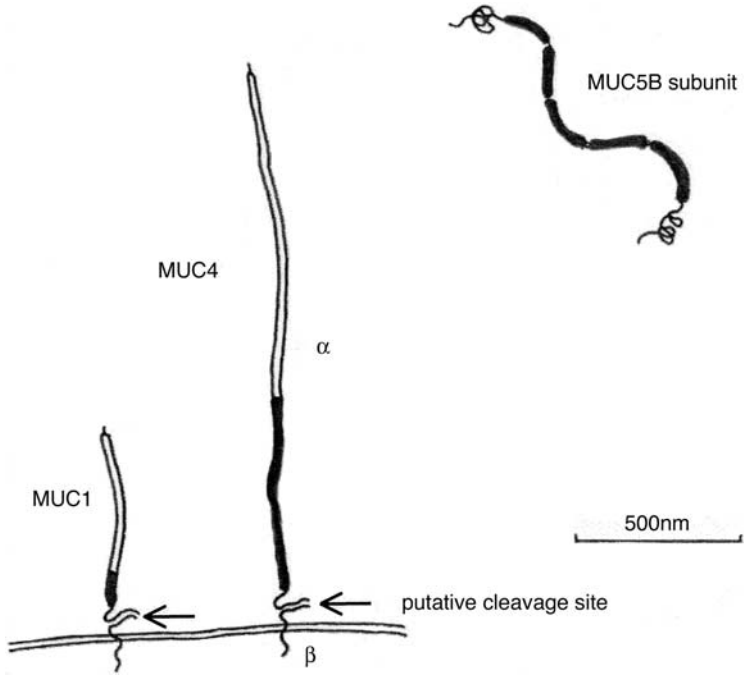

FIG. 1. Schematic drawing of MUC1, MUC4 and a MUC5B subunit. MUC1 and MUC4 are depicted as membrane-anchored mucins with a putative cleavage site close to the plasma membrane. The lengths of the glycosylated domains (thick lines) differ between alleles. The filled part indicates the shortest allele whereas the filled together with the open part represents the longest one. A MUC5B 'subunit' is depicted for reference and all three mucins are drawn to scale relative to each other. The lengths of the glycosylated domains are estimated with the assumption that the length/amino acid is 0.25 nm in this part of the molecule. This is likely to be an overestimation since the length of a MUC5B 'subunit' from cervical mucins is approximately 500 nm as visualized by electron microscopy (Sheehan & Carlstedt 1990).

et al 1999, Williams et al 2001), and in SMC such epidermal growth factor (EGF)-like domains can activate the ErbB2 tyrosine receptor kinase. This suggests that SMC plays a role in the regulation of signal transduction from the luminal surface into epithelial cells (Carraway et al 2000). The intracellular domains of cell-associated mucins have been most extensively studied in MUC1 where the cytoplasmic tail has been proposed to interact with proteins such as actin, β catenin and glycogen synthase kinase 3β (Li et al 2001).

Secreted mucins

The *bona fide* secreted mucins encompass MUC2, MUC5AC, MUC5B, MUC6 and MUC7. Except for MUC6, the full cDNA sequences of all these mucins are known.

The genes encoding MUC2, MUC5AC, MUC5B and MUC6 are found as a cluster on chromosome 11p15.5 (Pigny et al 1996) and all these mucins have been shown to occur as large oligomeric structures. After treatment of mucus with guanidinium chloride, we have shown that the oligomeric mucins occur as 'soluble' and 'insoluble' species. MUC5AC, MUC5B and MUC6 appear largely as soluble forms, whereas almost all colonic MUC2 is resistent to extraction and is found as a complex that is 'insoluble' in guanidinium chloride (Herrmann et al 1999). Reduction of disulfide bonds in MUC2 gives rise to discrete populations corresponding to mucin monomers and a series of oligomers suggesting that MUC2 apoproteins are assembled both via disulfide bonds and through an, as yet, unidentified reduction-resistant linkage (Herrmann et al 1999). Also, in saliva a significant proportion of the MUC5B mucins occur as an 'insoluble' complex (Wickström et al 2000).

MUC5AC and MUC5B from the respiratory and cervical tracts are polydisperse in size with values of M_r ranging from 2–30× 10^6 Da (Thornton et al 1997a). Cleavage of disulphide bonds gives rise to subunits (M_r 2–3× 10^6 Da) which correspond to mucin monomers while proteolytic digestion of reduced subunits yields proteinase-resistant fragments (M_r 3–5× 10^5 Da) corresponding to the mucin domains. Electron microscopy of intact MUC5AC mucins shows them to be long linear threads, many of which are in excess of 10 μm in length while the monomers are much shorter (average length 570 nm). Native MUC5AC mucins appear to be composed of up to 18 monomers linked end-to-end while MUC5B mucins often appear as heavily entangled filamentous structures (Sheehan et al 1999, 2000).

The MUC2, MUC5AC, MUC5B and MUC6 mucins show sequence homology in their N- and C-terminal domains, both to each other and to the von Willebrand factor (vWF). In MUC5B, sequences homologous to the cysteine-containing D1, D2, D' and D3-domains of the vWF comprise the first 1283 N-terminal amino acids, while a D4 domain and a 'cysteine-knot' (CK) domain occupy 809 amino acids at the C-terminal end of the molecule (Fig. 2). In addition to the cysteine-rich domains at the termini of the apoproteins, the large gel-forming mucins have cysteine-rich sequences interspersed between the mucin domains.

Mucin glycoforms

A hallmark of mucins is the abundance of highly diverse *O*-linked oligosaccharides in the mucin domains. These domains may contain several hundred different glycans, the structures of which are determined both by the cellular repertoire of glycosyltransferases and the sequence in which they act upon the apoprotein. In respiratory secretions, we and others have shown that while MUC5AC is usually present as an apparently single species, MUC5B is found as at least two

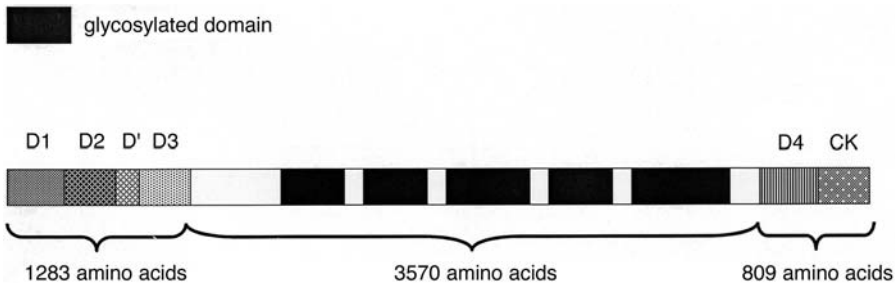

FIG. 2. Block diagram of MUC5B. The domain structure of MUC5B is shown with the D-domains, the cysteine knot (CK) and the five highly glycosylated domains indicated.

'glycoforms' which differ distinctly in their glycosylation (Thornton et al 1997b, Davies et al 1999, Wickström et al 1998). The more highly charged glycoform of MUC5B is recognized by an antibody against the sulfo-Le^C structure. This antibody also stains a subpopulation of the MUC5B-producing submucosal gland cells in the respiratory tract indicating that both a more and a less highly sulfated form of MUC5B are produced in the submucosal glands. The fact that mucins appear as distinct 'glycoforms' rather than a continuously heterogeneous population of differently glycosylated molecules lends weight to the suggestion that mucin-producing cells express distinct programmes of glycosylation and that cellular origin rather than apoprotein structure is the major determinant of glycosylation pattern. The levels of the glycoforms may vary in disease and it has recently been shown that the relative amounts of the less charged glycoform of MUC5B is increased in CF and chronic bronchitic sputum compared with healthy airway secretions (Kirkham et al 2002). The biological significance of the structural diversity of the oligosaccharides remains to be elucidated. However, the role of the simpler glycan structures may be merely to force the apoprotein into an extended conformation, whereas the more elaborate ones may have more specific functions, such as interfering with microbial colonization of mucosal surfaces or acting as receptors for non-mucin components that are part of the mucosal defence system. A major challenge is to understand the functional differences — if any — between mucin glycoforms.

Mucin polymorphism

The genetic polymorphism of the mucin apoproteins and the vast structural diversity of the oligosaccharides allow great inter-individual variations in the structure of the major matrix-forming molecules in mucus. Most likely, this diversity has allowed species to adapt to a changing environment — in particular to microbes and viruses that change their properties via mutations. Although the

'polymorphism' of mucins appears to be beneficial to the population as a whole, certain individuals may carry structures which make them more vulnerable to, for example, bacterial colonization or antigen penetration. This may, at least in part, explain the inter-individual differences in the susceptibility to infection and mucosal reactions to 'irritation' and environmental stress in general.

Mucins are proteolytically processed

Since the large oligomeric mucins share sequence homologies in their terminal domains with the oligomeric vWF, it has been proposed that MUC2, MUC5AC, MUC5B and MUC6 are assembled in a similar fashion to this protein. In vWF biosynthesis, the CK domain is involved in the disulfide-bond-mediated dimerization between the C-terminal parts of monomers. The D1 and D2 domains are involved in oligomerization via the N-terminal ends and are removed prior to secretion (Bonthron et al 1986). Previously, we have shown that both MUC2 and MUC5B monomers undergo cleavage in the C-terminal domains (Herrmann et al 1999, Wickström et al 1998) and in recent studies on salivary MUC5B, we have used antibodies recognizing the various N-terminal domains to investigate proteolytic cleavage of the molecules in this region. We have demonstrated the release of an N-terminal fragment which corresponds in size to the D1 and D2 domains, consistent with a cleavage event in the D′ domain (Fig. 3) (Wickström & Carlstedt 2001). This suggests that assembly of MUC5B occurs via a similar mechanism to that of the vWF. In porcine submaxillary mucin (PSM), a postulated MUC5B homologue, cleavage could not be demonstrated when the N-terminal domain was expressed in COS-7 cells (Perez-Vilar & Hill 1998). However, this may be explained by the fact that PSM differs from human MUC5B in that it does not contain a D′ domain and/or that the enzymes required for cleavage are not expressed in these cells. We have identified fragments consistent with further C-terminal cleavage events in the N-terminal of MUC5B suggesting that a major part of the N-terminal low-glycosylated part of the mucin is removed during assembly without affecting its oligomeric nature (Fig. 3). Differences in proteolytic processing were also seen between MUC5B mucins that are soluble in guanidinium chloride and those that appear as an insoluble complex. Similar findings have now been made for MUC5B isolated from respiratory secretions (C. Wickström, J. Davies & I. Carlstedt, unpublished results). Possibly, controlled proteolysis generates mucin species with different macromolecular structure and this then provides the basis for adapting the properties of secretions to meet local physiological demands.

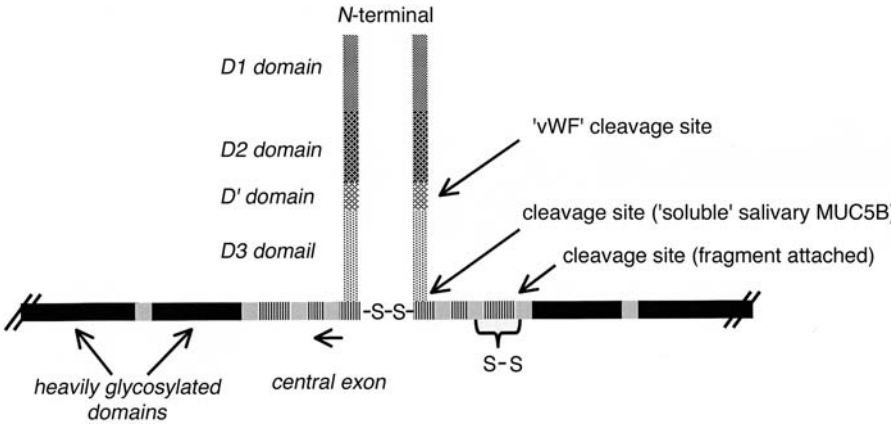

FIG. 3. Pictorial summary of postulated N-terminal cleavage events in MUC5B. Cleavage in the D′ domain generates a fragment that seems to correspond to the pro-polypeptide of the von Willebrand factor (vWf). Proteolysis in the D3 domain generates a second fragment from 'soluble' salivary MUC5B. A cleavage in the N-terminal portion of the 'central exon' gives rise to a fragment that is released after reduction and thus is linked to the mucin oligomers via disulfide bonds.

Mucin expression patterns in the respiratory tract

In normal adult respiratory tract, expression of nine mucin genes (*MUC1*, *2*, *4*, *5AC*, *5B*, *7*, *8*, *11* and *13)* has been demonstrated using Northern blotting or *in situ* hybrization (for a review see Copin et al 2001). *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC8* and *MUC13* are expressed in the surface epithelium whereas *MUC1*, *MUC2*, *MUC5B*, *MUC7* and *MUC8* are associated with the submucosal glands (Table 1).

Using antibodies developed in our laboratory that recognize MUC2, MUC4, MUC5AC, MUC5B and MUC7 mucins, we have shown that goblet cells produce mainly MUC5AC while MUC5B appears to be the dominating mucin in the submucosal glands, although some MUC5AC and MUC7 are usually present (Hovenberg et al 1996a, Wickström et al 1998). Antibodies against MUC4 react mainly with the surface epithelial ciliated cells (our unpublished observations). In addition, we and others have identified MUC5AC and MUC5B as major large gel-forming mucins in respiratory secretions (Thornton et al 1996, Wickström et al 1998, Davies et al 1999, Kirkham et al 2002). No cell-associated mucins have yet been isolated from the respiratory tract.

Several factors of relevance for respiratory disease including inflammatory mediators (interleukin [IL]1β, tumour necrosis factor [TNF]α, IL9 and prostaglandin E_2), tobacco smoke components such as acrolein, residual oil fly

TABLE 1 Mucins expressed in the respiratory tract

Mucin	*Membrane-associated?*	*Secreted?*	*Oligomeric?*
MUC 1	Yes	?	No
MUC4	Yes	?	No
MUC2	No	Yes	Yes
MUC5AC	No	Yes	Yes
MUC5B	No	Yes	Yes
MUC7	No	Yes	No
MUC8	?	?	?
MUC11	?	?	?
MUC13	Yes?	?	No?

ash and *Pseudomonas aeruginosa* exoproducts appear to up-regulate *MUC2* and/or *MUC5AC* expression (for a review see van Seuningen et al 2001). The promotor regions of *MUC2*, *MUC4*, *MUC5AC*, *MUC5B* and *MUC7* contain common *cis*-regulatory elements including binding sites for Sp1/Sp3, AP-1, glucocorticoid-response element and NF-κB. Thus, some factors may be involved in regulating more than one mucin gene, and mucus-secreting cells may be capable of expressing several mucin genes simultaneously. In apparently 'irritated' airways we and others (Wickström et al 1998, Davies & Carlstedt 2001, Chen et al 2001) have observed that, in addition to MUC5AC, MUC5B and MUC2 may be found in the goblet cells (Fig. 4). Staining for MUC5B is more marked than that for MUC2, suggesting that the more prominent change in irritated airways is from MUC5AC to MUC5B production. It is currently not known, however, whether MUC5B and/or MUC2 are produced together with MUC5AC or whether they originate from different cell populations.

High-turnover mucin species

Radiolabelling of airway cells and tissue in culture has been favoured as a method for monitoring the output of mucins in response to various stimuli. However, our studies using bovine airway tissue have shown that the large oligomeric mucins are poorly labelled with [^{35}S]sulphate and [^{3}H]glucosamine. In contrast, [^{35}S]sulphate was rapidly incorporated into high-molecular-mass mucin-like molecules, which were secreted into the medium (Svitacheva et al 1998). A similar component was

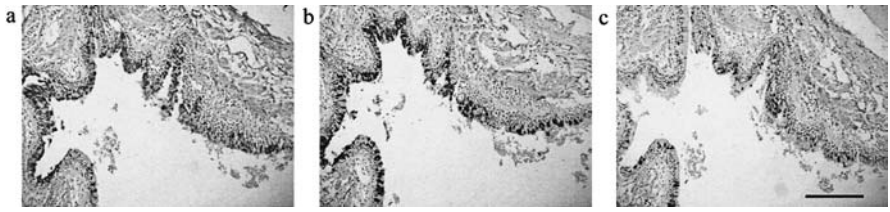

FIG. 4. Human airway stained with antibodies recognizing (a) MUC5AC, (b) MUC5B and (c) MUC2. Serial sections (4 μm) of human airway were stained with (a) the LUM5-1 antiserum (Hovenberg et al 1996b), (b) the LUM5B-2 antiserum (Wickström et al 1998) or (c) the LUM2-3 antiserum (Hovenberg et al 1996b). Sections were counterstained with Mayer's haematoxylin. The bar represents 200 μm.

identified from respiratory tract epithelial cells suggesting the molecules are continuously released from the epithelial surface (Svitacheva & Davies 2001). The molecules were monomeric and mucin-like in that they contained trypsin resistant domains and *O*-linked glycans. We propose that such molecules may provide a mechanism whereby 'shedding' of putative microbe receptors prevents the attachment of microorganisms to the luminal surface of the epithelial cells. These findings also highlight the fact that in studies where radioactive glycoprotein precursors are used to 'label' the mucins, results are more likely to reflect these monomeric mucin-like molecules rather than the large, oligomeric mucus-forming ones.

Summary and perspectives

In healthy airways, mucus provides a flexible and dynamic first line of defence against inhaled antigens, bacteria and particles. However, chronic bronchitis, asthma and cystic fibrosis are associated with an increased production of mucus, and airway 'irritation' has been shown to up-regulate the *MUC5AC* and/or *MUC2* genes. In addition, *MUC2* and *MUC5B* may be expressed in goblet cells where they are not normally found. Although the over-production of mucus is one important aspect of inflammatory airway diseases, the picture is likely to be much more complex than can be revealed through changes in mucin gene expression alone. We have shown that MUC5B is proteolytically processed during assembly. The physiological significance of this is not yet known but alterations in processing of mucins would affect their structure and may lead to changes in the physical properties of the mucus gel. Bacterial colonization of the airways is a significant feature of chronic bronchitis and cystic fibrosis and, since bacteria bind to carbohydrate structures, is likely to be strongly influenced by the glycan chains available in mucus. Respiratory mucins, most notably MUC5B, exist as different glycoforms, but the degree to which changes in the proportions of these is a

contributing factor to colonization in hypersecretory disease remains to be determined. As well as the mucus-forming mucins, monomeric mucins with an apparently high turnover have been identified in the epithelium. The role of these mucins in mucosal defence is obscure, but one possibility is that they act as cell-surface receptors for microorganisms and aid their removal through shedding from the epithelial surface. As yet, the presence of such mucins in human airway has not been investigated and it is not known whether they correspond to one of the known cell-associated ones. In addition to mucins, a range of other proteins involved in mucosal defence have been identified both in the sol phase of mucus and associated with the polymer matrix of mucus. From the complexity of mucus with its subtle blend of different mucin gene products and glycoforms combined with 'protective' proteins it is obvious that these secretions play a much more dynamic role in mucosal defence than simply providing a physical barrier.

Acknowledgements

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DISCUSSION

Basbaum: You described induction of MUC2 and MUC5B in the surface epithelium of irritated airways. Would you predict that the augmentation of these insoluble mucins would make a difference clinically?

Carlstedt: I don't know. The only way to get knowledge here is to make clinical investigations. We must define what is normal, and which changes occur in disease, and then relate this to how the individuals feel.

Basbaum: I think that John Fahy is actually doing this type of correlational work now.

Fahy: We took 15 non-asthmatic subjects living in San Francisco, most of whom were never smokers and all of whom had not smoked for 10 years, and who had smoked less than 10 pack years altogether. We found that the phenotype of their airway is fairly consistent in terms of the number of goblet cells and the volume of mucin within these cells. The principal airway gene that is expressed at the RNA level in the surface epithelium is *MUC5AC*. *MUC2* and *MUC5B* are also expressed, but at lower levels. This expression pattern is consistent between subjects. Even though these are people living in an urban environment, in comparing biopsies from healthy and asthmatic subjects we found that *MUC5AC* is up-regulated in asthma. We weren't able to dissect whether this was just a function of more goblet cells. When we get to individual goblet cells, we will be able to decide whether in disease there are gene expression changes at the individual cell level. To some extent, technology will help us as we get better at doing PCR for mucin genes and we can dissect out individual cells. I am not as worried as some others who have often asked how we can look at the mucus phenotype when so many things change it. I think that in disease in general — be it asthma, chronic bronchitis or CF — the disease-associated change is so large compared to environmental change that I don't think we need to be too worried about environmental noise *per se*.

Rose: In your histology of the irritated epithelium, which was from one patient, you showed staining in the airway epithelium in the goblet cells of three different mucin genes. I think this is the first time I've seen data showing that a goblet cell may express more than one mucin gene product.

Fahy: We have also seen expression of more than one gene in the goblet cells. We have seen the same cell express *MUC5AC*, *MUC2* and *MUC5B*. But *MUC5AC* is the predominant gene.

Carlstedt: I was not showing serial sections but adjacent ones. I have not done any double staining, so I can't really say whether one goblet cell is actually making two or more mucins. It could be two adjacent goblet cells producing different mucins.

Rose: The patterns are very similar. In mouse models of allergic asthma, there are lots of goblet cells in the airway surface epithelium. In our hands, not all goblet cells stain with the affinity-purified anti-MUC5AC:TR3A antibodies, which recognize murine MUC5AC mucins (Berger et al 1999). Just recently we showed that goblet cells in an OVA-model of allergic asthma express *Muc5a* and *Muc2* mRNA (S. Shahzeidi & M. C. Rose, unpublished work). Wu and colleagues have shown that *Muc5b* mRNA is also expressed in this model (Chen et al 2001).

Fahy: I don't have protein data yet, so it is conceivable that they could differ at the protein level.

Basbaum: When diverse mucin genes are expressed in the same tissue, how do they interact in the gel? Do they segregate?

Rose: The only work that addresses this is Sam Ho's work that Alan Jackson quoted earlier, in which Ho showed that MUC5AC and MUC6 mucins remained segregated within the gastric mucus gel in a laminated linear arrangement (Ho et al 2000).

Basbaum: What was the preparation?

Jackson: It was a cryo-preserved *in vivo* preparation from human stomach.

Fahy: Aren't there data that these mucin genes associate with different trefoil factors? I was under the impression that *MUC5AC* associates with *TFF3*, and this has consequences for viscosity also.

Carlstedt: It might, but personally I don't regard these data as particularly strong.

Barnes: I would like to know more about the enzymes that regulate glycosylation, as they may have effects on mucus quality. Perhaps these enzymes might be targets for future treatment to change mucus viscosity, for example.

Carlstedt: Not in specific terms. We know that there are quite a few glycosyl transferases. I would be surprised if the details in the glycosylation would dramatically influence the *physical* properties of the mucus. Once the potential glycosylation sites are substituted by a mono-, di- or trisaccharide, the mucin domains are probably fully expanded and become as 'space-filling' as possible.

But what can be introduced are sites for other things to bind to. Matthias Salathe was talking about hyaluronic acid earlier, and how this molecule acts as a matrix for other protecting components thus keeping them in certain locations. Mucus is most likely a matrix for many protective agents and in this context I think glycosylation may affect the properties.

Verdugo: Let us consider that mucins are secreted condensed in discrete packages, and when these hydrate and come out, they form a single layer. That is, the discrete packages anneal together and polymers from one network migrate, interpenetrating into the other neighbouring network, forming an interwoven carpet. Is this compatible with a fully cross-linked structure? If mucins are constantly cross-linked in the granules I would say that the mucus should be a collection of small individual gels with no chains that can interconnect them. But it turns out that it is not this way. For me, it remains a problem to correlate the complications coming from degenerative products studied in biochemistry to the actual physical topology of the mucous network.

Sheehan: We have looked at mucins from cell cultures (as has Ingemar Carlstedt) and also mucins from secretions. They are characterized typically by a polydispersity. What you are saying could be true: you could have types of mucin that Ingemar Carlstedt describes as insoluble, as defined by a sedimentation rate that would relate to a particular morphology and mass, as against species that were small enough to dynamically migrate through that background. You may have gels that are heterogeneous in morphological type (certainly, all the studies that I have done on fractionation would indicate that polydispersity is built in to the secreted mucin phenotype).

Carlstedt: Possibly within the mucous granule the mucins are much more 'insoluble' than they are after secretion. Maybe there is an active mechanism — perhaps a protease that turns 'insoluble' mucins into 'soluble' ones. We haven't found these proteases yet, but we have identified proteolytic cleavage events. Depending on which proteases are present and the extent to which they work, we may end up with different final products.

Sheehan: I agree, but I think there's also another feature that we have to consider from our data. It takes a couple of hours at least to make and secrete a mucin. After a 20 min pulse, we start to see evidence of labelled mucins leaving the cell after an hour or so, but mucins synthesized in that time are still coming out from the cell up to 100 to 200 hours later. All the evidence is that these are mature, so they are made in a certain form. My conclusion is that what is coming out from the cell at any particular moment is a distribution of mucin sizes, some of which are recently made, others made a long time ago. This fits nicely onto what Ingemar Carlstedt is talking about in terms of doing labelling experiments.

Verdugo: With isolated granules that are electroporated and allowed to swell or disperse, there is a tremendous variation of swelling kinetics. It could take days to

fully hydrate granules from goblet cells of the colon, for example, while in granules from the airway hydration is fairly fast. In granules from slugs, within 13 ms the mucus expands from 6 μm^3 to 300 μm^3: it's an explosive hydration. In essence, the topology of the system should be such that it will allow this kind of variation. In fully cross-linked networks you wouldn't really expect to have this range of dispersions.

Sheehan: Of particular interest with regard to this would be the sialyl transferases and sulfyl transferases. I would have thought, on the basis of the kind of model that Pedro Verdugo is building up, that the expansion is to some degree related to the amount of electrostatic charge on the mucins that is to become unshielded.

Verdugo: And the length. In order for the mucin to expand, the chains of mucin have to be pulled thoroughly apart.

Sheehan: So if we could make an inhibitor of that particular group of modifications, we might affect the ability of the mucins to hydrate and therefore form mucus.

Basbaum: So from what you and Pedro Verdugo have said, it seems that there is a testable hypothesis here. In other words, there is a spectrum from the slug, to the airway, to the gut in terms of mucin hydratability. There are a few possible parameters that could be responsible, including charge and length.

Sheehan: But more than anything, the sort of thing that Ingemar is talking about would be relevant here: cross-linking and proteolytic cleavage.

Basbaum: Would it do any good to compare granules from airway with granules from gut in a systematic fashion? Would you want to have the same mucin gene present?

Sheehan: You would need a lot of description to decode all of that.

Basbaum: I am trying to think how we could make them comparable, but perhaps it is not worth the effort. Maybe in making them comparable we would lose what we are looking for.

Engelhardt: I found Pedro Verdugo's comments interesting. I wonder whether differences in the types of mucins expressed in the surface airway epithelium and the glands may be due to different requirements for hydration. Obviously, if there are granules coming out of the ends of mucous tubules in glands, it would be disadvantageous to have them hydrate before they reached the airway surface.

Verdugo: There is also an element of folding. If we think of this system as a tangled network in condensed phase, that is going to expand in less than 15 ms to 600 times its volume, this would be like pulling a tangled fishing line. You would end up with many tight knots and many long loops. The implication is that the section between two tangles could be folded, just as is seen in DNA. In this sense, mucins can behave as block copolymers. They contain regions that are highly hydrophobic alternating with hydrophilic domains. This is like a set of perfectly ordered, interconnected domino pieces folded together forming an

accordion-like topology. This liquid crystalline order allows these networks to rapidly unfold and expand with very little frictional dissipation. Nematic liquid crystalline orders are usually revealed by X-ray diffraction but in mucus they are so large that they can be seen by simple polarized light microscopy (Viney et al 1993).

Carlstedt: What would happen if you included a pair of scissors in that system?

Verdugo: If you cut the polymer into shorter pieces the time for dispersion is going to decrease with the square of the length. It takes very little cutting to disperse it completely.

Rose: Has anyone figured out a way to isolate secretory granules?

Verdugo: Yes. We are doing this routinely. We sonicate the cells, mark the granules with quinicrine and then sort them in the flow cytometer.

Rose: Pedro, you mentioned slug secretory granules. Do they contain mucin glycoprotein polymers?

Verdugo: They are mucins.

Rose: Ingemar Carlstedt, you have been looking at the D-domains in secretory mucins and talking about where they are cleaved. Have you looked at the literature on the Weibel–Palade secretory granules in endothelial cells where vWF is cleaved and packaged before being secreted into the blood stream? This system has been worked out, so that your data showing cleavage of one or two D-domains in a secretory mucin was not surprising. This is how vWF, a high molecular weight glycoprotein synthesized in endothelial cells, is processed before secretion. This system may provide you with insights into enzymes that may also be present in secretory granules.

Carlstedt: We can probably learn a lot more by looking into the vWF system. We haven't done that. For instance, if there is a protease involved, it may be a similar one.

Sheehan: Putting my data together with Ingemar Carlstedt's, it could be that we are getting a glandular mucin phenotype from the hyperplastic mucin-secreting cells. The mucus is of the wrong type, is in the wrong place, and there is too much of it.

Basbaum: What about the ectopic expression of mucins where they don't belong? This applies to MUC2: it is insoluble but can be moved by intestinal peristalsis. Mucins that interact directly with airway cilia may have to be a bit more soluble.

Rose: This goes along with the structure of MUC5AC and MUC5B mucins versus MUC2 mucin. MUC2 has a very large internal domain, whereas 5AC and 5B mucins have several cysteine-rich domains interspersed with the tandem repeats.

Randell: Ingemar Carlstedt raised an important point: an emerging area is the regulated secretion of the membrane-bound mucins MUC1 and MUC4. We

know very little about whether their turnover is enhanced in chronic disease and their role in the bronchioles.

Basbaum: Do MUC1 and MUC4 turn over faster in disease?

Carlstedt: I can't say that. There are mucins that apparently turn over very fast. They seem to be monomeric and are dominated by one long glycosylated domain.

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Development and validation of a lectin-based assay for the quantitation of rat respiratory mucin

Alan Jackson, Phil Kemp, June Giddings and Rosemary Sugar

Novartis Respiratory Research Centre, Wimblehurst Road, Horsham, West Sussex RH12 5AB, UK

Abstract. The significance of a mucus hypersecretory phenotype to the pathogenesis of airways disease is controversial, but increasing evidence suggests that it can negatively impact upon patient health status. A critical aspect of developing our understanding of the role of mucus hypersecretion in disease is the development and appropriate use of methods permitting detection and quantitation of mucins in biological samples. However, the unique biophysical and biochemical properties of this class of glycoproteins do not lend themselves to routine quantitation. Individual pure mucins are not commercially available, the reactivity profile of commonly used reagents is generally not well characterized and assay development and validation is rarely covered adequately in the literature. Therefore quantitation of mucin in biological samples relies upon careful histochemical and biochemical characterization and partially purified mucin preparations. The absence of tools considered essential for assay development in other areas means that this characterization process does not generally lead to proof of selective detection of mucin, but rather to a level of confidence that mucin is detected and defined contaminants are not. This chapter provides an example of the process of development and validation of a lectin-based assay for quantitation of mucin in untreated complex biological samples.

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Mucin biophysical/biochemical properties

Every researcher involved in this symposium is interested in mucins or mucus either scientifically, clinically or both. Of the 15 abstracts published as pre-information for the symposium, only three including this one alluded to methods of detection and/or quantitation of mucin. One inference is that mucin quantitation methods are considered sufficiently routine that they do not require specific mention. A number of factors suggest that this is not true. Assay development and characterization is rarely if ever adequately reported in the literature. While for most assay development purposes the availability of pure target reagents would be considered a requirement, pure mucins are not available. Furthermore, the specificity of commercially available antibodies for

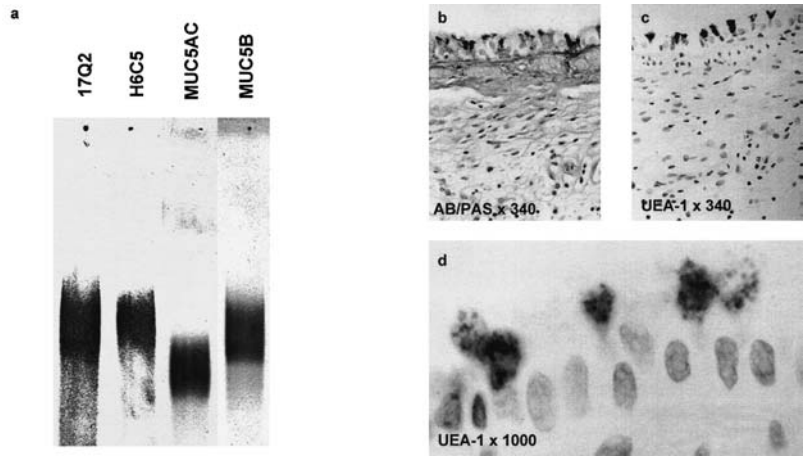


FIG. 1. (a) Western blots of HBEC mucin on 1% agarose gels. The mobilities of MUC5AC and MUC5B can be discriminated using specific antibodies, and indicate that 17Q2 and H6C5 selectively bound to MUC5B, but not MUC5AC. (b–d) Paraffin sections of rat airways stained with AB/PAS and biotinylated lectins visualized with the peroxidase substrate diaminobenzidine. (b, c) UEA-1 binds selectively to all AB/PAS stained goblet cells with negligible non-goblet cell staining. (d) UEA-1 selectively binds the mucin granules within goblet cells with negligible non-mucin granule staining. Agarose Western blot courtesy of L. H. Abdullah and C. W. Davis; specific mucin antibodies kindly provided by John Sheehan.

individual mucins is unclear. For example, recent results obtained from Western blots of 1% agarose gels indicate that distinct bands can be identified for MUC5AC and MUC5B using specific antibodies. These blots have been used to demonstrate that H6C5 and 17Q2, two antibodies generally considered useful for generic mucin assays (Lin et al 1989) (Fig. 1a) appear to be selective for MUC5B over MUC5AC (Jayawickreme et al 1999).

Although a number of methods exist for estimation of mucin concentrations in biological samples, most are time-consuming and require extensive sample preparation. For example, radiolabelled pulse chase requires dialysis to remove non-incorporated label (Meini et al 1993). Lectins and antibodies have been used to develop more convenient assay formats, however the characterization of these assays is rarely reported in detail. Notable exceptions include the reports of Lin et al (1989) and Jefcoat et al (2001). The lack of availability of individual pure mucins often leaves some doubt concerning the identity of material detected by commonly used reagents. For example, no published assays distinguish between detection of oligomeric gel-forming mucins versus non-oligomeric membrane associated mucins, both of which may be present in biological samples.

Development and characterization of assays for the quantitation of mucins depends upon an understanding of their biological, biochemical and biophysical

properties. In the present chapter we describe the development and characterization of a lectin-based sandwich assay for rat airway mucins using a lectin derived from gorse, *Ulex europaeus* agglutinin-1 (UEA-1). Our goal was to develop a safe, selective quantitation method for respiratory mucins derived from an *invivo* rat model of inflammation meeting a set of pre-defined criteria. In addition we aim to stimulate consideration of the relative strengths and weaknesses of mucin detection and quantitation methods.

Assay development criteria

Sample preparation

- No boiling sulfuric acid!
- No sample separations (columns/gels)
- No dialysis
- No reduction/deglycosylation/alkylation steps
- No serial dilutions (effective detection range should cover range of [brochoalveolar lavage mucin])

Assay performance

- Must detect secretory gel-forming mucins
- Must demonstrate selectivity against leucocytes, red blood cells, plasma
- Must demonstrate protein independence over the range 0–500 μg ml^{-1}
- Must demonstrate intra- and inter-assay coefficient of variance $< 20\%$

Assay development: histology and selectivity

One of the few features of secretory gel-forming mucins that aids the development and validation of tools and reagents for their quantitation relates to their production. Mucins are stored in histologically distinguishable granules within dedicated mucin-producing cells (Jeffery & Li 1997). For example, in rat intrapulmonary airways the secretory gel-forming mucins are thought to be almost exclusively produced by, and stored in, surface epithelial goblet cells (Rogers et al 1991). Therefore lectins that selectively react with these cells may be considered to be candidates for the development of enzyme linked lectin assays (ELLAs). Analysis of the binding patterns of a panel of lectins screened against paraffin sections of rat lungs demonstrated that UEA-1 bound all epithelial goblet cells detected by alcian blue/periodic acid–Schiff (AB/PAS) staining (Figs 1b,c). Higher power visualization confirmed that UEA-1 bound exclusively to the mucin granules (Fig. 1d). Other lectins tested bound to mucin granules of surface epithelial goblet cells, however their binding patterns were less suitable for

development of an assay for quantitation of mucins within rat bronchoalveolar lavage (BAL) fluid. *Helix pomatia* agglutinin (HPA) stained alveolar macrophages in addition to goblet cells suggesting that an assay developed using this reagent might be unduly influenced by changes in inflammatory cell components (data not shown). A lectin derived from *Tetragonolobus purpureas* selectively stained a subset of goblet cells suggesting that it might be too selective for a generic mucin assay (data not shown). This phenomenon has also been reported for antibodies (St George et al 1985).

Selective goblet cell mucin granule staining is a useful starting point for the selection of reagents which might be used for the development of assays for mucins in biological samples. Exclusion of reagents from further assay development on the basis of non-selective histological staining profiles should take account of the model systems to be assayed. HPA may be inappropriate for use in an assay for rat BAL, based on its reactivity with rat alveolar macrophages. However, it may be useful for mucin quantitation assays used in *in vitro* models. A caveat to the use of histological screening for the selection of reagents for assay development is that the reactivity of tissue components may change during processing. In one study 192 hybridomas producing antibodies against a lyophilized rhesus monkey mucin preparation resulted in 119 antibodies reactive by direct ELISA, but only 61 that reacted with secretory cells in sections (St George et al 1985). These discrepancies may be partially explained by loss of reactivity following tissue processing. Similarly, non-reactivity of reagents with the apical borders of non-goblet epithelial cells does not prove lack of reactivity with native membrane bound mucins. Finally, selective positive staining of goblet cell mucin granules does not equate with proof of reactivity with mucins since these granules contain non-mucin components.

Agarose gel electrophoresis and size exclusion chromatography

UEA-1 reactivity with rat BAL components on 1% agarose gels

Intact mucin sub-units have a molecular weight of approximately 2×10^6 Da or greater and are not resolved by 4–20% polyacrylamide gels (Carlstedt & Sheehan 1984) routinely used for protein separations. Molecular weight can help to distinguish mucins from most other components of biological fluids, however other high molecular weight molecules such as hyaluronic acid and chondroitin sulphate may also be present (Berger et al 1999). These molecules can be distinguished from mucins due to their differential sensitivities to enzyme digestion. We have used Western blots of 1% agarose gels with 0.1% SDS to examine the reactivity of UEA-1 with high molecular weight components of rat

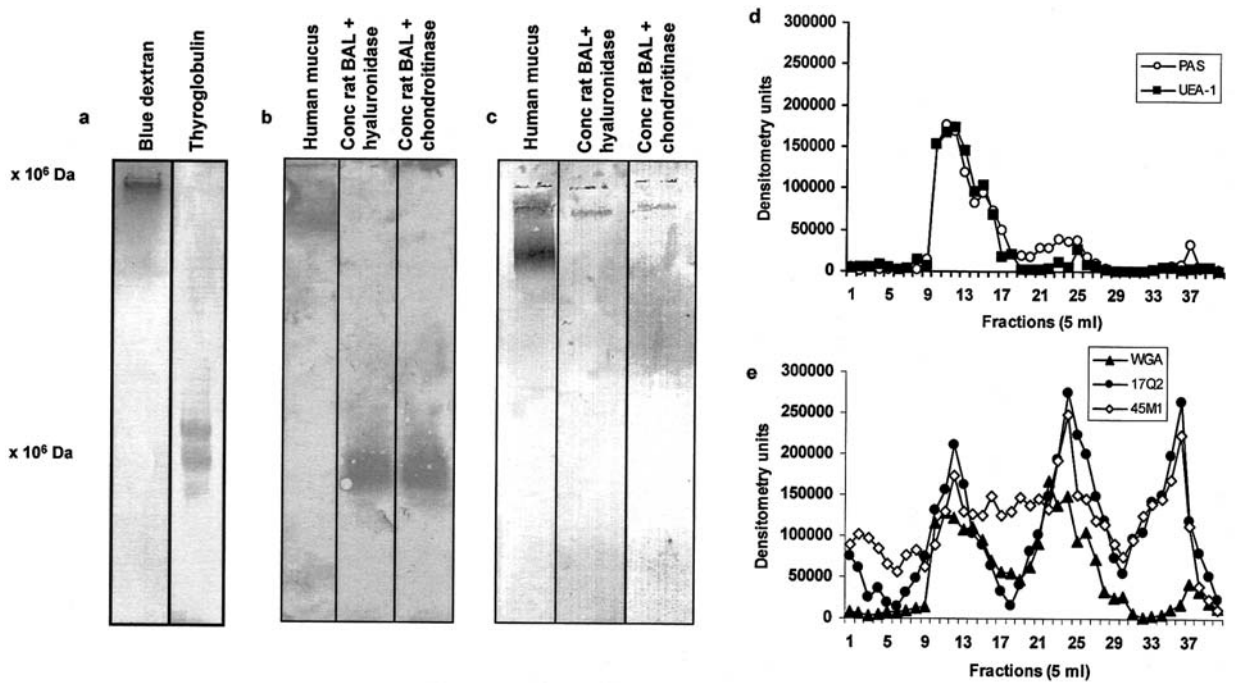


FIG. 2. (a–c) Western blots of 1% agarose gels. (a) Molecular weight standards: PAS staining of blue dextran (2×10^6 Da) and Coomassie Blue staining of thyroglobulin (0.67×10^6 Da). (b, c) PAS & UEA-1 staining respectively, of a human mucus standard, concentrated rat BAL after hyaluronidase digestion (2 h) and concentrated rat BAL after chondroitinase ABC digestion (2 h) demonstrating selective detection of high molecular weight mucin-like material by UEA-1, but additional staining of lower molecular weight material by PAS. (d, e) Dot blot densitometry of a human sputum sample pretreated sequentially with hyaluronidase, chondroitinase ABC, heparinase and DNase 1 and separated on a Sepharose CL-4B column. (d) Demonstrates that PAS and UEA-1 primarily detect material contained within the void volume. (e) Demonstrates in addition to the high molecular weight material detected in the void volume 45M1, 17Q2 and WGA detect additional major peaks consistent with the minor peaks detected by PAS.

BAL compared with a human mucin standard. PAS and Coomassie Blue staining was used to demonstrate the relative mobility of two molecular weight markers (Fig. 2a), blue dextran (2×10^6 Da) and thyroglobulin (670 000 Da) to give an impression of the molecular weight of material detected by PAS and UEA-1. Care should be taken, however, in interpreting the molecular weight of unknown material using dissimilar molecular weight markers since their mobility is dependent upon size, molecular weight and charge on the gels used in this study. PAS staining detected high molecular weight material in a human mucin standard preparation and also in a concentrated rat BAL sample (Fig. 2b). This reactivity in rat BAL was resistant to hyaluronidase digestion for 2 h or to chondroitinase ABC digestion for 2 h (Fig. 2b). UEA-1 also detected high molecular weight material insensitive to digestion with hyaluronidase or chondroitinase ABC (Fig. 2c). The material detected by PAS (Fig. 2b) had a substantially greater mobility than that detected by UEA-1 (Fig. 2c). These results indicate that PAS and UEA-1 detected high molecular weight material consistent with mucin glycoproteins although the reason for the different mobilities of the material detected by each reagent is unclear.

UEA-1 reactivity with human sputum fractions

Due to the ready availability of human sputum, we have chosen to use a human mucin standard for 'quantitation' of the mucin content of rat BAL, therefore we have examined the reactivity of UEA-1 with human sputum components. Human sputum was pre-digested with hyaluronidase (24 h), chondroitinase ABC (5 h), heparinase (6 h) and DNase I (1 h) as described by Goswami et al (1994) and then passed down a Sepharose CL-4B column. Densitometry was used to examine the reactivity of UEA-1 with consecutive 5 ml fractions dot-blotted onto nitrocellulose filters (Fig. 2d). The molecular weight of intact mucins is such that they are not retarded by Sepharose CL-4B and therefore would be expected to elute in the void volume. PAS and UEA-1 gave an almost superimposable pattern of reactivity and both reagents detected predominantly void volume material. We compared the reactivity of PAS and UEA-1 with the pattern of reactivity obtained using three other reagents commonly used to detect and quantify mucins (Fig. 2e). Two antibodies, 17Q2 and 45M1, generated against purified rhesus monkey tracheal mucin and human ovarian cyst mucin preparations respectively and one lectin, wheat germ agglutinin (WGA), showed good detection of the UEA-1 and PAS reactive void volume material. However, all three reagents detected a second major peak of reactivity with a molecular weight lying between 158–669 kDa. The antibodies 17Q2 and 45M1 also detected a third major peak with a molecular weight of less than 158 kDa. Interestingly all three of these peaks were detected by PAS, 17Q2, 45M1 and WGA (to differing levels),

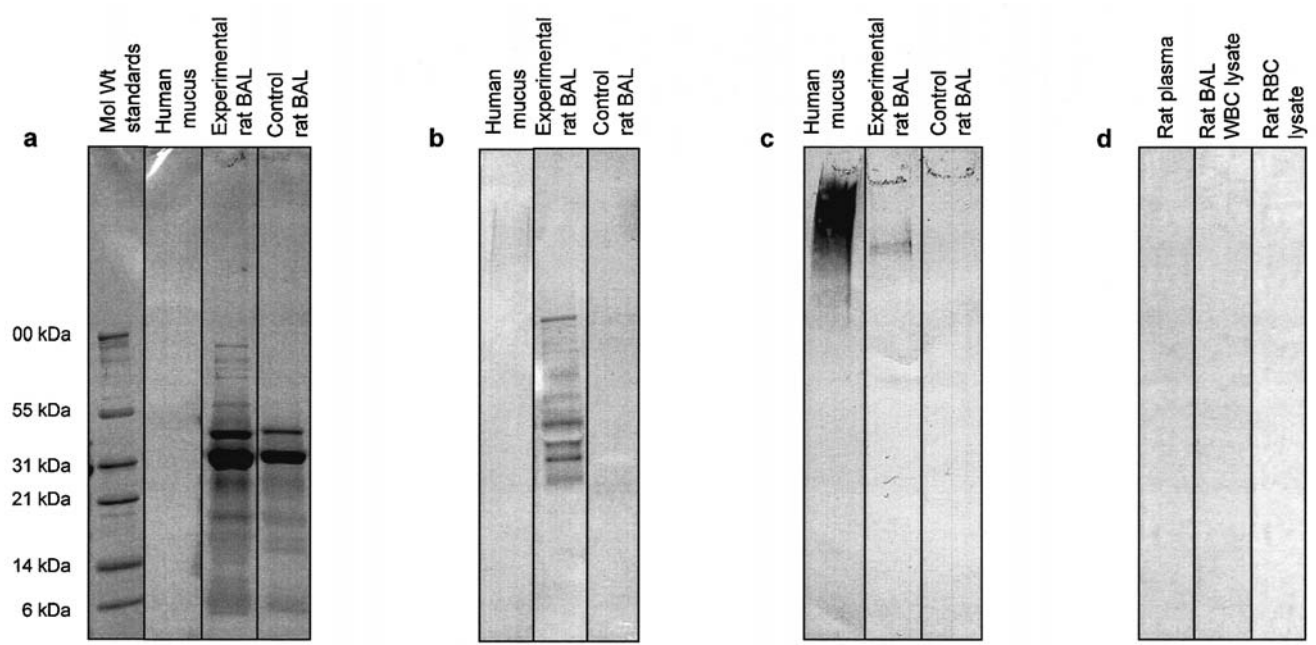


FIG. 3. (a) Coomassie Blue staining of a 4–20% polyacrylamide gel demonstrating proteins with a molecular weight range inconsistent with intact mucin in experimental and control rat BAL. (b–d) Western blots of 4–20% polyacrylamide gels. (b) Demonstrates PAS staining of low molecular weight material in rat BAL inconsistent with intact mucin. (c) Demonstrates selective staining by UEA-1 of high molecular weight material in both a human mucin standard and experimental rat BAL, but not in BAL from control rats which express few goblet cells. (d) Demonstrates a lack of reactivity of UEA-1 with neat rat plasma, BAL WBC lysate and RBC lysate.

whereas UEA-1 detected only the first two. It might be expected that assays developed utilizing these reagents would incorporate different biases into 'mucin' quantitation. For example UEA-1 appears to be biased towards detection of material contained within the void volume i.e. the very high molecular weight material. One interpretation of this might be that it may discriminate between intact mucins and those that have been degraded in some way. It is known that mucin subunits consist of alternating heavily glycosylated, protease resistant and poorly glycosylated, protease sensitive regions and that proteolytic cleavage can lead to production of fractions with molecular weights as low as 300–500 kDa (Carlstedt & Sheehan 1984). Alternatively the additional peaks may represent non-mucin material. The void volume fractions were pooled for use as a standard in future UEA-1-based assays.

SDS-PAGE and selectivity

Neither agarose gel electrophoresis nor size exclusion chromatography is sufficient to prove that UEA-1 selectively detects mucins. An intrinsic feature of both methodologies is the separation of sample components on the basis of charge and/or size. UEA-1 reactivity with some highly charged and/or low molecular weight components is, therefore, not assessed. Binding specificity is addressed only partially on paraffin embedded sections where tissue fixation artefacts could influence lectin binding patterns and titration of detection reagents could lead to subjectivity in interpretations of staining patterns. Therefore additional characterization of selectivity is critical. The materials against which selectivity is assessed should depend upon the uses for which the assay is intended. Our aim was to develop an assay that would be useful for the quantitation of mucins in rat BAL fluid, which contains mucin and non-mucin components of varying molecular weights. We used 4–20% gradient Tris-Glycine SDS-polyacrylamide gels and Western blotting to examine the reactivity of PAS and UEA-1 with components of rat BAL samples. It has previously been reported that Coomassie Blue staining does not effectively detect mucin (Tytgat et al 1995), and in our hands it did not detect a human mucin standard on a 4–20% gel. However, Coomassie Blue staining did demonstrate a considerable amount of proteinaceous material over a wide molecular weight range in BAL samples from both experimental and control rats. Experimental rats were pretreated with LPS (500 $\mu g\,kg^{-1}$; intra-tracheally [i.t.]) 48 h prior to collection of BAL. Control rats were pretreated with saline (Fig. 3a). PAS weakly detected the human mucus standard on Western blots, but detected a considerable amount of material in BAL samples from experimental rats over a molecular weight range inconsistent with intact mucins. Substantially less PAS reactive material was observed in the BAL of control rats (Fig. 3b). In contrast to Coomassie Blue and PAS, UEA-1 sensitively detected the human

mucus standard and the only material detected by UEA-1 in rat BAL samples was one band of reactivity in the stacking gel (Fig. 3c). This UEA-1 reactive material had a molecular weight greater than 200 kDa and was present in the BAL samples derived from animals exposed to LPS, but not in BAL samples from control animals. The airways of control rats contained negligible numbers of goblet cells. However, this number increased dramatically following exposure to LPS (data not shown). This profile of reactivity is consistent with UEA-1, but not PAS, selectively detecting goblet cell derived high molecular weight mucin-like glycoproteins.

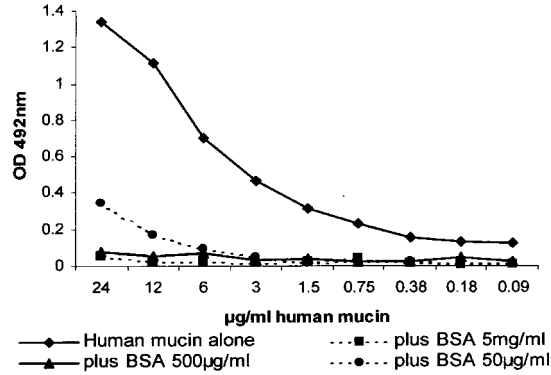
Experimental manipulation can lead to considerable changes in the composition of rat airway components, for example following plasma leakage or haemorrhage. In order to determine the likely tolerance of a UEA-1-based mucin assay to changes in BAL composition, we examined the binding of this lectin to components of rat plasma, rat BAL leucocyte lysates and rat whole blood lysates. UEA-1 showed no reactivity with undiluted rat plasma, lysates derived from BAL white blood cell (WBC) pellets or undiluted whole blood cell (RBC) lysate (Fig. 3d).

Assay development and validation

On the basis of these results UEA-1 was used to develop an ELLA for measurement of mucin in rat BAL fluid. Amongst the criteria that we set ourselves at the outset of the assay development process was that the assay should require a minimum of sample preparation and be independent of non-mucin protein up to a concentration of 500 μg ml^{-1}. Exposure (i.t.) of rats to inflammatory stimuli such as LPS leads to an increase in BAL protein concentration from approximately 50 to around 250 μg ml^{-1} in our hands (data not shown). We examined the effect of exogenously added bovine serum albumin (BSA) up to a concentration of 5 mg ml^{-1} on UEA-1 detection of a human mucin standard using either a direct binding or a sandwich ELLA format. BSA substantially interfered with detection of mucin at all concentrations examined (50, 500, 5000 μg ml^{-1}) in the direct binding assay, but did not significantly alter detection in the sandwich assay up to a concentration of 5 mg ml^{-1} (Figs 4a,b)

FIG. 4. (a, b) The effect of exogenous protein (BSA) on UEA-1 detection of a human mucin standard in two assay formats. (a) A direct binding ELLA (serial dilutions of human mucin standard coated directly onto a 96-well plate, detected with horseradish peroxidase conjugated UEA-1 [HRP-UEA-1]) demonstrates protein interference by concentrations as low as 50 μg ml^{-1}. (b) A sandwich ELLA (UEA-1 pre-coated onto a 96-well plate, serial dilutions of human mucin standard, detected with HRP-UEA-1) demonstrated no protein interference up to concentrations of 5 mg ml^{-1}. (c) Linear regression analysis of the relationship between rat BAL mucin content measured by UEA-1 sandwich ELLA and intrapulmonary goblet cell score in UEA-1-stained rat airways (r^2= 0.752, P<10.001).

a



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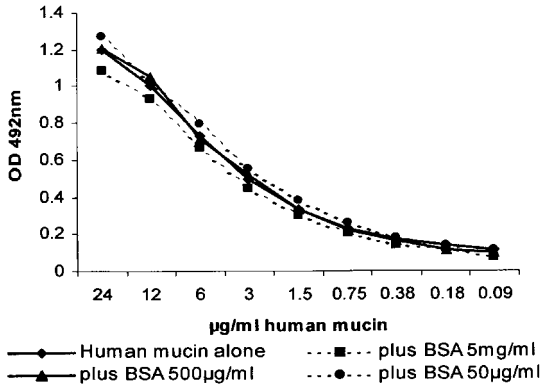

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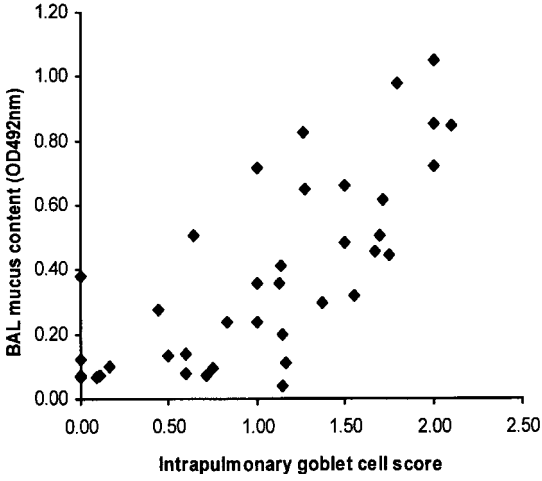

suggesting that the tolerance of a UEA-1 based sandwich ELLA was comensurate with its proposed use in untreated BAL samples. We optimized the assay for primary UEA-1 concentration, sample capture time, secondary UEA-1 concentration and incubation time, and incubation reaction temperature. The intra- (6–12%) and inter-assay (6–18%) variabilities were assessed using four concentrations of a human mucin standard (assessed gravimetrically).

As a final validation we reasoned that if the assay was detecting goblet-cell-derived mucin then the mucin content of the BAL fluid estimated using the assay should be related to the number of goblet cells in the airways. This hypothesis depends upon the goblet cells in all rats responding equally to the same stimulus, in this case the BAL procedure. It also depends upon the goblet cells only partially degranulating since total degranulation would be expected to lead to an increase in BAL mucin and a decrease in apparent goblet cell number. BAL mucin content was estimated using the UEA-1 sandwich ELLA and airway goblet cell score was estimated from paraffin sections of rat lung stained with UEA-1. Regression analysis indicated that BAL mucin content was significantly correlated with intra-pulmonary goblet cell score ($r^2 = 0.753$, $P < 0.001$) consistent with detection of goblet cell derived material by this assay (Fig. 4c).

Summary

The UEA-1 based sandwich ELLA described in this chapter selectively detected goblet cell derived, hyaluronidase and chondroitinase ABC resistant high molecular weight glycoprotein in rat BAL and in a human mucin preparation derived from sputum. The material detected by UEA-1 demonstrated an overlapping reactivity with PAS, 17Q2, and 45M1 consistent with mucin glycoproteins. UEA-1 did not react with components of neat plasma, white blood cell lysates or whole blood lysates on polyacrylamide gels. Therefore, blood components are unlikely to interfere with mucin detection in this model system. It should be noted that no attempt was made to retain whole blood cell membranes following lysis. However, UEA-1 did not detect blood cells in paraffin sections and cells are routinely spun out of BAL preparations prior to mucin analysis to minimize the potential for interference. This ELLA was unaffected by exogenous protein up to a concentration of 5 mg ml^{-1} permitting analysis of mucin content of essentially native biological samples. The assay is robust and has been formatted for 96-well plates. This assay therefore provides the opportunity to assess the mucomodulatory capacity of test compounds in rat lungs.

Finally, it is just as important to understand the weaknesses of an assay as it is its strengths. Despite extensive validation, lack of access to pure mucins or highly specific antibodies means that it has not been possible for us to determine the

relative selectivity of UEA-1 for MUC5AC and MUC5B. We know that UEA-1 stains human and rat submucosal glands and also human and rat goblet cells, suggesting that it may detect both of these mucins. However, similar staining profiles have previously been demonstrated for 17Q2, which appears to have at least some selectivity for MUC5B. In addition, detection of membrane bound mucins and non-mucin glycoproteins cannot currently be ruled out. Like all other assays based upon detection of carbohydrate moieties in mucins this assay is vulnerable to changes in glycosylation patterns (Enss et al 1995). Using such assays, a drug with activity against glycosyltransferases could reduce mucin detection without altering mucin concentration. Characterization of the selectivity profile of this assay will continue within our laboratory. In the future, a wider availability of well characterised mucin specific antibodies and pure mucin preparations will help us to further characterize and validate our assays and may even permit generation of assays specific for individual mucin species.

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DISCUSSION

Fahy: Do you know for sure that you are not going to pick up submucosal gland secretions with this assay?

Jackson: No, we believe that we do pick up these secretions. We have used the lectin in sections of nasal tissue in which it does detect glandular material. In the intrapulmonary airway glandular content is so small that we think that its contribution to mucus in the BAL is likely to be insignificant.

Fahy: What is your standard? You referred a few times to a human mucus standard.

Jackson: The human mucus standard was derived from hyaluronidase, chrondroitinase ABC, heparinase and DNAse I digested sputum from a healthy ex-smoker. The standard was produced by pooling the void volume components of sepharose CL-4B column separations.

Fahy: What is the answer to the problem of not having an inter-institutional standard? Do you think the lectin-based assay will pick up the Sigma bovine gastric mucin?

Jackson: Using commercially available 'mucin' preparations was the easiest option when we first started working on mucus in 1997/1998. At that time we were unhappy with the quality of the mucin preparations available — they were either highly impure or very degraded. We were not comfortable using this material as a standard in assays or as a reference for the development or characterization of reagents. I don't know whether this has changed.

Rubin: We have published very similar data to yours on studies we have done using an assay based upon a lectin from *Dolichos biflorus* (DBA), to detect both goblet cell and submucosal gland secretions from ferrets. We have looked at its responsiveness to these various factors. We have used as standards both bovine submaxillary mucin and porcine gastric mucin. We initially had problems with Sigma mucin, but it has got better. We have also looked at semi-purified human mucin from endotracheal tubes, from patients who have been intubated. We are fairly satisfied: it picks up *N*-gal-Nac and seems to be specific by immunohistochemistry.

Jackson: DBA was included in our lectin panel. We found it to be very good but somewhat less selective than UEA-1. It detected only goblet cells in the surface epithelium. We were slightly concerned that it did not detect all goblet cells, in the small airways. In addition DBA also detected collagen, elastin and cartilage. However, our assay is specific for rat, and we feel strongly that an assay should be validated for each model that it is going to be used with.

Rubin: We are using ferret, and we also used UEA.

Nettesheim: Wouldn't it be better if our chemists could make a synthetic standard mucus, instead of us being dependent on various biological materials?

Carlstedt: I don't think it is possible to do this. If you have a lectin-based assay, it will detect certain carbohydrates. The number of carbohydrate epitopes of a certain kind could easily vary from one mucin to another, between species and even between individuals. The only way to get a *bona fide* quantitation is to have a standard with exactly the same properties as the molecule you wish to measure. So if you are using rat, you need a rat mucin standard.

Jackson: I agree that ideally the mucin standard should be derived from the same species, strain and even model that the assay is being used on. This is not always easy and in our case, ethical issues have driven us to use a human mucin standard in place of rat in this instance. The human mucin standard provides a reference which permits comparison of mucin levels between treatments but does not provide full quantitation.

Carlstedt: There is another worry. You must have a way to solubilize the secretions. You spin out the cells. Mucus is a gel. Could you spin out a little bit of mucus also? Furthermore, what I call 'insoluble' mucins could vary from 90% down to 5%, so you need to standardize on how solubilization is performed.

Jackson: In our assay we do not routinely reduce or solubilize the mucins in rat BAL. We have previously tried to do this using dithiothreitol (DTT) and guanidine hydrochloride in antibody- and lectin-based assays (although not UEA-1) to detect human bronchial epithelial cell mucins. Our experience at that time was that guanidine and DTT substantially reduced signal to noise ratios. Having said this, I agree with your comment that we may not be detecting all of the mucin in our samples; I'm just unclear about how to improve this. Perhaps bicarbonate could be useful since Sarah Inglis and others have suggested that impaired bicarbonate transport in submucosal glands leads to inefficient solvation and gland duct blockage (Inglis et al 1998).

Basbaum: Those of us who are not chemists may be throwing away the baby with the bathwater, and coming up with quite unreliable biochemical data. With regard to the issue raised by Paul Nettesheim about a synthetic standard, if one particular lectin is recognizing Gal-Nac, why can't you just give a Gal-Nac standard and ask how much you need to see a particular intensity of reaction?

Sheehan: Because the Gal-Nac in the mucins is mainly cryptic, and it isn't seen at all unless you skim off most of the peripheral sugars.

Nettesheim: Why then does the Gal-Nac-based assay work?

Jackson: UEA-1 detects α-L-fucose, not Gal-Nac. This is a terminal sugar as opposed to the internal *o*-glycosidic sugar Gal-Nac.

Basbaum: So the UEA detects fucose? So why don't we just use fucose, then?

Sheehan: Because not all mucins have fucose. For instance, you would never have a significant assay for MUC5AC in the airway. We have never found much fucose associated with this molecule there.

Jackson: One reason that using fucose as a standard is unlikely to be effective is that in a sandwich assay, which is required for complex biological samples, the capture lectin would bind its target epitope on the sugar, leaving it unavailable to the detection lectin.

Nettesheim: Why would the assay work at all? What you are telling me is that this lectin assay would not work.

Carlstedt: It does work. The problem is that we cannot transform 'reactivity' into amount of mucins in a way that is valid for all samples. This is because the number of binding sites for lectins could be very different from one mucin to another.

Basbaum: That's OK, because then you would just have a shift to the left or the right with the given system that you are using.

Sheehan: Another example. We find big changes if we are dealing with a cancerous situation where the MUC5AC would not be seen by the same reagents as it would against the normal. You just want things to shift around, but when you actually want to assay a specific kind of molecule it could disappear in your assay, even though there is plenty of it.

Basbaum: From a biologist's point of view (as opposed to a chemist's), I don't know whether or not we care about absolute quantities. We just want to see whether there is a net change (in volume, viscosity, solubility, etc.). If we put in an irritant or drug, do these parameters change? Do relative amounts of MUC5AC and MUC2 change?

Sheehan: If you want to use a carbohydrate assay and they are changing in response to the irritants, you will not measure anything at all that is interesting unless you have an assay telling you how much the specific mucin protein secretion has changed.

Basbaum: Do you mean that we want the polypeptide and not the carbohydrate?

Sheehan: That is what you should reference against.

Basbaum: That's a problem. How do you get access to the polypeptide?

Carlstedt: There's one way for getting a good reference point: electrospray mass spectrometry. This is quite quantitative. It is not possible to use MALDI approaches because of the difficulties with ionization, and also there could be competition in how ions are released from the matrix. But in the future we should be able to take a sample, digest it and use 'reporter peptides', for, for example, MUC5AC and MUC5B.

Sheehan: We have spent a long time on this. We get very good report of markers, especially for MUC5B and MUC5AC. But relative quantitation of electrospray is difficult because there are ion suppression effects. This depends on how complicated the mixture is from which you are trying to assay. Let me throw another few difficulties in. Sometimes the biggest problem with mucus is solubilizing it. This is a critical issue. If you just go into a lung with a washing,

I'm sure you get almost none of the mucins out. They don't solublize. The liquid you take back up from the lung has only the most mobile and soluble components in it. Then, when you come to a real mucus, what happens? The only way you can solubilize it is by using chaotropic reagents such as guanidine together with disulfide-reducing agents. What happens when you come to a complex mixture, rich in proteins, with those two reagents? You turn the whole lot into a hydrophobic spaghetti: suddenly every protein has a size/hydrophobic contribution. Thus the large amounts of previously globular proteins completely dominate the situation and bind all the possible surface sites, creating a massive suppression of your mucin signal.

Jackson: That is with direct assay: what happens if instead you capture with a specific reagent?

Sheehan: Then it becomes a different situation. But you can't capture with a specific reagent out of 6M guanidine or urea, the reagents we use to solubilize mucins. The 6M guanidine/urea reducing agent will kill any of your specific capture reagents immediately. These are very serious underlying problems: getting at the mucus, solubilizing it and getting it fixed down onto a resin or matrix.

Verdugo: What is the pore size of the network, and what is the molecular weight of the lectin? In other words, does the lectin penetrate the network?

Jackson: The molecular weight was originally thought to be 170 kDa, however UEA-1 forms aggregates and the correct molecular weight is now reported as 68 kDa.

Verdugo: If your spy probe is able to get into the network, you might be able to track whatever sites it is recognizing. But if it is excluded, you are really binding to the surface of a glob of mucins.

Rubin: We have shown with confocal microscopy that it does get into the network to some extent. We have been able to stain down into the network with the DBA lectin. We have also stained the DNA network separately with YoYo1. John Sheehan, your point is very interesting. We have avoided BAL fluid completely. We have taken ferret trachea and measured the weight of a series of segments and measured their weight before and after induced inflammation with lipopolysaccharide (LPS). We have taken the trachea, incubated it and looked at the supernatant. We have also taken the trachea and incubated it in a solution with neutrophil elastase, in an attempt to quantify what is attached by what we can wash off and then what we can push out of it afterwards. This lets us see relative differences, and we can use it to look at secretagogue potential and stored/secreted mucin after chronic inflammation.

Jackson: There is an alternative to ELLA or ELISA type assays which may overcome many of the problems associated with measurement of mucus secretion. Histological measurement of either goblet cell number or goblet cell

area before and after stimulation gives a direct measure of goblet cell degranulation. Although this probably overcomes many of the assay difficulties we have been discussing, it is much more limited as far as pharmacological manipulation is concerned.

Fahy: We did that in a human model, in which we looked at the volume of stored mucin before and after an allergy challenge. It went up, rather than down. By this, we inferred that there wasn't an effect of an acute antigen challenge on goblet cell degranulation in human asthma. This was consistent with some lavage findings we had, which showed that there was no change in secreted mucin.

Basbaum: Confounding this discussion are the diverse scenarios in which we are trying to measure mucins. The simplest model is cell culture. By analysing the supernatant, which is a relatively simple fluid, measurements are straightforward. Measurements and their interpretation are much more difficult in human airways.

Davis: We have to take a very practical approach to this problem, one that depends on the goal of the project. In our own work we deal with simple systems, cell cultures of one kind or another, and we want to quantify the mucins that are secreted. If we perfuse a cell culture and stimulate secretion, it takes about 10 min for the mucin to appear in the perfusate. By video microscopy, exocytotic secretion occurs very rapidly — in seconds and minutes — so we assume that this delay is due to the need for the secreted mucin, or some fraction of it, to solubilize into the perfusion stream. In the case of SPOC1 cells we use a soybean agglutinin ELLA for mucin detection. By Western blotting and in cells this lectin stains only mucin glycoconjugates of high molecular weight and secretory granules.

Basbaum: Do the biochemists see any problem with this?

Carlstedt: No. You are entitled to do whatever you want in your defined system, as long as it is validated. The point that John Sheehan and I were making is that you cannot find something that is 'universal'.

Davis: I also wanted to share some difficulties we have had removing mucus from cultures. We have done a lot of work with human bronchial epithelial cells grown in air–liquid interface cultures, including one experiment in which we were trying to quantify secreted mucins. We sampled every third day by adding 0.5 ml of PBS in the lumen, re-pipetting it over the surface three times and taking it off. We then repeated this twice with fresh PBS and pooled the samples. In a high frequency of cases after sampling we could still see sheets and blobs of mucus floating on the culture surface! This experience emphasizes the need for standardizing conditions as much as possible, but even then we may still be fooling ourselves.

Jackson: We also work on human bronchial epithelial cell cultures similar to those that Bill is referring to. In our hands it takes a single rinse followed by four one-hour washes with culture medium to remove accumulated mucus from the culture surface. This procedure removes a considerable amount of mucus from

the cultures and establishes a stable baseline from which agonist induced mucus secretion can be measured.

Nadel: The point about validation is well taken. Alan Jackson, you are looking at the number of goblet cells and the amount of secreted material. I suggest that instead you should study the volume of the material that degranulates, plotting the volume of decrease on one axis against what is measured in the secreted fluid. You are plotting the number of cells, which doesn't seem as meaningful in this system. It is the loss of material that is seen visually when the cells are stained against the material that is measured in the supernatant that is important.

Jackson: Your point relates to the regression analysis of the airway goblet cell numbers verses BAL mucin content from rats challenged with LPS that we have used as part of our assay validation process. The analysis you suggest would be appropriate in a model system in which a measurable change in the area of goblet cell granule staining occurred for example in response to an acute mucin secretagogue, e.g. ATP or neutrophil elastase. Unfortunately we have not been able to measure such differences in our model even in response to intratracheal instillations of known mucin secretogogues such as ATP or methacholine.

Nettesheim: We have just finished a series of experiments using stimuli that increase mucus production, showing that there are situations where the number of mucus-containing cells does not change even though the amount of mucus produced is greatly increased. I think these correlations you described may occur under certain conditions, but there is no compelling evidence that more mucous cells are needed in order to produce more mucus. A mucous cell can produce a lot of mucus or just a little bit of mucus. I am worried about attempts to make this kind of a correlation.

Jackson: The data I have shown comes from an experiment in which rats were challenged with LPS to generate an increase in the number of airway goblet cells and sacrificed 48 h later. BAL was undertaken then the lungs were removed from the same animals for histology. We related the BAL mucin content estimated using our UEA-1 ELLA to the intrapulmonary goblet cell score estimated from UEA-1 stained sections. No additional stimulus was given prior to the BAL procedure to induce mucus secretion. We reasoned that an increased number of goblet cells in the airways, whether stimulated or unstimulated, would result in an increase in the amount of mucus in the BAL since even in the unstimulated state goblet cells release some mucous granules. As long as all animals used for this analysis were treated in the same way (which they were) and the goblet cells did not completely empty (which they did not), then one would expect to find a relationship between BAL mucin content and the number of airway goblet cells. Under these circumstances I believe that the regression analysis I have presented is entirely appropriate.

Basbaum: From the standpoint of a pharmaceutical company, perhaps the mechanism by which mucus production is increased doesn't matter. It may be enough as a first approximation just to know that when a drug is applied it decreases the amount of mucin being produced, whether this means fewer cells making mucin or each cell making less mucin.

Jackson: I disagree. I think we need to understand the mechanisms underlying changes in mucus secretion in response to experimental drugs.

Harkema: We normally measure the volume density of mucus, rather than individual mucous cells in the airway epithelium. I agree with what Paul Nettesheim is saying: I think it is possible to get a system in which there is quick release of mucus. Then by histopathology and image analysis you can look at the decrease in the stored product. But you have to also look at the recovery of stored product after the hypersecretory event. The trick is to adequately measure the hypersecretion on the airway surface. This has been relatively difficult for us to do in rodent airways. What you want is a reproducible system where you can adequately correlate changes in intra-epithelially stored product with changes in the airway mucus.

Basbaum: I don't know whether you have to have the whole thing or just a fixed reproducible fraction.

Harkema: But what I am saying is that we need to validate the secretion. There are numerous cases where there is an increase in stored product but we can't see an increase in the secreted airway mucus. It is a dynamic system.

Reference

Inglis SK, Corboz MR, Ballard ST 1998 Effect of anion secretion inhibitors on mucin content of airway submucosal gland ducts. Am J Physiol 274:L762–L766

Regulation of mucin secretion from *in vitro* cellular models

C. William Davis

Department of Cell and Molecular Physiology, and Cystic Fibrosis/Pulmonary Research and Treatment Center, 6009 Thurston-Bowles, University of North Carolina, Chapel Hill, NC 27599-7248, USA

Abstract. Conceptually, *in vitro* models for airway mucin secretion may provide useful information pertinent to many aspects of goblet cell biology/physiology. Such models may be especially useful in identifying potential secretagogues, probing the distribution of receptors between goblet cell apical and basolateral membrane domains, and revealing intracellular messenger pathways underlying receptor activation. We have focused most recently on human bronchial epithelial cell cultures grown as tracheal xenografts and SPOC1 cell cultures. These two models are remarkably similar with respect to the regulation of mucin secretion: luminal challenges with the P_{2Y2} purinoceptor agonists ATP or UTP elicit mucin secretion with EC_{50}s of about 3 μM and archetypal agonists to other purinoceptors test negative. P_{2Y2} purinoceptors typically couple via G_q to phospholipase C, suggesting that intracellular Ca^{2+} and protein kinase C (PKC) are important in activating intracellular pathways leading to goblet cell mucin release. Consistent with this notion, phorbol myristate acetate and ionomycin elicit mucin secretion from SPOC1 cells and HBE xenografts, whereas cyclic nucleotides do not. Delineation of the molecules comprising these receptor/messenger interactions and their supporting pathways remains an important challenge for the development of drugs effective in therapeutic interventions in mucin hypersecretory airway diseases; with these models we have initiated the process.

2002 Mucus hypersecretion in respiratory disease. Wiley, Chichester (Novartis Foundation Symposium 248) p 113–131

Because mucus and its hyperproduction figure prominently in all of the obstructive pulmonary diseases (chronic bronchitis, emphysema, cystic fibrosis and asthma), there is a rich history of investigation into the mucin secreting cells of the lung and their regulation (for reviews, see Verdugo 1990, Davis 1997). Briefly, mucin is secreted by two principal epithelial secretory cells situated in the superficial epithelium and submucosal glands; commonly, but not exclusively, these are called goblet and mucous cells, respectively. Whether these two cell types represent similar or different secretory cells is presently unknown (see Davis & Randell 2001). However, from a regulatory point of view, they are very different.

Mucous cells in submucosal glands are stimulated to secrete by many agents, chiefly of neural origin, including acetylcholine, substance P and vasointestinal peptide, as well as inflammatory agents such as histamine, bradykinin and PG/LT4. Goblet cells in the superficial epithelium, however, appear to be refractory to these agents (see Davis & Randell 2001, Finkbeiner 1999, Fung & Rogers 1997). Indeed, purinergic agonists active at apical membrane P_{2Y2} receptors, ATP and UTP, are the only agents known to elicit mucin secretion consistently from goblet cells (see Davis 1997, Davis & Randell 2001). In this paper, the experimental models that have been used to study goblet cell mucin secretion are briefly reviewed, and we then focus on the purinergic cellular messenger pathways underlying the secretory event.

Mucin secretion models and assays

Historically, mucin secretion has been studied in a wide variety of experimental *in vitro* models. The first employed isolated tracheas or large bronchi either in short-term experiments conducted with fresh tissue, or with explants of these tissues maintained for short periods of time in organ culture. Such studies supplied a rich amount of data (e.g. Florey et al 1932), but they were compromised by the fact that the preparations contained both the superficial epithelium and submucosal glands characteristic of intact, cartilaginous airways from large mammals — hence the mucins detected may have emanated either from both goblet or mucous cells. To avoid this problem, more recent efforts have been directed toward studies employing experimental models comprised selectively of one of the two mucin-secreting cell types. The efforts of our laboratory (see Davis & Abdullah 1997) have focused on mucin secretion from explants of superficial epithelium from canine trachea (Davis et al 1992) and human turbinates (Lethem et al 1993), cultures of SPOC1 cells (Doherty et al 1995, Abdullah et al 1996), and more recently, SPOC1 cells and human bronchial epithelial (HBE) cells grown in tracheal xenografts hosted by nude mice (Conway et al 2002).

We have also employed a variety of mucin secretion assays, attempting in each case to circumvent many of the technical problems associated with detection of mucin. Initially, with explants of superficial epithelium, we used video microscopy under differential interference contrast illumination to visualize mucin granule exocytosis directly. This assay avoids many of the uncertainties with specificity that plague biochemical mucin detection techniques; however, both the isolation and culture of the superficial epithelium, and the microscopy itself, are very labour intensive, which limits the number of experiments that can be completed in a given period of time. Hence, most of our work has employed binding assays based on the use enzyme-linked lectins (SBA-ELLA; Abdullah

et al 1996) or antibodies (the monoclonal antibody, H6C5-ELISA; Conway et al 2002). Additionally, we have recently developed a microtiter plate assay using a variant of periodic acid–Schiff (PAS) staining, namely periodic acid-biotin-hydrazide (PABH; Conway et al 2002). All of these assays, including the H6C5 ELISA suffer problems of specificity, namely, they do not detect specific mucins, they may detect materials other than mucin, and/or they may not detect all mucins. Nonetheless, with careful validation the assays can yield valuable data. Where possible, we attempt to reduce some assay uncertainties by implementing perfusion into the experimental procedure which minimizes the contribution of non-mucinous, extracellular materials to the signal and adds the element of time to aid analysis of the mucin secretion data.

Constitutive versus regulated, basal versus stimulated secretion

The gel-forming mucins secreted by goblet and mucous cells have monomer molecular weights exceeding 5×10^6 Da, about 90% of which is carbohydrate. This size and complexity has hindered greatly not only the study of mucin gene and glycoprotein structures, but also our understanding of mucin biosynthesis. Goblet and mucous cells have many structural features in common with many other secretory cells, however, about which much more is known regarding the secretory pathway. Until our specific knowledge of goblet cells increases substantially we must rely on the information from these other secretory cells to guide our understanding of the synthesis and secretion of mucin.

In endocrine and other exocrine cells, the secretory pathway supplies vesicles to two, independent exocytotic pathways (see Arvan & Castle 1998, Burgess & Kelly 1987, and Fig. 1). Along the *constitutive secretory pathway* vesicles budding from the *trans*-Golgi network (TGN) are directed immediately to the plasma membrane where they exocytose their contents to the cell exterior. This pathway is also responsible for the delivery of many integral or tethered membrane proteins to the plasma membrane. In contrast, vesicles budded from the TGN along the *regulated secretory pathway* are directed into an intermediate storage compartment, in which the vesicles and their contents mature into relatively large secretory granules. Granules are released from this pool to exocytose their products only when the cell is appropriately stimulated by extracellular agonists or other factors carrying signals from the nervous, endocrine or local regulatory systems.

From a cell biological consideration of the secretory pathway then, the term, *constitutive* potentially conflicts with its common use in physiology and pharmacology where it generally refers to a basal rate. Consequently, *constitutive secretion,* may mean, 'the release of secretions via the constitutive limb of the secretory pathway', or it may mean, 'the basal rate of secretion', with the actual pathway

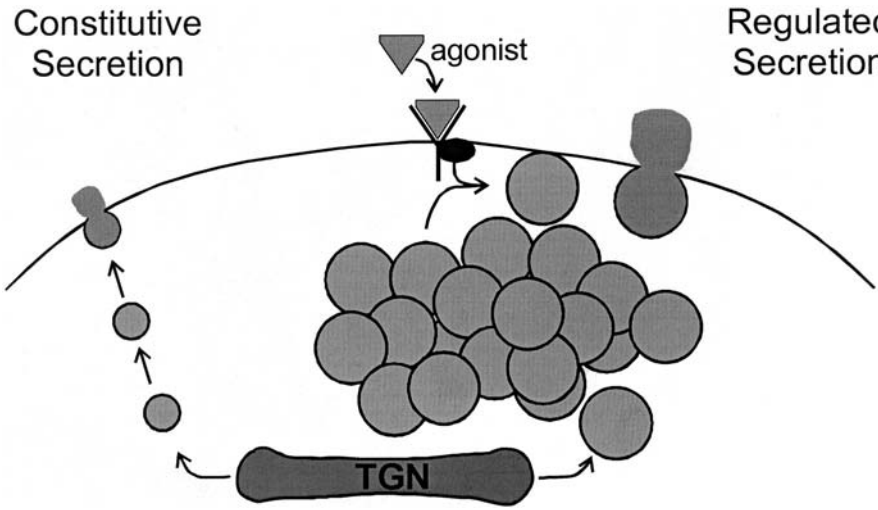


FIG. 1. Regulated and constitutive secretory pathways. The constitutive secretory pathway is virtually ubiquitous in eukaryotic cells and is responsible for the unregulated secretion of proteins exiting the *trans*-Golgi network (TGN) and for the insertion of integral proteins into the plasma membrane. The rate of secretion by the constitutive pathway is regulated solely by the rate of protein synthesis and its delivery to the TGN. The regulated secretory pathway is found in cells specialized for protein secretion. Its granules are directed from the TGN into a pool of storage granules, from which the material is released upon receipt of an appropriate external signal.

of basal release, constitutive or regulated, unstated or unknown. To confound the problem, the mechanism of *constitutive secretion* is a highly regulated event (Miller & Moore 1991). For clarity, *constitutive secretion* will refer to the mechanism of exocytotic release, *basal secretion* will refer to the baseline rate of release of mucin, and *regulated secretion* will refer to agonist-induced secretion from the regulated secretory pathway.

In experiments using video microscopy to monitor mucin granule (× 0.5–1.5 μm diameter) exocytosis at the apical membranes of individual goblet cells from canine trachea and human turbinates, we observed very low rates of baseline release of about three exocytotic events per hour (Davis et al 1992, Lethem et al 1993). By virtue of their large size, alone, the mucin granules exocytosed represent the regulated secretory pathway; hence, the mucins released by this route represent a basal level of secretion. The mucin granules are released either as a result of baseline exocytotic activity of the regulated pathway, and/or the pathway is responding to a baseline level of local agonist secretion from the tissue, e.g. ATP or UTP secretion that triggers secretion via P_{2Y2} receptors. Additionally, it is possible that mucins released from the constitutive secretory

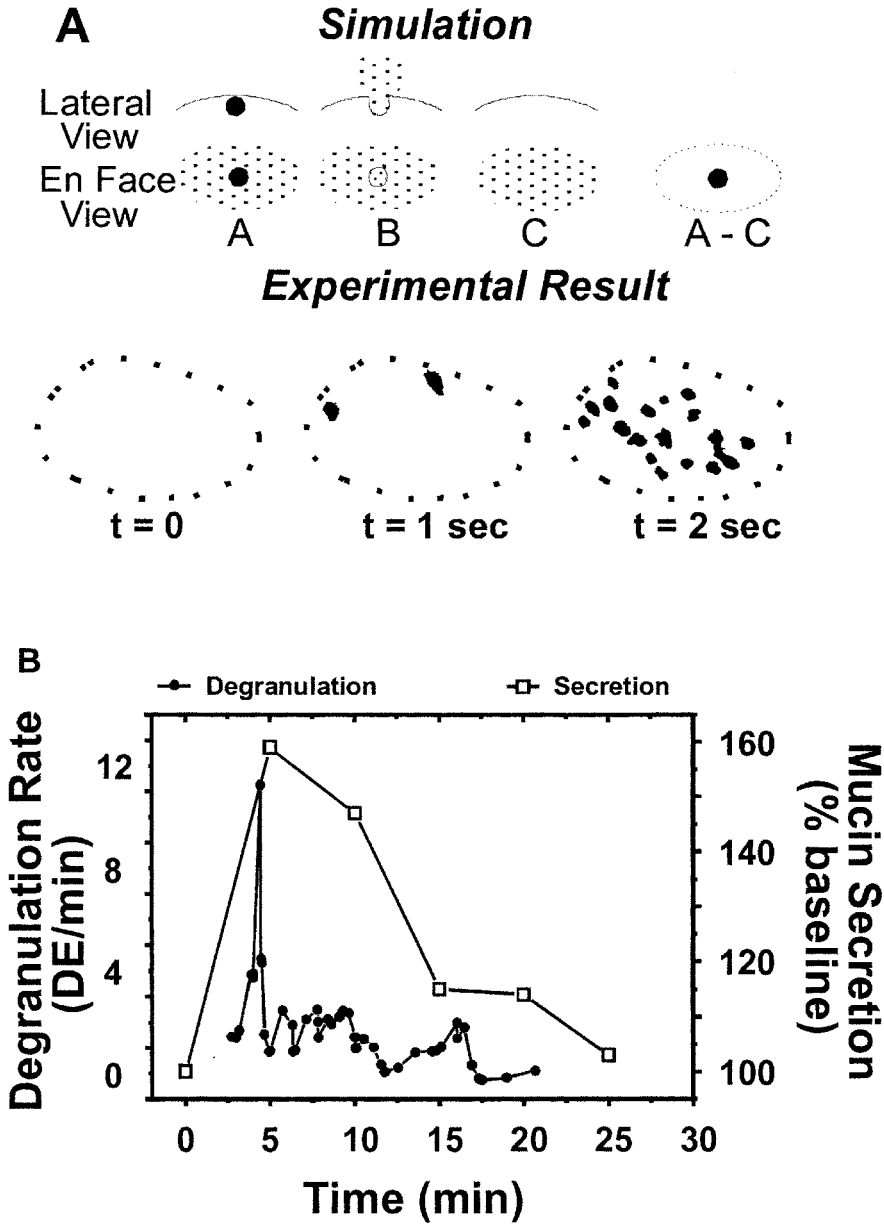
pathway contribute to the basal level of secretion. These mucins could be secreted via the constitutive pathway, or from immature granules which are exocytosed via the constitutive, or constitutive-like pathway before the granule membranes are remodelled to reflect mature mucin granules (Eaton et al 2000). Further work is obviously needed to clarify these pathways and their regulation in mucin-secreting cells.

Regulated mucin secretion

As noted above, in the absence of agonist, goblet cells in both canine trachea and human turbinates are fairly quiescent. When exposed to the P_{2Y2} agonists ATP or UTP, however, the cells respond in a nearly explosively manner. Figure 2A depicts the video microscopy degranulation assay strategy, and shows the results from an individual human goblet cell in the first two seconds of secretion. The rate of degranulation typically rises from baseline levels of 0.05 degranulation events (DE)/min to peaks of *tens* of DE/min occurring in the first couple of minutes (see Davis et al 1992, Lethem et al 1993). Following this burst of exocytotic activity, as seen in the result depicted in Fig. 2A, the rate then generally declines sharply, possibly due to a receptor desensitization phenomenon and/or depletion of a readily-releasable mucin granule pool. Mucin secretion, as assessed by detection of mucins collected from a perfusate, tracks the kinetics of exocytosis reasonably well (Fig. 2B). The delay between the peaks in exocytotic activity and apparent secretion can be attributed to the need for the mucins to hydrate, anneal and solubilize into the solution phase following release from the cell (see Verdugo 1990). Also favouring a close relationship between mucin granule exocytosis and mucin secretion are comparative experiments showing that the ATP concentration–effect relationships for degranulation and secretion are very similar, in terms of both EC_{50} and saturation dose (Davis & Abdullah 1997).

Purinergic regulation of mucin secretion

In goblet cell experimental models from several species, mucin secretion has been shown to be under powerful control by purinergic agonists acting through the P_{2Y2} receptor (Davis & Abdullah 1997, Kim et al 1997), and as noted above ATP and UTP are the only agonists which consistently activate these cells. Goblet cells appear to be unresponsive to agonists which typically activate mucin secretion from submucosal glands, including those which interact with adenylate cyclase-activating receptors (Davis & Randell 2001, Finkbeiner 1999, Fung & Rogers 1997). In recent experiments, we have tested the effects of airway secretagogues on mucin secretion from human bronchial epithelial cell cultures grown



in tracheal xenografts in the back of nude mice. Denuded tracheas from young chickens served as the surrogate support, the lumens of which were seeded with passage 2 HBE cells. Over a period of three weeks, the HBE cells differentiated into a pseudostratified epithelium with a full mucociliary phenotype. The xenografts implanted were pre-cannulated at each end, which allowed them to be mounted immediately for perfusion following their removal from the host. Following a two hour equilibration, the HBE xenografts responded robustly to P_{2Y2} receptor agonists with about a threefold increase in mucin secretion (Conway et al 2002). In this preparation, only nucleotide di- and triphosphate agonists stimulated mucin release. Other secretagogues tested, including permeant cyclic nucleotide analogues had no effect (Fig. 3). Hence, like goblet cells from other species, human airway goblet cells respond robustly to P_{2Y2} purinoceptors (Chen et al 2001, Lethem et al 1993), but poorly or not at all to other secretagogues characteristically present in the airways.

P_{2Y2} purinoceptors couple via G_q to phospholipase C, which generates inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol as cellular messengers. Consistent with expectations, treatment of goblet cells with agents which mobilize intracellular Ca^{2+} or which activate PKC also stimulate mucin secretion: ionomycin and phorbol myristate acetate (PMA) cause a stimulation of mucin secretion to levels similar to, or exceeding, those achieved with agonist, both from SPOC1 cells (Abdullah et al 1997) and from HBE xenografts (Fig. 4). In SPOC1 cells, we have recently shown nPKCδ, a Ca^{2+}-independent PKC isoform to be activated selectively upon agonist stimulation (Abdullah et al 2002). Hence, in goblet cells

FIG. 2. Goblet cell mucin granule exocytosis. (A) Visualization of exocytosis. *Top panel, Simulation*: lateral and en face views (A–C) depict the exocytosis of a secretory granule across the plasma membrane of a cell. Note that in the en face view, degranulation is observed as the disappearance of the granule. Hence, subtraction of Image C from Image A yields a resultant image of the granule as it appeared just before it was exocytosed (A–C). This technique was used to visualize the loss of granules from a human turbinate goblet cell during agonist (ATP)-stimulated secretion, as shown in the *Lower Panel, Experimental Result*. The dotted, circular line represents the cell margin. The ~ 0.8 μm 'blobs' which appear at $t = 1$ and 2 s represent mucin granules that were exocytosed in the respective interval (redrawn, with contrast reversal, from raw images in Lethem et al 1993). (B) Relationship between exocytosis (degranulation) and mucin secretion from human goblet cells. An individual goblet cell resident in an epithelial explant was monitored continuously by video microscopy (see Davis et al 1992), and the luminal effluent perfusate from the chamber was collected simultaneously at 5 min intervals, beginning at $t = -5$ min. The delay between secretagogue introduction at $t = 0$ and the first degranulation event (DE) represents the dead volume of the perfusion system, plus the approximately 35 s necessary for goblet cells to begin secreting. Degranulation events results are expressed as a rate (DE/min) to correspond to mucin secretion. Mucins in the perfusate fractions were assessed by an ELISA using the monoclonal antibody, 17Q2, and are expressed relative to those collected in the baseline period. (Lethem MI & Davis CW, unpublished results.)

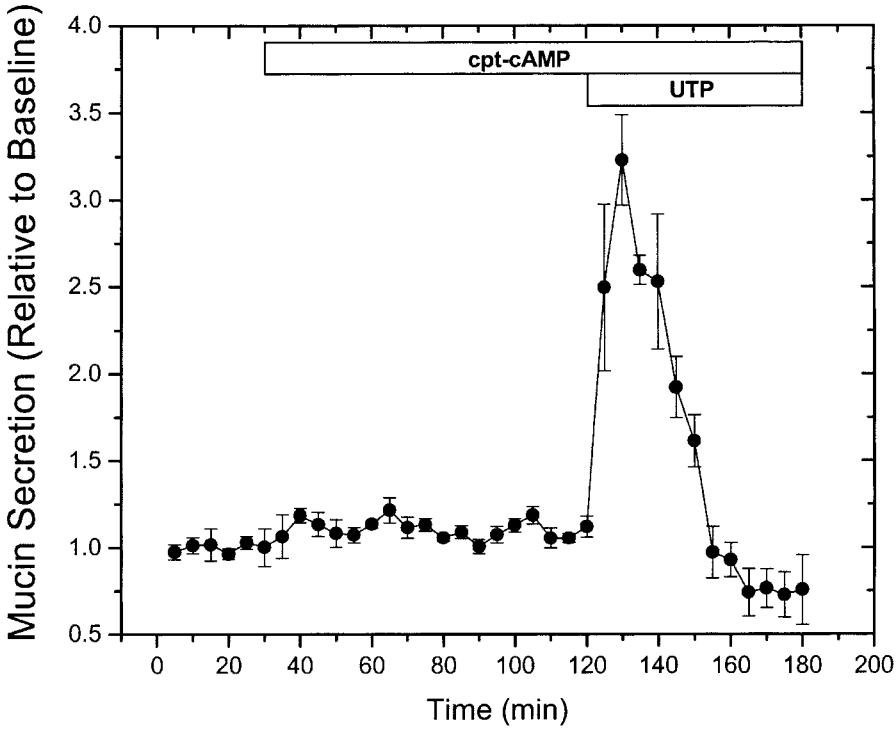


FIG. 3. Effects of a permeable cAMP analog (cpt-cAMP, 1 mM) and UTP (100 μM) on mucin secretion from human bronchial cell xenografts. Following removal from the host, HBE xenografts were perfused for an initial 2 h equilibration. The perfusate was subsequently collected in 5 min fractions for a 30 min baseline period, and then during exposures to the cyclic nucleotide, and cyclic nucleotide+ UTP. Mucins were detected in the fractions using an H6C5-based ELISA. The data are presented as mean ± SE (n= 3), and are expressed relative to baseline. (Redrawn from Conway et al 2002.)

the signal transduction pathway mediating mucin release in response to P_{2Y2} receptor activation appears to be phospholipase C (see also, Kim et al 1997).

PMA effects on mucin secretion, revisited

A curious feature of our previous results with PMA effects in SPOC1 cells was a rightward shift in its concentration–effect curve (Abdullah et al 1997): the EC_{50} was 75 nM and the effect saturated at 300 nM, a 10-fold higher level than is generally required to maximally stimulate PKC. In studies recently completed, we investigated the quantitative relationships between PKC translocation and mucin release from SPOC1 cells stimulated with purinergic agonist and with

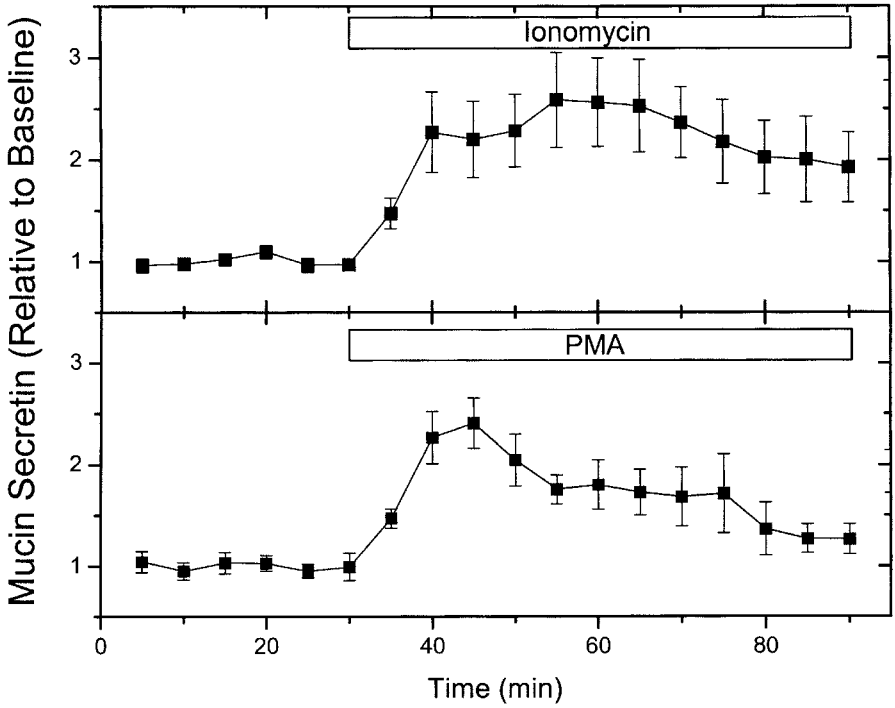


FIG. 4. Stimulation of mucin secretion from HBE xenografts by ionomycin (3 μM) and PMA (300 nM). Xenografts were treated as described in Fig. 3 (n= 3 each; redrawn from Conway et al 2002).

PMA (Abdullah et al 2002). With agonist stimulation, nPKCδ translocation into the membrane fraction saturated at the same agonist concentration, 100 μM, as did mucin secretion, and the EC_{50}s for each measurement were similar (2–3 μM). With PMA stimulation, however, the effects on nPKCδ (and cPKCα) saturated at 30 nM, whereas mucin secretion saturated at PMA concentrations above 300 nM. Hence, there appear to be PKC-independent effects of PMA to stimulate mucin granule exocytosis which are revealed at concentrations well above those necessary to maximally activate PKC.

In recent years, work in many laboratories has indicated the presence of other phorbol ester receptors in cells, principally proteins possessing C1 domains, the diacylglycerol-binding domain originally described for PKC (see Ron & Kazanietz 1999). The non-PKC, PMA-responsive protein most likely situated in a position to stimulate mucin secretion is MUNC13, a obligate, exocytosis accessory protein thought to be essential for secretory granule docking and/or priming (Brose et al 1995). We therefore probed SPOC1 cells for mRNA expression of both MUNC13

and DOC2, the granule membrane MUNC13 binding partner (Duncan et al 2000). Expressed, were ubMUNC13-2, the ubiquitous splice variant of MUNC13-2, and DOC2γ. MUNC13-4 was also found in SPOC1 cells, but since this isoform lacks a C1 domain (Koch et al 2000), it is unlikely to participate in regulated mucin secretion.

A working model for the regulation of mucin granule exocytosis

Figure 5 presents a model for the regulated exocytosis of a mucin granule. The series of events begins with intracellular messenger generation following receptor activation, and it ends with an exocytotic event. The model was compiled from the current body of knowledge available for airway goblet cells (Davis 1997) and other secretory cells (see Trifaro et al 2000, Martin 2002). Cortical microfilaments, which are located in the apical pole of epithelial cells, have long been known to participate in the regulation of exocytosis. Lying between the plasma membrane and secretory granules, they either function simply as a physical barrier to the approach of granules to their plasma membrane docking sites, and/or they serve as scaffolds for other molecules which serve to regulate granule docking. In either case, disruption of cortical microfilaments is a key step in regulated exocytosis (Muallem et al 1995). Two proteins are postulated to participate in microfilament disruption, MARCKS and scinderin. MARCKS, the myristolated, alanine-rich C kinase substrate, inserts into the plasma

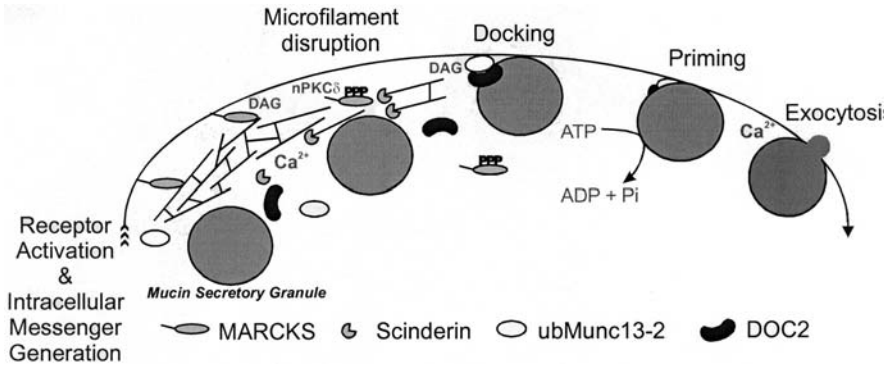


FIG. 5. A model for the regulated mucin granule exocytosis pathway in airway goblet cells. Shown are the intracellular messengers generated by P_{2Y2} purinoceptor activation, diacylglycerol (DAG) and Ca^{2+}, along with their downstream effector molecules thought to be active in triggering regulated exocytosis. These molecules include actin microfilaments, unlabelled and depicted as linear, linked filaments. Not shown for clarity are the SNAP, SNARE, and other accessory proteins comprising the core of the exocytotic complex.

membrane in its unphosphorylated state. The phosphorylation of MARCKS by PKC results in the disruption of cortical microfilaments, by one of two possible means[1]. First, it may anchor microfilaments into the plasma membrane directly (Hartwig et al 1992), in which case its phosphorylation would destroy microfilament anchorage sites (Trifaro et al 2000). Alternatively, MARCKS may function to sequester phosphatidylinositol-4,5-bisphosphate (PIP_2); when released following MARCKS phosphorylation, PIP_2 would then trigger disruption of the cytoskeleton (Arbuzova et al 2002). The other protein involved in microfilament disruption, scinderin, is a Ca^{2+}-activated enzyme related to gelsolin that severs and caps microfilaments in the period preceding secretory granule docking (Trifaro et al 2000). Following disruption of the cortical cytoskeleton, mucin secretory granules dock on the plasma membrane and are primed, a function in which MUNC13 appears to play a critical role (Martin 2002). The final steps of exocytosis, fusion of the granule and plasma membranes through the formation of the SNARE-mediated fusion pore (Gerst 1999), now require nothing more than a Ca^{2+} trigger (Martin 2002, Sugita et al 2002).

While the role of Ca^{2+} in activating exocytosis has long been appreciated, more controversial is its source. The endoplasmic reticulum is the more conventional store, from which Ca^{2+} is liberated by $InsP_3$ following receptor activation. In secretory cells, however, granules have also been implicated as an intracellular Ca^{2+} store (Petersen 1996, Yoo 2000). Perhaps the best evidence for $InsP_3$-releasable Ca^{2+} from secretory granules comes from goblet cells: independent Ca^{2+}-sensitive fluorescent dyes loaded into granules and cytosol indicated a simultaneous loss of Ca^{2+} from granules as it increased in the adjacent cytosol (Nguyen et al 1998).

In conclusion, our understanding of regulated mucin secretion has increased substantially over the past 10–15 years. We have, however, just reached the point where our model systems are sufficiently robust as to inject an element of rigour that has been generally lacking in the field. It will be in the next 10–15 years that we will see our knowledge of goblet cell mucin secretion advance to the point where it is on par with that presently available for other secretory cell systems. Hence, these are exciting times for the field; there is great hope that some of our future excitement will come from the development of successful drug intervention strategies targeting mucus hypersecretion in obstructive lung disease.

[1]A recent paper proposed a different/additional role for MARCKS in regulated mucin secretion: once liberated to the cytoplasm, MARCKS was suggested to bind to mucin granules, to thereby allow microfilaments to attach and guide them to the plasma membrane (Li et al 2001). While this hypothesis is intriguing, it differs sufficiently from observations on many other secretory cell models and requires testing in other systems.

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DISCUSSION

Jackson: In the SPOC1 cells, how do you know it is P_{2Y2} and not P_{2Y4}? As you know, ATP and UTP are equipotent at both receptors in rat, although not in human.

Davis: We can't exclude the P_{2Y4} receptor at this point. We have identified the P_{2Y2} receptor in SPOC1 cells by PCR, but we haven't checked for the P_{2Y4} receptor.

Nadel: What is the mechanism for degranulation by neutrophil elastase? Is it a proteolytic effect? It is a potent effect, and neutrophils are believed to be a potent source of degranulation. The degranulation is due to a serine-active site.

Davis: I was expecting you to ask that question! It would be nice if there was a thrombin-like receptor.

Nadel: It doesn't seem to act like that. One possibility is that elastase is cleaving some protein signal on the surface (e.g. a receptor). This could be activating a process that communicates intracellularly and doesn't appear to be Ca^{2+} dependent. For example, molecules such as pro-tumour necrosis factor (TNF)α, pro-transforming growth factor (TGF)α and even epidermal growth factor (EGF) receptors themselves, which are on these epithelial cells, might activate a process inside the cell when they are cleaved.

Davis: One possibility that we are working on would involve PKCδ. Of all the PKC isoforms, it is the only one that can be activated by tyrosine phosphorylation. One of the questions that I always ask is that when metaplastic conditions are induced in the airway, resulting in a lot of new goblet cells, what is it that causes them to secrete? Perhaps the tyrosine phosphorylation networks in these cells that metaplastically transform them can also cause them to secrete by activating the secretory machinery. If this is the case, PKCδ could be a player.

Basbaum: What do you believe is the relevant P_2 receptor agonist in the airway?

Davis: ATP, coming from the epithelium.

Basbaum: Under what conditions?

Davis: We can't define that too well. Others in our group have shown that nucleotides are secreted by almost all cells, and especially airway epithelium across both apical and basolateral membranes.

Basbaum: Is this controlled?

Davis: ATP secretion is easily stimulated by mechanical perturbation.

Verdugo: The granule is loaded with ATP.

Basbaum: So you would see a feedforward type of process.

Davis: That would be another independent process. You can take cultures that don't have any goblet cells in them and still see ATP release when they are mechanically disturbed. Each time you change the culture medium in the culture dishes or bump the plate, you are stimulating ATP secretion that lasts for 30 min or so.

Basbaum: What about *in vivo* where all the surfaces are buffeted by all sorts of things, including mucus itself? Should we envision epithelial cells secreting ATP all the time? I suppose this could contribute to 'baseline' secretion.

Vargaftig: If ATP is so important, can you block secondary secretions with apyrase?

Davis: Theoretically, yes. We haven't tried this manoeuvre, however, when studying mucin secretion.

Vargaftig: Under laboratory conditions, if you keep clean mice and immunize them with ovalbumin and then challenge them with ovalbumin, this increases the number of goblet cells markedly. There is absolutely no secretion though. Then if you challenge secondarily with just about anything, for instance another antigen or a cholinergic stimulus, there is a large release of mucus.

Davis: Duncan Rogers has done a lot of work in this area. From his work, I believe the different parts of the epithelium communicate. You say that acetylcholine causes goblet cell secretion in a mouse model where submucosal glands are nonexistent. We have never been able to get a goblet cell in culture to respond to a cholinergic challenge.

Vargaftig: That is incredible. We had an occasional experience where, in order to save time and money, we were provoking the animals with methacholine, keeping the animals for three days and then doing the goblet cell analysis by microscopy. At that time, people in the lab said that they no longer found any goblet cells. It turned out that methacholine is so potent in those mice that it completely emptied the goblet cells. After three days when we looked it appeared that there was no goblet cell production, but this was because everything had been secreted.

Basbaum: There's an old literature about pilocarpine and a debate regarding whether or not cholinergic receptors exist on goblet cells.

Rogers: Bill Davis mentioned our work on this (Kou et al 1992, Tokuyama et al 1990). We looked at guinea-pig trachea because it contains 'robust' goblet cells as opposed to the rat trachea, which has more delicate goblet cells. Guinea-pigs give a good window of opportunity for studying goblet cell discharge. We stimulated the vagus nerve in anaesthetized guinea-pigs *in vivo* and demonstrated a discharge of goblet cells by histology. In this specific circumstance we observed goblet cell discharge in response to cholinergic nerve stimulation. There are nerves in the epithelium which you can show by electron microscopy.

Davis: In human airways, isn't the superficial epithelium sparsely innervated?

Jeffery: It is highly variable between species. It is well innervated in the rat, and not at all in the large airways of the mouse. It is sparsely innervated in the guinea-pig.

Davis: SPOC1 cells come from rat. We can throw cholinergic agonists at them all day and they will just sit there.

Rogers: Do you think that you have lost receptors in the culturing process?

Davis: That is always a possibility.

Fahy: One of the issues about degranulation *in vivo* in humans is that there must be a mechanism in place that normally prevents degranulation, because we showed that patients with asthma challenged with airway allergen have airway inflammation without associated goblet cell degranulation.

Basbaum: Could you detect any mucin in their airways after the allergen challenge?

Fahy: That's a difficult measurement, as we have already discussed, but ELISA-based measures of mucin in lavage did not change with allergen challenge. More convincing than this, though, we couldn't show a decrease in the volume fraction of stored mucin in tissue sections of airway mucosal biopsies.

Basbaum: I think that it is more subtle than this. You could well have goblet cells that are secreting a lot and also resynthesizing.

Fahy: We have three separate lines of evidence suggesting that they didn't secrete very much. One is that that in these patients the β agonist normalized their lung function. Second, we didn't find any increase in mucin signal in the lavage. Third, we didn't find a reduction in the volume fraction of stored mucus; it was more towards an increase. I conclude that allergen challenge in asthmatics usually doesn't cause goblet cell degranulation. When asthmatics have acute exacerbations or fatal events, there must be a combination of events in the airway resulting in smooth muscle contraction and goblet cell degranulation. We don't understand these events.

Jeffery: I want to make a comment about where in the lung, anatomically, the goblet cells are. This is an important issue in terms of whether or not there is a fatal event. In those cases where there is a fatal event, there is mucous metaplasia and small airways are involved. In this situation there is stimulation of mucus release and concordant smooth muscle contraction. But I consider that many asthmatics do not have mucous metaplasia of their small airways, particularly early on in their history. Therefore I would suggest they are less at risk during airway contraction than those that have a metaplastic change in the small airway. It is not just the number of goblet cells that is important, but also where they appear and increase anatomically.

Verdugo: We don't find an effect of acetylcholine in cultures either. But this doesn't imply that *in vivo* the situation couldn't be different. For those who are developing assays to screen different drugs, to what extent will an *in vitro* preparation work? The expression of receptors varies enormously in cell lines, and in this regard it is important to establish the validity of our preparations, particularly for screening assays. However, we are asking questions about the transmission of message beyond the receptor inside a cell. It is important to focus on this issue.

Barnes: Muscarinic M_3 receptors disappear in tissue culture as the gene switches off (for unknown reasons). M_2 receptors persist, however. This may change the pharmacological response of cultured cells to acetylcholine. The other issue is that epithelial cells (including human) synthesize acetylcholine and inflammatory stimuli such as TNF increase expression of choline acetyltransferase, and therefore induce acteylcholine production. This may be one reason why anticholinergic drugs may be more effective than β agonists as treatments for chronic obstructive pulmonary disease (COPD).

Basbaum: It seems that the weight of the evidence suggests there is an M_3 receptor that stimulates goblet cell secretion *in vivo*, at least in some species.

Davis: Have they ever been shown to be expressed on goblet cells in the superficial epithelium?

Barnes: We found expression of M_3 receptors in epithelium of human airways, and this appears to include goblet cells. By contrast, we did not see expression of β receptors on goblet cells.

Basbaum: Bill Davis, is your information consistent with the presence of muscarinic receptors driving the whole thing? Is there anything inconsistent?

Davis: I have no evidence that there is any effect of muscarinic agonists, but if the receptors aren't there then I wouldn't see it.

Verdugo: You have to remember also that ATP is a common currency in the airway. The possibility exists that acetylcholine-like drugs could be acting indirectly via ATP release from some other source.

Davis: ATP is an extremely important agonist in the airway. One endocrinology text talks about a 'lumone': a substance like a hormone that is secreted into the lumen and acts downstream. ATP is a lumone in the airway, and it may coordinate mucociliary clearance in terms of fluid secretion, mucin secretion and ciliary beating. This is not to say, however, that acetylcholine doesn't work from a basolateral site.

Basbaum: Our own data support what you are saying with respect to the importance of ATP as a lumone. We find that viruses cause epithelial cells to release ATP. We also find that bacteria cause epithelial cells to release ATP, and as bacteria die (as a result of host defence or antibiotics) there is significant release of ATP. Levels are inevitably raised in the lumen of the airway in pathological conditions, in addition to the baseline levels. Our interest is in the fact that these nucleotides definitely contribute to mucin synthesis. You are saying that they definitely stimulate mucin exocytosis, and we find that they also stimulate mucin synthesis.

Verdugo: This opens up another issue in a sense that there is a window of pathology: if this message floats around all the time, you could have an epithelium that is overstimulated all the time. There should be some mechanism present for dephosphorylating ATP. Failure of the ATP ecto-metabolism system could have pathological consequences.

Davis: Maryse Picher in our group has done degradation experiments with UTP. If we extrapolate her results down to a 30 μm deep film on the airway, and introduce a saturating dose of UTP, it is degraded beyond detection in just 30 seconds. This is a very active degradation system!

Nadel: Cholinergic mechanisms *in vivo* are potent sources of secretion in the glands. They are very variable in the surface epithelium, depending on the species. In discussing mechanisms of hypersecretion, glands are likely to be potent sources of cholinergic secretion, unlike sources of secretion in the airway epithelium.

Sheehan: Thinking back to the previous discussion and how it meshes with this one, I'd like to relate a couple of our observations. Some years ago we worked with lipid toxin substances coming from *Pseudomonas*, putting them onto cat airway. What struck me then was the extent of release of PAS-staining material. It turned

out to be mucin-like, but we think it was membrane-bound mucins that were being released. We were initially looking for fresh synthesis, but we concluded that 'secreted' mucins were probably already there and were stimulated out of the membrane by the insult we had applied. Another point: we mucinologists are typically biased to believe that substances detected by PAS-like reagents are secreted from goblet cells. Histology is very good at showing us goblet cell granules, but a lot of other cell types can be very busy secreting mucins constitutively and these mucins or mucin-like substances will not necessarily be readily identifiable histologically using PAS.

Basbaum: Proportionally, how abundant are the membrane-bound mucins with respect to the secreted mucins?

Sheehan: We don't know. We just picked them up in a PAS-related assay and showed that they were these kinds of mucins. But the antibodies against MUC4 and MUC1 are not very good in these kinds of assays. We can't rely on them to show us anything quantitative. Also, there are many other membrane-bound mucins that have not yet been characterized. The contribution that all these substances are making is currently undefined. This would not be picked up by relationship to all this talk about goblet cells and secretion. Bill Davis could be tuning into a whole set of other mucins that are not yet defined in terms of gene products and how they are being released in this system.

Basbaum: Is the release of the cell surface mucins under any form of control?

Carlstedt: Not that we know of. There is so little known about their role.

Jackson: One of the validation steps that Mike Lerhem and Bill Davis have used for one of their lectin based assays is to quantify mucin granule exocytosis using video microscopy and compare that with detection of mucus by a lectin-based assay. The results were virtually identical, suggesting that at least some of these assays are detecting exocytosis rather than surface-bound mucins.

Nettesheim: I hear a lot about exocytosis, but what I don't hear enough about are two other issues that I think are equally (or more) important. One has to do with synthesis. I don't care about exocytosis, because if you don't synthesize more mucus in response to a stimulus, the exocytosis event is going to be over quickly and everything is done with. We are underemphasizing the role of regulation of synthesis. I haven't heard a word about the coupling of exocytosis and synthesis. I would propose as a hypothesis that if a cell exocytoses its material, a feedback mechanism will compel the empty cell to synthesize more mucin. If the cell is full of mucus it doesn't synthesize much mucus. I believe we need to focus more than we have on synthesis and the potential for coupling between exocytosis and synthesis.

Nadel: When a cell degranulates, how does it know how to re-granulate? In your view, when cells degranulate, do they regranulate?

Nettesheim: I have no idea.

Nadel: I have a proposal. When cells in the airway epithelium degranulate, perhaps they signal interleukin (IL)8 production. If IL8 production is signalled by degranulation of epithelial cells, this could be a sufficient signal for regranulation.

Vargaftig: The best way to test this interesting idea experimentally is to immunize an animal with two proteins, such as ovalbumin and BSA. Then you provoke the animal with one and cause discharge with the other. The reason for using two is that if you use the same antigen you can always argue for desensitization. In the middle of this, if it works, you can apply inhibitors such as IL8.

Nettesheim: I would propose that one way to look at this is to test whether exocytotic agents increase transcription or translation of mucins.

Basbaum: I think you should do it. This issue comes up every 20 years and no one ever feels like doing the experiments!

Tesfaigzi: Bill Davis posed the question as to whether it is worth inhibiting the secretion. What would be a good way to inhibit secretion?

Davis: There are no blockers for these processes that we know of. The only thing I would suggest is using a lot of apyrase, which breaks down ATP and ADP.

Basbaum: Presumably this doesn't get inside the cells.

Davis: No, it doesn't.

Vargaftig: We did that *in vivo*, and it is devoid of toxicity. You can destroy ATP and have compatible results.

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Oscillations of pH inside the secretory granule control the gain of Ca^{2+} release for signal transduction in goblet cell exocytosis

Wei-chun Chin*, Ivan Quesada, Thuc Nguyen and Pedro Verdugo[1]

*Department of Bioengineering, University of Washington, Seattle, WA 98195 and *Department of Chemical Engineering, Florida State A&M University, Tallahassee, FL 32310, USA*

Abstract. Although Ca^{2+} plays a critical function in relaying intracellular messages, the role of subcellular organelles in the dynamics of intracellular Ca^{2+} still remains largely unexplored. We recently demonstrated that secretory granules can signal their own export from the cell by releasing Ca^{2+} to the cytosol. Oscillations and release of Ca^{2+} in/from the granule result from the combined action of a Ca^{2+}/K^+ ion exchange process that occurs in the granule's matrix, and the sequential activation of two Ca^{2+}-sensitive ion channels: an inositol 1,4,5-trisphosphate receptor Ca^{2+} channel ($InsP_3R$) and an apamin-sensitive Ca^{2+}-activated K^+ channel (ASK_{Ca}). The results reported here from studies using isolated mucin granules indicate that intralumenal granular Ca^{2+} oscillations ($[Ca^{2+}]_L$) and the corresponding cyclical release of Ca^{2+} to the cytosol induced by $InsP_3$ are accompanied by corresponding intragranular pH_G oscillations. Our data show that K^+-induced unbinding of Ca^{2+} from the mucin matrix increases as the pH_G declines. These observations suggest that oscillations of pH_G can modulate the gain of the Ca^{2+}/K^+ ion exchange process, thereby controlling the amplitude of $[Ca^{2+}]_L$ oscillations and the granule–cytosol release gradient of $[Ca^{2+}]$.

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A ubiquitous feature of virtually all types of secretory cells is that the polyanionic networks that form the matrix of secretory granules can exchange monovalent ions with Ca^{2+} ions normally bound to the matrix (Uvnas & Aborg 1977, Verdugo 1990, Nguyen et al 1998). New advances in polymer gel theory (Katchalsky et al

[1]The chapter was presented at the symposium by Pedro Verdugo, to whom correspondence should be addressed.

1951, Tanaka et al 1980, Tanaka 1981, de Gennes & Leger 1982, Edwards 1986) provide a powerful scaffolding of testable physical principles of remarkable predictive power to investigate how secretory granules store and release their products, and how they function as Ca^{2+} oscillators for signal transduction in secretion.

Thanks to the unique properties of the matrix, hormones and other products can be stored at near molar levels in membrane-bound granules (Verdugo 1994) and still remain in osmotic equilibrium with a cytosol that rests at only ~ 250 mOsm. In granules of the nerve terminal for instance, acetylcholine can reach up to 0.5 M (Johnson et al 1979). In conflict with basic principles of physical chemistry, secretory granules had been thought to contain 'a soup' and to exocytose their 'cocktail' to the extracellular space (Gerdes et al 1989). The application of theory and methods of polymer physics revealed that secretory products are not in a 'soup'. Instead, they are entrapped in a condensed polymer matrix that fully constrains their mobility reducing the intralumenal osmotic activity to negligible values (Verdugo 1994). During exocytosis the secretory pore forms a water bridge allowing the diffusion of extracellular Na^+ ions into the granule lumen. A massive Na^+/Ca^{2+} ion exchange follows (Verdugo 1990, Thirion et al 1999). Na^+ displaces Ca^{2+} bridges from the condensed secretory matrix triggering a characteristic polymer gel phase transition that results in quick swelling of the matrix and the release of the entrapped moieties to the extracellular space (Verdugo 1990).

In signal transduction, secretory granules can function as intracellular Ca^{2+} oscillators relaying their own release from the cell (Nguyen et al 1998, Quesada et al 2001). Indeed, granular Ca^{2+} was shown to be involved in the initiation of exocytosis (Scheenen et al 1998, Mundorf et al 2000). In this instance, a scaled down but very similar K^+/Ca^{2+} ion exchange process raises the intralumenal granular Ca^{2+} concentration ($[Ca^{2+}]_L$) increasing the intralumenal/cytosol $[Ca^{2+}]$ gradient ($\Delta[Ca^{2+}]_{L-C}$) and the flow of Ca^{2+} to the cytosol that signals exocytosis. While total Ca^{2+} can reach high levels inside secretory vesicles (~ 100–150 mM; Hutton et al 1983), most of it is tightly bound forming Ca^{2+} bridges in the matrix (Uvnas Aborg 1989).

In the mucin granule, the concentration of free ionized $[Ca^{2+}]_L$ reaches only 10–25 μM (Nguyen et al 1998) and represents < 0.005% of the total vesicular Ca^{2+} that can reach up to 100–150 mM (M. Villalon & P. Verdugo, unpublished work). Although low resting $[Ca^{2+}]_L$ has thermodynamic advantages as it reduces the osmotic load across the granular membrane it limits the $\Delta[Ca^{2+}]_{L-C}$ and the rate of release of Ca^{2+} to the cytosol. However, we recently demonstrated that binding of ATP to its plasma membrane receptor starts a chain of intracellular events that drastically raises $[Ca^{2+}]_L$ leading to granule/cytosol Ca^{2+} release and oscillations of cytosolic $[Ca^{2+}]_C$ (Nguyen et al 1998). ATP triggers the production

of inositol 1,4,5-trisphosphate ($InsP_3$) that can readily activate its own receptor in the granular membrane. $InsP_3$ binding opens the vesicular $InsP_3R$ channel starting an initial release of Ca^{2+} to the cytosol that is driven by the resting levels of $[Ca^{2+}]_L$. Increased cytosolic $[Ca^{2+}]_C$ opens a recently discovered apamin-sensitive Ca^{2+}-activated K^+ channel (ASK_{Ca}) present in the granule's membrane (Nguyen et al 1988, Quesada et al 2001). Cytosolic K^+ can then move into the granule resulting in a K^+/Ca^{2+} exchange that can readily unbind Ca^{2+}, significantly rising $[Ca^{2+}]_L$ and the $\Delta[Ca^{2+}]_{L-C}$ gradient for Ca^{2+} release to the cytosol. Increased outflow of Ca^{2+} decreases the $[Ca^{2+}]_L$ and augments the local $[Ca^{2+}]_C$, closing the $InsP_3R$ channel while the ASK_{Ca} remains open, leading to an increase in $[Ca^{2+}]_L$. However, as Ca^{2+} diffuses away from the $InsP_3R$ channel or it is buffered in the cytosol, the $InsP_3R$ channel opens again while the ASK_{Ca} channel inactivates, repeating the cycle that generates a standing train of $[Ca^{2+}]_L$ oscillations and cyclic Ca^{2+} release to the cytosol that exhibits a period of about 9 s and lasts for as long as the $InsP_3$ receptor remains occupied (see Figs 1 and 4).

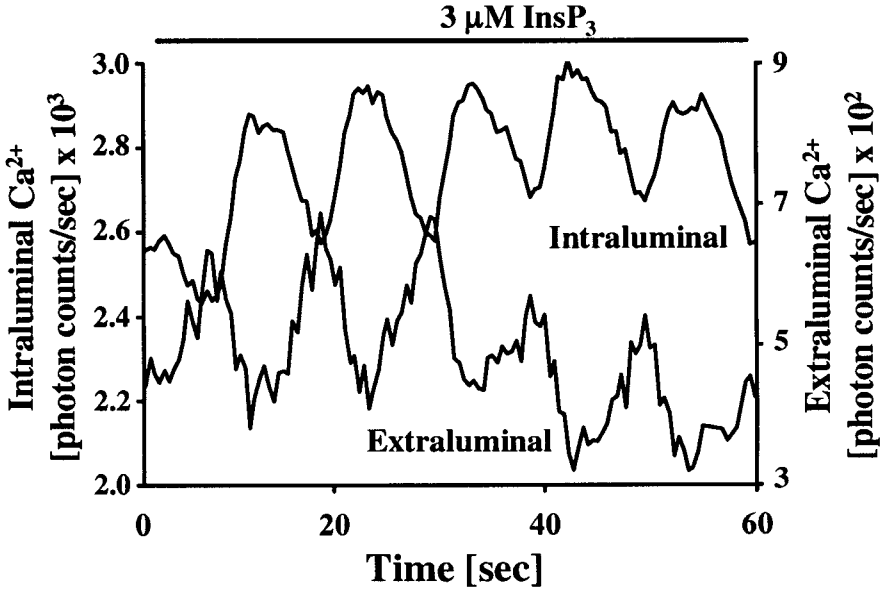

FIG. 1. Oscillations of intralumenal $[Ca^{2+}]_L$ and Ca^{2+} release (extralumenal) in isolated mucin granules upon addition of 3 μM $InsP_3$. The frequency of $[Ca^{2+}]$ oscillation is about 0.13 Hz. Notice that oscillations of $[Ca^{2+}]_L$ and extralumenal $[Ca^{2+}]$ are ~ 180° out of phase (n = 14).

The experiments reported here using optical sectioning in isolated mucin granules indicate that $[Ca^{2+}]_L$ oscillations are accompanied by a corresponding train of intralumenal pH_G oscillations. Our results show that pH_G fluctuations can control the gain of K^+/Ca^{2+} exchange. Low pH_G increases the yield of K^+/Ca^{2+} exchange drastically rising the $\Delta[Ca^{2+}]_{L-C}$ that drives Ca^{2+} release and the corresponding $[Ca^{2+}]_C$ oscillations in the cytosol.

Experimental procedures

Mucin granule isolation and dye loading

Granules were isolated following procedures published previously (Nguyen et al 1998). Briefly, mucin granules in intact goblet cells were labelled with LysoSensor Green DND-189 ($pK_a = 5.2$) to monitor pH_G changes, or else with Calcium Orange 5N ($K_d = 20\,\mu M$) to monitor $[Ca^{2+}]_L$. These two different pools of cells were then suspended in an intracellular buffer — 140 mM potassium glutamate, 500 nM Ca^{2+} (EGTA-buffered), 20 mM Tris, pH 7.6 — while the cell membrane and cytoskeleton were disrupted by sonication. To detect Ca^{2+} release, we separated the granules by centrifugation and resuspended them in intracellular buffer containing either 10 μg/ml of dextran-conjugated Calcium Green 1 ($K_d = 190$ nM) or 10 μg/ml of dextran-conjugated Calcium Crimson ($K_d = 185$ nM), both non-permeant Ca^{2+} probes (Molecular Probes, Eugene, Oregon). Granule suspensions were then allowed to attach to polylysine-coated glass chambers and stimulated by exposure to 3 μM $InsP_3$. Oscillations of pH_G and Ca^{2+} release or of $[Ca^{2+}]_L$ and Ca^{2+} release were monitored in cells labelled with LysoSensor Green DND-189/Calcium Green 1 or Calcium Orange 5N/Calcium Crimson, respectively.

Optical sectioning

Granules were imaged by a Nikon Diaphot inverted microscope using a 100 oil immersion objective with a numerical aperture of 1.4. Images were captured by a thermoelectrically cooled low dark noise, 336× 243 CCD matrix digital camera (Spectra Source Model 400, Westlake Village, CA). The camera was mounted in the photoport of the microscope using a 20× relay lens yielding a 10 pixels/μm resolution. Two-line scans resolving an area of 0.2× 30 μm were sampled every 300 ms. Thin optical sections (~ 200 nm) were obtained by a no-neighbours deconvolution algorithm (Nguyen et al 1998) allowing us to measure directly the fluorescent intensity (average photoelectron count per pixel) as function of time inside and outside secretory granules. Validations of the optical sectioning method have been published elsewhere (Nguyen et al 1998, Quesada et al 2001).

Ca^{2+}/K^{+} ion exchange

Isolated mucin granules were loaded with Calcium Orange 5N (K_d= 20 μM) and equilibrated in ATP-free intracellular buffer containing 100 nM free Ca^{2+} (EGTA buffered), 20 μg/ml heparin and 100 nM apamin. Under these conditions the $InsP_3R$ channel, the ASK_{Ca} channel, and the SERCA pumps are rendered inoperative and resting $[Ca^{2+}]_L$ remains stable at about 25 μM (Nguyen et al 1998). To evaluate the pH-dependence of Ca^{2+}/K^{+} ion exchange, 10 μM nigericin, a K^{+}–H^{+} ionophore, was used to equilibrate K^{+} and H^{+} across the membrane. The $[K^{+}]$ in the intracellular buffer was varied from 1 mM to 140 mM at pH 5.5 and 6.5 [buffered with 10 mM MES (2-[*N*-morpholino]ethanesulfonic acid). The ionic strength and osmolarity were kept constant by adjusting the concentration of *N*-methyl-D-glucamine (NMG) in the intracellular solution. Ca^{2+} probe K_d was computed following procedures described elsewhere (Kao 1994).

Results

Mucins, the polyanions that make the matrix of goblet cell granules, can function as ion exchange resins releasing bound Ca^{2+} in the presence of monovalent cations (Forstner & Forstner 1975). To investigate the effect of pH on Ca^{2+}/K^{+} exchange in mucin granules, we equilibrated isolated mucin granules loaded with the Ca^{2+}-probe Calcium Orange 5N in an intracellular solution while we increased the $[K^{+}]$ from 1 mM to 140 mM at pH 5.5 and 6.5. As shown in Fig. 2, rising $[K^{+}]_G$ produced a characteristic non-linear increase of $[Ca^{2+}]_L$. Notice that the same change of $[K^{+}]$ produces twice as much increase of $[Ca^{2+}]_L$ at pH 5.5 than at pH 6.5. We have reported, that the influx of K^{+} into the granule can mobilize bound Ca^{2+} from the mucin matrix readily increasing $[Ca^{2+}]_L$ and $\Delta[Ca^{2+}]_{L-C}$ (Nguyen et al 1998). Here we show that changes of pH_G can further increase the gain of the Ca^{2+}/K^{+} ion exchange operating inside the granule.

Exposure of granules to an intracellular buffer containing 3 μM $InsP_3$ triggered a train of oscillations of $[Ca^{2+}]_L$ and pH_G (Fig. 3). As shown in Fig. 1, the oscillations of $[Ca^{2+}]_L$ are in opposite phase with corresponding oscillations of $[Ca^{2+}]$ outside the granule. Conversely, the oscillations of pH_G are in phase with increases of $[Ca^{2+}]$ outside the granule (Fig. 3). Notice that the pH probe increases its fluorescence as pH decreases. Thus, the periodic increase of $[Ca^{2+}]_L$ is associated with corresponding periodic intragranular acidification, ruling out a Ca^{2+}/H^{+} exchange since replacement of Ca^{2+} by H^{+} should result in increased pH with Ca^{2+} unbinding.

Discussion

The progressive acidification of the subcellular compartments of the secretory pathway is now well established. The pH decreases from ~ 7.5 in the endoplasmic

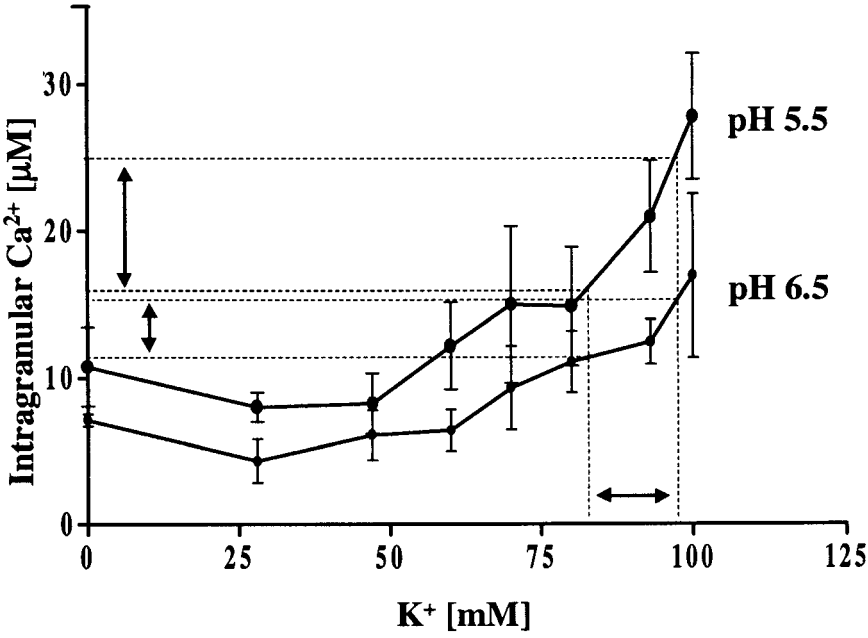

FIG. 2. pH effect on Ca^{2+}/K^+ ion-exchange in mucin secretory granules. Increased $[K^+]$ in the granular lumen results in an increase in $[Ca^{2+}]_L$. Note that at lower pH, the same $[K^+]$ concentration can mobilize more bound Ca^{2+} from matrix networks (n= 6).

reticulum (ER), to ~ 6 in the Golgi and to ~ 5.5 in secretory vesicles (Hutton 1982, Wu et al 2001). However, the mechanisms that control pH in secretory granules, and the multiple roles of intralumenal pH still remain poorly understood. The idea that Golgi and secretory granules control their acidic pH values by altering their conductances to Cl^- (Barasch et al 1988, 1991) has now been questioned by new observations that indicate that the decrease of pH from the ER to the Golgi and to the granules probably results from a corresponding increase in the density of active H^+ transport pumps and a concomitant decrease of H^+ permeability (Wu et al 2001).

Acidification is important for sorting and processing of prohormones (Gerdes et al 1989, Wu et al 2001), and the findings that granules can signal their own exocytosis by releasing Ca^{2+} to the cytosol is attracting attention to the role of granules and their secretory matrix in the control of the dynamics of $[Ca^{2+}]_L$ (Yoo & Albanesi 1990, Gerasimenko et al 1996, Nguyen et al 1998, Quesada et al 2001). To explain intralumenal Ca^{2+} unbinding, Yoo et al (2001) have focused on pH modulation of specific interactions of the secretory matrix with the $InsP_3$

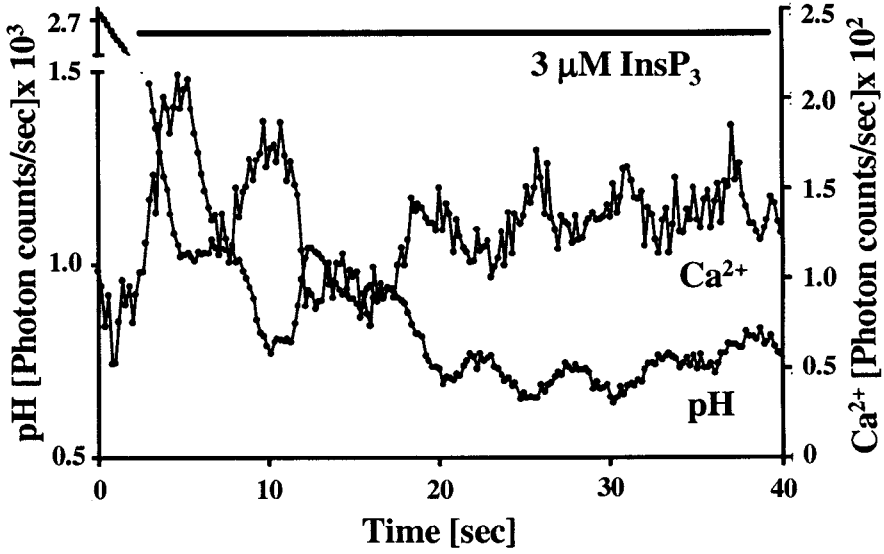

FIG. 3. Intralumenal pH fluctuations and oscillations of Ca^{2+} release in an isolated mucin granule induced by 3 μM $InsP_3$. Drop in photon counts represents an increase in pH. The frequency of oscillation is approximately 0.13 Hz (n= 13).

receptor while our studies have been focused on the ion exchange properties of the polyanionic matrices present inside the granule (see Fig. 4). We demonstrated that $[Ca^{2+}]_L$ oscillations and corresponding oscillations of Ca^{2+} release to the cytosol result from a highly cooperative Ca^{2+}/K^+ ion exchange process rather than from Ca^{2+} transport into the lumen. This new paradigm for storage and release of Ca^{2+} has been found to operate not only in mucin granules but also in mast cell granules (Nguyen et al 1998, Quesada et al 2001). It requires the coordinated interaction of three molecular components: a polyanionic matrix that functions as an ion exchanger, and two Ca^{2+}-sensitive channels with opposed Ca^{2+} sensitivity: one to release Ca^{2+} to the cytosol, and other to import K^+ into the secretory granule. The results presented here further support the model validated by our previous observations and are consistent with recent observations in mast cells that indicate that exocytosis is preceded by pH changes and Ca^{2+} fluctuation (Williams et al 2000). Our results show that in mucin granules, activation of the $InsP_3R$ channel produces both $[Ca^{2+}]_L$ and pH_G oscillations. The periodic increase of $[Ca^{2+}]_L$ is associated with a corresponding periodic intragranular acidification, ruling out a Ca^{2+}/H^+ exchange since replacement of Ca^{2+} by H^+ in the mucin matrix should result in decreased proton concentration and increased pH as Ca^{2+} unbinds. The source of H^+ could be

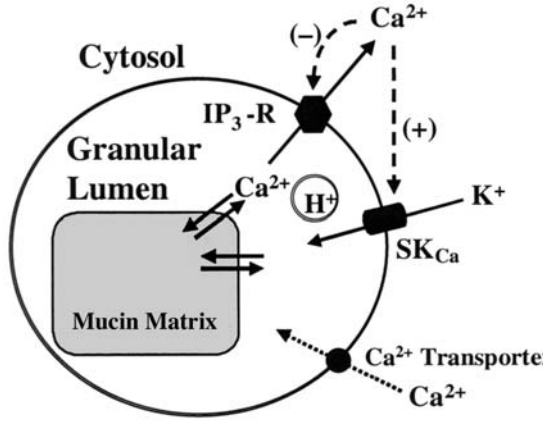

FIG. 4. Working model for the dynamics of Ca^{2+} storage and release in/from secretory granules. Two pools of Ca^{2+} are found inside the secretory granule: one bound to the secretory matrix, and a free ionized Ca^{2+} pool (Meldolesi & Pozan 1995). Oscillations of $[Ca^{2+}]_L$ require the functional interaction of the granule polyanionic matrix, operating as a Ca^{2+}/K^+ ion-exchanger, and two Ca^{2+}-sensitive ion channels probably located in close vicinity in the granular membrane: an ASK_{Ca} that imports K^+ into the granule, and an $InsP_3R$–Ca^{2+} channel to release $[Ca^{2+}]_L$ to the cytosol (Nguyen et al 1998, Quesada et al 2001). Stimulation of the secretory cell results in production of $InsP_3$ that binds to $InsP_3R$ in secretory granules opening $InsP_3R$–Ca^{2+} channels and prompting a release of lumenal Ca^{2+} to the cytosol that induces an initial decrease of $[Ca^{2+}]_L$. The subsequent increase of $[Ca^{2+}]_C$ in the vicinity of the granule (Fig. 1) inactivates the $InsP_3R$ channel while opening the ASK_{Ca} channel. Inflow of K^+ into the lumen results in Ca^{2+}/K^+ exchange and unbinding of Ca^{2+}, which jointly with the closure of the $InsP_3R$–Ca^{2+} channel produces an increase of $[Ca^{2+}]_L$. As the local increase of $[Ca^{2+}]_C$ dissipates by diffusion into the cytosol or by binding to cytosolic buffers the $InsP_3R$ opens again, starting a new cycle that continues for as long as the $InsP_3R$ remains occupied. Although the mechanism of the corresponding train of intragranular fluctuations of $[H^+]$ remains to be investigated, the observed oscillations of pH_G can readily potentiate Ca^{2+} release from the granule.

explained by the formation of potassium salt of intralumenal ATP, with the inflow of K^+ inducing the release of H^+ from phosphate residues. Whether pH changes are necessary and/or sufficient for the granule to function as an intracellular Ca^{2+} oscillator cannot be established from the present observations. However, in the light of physical chemistry theory our results suggest that pH probably affects the coordination of Ca^{2+} bound to the matrix. Proton dislodging of one of the paired electrostatic links of Ca^{2+} increases the probability of K^+ unbinding Ca^{2+} from the network. This synergistic ion exchange effect could increase the net gain of the Ca^{2+}/K^+ exchange process and the corresponding amplification of the $\Delta[Ca^{2+}]_{L\text{-}C}$ that drives Ca^{2+} release for signal transduction in exocytosis.

Acknowledgement

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DISCUSSION

Basbaum: I don't recall you talking about the $InsP_3$ receptor in your model before. Is that something relatively new?

Verdugo: Yes. It was published in *Nature* a couple of years ago (Nguyen et al 1998, Quesada et al 2001).

Basbaum: My recollection of your model was that the polyanion-rich sites on mucins are mutually repulsive, but the presence of Ca^{2+} in the granule shields these sites sufficiently to keep everything relatively compact. Then, at the time of fusion and fission of the granule membrane with the plasma membrane, Na^+ exchanges for Ca^{2+}, but that Na^+ is an inadequate shield. Is that correct?

Verdugo: Inflow of Na^+ exchange with bound Ca^{2+} reduces cross-linking of the mucin polyanionic polymers, Ca^{2+} is released, and the network undergoes a typical polymer gel phase transition from condensed to hydrated phase, and swells.

Basbaum: So now you are introducing the idea of the $InsP_3$ receptor, which means that intracellularly the Ca^{2+} can be lost to the cytoplasm. Does this imply that the granule will swell in the cytoplasm?

Verdugo: Yes, that is exactly what I said. There is release of Ca^{2+} from the granule to the cytosol. Under normal conditions this small Ca^{2+} release functions as a signal for exocytosis but does not decondense the mucin network. Of course, there is a potential for pathology as the granule can prematurely decondense inside the cell. Petersen and others (Raraty et al 2000, Krüger et al 2000), who work in the exocrine pancreas, have suggested that this may be the case in acute pancreatitis.

Basbaum: Has swelling of granules been seen in this disease?

Verdugo: I don't know. However, if in isolated granules you use an ionophore for K^+ to increase the intraluminal concentration of K^+ sufficiently, the granule will eventually explode.

Basbaum: Presumably, it is not the K^+ directly, but the resulting extrusion of Ca^{2+} from its cross-linking sites in the network.

Verdugo: That is correct. Like in any critical phenomenon, there is a critical point where phase transition takes place. However, there is a large range of K^+/Ca^{2+} exchange within which bound Ca^{2+} can still be extracted from the network without phase transition occurring. If I had been in A. C. Burton's Imaginary Celestial Committee in charge of designing this particular system, I wouldn't have used this design because of its inherent high risk. However, what mitigates the risk of the matrix undergoing transition before release is that there is a large excess of Ca^{2+} bound in the granule. In signal transduction, the amount of Ca^{2+} that needs to change hands from the lumen of the granule to the cytosol in order to raise cytosolic Ca^{2+} concentration from $\sim 10^{-7}$ M to say 1 μM, is very small. Therefore the amount of Ca^{2+} that is exported from the granule is in the range of nano- to micromoles — very small amounts.

Basbaum: What is the purpose of this happening inside the cell?

Verdugo: This is like the granule having its own passport to get out. The granule receives an $InsP_3$ message and it starts releasing Ca^{2+}, signalling to the cell's shuttle system to move it out.

Davis: What Pedro Verdugo is suggesting is that the granule is acting as the intracellular Ca^{2+} store for agonist-dependent exocytosis, rather than the ER.

Basbaum: So the granule releases its own Ca^{2+}, to allow the neutralization of granule–plasma membrane mutual repulsion.

Verdugo: The repulsion idea has not been experimentally verified but the release is correct; the granule oscillator machinery consisting of a mucin ion exchange matrix and two ion channels with opposite sensitivities to Ca^{2+} can indeed release periodic pulses of Ca^2 signalling the cell to initiate its export. At the membrane exit site, together with neutralizing membrane repulsion this Ca^{2+}-signal can also activate the formation of the molecular scaffolding for granule docking, membrane fusion and subsequent formation of the secretory pore (Rahamimoff & Fernandez 1997).

Davis: It has a mole of Ca^{2+} per kilogram of dry weight. The small amount that is mobilized during this process shouldn't really affect overall Ca^{2+} levels inside the granule.

Sheehan: The other interesting characteristic of this model is that it shows keenly the difference between Na^+ and K^+ as effectors of that expansion. K^+ is the intracellular ion, but it is most important that this globule doesn't start to expand when it is still inside the cell in the dramatic way that it does when it leaves the cell. It only really comes under a Na^+-dominated influence as it leaves the cell.

Basbaum: Why is there such an important functional distinction between Na^+ and K^+?

Sheehan: Na^+ and K^+ are different with respect to their interactions in polyanionic networks. This is mainly because the energy required to de-shield these ions from water differs significantly. K^+ ions can often bind more strongly to COO^- and SO_4^- groups and thus desolvate them. So whether they get coordinated into the macromolecular environment to form complexes or remain hydrated and thus draw water into the biomolecule to form gels, there is a subtle balance.

Basbaum: So Na^+ has the more explosive effect, and K^+ just some low oscillatory effect.

Rogers: The swelling that Pedro Verdugo has seen — and the potential for it to happen intracellularly — is very interesting. A few years ago we were studying electron micrographs of goblet cells, both prestimulated and stimulated (Newman et al 1996). We found stimulated cells demonstrated intracellular granules that had become fused together before expelling secretions onto the surface. In fact, the granules that were fused intracellularly were larger in cells where the granules were not fused together. Perhaps in the stimulated cells these granules can swell intracellularly.

Verdugo: My feeling is that the name 'goblet cell' is derived from a histological artefact. If you rapid-freeze the cells, they don't look like goblets at all. They are very small. The expansion of granules that is seen in histology is an artefact of fixation, and this is what makes the cells look goblet shaped.

Rogers: I would agree with this, because rapidly frozen cells do not look as much like goblets as they do in chemically fixed tissue (D. F. Rogers & T. M. Newman, unpublished results).

Jeffery: Why does one observe some secretory cells, such as the serous cells, that do not exhibit this so-called fixation artefact? We are all (histologists) looking at artefacts, but these artefacts are telling us something, surely? Can we not relate this morphological difference to a biochemical difference?

Verdugo: The kinetics and stability of cross-linking by the fixative of one matrix to another could be very different. The 'dense core granules' for me are nothing but the observation that the periphery was decondensed and the centre remains cross-linked. It is a half-way expanded granule. The degree to which the granule will expand is the ratio between the diffusion of the cross-linking agent coming onto the network and the expansion of the network. You might find in certain networks that the cross-linker could have a hard time getting in and the granule can then expand fast. In others, the cross-linking fixative can penetrate fast and the granule hardly expands at all.

Jeffery: The extent of the cross-linking is telling you something about differences in the way the molecules are compacted in those two situations. The fixative is having a different effect on different cells in the same tissue. It has its in-built control. It must be reflecting something that is different biochemically or biophysically.

Verdugo: The content of the granules is different.

Davis: There was a nice study some years ago by a Japanese investigator who worked with intestine goblet cells, slam-freezing them. The tissue is slammed down on to a copper block frozen to cryogenic temperatures. He observed a gradient of fixation inside a cell. The deeper in the cell you went from the direction of freezing, the more 'intragranular fusions' there were. The deeper granules were apparently hydrating and the matrix expanding during the freezing to the point where the granules were breaking.

Jeffery: Bill Davis, I was interested in the pictures you were showing earlier on. These were describing what we used to refer to as a merocrine secretion. In contrast, with goblet cells granules fuse and it is a bit like squeezing a toothpaste tube. There was actually apocrine secretion where a lot of granules were fusing both within the cell and during the expulsion of a leading granule.

Davis: For the slam-freezing paper, I was describing goblet cells in the GI tract, which do exhibit compound exocytosis.

Jeffery: What do you think is happening in the airway? Is the secretion always merocrine, or can we believe in apocrine secretion in the airway also?

Basbaum: You'd better define for everyone what 'merocrine' and 'apocrine' mean.

Jeffery: Apocrine secretion is where some of the cytoplasm of the cell is released at the same time as the secretory granules. In other words, there is some loss of cell. Merocrine secretion takes place granule by granule, without loss of cytoplasm.

Verdugo: In the slug the system is very interesting. The slug mucus secretory cells have a compartment in which the granules are stored intact. Granules move from this compartment when the cell secretes: the smooth muscles around the cell contract and the granules are secreted intact. They are the size of an erythrocyte, and they have two membrane layers. They are in effect time bombs that explode outside the cell. They are sensitive to shear and ATP. In this system the expansion takes less than 16 ms.

Jeffery: Is this in any way allied to mast cell degranulation, where lacunae are formed?

Basbaum: That is true compound exocytosis. Marian Neutra 'wrote the book' on goblet cell secretion, albeit in the gut. Did she show compound exocytosis?

Jeffery: She showed both, but they differed depending on the stimulus. Acetylcholine, for example, did not result in compound exocytosis. It was straight exocytosis, preferentially from one intracellular part of the cell rather than another.

Basbaum: I presumed that airway goblet cells would behave in the same way as gut goblet cells.

Rogers: We have seen both apocrine and merocrine secretion in guinea-pig trachea organ culture (Newman et al 1996). We have performed 'slam' freezing and we saw simple exocytosis, compound exocytosis and merocrine-like expulsion of cytoplasmic content. We also had some data in human bronchus where we could see similar things. This was after stimulation with ATP.

Davis: Weren't you using tannic acid?

Rogers: We had tannic acid delay in our guinea-pig trachea studies (Newman et al 1996). There's the possibility of an artefact. Nevertheless, you wouldn't expect tannic acid overlaying the surface of the epithelium to induce an apocrine-like secretion, and we did not observe it in control cells.

Plopper: I think it depends a lot on how the samples are treated. You can freeze them slowly and get all the granules to coalesce, or you can slam freeze them and mostly they are pretty tightly bound.

Basbaum: The gold standard is the rapid freezing. Are you saying that with rapid freezing you do not see this compound exocytosis (i.e. fusion of granules within the cytoplasm)?

Plopper: We don't see this in unstimulated cells.

Davis: I suggest that the gold standard is watching living cells. I have spent hours watching video microscopy and have never seen a compound exocytosis. I always saw single granules fuse to the apical membrane and pop.

Basbaum: But you don't see the inside of a cell.

Davis: We can focus anywhere in the cell. Occasionally, when we were working with a goblet cell that was less than half full of granules, we'd see an exocytotic event create a vacant space, and then another granule would 'rise to the surface' to fill the void.

Rogers: Your *in vitro* culture of goblet cells is likely to be different from what is happening *in vivo* in the airways.

Davis: The cells we were watching were in an explanted epithelium. Hence, they represent native goblet cells. It is highly unlikely that there will be a dedifferentiation to the extent you suggest over a period of two days.

Verdugo: Manuel Villalon did his thesis on electron probe microanalysis. To do X-ray microanalysis rapid freezing is a requirement. We never saw any compound exocytosis in quick-frozen material. We always found individual granules inside. They are very dense. There is no goblet shape of the cell: they are columnar. There is no question in my mind that the fixative is permeabilizing the granules and they are expanding during this process.

Nettesheim: That is why I am proposing to do away with the term 'goblet' cell. There are mucous cells and other cells.

Nadel: What is the physiological significance of the different secretion outcomes that we are discussing here? I can see that the explosive expansion inside the cell could kill it, but what are the other physiological issues here?

Jeffery: We are trying to establish whether there are two different mechanisms operating. If there is merocrine versus apocrine secretion taking place, then two different mechanisms may be operative. This might be important.

Verdugo: You may have a point here. There was a paper published by someone in Paris working on the goblet cells of the oviduct of the quail. He demonstrated by rapid freezing that there were free granules intact outside the cells.

Basbaum: Apocrine secretion could lead to that. Apocrine, we used to say, is the breaking off of the top of the cell. Higher organisms would work hard to avoid this, presumably.

Jeffery: Maybe, maybe not. Perhaps it is a mechanism for turnover. In apocrine secretion the cells are presumably lost not through apoptosis, but by sloughing. With merocrine secretion, the cell could go on and on producing mucin for relatively longer periods.

Basbaum: So what is holocrine secretion?

Jeffery: Loss of the entire cell.

Davis: The discharge of hagfish slime is by holocrine secretion from the slime cells: whole cells are shed.

Rogers: One of the advantages of fusion of granules within the cell, as opposed to multiple exocytotic events on the surface of the cell in response to stimulation, is that if you have many granules fusing with the apical membrane, you are increasing the surface area of the apical membrane. This leads to destabilization of the cell. If there is one exocytotic pore on the apical surface through which fused intracellular granules can extrude mucus, in a toothpaste fashion, this retains membrane integrity.

Basbaum: What happens to the membranes of all those granules?

Rogers: They may go back to form granules again, or they may be recycled. Theoretically, there is an advantage to having one secretory outlet as opposed to many.

Basbaum: I don't think so. What Marian Neutra showed is that a cavity forms. The patches of secretory granule membrane that used to encompass each of the secretory granules open out into the plasma membrane and there is a short period of time during which you can see a membranous cavitation in the goblet cell. Then, over time, the granule membrane is recycled.

Davis: Once again, that is in the gastrointestinal tract. This can be seen in HT29 cells, a cell line derived from colonic epithelium.

Basbaum: Because of the common embryonic origin of gut and respiratory epithelium, until it is proved otherwise I would assume that it is a similar mechanism.

Davis: We have never seen any evidence for that in airway, by the same techniques, that Carol Bertrand uses to observe compound exocytosis in HT29 cells.

Verdugo: For as long as quick freezing doesn't prove this, I won't believe a word. The chemistry of histology is fairly primitive. It involves the formation of cross-links inside the secretory matrix and in other biopolymer networks found in the cell (cytoskeleton, nuclear matrix, calsequestrin networks of the ER, etc.). Of course, we can learn many things from these preparations, but we need to keep in mind that we are dealing with a highly artefactual picture.

Jeffery: It's important to investigate this further and establish whether there is more than one mechanism or not. Reflecting back to primitive animals, I do believe that the explosive release of mucin can be a very important defence mechanism and one that might well be maintained, and used appropriately by the respiratory tract. Slow release might have another purpose. Bill Davis' system seems ideal for investigating this question. By trying different agonists and scenarios, you may well discover that there is explosive release in certain circumstances controlled by entirely different mechanisms and responsive to different forms of treatment than merocrine secretion.

Basbaum: Is there an implication that Bill Davis' and Pedro Verdugo's results are inconsistent?

Jeffery: Not at all. The nature of what is being investigated is related but different. Bill's system is perhaps an easier one for investigating this question.

Barnes: Pedro Verdugo, do you see a graded response to activating stimuli in an individual cell, or is it all-or-nothing? When Duncan Rogers was looking at this, he either counted goblet cells as absent or present with Alcian blue staining. I can see how that can be a dose–response if mucus secretion is measured mucus, as it would reflect the number of cells that discharge, but in an individual cell, is there a gradation of response?

Verdugo: Our results were from mechanical stimulation. These cells were grown with a dialysis membrane on top of them. When this membrane is pulled off, the cells start secreting immediately. They probably have surface mechanical receptors. You can also induce secretion with ATP in these cells. We haven't really seen whether they expel all the granules. However, when we used quinicrine to fluorescently label granules, we observed that once the cells start degranulating, few granules are left behind. The rate of hydration of the released granules is very consistent. But if we disrupt the cell by ultrasonification and electroporate the isolated granules we find that the isolated granules differ in their swelling kinetics. There must be some final step of glycosylation or post-translational modification that takes place in the granule, and perhaps those granules that are immature are not released.

Davis: I can answer Peter Barnes' question directly. It took us six months to do a dose–response curve, and we observed a beautiful saturating relationship between the number of degranulations and [ATP].

Engelhardt: Is it thought that the interaction between the membrane of the vesicle and the apical membrane required to initiate exocytosis is a passive event? Has anyone studied which syntaxins or Rab proteins might regulate this event? It seems that this would be fairly important to the initial step of this process. Is it activated by membrane receptors stimulated by ATP?

Verdugo: There is a great deal of interest and effort in this field. A few years ago in the Biophysical Society meeting someone mentioned that there had been

at least 50 proteins that had been implicated in granule docking and membrane fusion, with many of them often being dis-implicated by later publications. This is a complex field with rather low signal-to-noise ratio.

Rogers: To answer John Engelhardt's question, there's one publication looking at docking proteins in epithelial secretory cells (Koch et al 2000). This is a new field for airway goblet cells.

Engelhardt: It seems that there could be a lot learned from all the dominant negative reagents available to modulate vesicular transport. If you could identify one specific pathway that initiates the fusion event, and you prevented it, this would have obvious therapeutic potential.

Disse: Is there any evidence of relevance of this expansion/explosion mechanism of goblet cells in disease? You mentioned as an example acute pancreatitis.

Verdugo: I don't know. My work focuses on the polymeric material inside the granule. The cell biology is not my priority. I know that Ole Petersen and colleagues (Raraty et al 2000) have been working on the exocrine pancreas, and this is one of the things that they have found: release under particular conditions inside the cell which kills the cell.

Disse: Under conditions of massive clogging with mucins, as occurring during status asthmaticus, is it known whether this is a very fast process or whether mucus accumulates with time?

Randell: I'd like to get back to Dr Nadel's question. I think we agree that preventing goblet cell hyperplasia and metaplasia might be a good thing therapeutically. But would it be a good therapy to inhibit granule secretion? Would this prevent the accumulation of mucus or would it have some untoward effect on cell physiology?

Basbaum: Any kind of inhibitor could be titrated. In addition, we are going to want to select targets that are as specific as possible.

Davis: As we become more comfortable with the proteins that are involved in this process, we are identifying targets.

Basbaum: Would inhibiting them be disruptive to the organism?

Davis: The problem is that the proteins are likely to be similar in other secretory cells, such as type 2 cells. We certainly don't want to inhibit surfactant secretion! This is one reason why we are putting some emphasis on PKCδ: different secretory cells appear to use different PKC isoforms in their regulation.

Basbaum: Is PKCδ relatively unique to the goblet cells?

Davis: It is the first instance of it participating in the secretory event.

Nettesheim: PKCδ is involved in many other cell systems and completely different cellular functions.

Davis: That is correct: it appears to be the most common PKC isoform in human airway epithelium. Hence, if selective inhibition of PKCδ inhibits

mucin secretion, we would the have to test for short- and long-term side effects.

Jeffery: If you produce extra secretions in the first six to eight generations of airways, cough will remove it and its entrapped noxious particles and gases, and this is helpful to the respiratory tract. The key issue is the mucous metaplasia that occurs distally rather than the increased secretion centrally. It is a question of targeting the treatment to the right anatomical zone. It is difficult to remove mucus from small airways by cough.

Salathe: One of the major secretagogues is ATP, but we don't know how it is released except from the mucus granules themselves. This may also be a drug target. We need to figure out how it is actually secreted into the airways.

Basbaum: There is an idea that ATP exits cells through the cystic fibrosis transmembrane conductance regulator (CFTR).

Verdugo: This was one of the ideas that has been proposed. However, if we consider that there are millimolar concentrations of ATP in secretory granules and that goblet cells respond to micromolar concentrations of this agonist, there is plenty of ATP for goblet cells to talk to each other by releasing their granules (Verdugo 1991).

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A calcium-activated chloride channel blocker inhibits goblet cell metaplasia and mucus overproduction

Yuhong Zhou, Michael Shapiro, Qu Dong, Jamila Louahed, Christine Weiss, ShanHong Wan, Qiming Chen, Carl Dragwa, Dawn Savio, Minxue Huang, Catherine Fuller*, Yaniv Tomer, Nicholas C. Nicolaides, Michael McLane and Roy C. Levitt[1]

*Genaera Corporation, 5110 Campus Drive, Plymouth Meeting, PA 19462 and *University of Alabama, 1918 University Boulevard, Birmingham, AL 35294, USA*

Abstract. We have previously shown that expression of a Ca^{2+}-activated Cl^- channel (*mCLCA3* in mice and *hCLCA1* in humans) is up-regulated along with goblet cell metaplasia and mucus overproduction in the lungs of interleukin 9 (IL9) transgenic mice, and in human primary lung cultures by IL4, IL13 and IL9. We show here that *hCLCA1* expression in NCI-H292 cells specifically induces soluble gel-forming mucin production. Moreover, niflumic acid (NFA), a blocker of *hCLCA1*-dependent Cl^- efflux, inhibits MUC5A/C production in these cells. NFA treatment during natural antigen-exposure, where *mCLCA3* is greatly up-regulated in the lung, significantly reduces airway inflammation, goblet cell metaplasia and mucus overproduction *in vivo*. These data suggest that this Ca^{2+}-activated Cl^- channel plays an important role in epithelial-regulated inflammatory responses, including goblet cell metaplasia, and represents a potential novel therapeutic target for the control of mucus overproduction in chronic pulmonary disorders.

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Airway epithelial cells provide the first line of defence from invading organisms and other environmental challenges (Takizawa 1998). Epithelial cells elaborate a large variety of mediators in response to environmental activation, including defensins, providing innate immunity (Zasloff 1992), as well as endothelin (Nakano et al 1994), lipids (Holtzman 1991), growth factors (Sacco et al 1992), cytokines and chemokines (Ohtoshi et al 1991, Takizawa et al 1992, Nakamura

[1]This paper was presented at the symposium by Roy C. Levitt, to whom correspondence should be addressed.

et al 1991) that are important in the pathogenesis of airway disorders. Moreover, epithelial cells also represent a mechanical barrier to the environment. A critical component of this defensive barrier is the mucous glycoprotein layer (Pullan et al 1994, Matsui et al 1998) and, in the lung, the ciliary escalator. Throughout the body, these glycoproteins are made up of a series of high molecular weight muco-glycoconjugates coded for by as many as 16 known *MUC* genes (Moniaux et al 2001).

Both membrane-bound and soluble forms of mucin proteins have been identified. The soluble forms including MUC2, MUC5B, and MUC5A/C are gel-forming and generally appear to be coordinately up-regulated in airway disorders characterized by the overproduction of mucus such as cystic fibrosis (CF) (Davies et al 1999, Voynow et al 1998, Li D et al 1997), chronic bronchitis (Davies et al 1996), asthma (Longphre et al 1999) and chronic sinusitis (Li D et al 1997, Voynow et al 1998). Mucus overproduction is a leading cause of airway obstruction in cystic fibrosis, asthma and chronic bronchitis, which together account for nearly 25 million patients in the USA. In CF, for example, it has been suggested that bacterial colonization may influence the mucins present in the CF airway and that exoproducts from *Pseudomonas aeruginosa* may up-regulate *MUC2* and *MUC5A/C* genes in bronchial tissues and cells *in vitro* via activation of MAPK signalling, which leads to NF-κB activation of *MUC* gene transcription. Mucus overproduction may then contribute to additional bacterial colonization in a positive feedback loop (Li JD et al 1997, 1998, Dohrman et al 1998). It is generally accepted that therapeutic modulation of the overproduction of gel-forming mucins would be beneficial in these chronic obstructive airway disorders. Recent advances in our understanding about the mechanisms of gel-forming mucin gene up-regulation by various noxious stimuli may aid us in the treatment of these disorders (Basbaum 2002, this volume).

Recently, the *hCLCA1* (human Ca^{2+}-activated Cl^- channel 1) gene and murine *Gob5* gene (later renamed as *mCLCA3*) have been cloned as novel members of the growing family of Ca^{2+}-activated Cl^- channels (Zhou et al 2001, Pauli et al 2000). The members of this family share a high degree of homology and appear to mediate apical Ca^{2+}-activated Cl^- conductance in a variety of tissues (Gruber et al 1998). Sequence homology, a similar pattern of tissue distribution, and induction in response to selected cytokines confirms that *hCLCA1* and *mCLCA3* are counterparts in the human and mouse (Nakanishi et al 2001, Zhou et al 2001). Both *hCLCA1* and *mCLCA3* are expressed in basal crypt epithelia and goblet cells of the small and large intestine (Gruber et al 1998), but in contrast to other channel family members, their expression was not observed in the healthy lung. *mCLCA3* expression, however, was found to be strongly induced in several murine asthma models (Nakanishi et al 2001, Zhou et al 2001) and *hCLCA1* was recently shown to be induced in the lung in human asthma (Toda et al

2002). These observations suggest than an hCLCA1 inhibitor may represent an important new mucoregulator therapeutic strategy.

The goal of this study was to examine the potential impact of niflumic acid (NFA), a known channel inhibitor, on MUC5A/C production *in vitro* and in an inflammatory model of lung disease characterized by prominent goblet cell metaplasia and mucus overproduction.

Experimental procedures

Mice. The following studies conformed to the principles for laboratory animal research outlined by the Laboratory Guide for Animal Use and were approved by the Institutional Animal Care and Use Committee. Interleukin (IL)9 transgenic mice were generated in a FVB/N background as described previously (Renauld et al 1994). (C57BL/6JXDBA2/J)F1 (BDF1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6D2F1 mice 6–8 weeks of age were used in this study. Mice were maintained in non-pathogen-free conditions. The animal housing facilities were maintained at 22 °C (range 19–24 °C) with a light cycle of 12:12 hours light:dark. Food and water were provided *ad libitum*.

Expression constructs and cell lines. As previously described (Zhou et al 2001), the *hCLCA1* gene was cloned and ligated into the mammalian expression vector pCDNA3 (InVitrogen). The gene was inserted in the sense orientation (relative to the promoter) to direct overexpression of message in the sense orientation and allowing overexpression of the hCLCA1 protein. As a control, the gene was inserted in the antisense orientation. The NCI-H292 cell line was purchased from the American Type Culture Collection (Manassas, VA) and cultured at 37 °C in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin (GIBC/BRL) in humidified air supplemented with 5% CO_2. Transfections were carried out using the Fugene6 reagent according to the manufacturer's instructions (Roche). Stable transfected cell lines were selected in the presence of 0.4 mg/ml G418.

Antigen sensitization and pulmonary functions. Antigen sensitization was carried out essentially as described previously (Mehlhop et al 1997, Kurup et al 1992, McLane et al 1998). Mice were anaesthetized by methoxyflurane inhalation and 25 μl of *Aspergillus fumigatus* (Af) (Bayer Pharmaceuticals, Elkhart, IN) extract antigen were applied to the left nare. BDF1 mice were immunized once per week for 3 weeks and on day 22 (i.e. days 0, 7, 14, 21 and 22) and were phenotyped approximately 12 hours after the last immunization. Steroid treatment consisted of interperitoneal injection of dexamethasone (Sigma, 1 mg/kg) once per day for 23 days.

To determine the bronchoconstrictor response, respiratory system pressure was measured via a port in the tracheal cannula and continuously recorded before and during exposure to a bronchoconstrictor approximately 12 hours after the last immunization. Mice were anaesthetized and instrumented as previously described (Levitt et al 1988, Nicolaides et al 1997, McLane et al 1998). The bronchoconstrictor response to 58 μg/kg of 5-hydroxytryptamine (5-HT, Sigma, St Louis, MO) was assessed by the change in peak inspiratory pressure (P_{pi}) integrated over time (5 min post-bronchoconstrictor). This parameter termed the Airway Pressure Time Index (APTI) is a simple and repeatable measure of the change in P_{pi} and is highly correlated with respiratory system resistance and elastance following a bronchoconstrictor challenge (Ewart et al 1995).

Mice were anaesthetized with methoxyflurane, and niflumic acid (100 μg/20 μl) or PBS (in control animals) was administered intratracheally daily throughout the antigen-exposed period (22 days).

Bronchoalveolar lavage (BAL). After measurement of lung function parameters, lungs were lavaged with three 0.5 ml washes of 0.9% sterile saline (room temperature). The washes were combined and the lavage fluid was centrifuged (2800× g for 10 minutes at room temperature in an Eppendorf microcentrifuge 5415C; Hamburg, Germany), and the cell pellet was resuspended in 1 ml of Dulbecco's phosphate-buffered saline (without Ca^{2+} or Mg^{2+}). Cells were then counted using a Coulter Counter. Differential cell counts of BALs were made from slide preparations (Cytospin 3, Shandon, Pittsburgh, PA) stained with Kwik-Diffs stains (Shandon, Pittsburgh, PA). Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard morphology using light microscopy and at least 200 cells were counted under 400× magnification. The percentage and absolute numbers of each cell type were then calculated.

Northern blot and RT-PCR. Total RNA was isolated from mouse tissues and cell lines using Trizol reagent (InVitrogen) following the manufacturer's protocol. Northern blot analyses were performed by separating by gel electrophoresis 10 μg of total RNA on 1% formaldehyde gels and transferring RNA to GeneScreen Plus membranes (NEN Life Sciences). Membranes were probed with $\alpha[P^{32}]$-dCTP random radiolabelled *hCLCA1* or *mCLCA3* fragments generated by restriction digestion of the corresponding cDNA clones. RT-PCR was performed by reverse transcribing 1 μg of total RNA and amplifying cDNA with the appropriate primers by PCR. Products were separated by electrophoreses on 2% agarose gels and visualized by ethidium bromide staining. A ubiquitously expressed housekeeping gene, *hPMS2*, was assayed as an internal control. Primer pairs used to generate *hPMS2* and mucin messages are listed in Table 1.

TABLE 1 Primer pairs used to generate hPMS2 and mucin messages

Gene (accession #)	*Sense primer (5′ ⟶ 3′)*	*Reverse primer (5′ ⟶ 3′)*
hMUC1 (J05582)	GCCAGTAGCACTCACCATAGCTCG (3113–3136)	CTGACAGACAGCCAAGGCAATGAG (3627–3605)
hMUC5A/C (AF015521)	GTGGAACCACGATGACAGC (610–629)	TCAGCACATAGCTGCAGTCG (1428–1408)
hPMS2 (U13696)	GGACGAGAAGTATAACTTCGAG (2133–2154)	CATCTCGCTTGTGTTAAGAGC (2505–2485)

Numbers in parentheses refer to oligonucleotide position contained within the published cDNA.

Western blot. Cells were directly lysed in sample buffer containing 2% SDS and 5% β-mercaptoethanol. Lysates were passaged three times through a 26 gauge needle and then heated for 10 min at 65 °C. Lysates were fractionated on 4–12% polyacrylamide Bis-Tris gels (Novex) and electrophoretically transferred to PVDF (Biorad, Hercules, CA). Membranes were blocked in 5% non-fat milk and probed with affinity-purified polyclonal hCLCA1 antiserum (the peptide CEELSKMTGGLQTYASDQNNGLID was used to immunize rabbits and for purification). Bands were visualized with a horseradish peroxidase-linked anti-rabbit secondary antibody (Biorad, Hercules, CA) and a chemiluminescent detection kit (Pierce, Rockford, IL).

Specific ELLA. MUC5A/C secretion in the culture medium was quantified by a modified enzyme-linked lectin assay (ELLA). Briefly, MUC5A/C antibody (1-13M1, NeoMarkers, Freemont, CA) was used to coat plates. After blocking the plates with BSA, cell culture supernatants were incubated with the antibody for 2 hours. Specifically bound MUC5A/C was then detected by HRP-Lectin (Sigma, St Louis, MO) and absorbance at 450 nM was read after incubation with HRP substrate (TMB peroxidase kit, Biorad, Hercules, CA).

PAS staining. NCI-H292 cell lines were cultured on cover slides in six-well plates for 3 days to reach confluence. For Cl^- channel inhibitor studies, cells were cultured in the presence of either 100 μM NFA (Sigma, St Louis, MO) for 24 hours prior to fixing. Lung tissue derived from various mice were dissected free of hilar lymph nodes, and snap frozen in liquid nitrogen immediately. The tissues were embedded in Cryomatrix embedding resin (Shandon, Pittsburgh, PA). Frozen sections (8 μm) were made on an IEC Microtome plus at – 20 °C. Sections were transferred onto silane-treated glass slides, and allowed to dry at room temperature for 1 hour. Cells and tissue sections were fixed in 5% formaldehyde in ethanol. Sections were stained with alcian blue/periodic acid–Schiff reagent (AB/PAS) and counterstained with haematoxylin (HE) (Sigma, St Louis, MO). Cells were stained with PAS.

Patch clamping. Patch clamping studies utilized HEK293 cells transfected with *mCLCA3* and Cl^- conductance was measured as described previously (Gruber et al 1998). The cells were incubated with ionomycin to trigger Ca^{2+}-activated Cl^- conductance in the presence or absence of diclofenac (100 μM).

Statistical analyses. One way analysis of variance (ANOVA) with subsequent comparison by Tukey's test were used for analyses of airway hyper-responsiveness and lung cellularity. Homogeneity of variances was tested using Levene's test and

data were transformed appropriately if necessary before applying ANOVA. Data were considered significant if $P < 0.05$.

Results

hCLCA1 is both necessary and sufficient to up-regulate soluble mucin production in vitro

Stable NCI-H292 cell lines overexpressing the *hCLCA1* gene product (sense orientation) were established and compared to both cell lines overexpressing *hCLCA1* in the antisense orientation and with control cells transfected with empty vector (expression vector with no gene insert). Northern and Western analyses demonstrated channel expression only in stable lines transfected with the *hCLCA1* sense construct (Figs 1 and 2).

RT-PCR analysis showed *MUC5A/C* expression in the *hCLCA1* sense-expressing cells, while *MUC5A/C* was below detection in the anti-sense expressing and control cells (Fig. 2A). *MUC1* expression was present at an

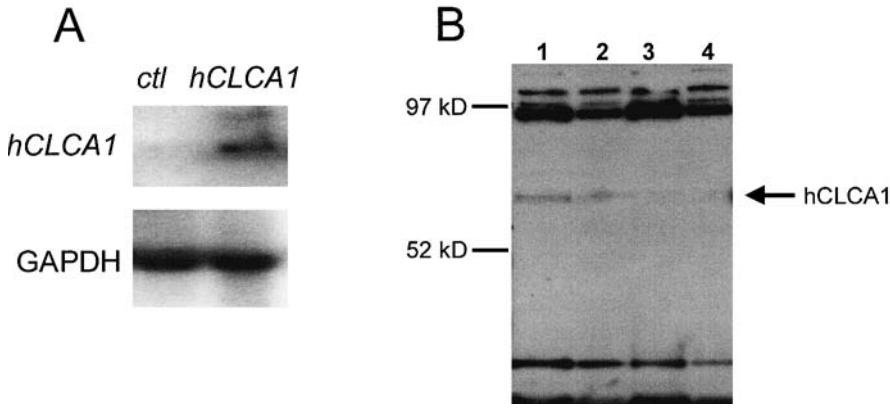

FIG. 1. Expression of *hCLCA1* in NCI-H292 cells. (A) Northern blot analysis of NCI-H292 control cells (transfected with empty expression vector; see methods) or cells transfected with an *hCLCA1* expression vector (see methods). Total RNA (10 μg) was loaded on gels. Blots were subsequently probed for *hCLCA1* or *GAPDH*, a housekeeping control gene. (B) Western blots of total cell lysates were probed with an antibody to *hCLCA1*. Lysates from *hCLCA1* expressing cells, lanes 1–2; lysates from parental NCI-H292 cells, lanes 3–4. 25 μl of lysate was applied to lanes 1 and 3; 12.5 μl to lanes 2 and 4. The hCLCA1 protein migrates at a lower molecular weight than predicted by its sequence. Proteolytic processing to a lower molecular weight was observed previously (Gruber et al 1998). In lysates from HEK293 cells transiently transfected with *hCLCA1* (and not in control transfections), the same band observed in NCI-H292 cell lysates was detected as well as a higher molecular weight band corresponding to the predicted molecular weight (not shown).

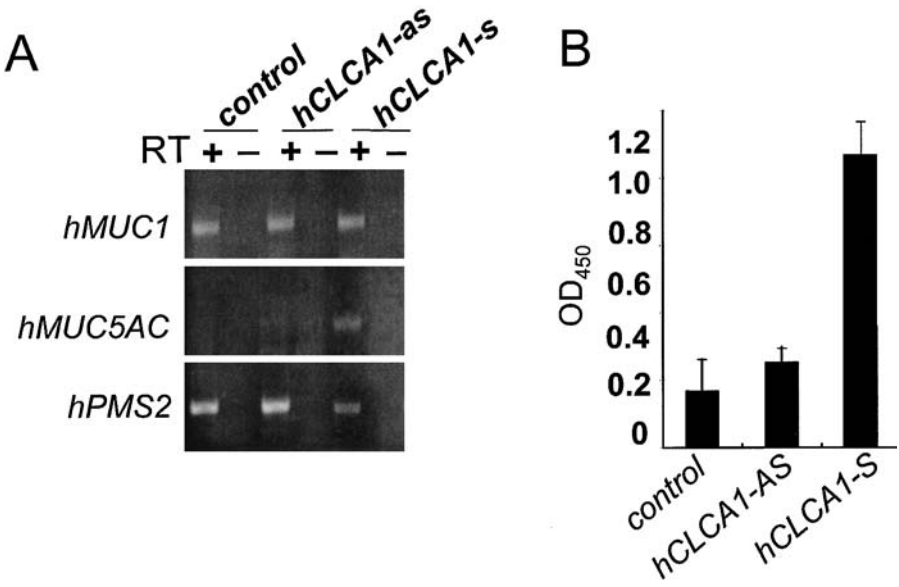

FIG. 2. Effects of *hCLCA1* on mucin gene expression in NCI-H292 cells. (A) RT-PCR analysis of mucin gene expression from control, *hCLCA1* sense (hCLCA1-S) and *hCLCA1* antisense (hCLCA1-AS) expressing cells. RT-PCR amplification was performed for mucin gene expression using specific primers listed in Table 1. Amplification of *hPMS2* was used as an internal control. Samples where reverse transcriptase was omitted (– lanes) were used as negative control for each condition. (B) Specific-ELLA analysis of relative MUC5A/C production from control, *hCLCA1* sense (hCLCA1-S), or *hCLCA1* antisense (hCLCA1-AS) expressing cells. Number represent mean± STDEV (n=3). Results were representative of three independent experiments.

equivalent level in all cell lines (Fig. 2A). Specific enzyme-linked lectin analysis (ELLA) also revealed a dramatically elevated MUC5A/C protein production and secretion in *hCLCA1* sense-expressing clones compared to control or antisense-expressing cells (Fig. 2B).

To determine whether this increased mucin gene expression resulted in increased mucoglycoconjugate production (mucus), we stained cells with periodic acid–Schiff reagent (PAS). Control NCI-H292 cells (Fig. 3), or antisense-transfected cells (data not shown) displayed minimal PAS staining. In contrast there was a dramatic increase of PAS-staining cells and mucus-containing granules in cells expressing *hCLCA1* (sense construct) (Fig. 3B). Similar results were found in *hCLCA1* sense-expressing Caco-2 cells (data not shown).

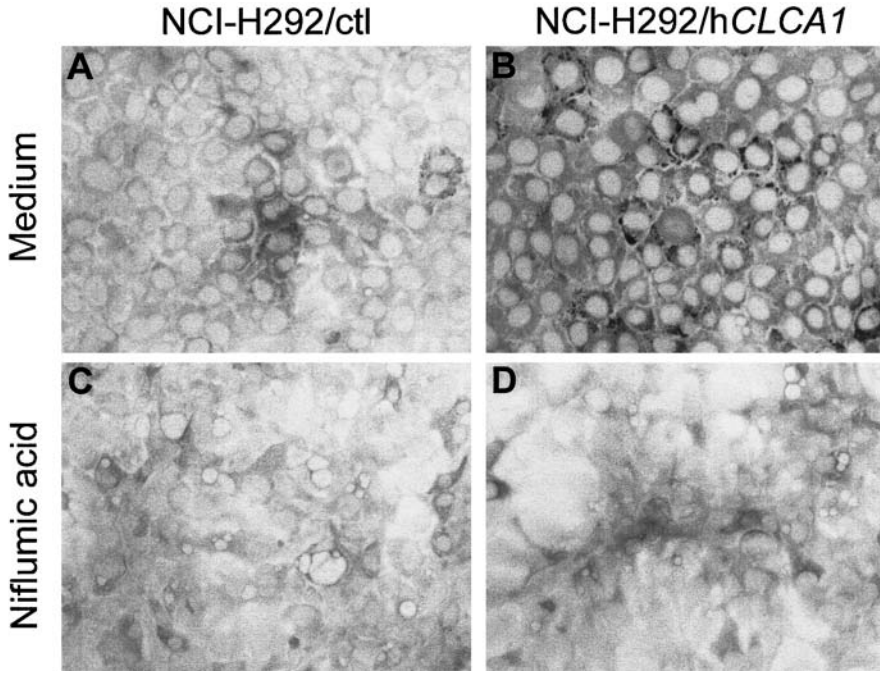

FIG. 3. Enhanced mucus production in NCI-H292 cells expressing *hCLCA1*. PAS staining was performed on NCI-H292 cells. Panels A and C were control cells transfected with empty vector. Panels B and D were cells transfected with *hCLCA1* expression vector (sense). Cells in panels A and B were treated with medium and vehicle and cells in panels C and D were treated with medium and 100 μM niflumic acid (NFA) in vehicle. Cells were cultured for three days to confluence. Results of *hCLCA1*-expressing cells were representatives of two independent cell lines.

NFA inhibits hCLCA1-induced mucin up-regulation in vitro

We next asked whether enhanced mucus production and *MUC* gene up-regulation by *hCLCA1 in vitro* could be selectively modulated by NFA, a known inhibitor of Ca^{2+}-activated Cl^- efflux due to *hCLCA1* (Gruber et al 1998). Control and *hCLCA1*-expressing NCI-H292 cell lines were cultured in the presence of NFA or diluent and monitored at confluence for mucus production. NFA at 100 μM concentration blocks *hCLCA1* function nearly completely (Gruber et al 1998). NFA treatment (100 μM) of *hCLCA1*-expressing cells significantly reduced PAS staining as compared with untreated cells (Fig. 3D versus 3B); whereas PAS staining of inhibitor treated control cells showed virtually no difference compared to untreated cells (Fig. 3C). MUC5A/C production as measured by ELLA was also inhibited significantly by this concentration of NFA (Fig. 4).

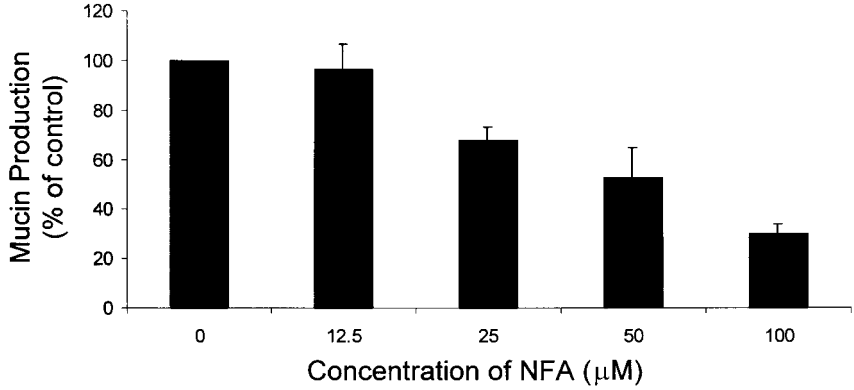

FIG. 4. Suppression of mucin production in NCI-H292 cells expressing *hCLCA1* with a Cl^- channel inhibitor. *hCLCA1*-expressing NCI-H292 cells were grown to confluence and then incubated for 48 h in OptiMem media (Gibco/BRL) supplemented with the indicated concentrations of NFA. The concentration of vehicle (DMSO) was adjusted to 0.1% in all solutions. Conditioned medium was collected and mucin production measured by MUC5A/C-specific ELLA (see methods). Number represent mean ± STDEV (n= 2) and were normalized to mucin produced in the absence of NFA.

Tissue distribution of mCLCA3 gene before and after allergic inflammation

We next determined the tissue distribution of mCLCA3 and the effects of natural antigen-exposure on this gene's induction in a murine model of lung goblet cell metaplasia and mucus overproduction (McLane et al 1998). In naïve BALB/c mice, high levels of *mCLCA3* expression (Fig. 5, upper panel) was observed in colon and small intestine by Northern blotting. No expression of *mCLCA3* was detected in lungs from naïve BALB/c mice. However, after intranasal antigen-exposure, lung *mCLCA3* up-regulation was observed (Fig. 5) along with significant airway inflammation as evidenced by increased airway hyper-responsiveness (APTI), broncheoalveolar lavage (BAL) inflammatory cell influx including eosinophils and total cells, and intense PAS staining indicating goblet cell metaplasia and luminal mucoglycoconjugates (Fig. 6). When the mice were treated with steroid along with antigen, *mCLCA3* was still up-regulated (Fig. 5), suggesting that steroid treatment alone might not be sufficient to block *hCLCA1* up-regulation and accompanying mucin overproduction in respiratory disease.

In vivo suppression of goblet cell metaplasia, mucus overproduction and airway inflammation by NFA

We next evaluated the effects of intratracheal NFA on inflammatory endpoints in this allergic lung model (McLane et al 1998). A single daily administration of

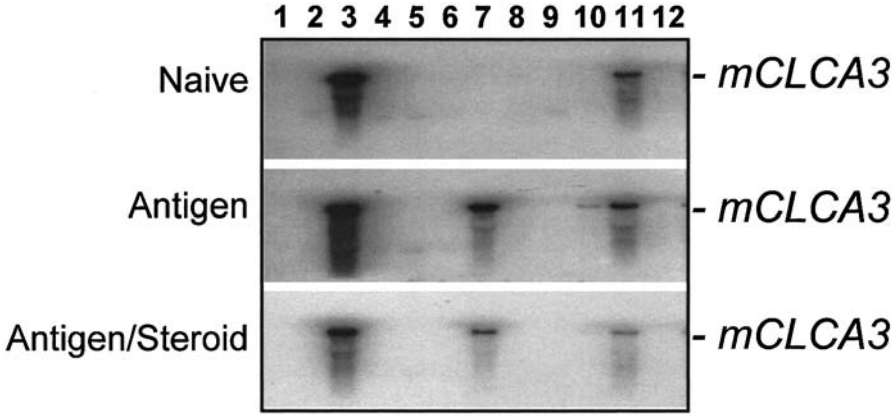

FIG. 5. Induction of *mCLCA3* expression in lung of antigen-exposed BALB/c mice. Northern blot analysis of total RNA from different tissues of naïve, antigen-exposed and antigen-exposed steroid-treated BALB/c mice. Total RNA (10 μg) was hybridized with a specific *mCLCA3* probe recognizing a 3 kB band. Lanes 1–12 represent bone marrow, brain, colon, heart, kidney, liver, lung, lymph notes, skeleton muscle, ovary, small intestine, and spleen, respectively. Blots were stripped and reprobed for *GAPDH* as a control (not shown) demonstrating similar loading for all samples.

NFA significantly reduced antigen-induced mucus-containing cells in airway epithelia (Fig. 6C). BAL eosinophils were also significantly reduced by NFA treatment (Fig. 6B). In addition, NFA treatment tended to reduce antigen-induced airway hyper-responsiveness (Fig. 6A), without affecting elevated total IgE production (data not shown). *mCLCA3* gene expression was not affected by NFA (data not shown).

In addition to being a well established inhibitor of Cl^- conductance mediated by hCLCA1 and related channels (Gruber et al 1998), NFA is also known to exert anti-inflammatory effects through inhibition of cyclooxygenase (COX) (Barnett et al 1994). To determine whether NFA-mediated inhibition of mucin over-production could be due to COX inhibition rather than channel inhibition, we performed studies with the non-steroidal anti-inflammatory drug diclofenac. Diclofenac is similar in structure to NFA and is a potent COX inhibitor (Barnett et al 1994). However, diclofenac was not found to inhibit mCLCA3-mediated Cl^- conductance in patch-clamping experiments (data not shown). Further, diclofenac failed to suppress mucus production *in vitro* in *hCLCA1* sense-expressing cells by RT-PCR analysis and in antigen-exposed mice (data not shown). Hence, COX inhibition does not appear to be sufficient to inhibit mucus overproduction or goblet cell metaplasia in experiments in which NFA had inhibitory effects,

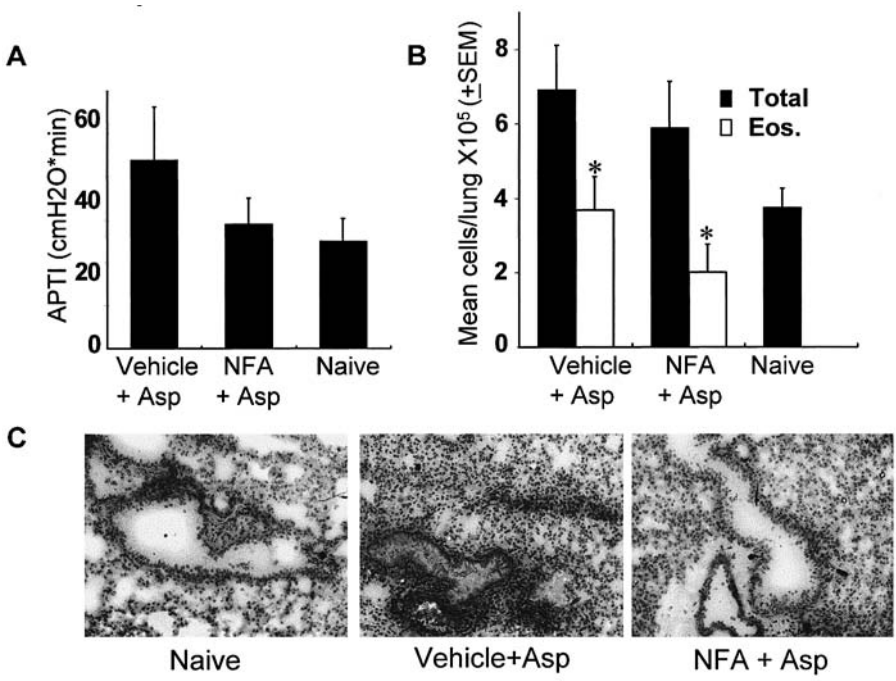

FIG 6. Suppression of antigen-induced airway hyperresponsiveness (AHR), airway inflammation and mucus production with a Cl^- channel inhibitor. Naïve, antigen-exposed vehicle treated (Vehicle+ Asp), and antigen-exposed NFA-treated (NFA+ Asp) mice were studied. (A) Bronchial responsiveness was assessed as the airway pressure time index (APTI) to 5-hydroxytryptamine challenge. (B) Total cells and eosinophils were counted from bronchoalveolar lavage (BAL). Total BAL cells were collected from the lungs of mice and stained with Kwik-Diff (Shandon, Pittsburgh, PA) and counted for eosinophils (200 cells/animal). Numbers of total cells are shown as filled bars, whereas numbers of eosinophils are shown as open bars. $P < 0.05$ between groups is denoted by an asterisk (*). The numbers represent mean± SEM, $n = 9$–10 mice from each group. (C) AB/PAS staining on representative lung sections from each group of mice are shown. Nuclei were counterstained with H&E (magnification × 16).

suggesting that these effects more likely resulted from channel inhibition than COX inhibition.

Discussion

Mucus overproduction in a variety of chronic respiratory conditions continues to represent an important medical challenge. While certain forms of mucin, like MUC1, are membrane-bound and provide protection, lubrication, and help maintain hydration of our epithelial surfaces, gel-forming, secreted types are

implicated in disease. These secreted forms appear to be up-regulated in disease states and their overproduction may contribute to airway obstruction, dysfunction and complications including infections (see paragraph after abstract).

We and others have recently described the regulation of soluble gel-forming mucin production by a Ca^{2+}-activated Cl^- channel (Zhou et al 2001, Levitt et al patent applications 1999, Nakanishi et al 2001). While there is still a great deal to learn about the structure and functions of hCLCA1 and the murine homologue mCLCA3, these channels clearly differ from other Ca^{2+}-activated Cl^- channels. Unlike other known Cl^- channels expressed at baseline in lung epithelia (Gandhi et al 1998), *hCLCA1* and *mCLCA3* are virtually absent in human and murine normal lung epithelium and are highly induced by inflammation (Nakanishi et al 2001, Zhou et al 2002, Toda et al 2002, Komiya et al 1999). Expression of *hCLCA1* in bronchial biopsies is also significantly greater in allergic asthmatic patients compared to non-asthmatic controls and tightly associated with mucus up-regulation (Toda et al 2002). This tight association between *hCLCA1* and *mCLCA3* lung expression and mucus overproduction suggests this channel may represent a therapeutic target in chronic obstructive lung disorders such as cystic fibrosis, chronic bronchitis, sinusitis and asthma.

The present report demonstrates the inhibition of goblet cell metaplasia and selective down-regulation of secreted gel-forming mucin expression by the modulation of these Ca^{2+}-activated Cl^- channels. Importantly, these results confirm that the induction of MUC5A/C in *hCLCA1*-expressing NCI-H292 cells is due specifically to this Ca^{2+}-activated Cl^- channel. NFA, a small molecule inhibitor of hCLCA1, was able to modulate the expression of MUC5A/C. Importantly, both gene down-regulation and decreased MUC5A/C protein production was observed in the absence of an effect on MUC1 expression in airway epithelial cells.

Moreover, NFA was able to modulate goblet cell metaplasia and mucus production, and produce a desirable anti-inflammatory effect in an antigen model of asthma. NFA is still commonly used as a non-steroidal anti-inflammatory agent because of its COX-inhibiting activity. However, the down-regulation of mucus overproduction was most likely not due to COX inhibition because the structurally related and potent COX inhibitor, diclofenac (Barnet et al 1994, Turini et al 2002) failed to block hCLCA1 activity nor inhibit *MUC* gene regulation. Taken together, these data support the hypothesis that mucus over-production in this animal model occurs via induction of *mCLCA3*, and inhibitors of this channel can reverse these pathologic events. Our findings are similar to those of Nakanishi et al (2001), in that *hCLCA1* and its murine counterpart, *mCLCA3*, are shown to be important in regulating MUC5A/C production *in vitro* and *in vivo*. These data are consistent with this channel being both necessary and sufficient to selectively regulate the production of soluble gel-forming MUC5A/C.

This report describes the modulation of goblet cell metaplasia and selective down-regulation of secreted gel-forming mucin expression with a small molecule blocker of a calcium-activated chloride channel. These results suggest that an orally absorbed, well-tolerated, small molecule blocker of hCLCA1 may selectively inhibit soluble gel-forming mucin overproduction and represents an attractive innovative therapeutic strategy to control mucus overproduction in multiple disease states.

Acknowledgements

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DISCUSSION

Basbaum: In the IL9-overexpressing mouse, were there any tissues other than respiratory tract that overexpressed the CLCA1 channel?

Levitt: In the model we used, IL9 expression was driven by a PIM promoter that was supposed to be T cell specific, but it went systemic and IL9 was expressed in all tissues. This is not true of Richard Flavell's IL9 transgenic model (Temann et al 1998), which used a CC10 promoter and was created independently.

Basbaum: But what about CLCA1?

Levitt: The channel seems to be expressed in gut, urogenital and other mucosal tissues that are exposed to the environment, but not the lung. So bacterial stimulation may be another (other than allergen) noxious stimulus that turns on channel expression. But in the gut, which is constantly exposed to bacteria this channel is present constitutively. This is how it was found: the Gob5 paper (Komiya et al 1999) described expression of the channel in the goblet cells in the gut, but they didn't find it in the healthy lung. It is only after we were looking at diseased tissues that we found this channel up-regulated in the lung.

Basbaum: Besides the lung, with respect to the IL9 overexpressor, were there any other tissues that showed increased expression of IL9 but not Gob5?

Levitt: Yes, there is some discordance between IL9 expression in the transgenic mouse that occurs in all tissues, and channel expression that only occurs in epithelial cells. So there are a limited number of tissues that up-regulate channel production and also seem to have increased mucin stores with IL9 overexpression. I can't remember exactly what the transgenic tissue blots looked like, but the restricted pattern of expression of the channel to mucosal surfaces produces some discordance. The tissue distribution of Cl^- channel in the IL9 transgenic is included in our publication (Zhou et al 2001).

Fahy: Apart from safety, is the primary outcome variable going to be FEV_1 (forced expiratory volume in one second) in the asthma trial?

Levitt: We are looking at a number of pulmonary functions. Most people are focused on mucous metaplasia in the small airways in terms of pulmonary

function. We are trying to monitor small airway function also. The primary endpoint is really safety, however.

Fahy: If the functional effect of the channel is mainly on goblet cells, it may not be manifest in pulmonary function tests.

Levitt: The channel is also expressed in other mouse tissues including submucosal glands. To the extent that we have metaplasia and mucus overproduction in the small airways, especially in moderate asthmatics, we could see something in pulmonary function measures, especially widely available measures of small airway function.

Fahy: But you are not going to look directly at the mucociliary apparatus.

Levitt: No, not in this early safety study. But we'll probably collect biopsies and other endpoints in future studies.

Barnes: I don't understand the link between an effect on mucus secretion and on eosinophils and airway hyperreactivity. Do you think that all this is secondary to airway obstruction or a result of mucus secretion, or is this Cl^- channel expressed in other cells such as a ciliated epithelial cells, or airway smooth muscle cells?

Levitt: I didn't see the link either, although these were standard endpoints in our models. We were curious, looking at our data and those of Nakanishi et al (2001) as to why it should affect bronchial hyperresponsiveness. I suppose it could be because you are having an effect on airway epithelium in terms of its differentiation and role in mucin production. What happens with noxious stimuli? Perhaps chemokine production and other events like NO production related to the control of bronchial hyperesponsiveness by the epithelia are affected. These are relatively minor, clinically irrelevant endpoints to follow. We are really focused on mucus production.

Barnes: But you are not measuring mucus production in your clinical study.

Levitt: Not yet; this is a safety study and right now there's no easy way to do this.

Rogers: What was the background to using ibuprofen as a control?

Levitt: The clinicians were looking for an anti-inflammatory compound that could be given as a control that might have some clinical benefit for FEV_1. This can be used as a control in terms of gastrointestinal tolerance for an oral compound, as well as an anti-inflammatory.

Vargaftig: Niflumic acid is a potent COX inhibitor. Ibuprofen is too. I presume that MSI-1995 is a COX inhibitor.

Levitt: We can clearly divorce COX inhibition from inhibition of gel-forming mucins. This is what we are trying to do, because in asthmatics COX blockade by itself wouldn't be a good thing.

Vargaftig: Does IL13 induce the same channel as IL9?

Levitt: Yes. It seems that a number of cytokines can turn on the channel, as can a number of natural stimuli. IL4, IL13 and IL9 are the key cytokines.

Barnes: It seems that you have this drug which you hope will work in asthma by inhibiting mucus, and yet you are not measuring mucus secretion.

Levitt: We are going to have to look at mucus production eventually. Probably a lung lavage coupled with an ELISA we are working on will be important endpoints to follow. Also I would biopsy and look at channel expression in different patients, and try to couple this with treatment effectiveness.

Rogers: It is a fascinating story. You are saying that you have a little channel in the epithelium — a Cl^- channel activated by Ca^{2+} — and the activation of this channel leads to a myriad of events, eventually resulting in asthma. How can the channel do this? You alluded to the fact that it is going through epidermal growth factor (EGF) and MAP kinase. Have you got a plan in your mind of how this all fits together?

Levitt: No, but I wish we had the entire pathway. What we can provide is an insight that this seems to be relevant, from probing it in a number of different ways, including using antagonists of the channel.

Rogers: Is the channel intimately linked with some other receptor?

Levitt: It very well could be.

Danahay: You are calling Gob5 a channel, but are you aware of any electrophysiological evidence actually showing that it is a Ca^{2+}-activated Cl^- channel? There's also a certain amount of debate among the Cl^- channel community as to whether hCLCa1 is actually a Cl^- channel (Jentsch et al 2002). This comes back to Duncan Rogers' point that this family of putative channels bear a strong structural homology to a family of adhesion molecules (Pauli et al 2000).

Levitt: You raise a good point. We don't really know what these channels do and where they fit into this pathway. However, they seem to be linked to the production of mucus. They have some resemblance to these adhesion molecules, especially the bovine form. They don't appear to be terribly relevant in terms of Cl^- conductance, although we do have some data that suggest that there is a Ca^{2+}-dependent Cl^- efflux in cells forced to express the channel, as indicated in our work and that of Gruber et al (1998).

Davis: Can you correlate the channel activity to that conductance? There are multiple Ca^{2+}-activated Cl^- channels in the cell. You are measuring whole cell current.

Levitt: We have done the controlled experiments in which the channel is expressed selectively versus control vector. In these experiments you can see Ca^{2+}-dependent Cl^- efflux.

Vestbo: This is absolutely out of my field, but I've just been at a meeting that tried to get epidemiologists working on asthma to communicate with geneticists: it was quite clear that the geneticists had overwhelming knowledge on genetics but were left with little help on phenotypes to work with in their asthma genetics research. When you are discussing how to apply this experimental research to

patient populations, you will probably need a very good description of your patient material if you want to look at metaplasia in small airways. You would need information such as disease duration and previous treatment, or else you would risk wasting all your fancy science.

Nadel: Presumably downstream, but definitely in this system, the EGF receptor is found activated by tyrosine phosphorylation. You believe that you have a molecule that is expressed, and downstream somewhere there is a mucin being produced. The EGF receptor is not constitutively expressed in the lower airways of normal humans, and it is probably not expressed in your animals. One thing you need to do is figure out whether there are inflammatory pathways that are key to the production downstream. You might look to see whether the EGF receptor is expressed, and whether it is phosphorylated. If it is phosphorylated, is it phosphorylated by a ligand? A likely ligand is transforming growth factor (TGF)α in the airway epithelium. Then you can dissect to see whether there are free radicals or elastases from neutrophils that are involved in the cascade.

Jeffery: One of the researchers in our lab, Jie Zhu, has been describing IL4-producing cells in and around the glands in patients with chronic obstructive pulmonary disease (COPD) (Zhu et al 2001). Whether or not there is a causal link with mucus hypersecretion, I don't know. But listening to you, it struck me that it might be worth studying COPD patients, who are regular producers of mucus, rather than asthmatics who are variable in their production of mucus.

Basbaum: I think the rationale for studying asthmatics is that the channel was found to be up-regulated in two independent models of asthma in the mouse.

Randell: Would it be a good idea to test Roy Levitt's compound in human primary cells *in vitro* or in bronchial xenografts in nude mice, before jumping ahead to the clinical trials?

Basbaum: Roy Levitt sent us some of his compound and we have several irritant mucin assays up and running in our lab, using human cells. We see compelling inhibition of mucin induction by irritants when we apply his compound. We don't understand the mechanism. It is kind of a black box right now. I was interested to hear someone say that these so-called channels may actually be adhesion molecules. We should be open-minded at this point: we are fortunate that this has dropped into our laps.

Jackson: It is not a very potent compound. You have used intratracheal administration in order to generate a sufficiently high concentration to get an effect in mice. However, you are aiming for an oral administration route in your clinical studies. Have you administered this compound orally in mice and generated high enough concentrations in the right place to have an effect in the airways?

Levitt: We are getting these data in humans now. Remember, of course, that what I presented was intratracheal NFA data as an early proof of concept. We are

not using NFA acid in the clinic. MSI-1995 is a much more potent drug and well tolerated orally.

Jackson: Have you done this in mice?

Levitt: We certainly see evidence of our mucoregulator compound absorbed into the blood of mice, and more recently into humans.

Jackson: Did you see an effect with an oral dose?

Levitt: Yes. We observed airway effects with an oral dose as demonstrated in my presentation. Remember MSI-1995 produced decreased BAL MUC5A/C production in the allergen-treated mice, and healthy growing cystic fibrosis mice, where the controls all died.

Basbaum: Is CLCA1 directly inducible by cytokines *in vitro*?

Levitt: We can do intratracheal instillation with a series of cytokines. This was done in the studies I alluded to in my talk (Zhou et al 2001). We can up-regulate channel expression with IL4, IL9 or IL13 instillation. We have tried to stimulate channel expression in cell culture in transformed cell lines and so far have for the most part failed. We force the cells into apoptosis when we try overexpression of the channel in most transformed lines *in vitro*. Similarly, cytokine stimulation *in vitro* in transformed lines really doesn't produce channel over-expression or abundant mucin production. Primary cells are required.

Fahy: Does an inhibitor of TGFα prevent the effect of the channel on mucin expression? It's interesting that the EGF receptor tyrosine kinase inhibitor prevents the effect. This receptor is on the surface of the cell as is the channel. If the expression of this channel increases mucin production without stimulation of any sort, then the activation of that surface molecule in some way is initiating a signal that comes back through the EGF receptor. One possibility is that TGFα is involved.

Verdugo: What happens if you put a Cl^- ionophore on these cells? Where is this channel acting? It is located at the cell membrane and is importing Cl^- into the cell. What if you use a Cl^- ionophore?

Basbaum: If would be great if we had organic compounds that resembled each other except for the fact that some of them inhibit Cl^- transport and others don't.

Levitt: We have such molecules.

Basbaum: Do they all inhibit mucin production?

Levitt: No. I showed an example of two closely related compounds where the one that lacks channel inhibition didn't have an effect on mucin.

Davis: Have you done something as simple-minded as taking the Cl^- out of the medium bathing your cultured cells?

Levitt: No, we don't do the simple-minded experiments, just the tough ones!

Barnes: Cromoglycate and redocromil sodium are thought to work by an effect on Cl^- channels, which were presumed to have increased expression in asthma. Roy Levitt, have you looked at these drugs in your system?

Levitt: It is possible that their could be some overlap in activity of channel inhibitors, but the unique feature of these channels is that they are very restricted in terms of their expression to mucin-producing cells. Thus, the Cl^- channel we are working with is not expressed in mast cells and other inflammatory cells so the mechanism is likely to be different than cromolyn sodium. Moreover, as far as I am aware, cromolyn has no mucin inhibiting activity in our assays.

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Mechanisms by which Gram-positive bacteria and tobacco smoke stimulate mucin induction through the epidermal growth factor receptor (EGFR)

Carol Basbaum, Daizong Li, Erin Gensch, Marianne Gallup and Hassan Lemjabbar

Biomedical Sciences Program, Department of Anatomy and Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0452, USA

Abstract. Mucin, the major macromolecular component of mucus, is generally considered to be a protective substance. When overproduced in a variety of lung diseases, however, mucin gives rise to clinical problems such as airway obstruction and recurrent infection. Our approach to identifying drug targets for the control of mucin overproduction is the analysis of cellular signalling pathways linking stimuli in the diseased lung to mucin transcription. Here we show that mucin transcription in response to both Gram-positive bacteria and tobacco smoke is mediated through activation of the epidermal growth factor receptor (EGFR). The mode of activation of EGFR in response to bacterial lipoteichoic acid involves cleavage of the transmembrane ligand HBEGF by ADAM 10, whereas the activation of EGFR in response to smoke involves cleavage of amphiregulin by ADAM 17.

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Mucin production is a protective mechanism with ancient phylogenetic origins. It is used by host cells to provide a barrier between themselves and environmental threats. The mammalian lung is confronted with threats in the form of bacteria, viruses, fungi, and particulate components of tobacco smoke and air pollution. Over the course of evolution, cell surface receptors and intracellular signalling pathways have developed so that host cells can detect these noxious stimuli and respond to them. Many of these responses involve the up- or down-regulation of specific genes. Mucin is one of these. Despite its adaptive nature, mucin's overproduction in diseases such as cystic fibrosis, chronic bronchitis and asthma results in significant pathology. Through understanding the specific receptors, signalling molecules and gene expression mechanisms pertinent to each disease process, it

may be possible to modulate them so that mucin is produced in moderate, rather than excessive, quantities.

Although the diversity of noxious stimuli in inspired air requires diverse receptors at the cell surface, evolution has provided for such receptors to transduce signals through a relatively small group of downstream signalling molecules. The integration of input from these receptors can begin with signal transduction to a limited number of 'secondary' receptors including the epidermal growth factor receptor (EGFR), ATP receptors, and Toll-like receptors (TLRs). That each of the latter is 'hard wired' to the stress-response elements NF-κB and /or AP-1, provides a conduit for the activation of stress-responsive genes such as mucin. This report focuses on how EGFR and associated downstream signalling mechanisms are activated by dissimilar stimuli deriving from Gram-positive bacteria and tobacco smoke.

EGFR is typical of the family of growth factor receptors referred to as receptor tyrosine kinases (Leserer et al 2000). This name denotes the presence of intrinsic tyrosine kinases located in the cytoplasmic domains of the receptors. Typically, ligand binding induces dimerization of two receptor molecules, bringing each kinase into proximity with the cytoplasmic tail of the other receptor monomer. The cytoplasmic tail contains multiple sites for tyrosine phosphorylation, each of which, when phosphorylated, provides a binding site for other proteins containing SH2 domains. Such interacting proteins include effector proteins such as PI3K and PLCγ but also include adaptor molecules such as Shc and Grb2. The pattern of phosphorylation induced by a stimulus is what dictates the nature of the evoked cellular response.

We (Gensch et al 1999, Lemjabbar & Basbaum 2002) and others (Takeyama et al 1999) have shown that diverse noxious stimuli activate EGFR phosphorylation in lung epithelial cells. A consequence of this is transcription of the mucin genes *MUC2* and *MUC5AC*. This suggests that mucin overproduction associated with the exposure of the lung to noxious stimuli can potentially be modulated by interrupting the sequence of events preceding or following phosphorylation of EGFR. Here we examine the events preceding EGFR activation by Gram-positive bacteria vs. tobacco smoke.

Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* are limited by cell walls containing structural molecules called teichoic acids. We observed that lipoteichoic acid (LTA) from *S. aureus*, in an example of molecular mimicry, bound to and activated G-protein coupled receptors for platelet-activating factor (PAF) on the surfaces of epithelial cells (Lemjabbar & Basbaum 2002). By mechanisms that remain unclear, PAF receptor (PAFR) activation led to the cleavage of an endogenous transmembrane ligand called heparin-bound EGF (HBEGF). This cleaved ligand bound to EGFR, activating the classical growth factor signalling pathway including activation of Ras and the MAP kinase erk1/

2. Using morpholino antisense oligonucleotides to specifically delete members of the ADAM family of metalloproteinases known to cleave such ligands, we determined that the metalloproteinase activated in response to LTA was ADAM 10, a homologue of the *Drosophila* protease, kuzbanian. This identified three key control points mediating activation of EGFR by bacterial LTA (PAFR, ADAM 10 and HBEGF).

Dissecting the pathway activated by tobacco smoke was more difficult. We were unable to obtain evidence for the involvement of PAFR or any other G protein-coupled receptor. EGFR phosphorylation has been shown, in some instances, to be triggered intracellularly by non-receptor kinases including c-Src (Parsons & Parsons 1997) and Jak2 (Yamauchi et al 1997). To determine whether phosphorylation of EGFR by smoke was occurring intracellularly or instead by a process involving ligand interaction at the receptor ectodomain, we asked whether smoke could phosphorylate EGFR in the presence of an antibody blocking the ligand binding site. Results showed that with this part of the receptor blocked, smoke did not stimulate receptor phosphorylation. This implied that a ligand of some kind was necessary for smoke-induced receptor activation. Since NCIH292 lung epithelial cells were the only cells present in our cultures it was clear that any such ligand must have originated from the NCIH292 cells themselves. To directly assay for the cleavage and release of an EGFR ligand from these cells, we designed a study using reporter cells (SKBR3) which lacked the ability to phosphorylate EGFR in response to smoke itself, but could respond if exposed to a soluble ligand. We found that the transfer of medium from smoke-exposed NCIH292 cells caused EGFR phosphorylation in the reporter cells, supporting the idea of a transferable ligand cleaved by smoke-induced mechanisms.

EGFR can be stimulated by at least six molecular ligands comprising the family of EGF-like growth factors (EGF, TGFα, HBEGF, amphiregulin, betacellulin and epiregulin). These are known to activate EGFR following their cleavage from transmembrane precursors. Cleavage is mediated by cell surface proteases known as ADAM metalloproteinases. Collectively, these proteases are blocked by broad-spectrum inhibitors such as GM6001. The inhibition of EGFR phosphorylation by GM6001 indicated that receptor activation by smoke involved a similar process. To identify the specific protease involved in the response, we again used morpholino antisense oligonucleotides directed against ADAM family members. In contrast to our earlier results with LTA, which implicated ADAM 10 in the response (see above), the results from experiments with tobacco smoke showed that smoke signalling was blocked by anti-sense ADAM 17 (also referred to as tumor necrosis factor [TNF]α-converting enzyme, TACE).

To determine which of the six EGFR ligands (see above) was cleaved by ADAM 17 during smoke exposure, we immunoblotted concentrated medium

from NCIH292 cells exposed to smoke or the bacterial cell wall component LTA. Using antibodies directed against each of the six known ligands, we determined that the ligand cleaved in response to smoke exposure was amphiregulin. This contrasts with results obtained after exposure to LTA, in which immunoblot data showed abundant cleavage of HBEGF. Thus, two different stimuli activate EGFR by distinctly different mechanisms in the same cell type: one involves ADAM 10 and HBEGF, the other involves ADAM 17 and amphiregulin.

Seeking the signal transduction mechanisms operating upstream of ADAM 17, we monitored smoke-induced amphiregulin cleavage in the presence of many signal transduction inhibitors. Although inhibitors of PKC and PKA had no effect on the response, antioxidants strongly blocked it. This was true whether we used the oxygen radical scavenger DMTU (dimethylthiourea) or *N*-acetylcysteine (N-Ac), a glutathione precursor. Consistent with this, antioxidants also inhibited smoke-induced phosphorylation of EGFR.

The antioxidant results suggested that smoke might stimulate production of reactive oxygen species (ROS) in the epithelial cell. We confirmed this by experiments in which NCIH292 cells were loaded with the ROS-sensitive dye dichlorodihydrofluorescein (DCF). This compound becomes fluorescent when exposed to oxygen radicals, and is rapidly (< 2 min) activated within cells by smoke exposure. ROS can originate in at least two ways within the cytoplasm: (i) leakage from mitochondria, and (ii) generation by cell surface NADPH oxidase. We found that cells pretreated with diphenyliodonium chloride (DPI), an NADPH oxidase inhibitor, but not with two inhibitors of mitochondrial electron transport (rotenone and antimycin A) fail to generate ROS upon smoke exposure. Thus far, the stimulation of NADPH oxidase by smoke is the earliest known event in the signal transduction cascade leading to mucin induction by tobacco smoke. A comparison between the EGFR activation mechanisms activated by smoke and LTA is shown in Fig. 1.

In separate experiments, we determined that the cytoplasmic signalling cascade initiated by smoke terminates in the activation of *MUC5AC* transcription by activating an AP-1 site (TRE). This was determined by a combination of methods including deletion mutation of the *MUC5AC* 5′ upstream flank. After narrowing the response element to a 50 nucleotide fragment containing an AP-1 site, we mutated this site and found a major reduction in smoke-induced gene activity. Gel shift analysis showed smoke-induced protein binding to a probe containing the AP-1 site. An antibody recognizing all jun family members shifted the DNA-protein band upward, to a position of lower mobility, confirming that jun proteins were induced to bind the AP-1 site under conditions of smoke exposure. Is AP-1 activation a consequence of EGFR stimulation? Although EGFR signalling is known to activate members of both the jun and fos families of protein transcription factors, the effects of smoke on AP-1 do

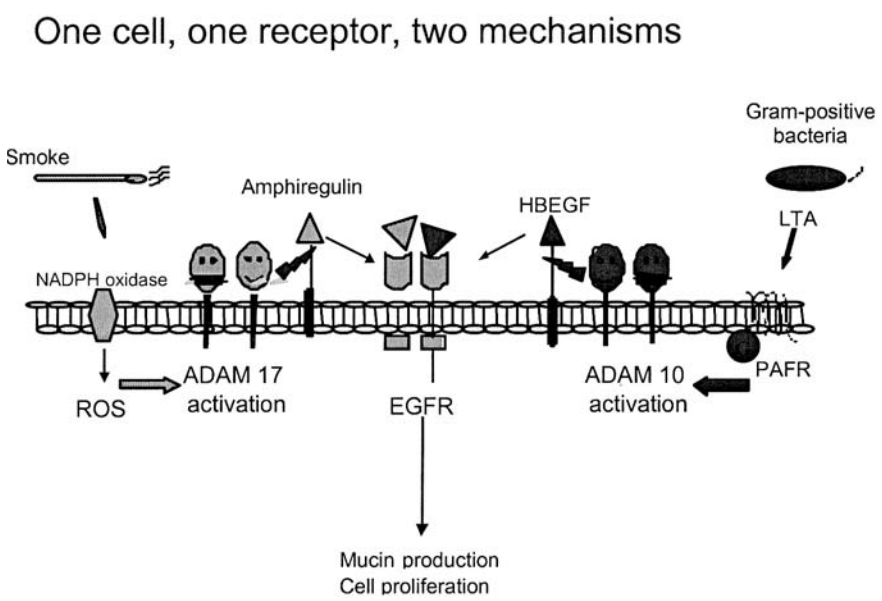

FIG. 1. The model depicts activation of EGFR by tobacco smoke and the Gram-positive bacterial component lipoteichoic acid (LTA). In both cases, EGFR activation requires the cleavage of a transmembrane ligand. In response to tobacco smoke, amphiregulin is cleaved by ADAM 17 (TACE), which is stimulated by an as yet unclear mechanism involving oxygen radicals (ROS) produced by NADPH oxidase. In contrast, in response to LTA, HBEGF is cleaved by ADAM 10 (kuzbanian), which is stimulated by an as yet unclear mechanism involving G proteins. The activation of EGFR by noxious stimuli in the environment leads not only to mucin production, but also to cell proliferation.

not seem to be wholly attributable to signalling through EGFR. Thus, AG1478 and dominant negative (DN) EGFR in our hands only inhibit ~ 50% of the induction of mucin by smoke. This is consistent with the fact that EGF, in concentrations that produce receptor phosphorylation equal to or higher than that produced by smoke, result in only a fraction of the intensity of mucin induction.

To summarize, mucin overproduction in some pathogenic settings is (partly) mediated by activation of the growth factor receptor, EGFR. EGFR acts as an integrator of noxious stimuli through its receptiveness to a variety of ligands cleaved in a variety of stimulus-specific ways. These mechanisms have likely evolved to exploit EGFR's downstream linkages with NF-κB and AP-1, major control points for mucin induction and other defensive responses. An understanding of how EGFR controls mucin production may lead to strategies for therapeutic intervention in patients with mucus hypersecretion.

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DISCUSSION

Nadel: About 25 years ago, Cohen discovered the EGFR. For the next 15 years its application focused on cell multiplication in cancer. In retrospect, a lot of this had to do with the fact that he was given cultures that had high concentrations of EGFR on the surface. It was very natural for him to go on and study cancer, and he won the Nobel prize for it. When we stumbled on to the observation that EGFRs in normal airway tissue cause cells to differentiate but not multiply, we called him. At the time he said he thought this should be published, and so we did. Since then we have shown that it is involved in a whole range of aspects of mucin production, including responses to wounding, antigens, interleukin 13, neutrophils, elastase, cigarette smoke and bacteria. What is most interesting about it to me is what actually makes an airway epithelial cell differentiate, and what makes it multiply? Stephen Holgate's work has shown that if cells are wounded, they migrate. Then they multiply. Some of these cells then differentiate. What is it about the normal structure of cells that prevents the extreme phosphorylation by EGFRs that inhibits the downstream cycling pathway, turns the cells into G arrest and enhances cell differentiation?

Basbaum: One interesting possibility is that there are different ways to stimulate a receptor, and different ways to release ligand to bind to the receptor. There could well be different patterns of tyrosine phosphorylation on the cytoplasmic tail in response to different stimuli that could easily explain why the downstream consequences of activating EGF by one stimulus would lead to proliferation, and the next would lead to the turning on of phenotype-specific genes.

Nadel: The strength and duration of phosphorylation of the EGFR is a major determinant of whether the cell goes into an active cycling pathway or differentiates. If phosphorylation is reduced, the cells tend to differentiate.

Basbaum: Another possibility that has not been raised is the presence of four different EGFR subtypes. You are inevitably going to see different downstream consequences in response to activation of different heterodimers. In cancer, for example, there are abnormally high levels of the EGFR subtype referred to as erbB2 (Her2/neu).

Vargaftig: I'm very interested in the role of the PAF receptor, because many years ago a lot of drugs failed in asthma trials because of specific PAF antagonists. The idea at the time was that PAF was involved with hyperreactivity. I understand that you use PAF antagonists. One or two of them were phospholipids. Have you tried using an antagonist that was not a phospholipid to see the specificity of the effect?

Basbaum: We know that the PAF receptor is involved because in the presence of LTA the PAF receptor is phosphorylated, and also it clusters and is internalized (just as it is in response to PAF).

Vargaftig: LPS does not do that.

Basbaum: Correct.

Vargaftig: Long ago when the anti-PAF reagents were first tested, a fortune was spent demonstrating protection against Gram-negative bacteria, and never against Gram-positive.

Barnes: You must have looked at, or considered the possibility of synergy between these different mechanisms that converge on a common pathway. This could be highly relevant in disease. If passive smoking is insufficient to activate it alone, in the presence of another activating stimulus, it could become important. This may also be relevant to the development of future therapies. You may only need to block one of these mechanisms to take out the amplification, and thus have a larger effect than predicted. Did you look at the interaction of these different activating mechanisms?

Basbaum: No.

Nettesheim: The regulation of the EGFR-mediated responses has been extensively studied in multiple cell models, including epidermal cells. There is good evidence that as cells become confluent, negative growth regulators such as transforming growth factor (TGF)β are being produced. TGFβ regulates a number of cell cycle arrest genes such as Rb and p53. In response to Jay Nadel's question, there are extensive studies that explain how the system driven by the EGFR in terms of proliferation then shuts down and growth arrest occurs.

Davis: Paul Nettesheim, do you have to decrease EGF concentrations to get HBE cells to differentiate?

Nettesheim: We haven't studied differentiation. I think what you are referring to is that if the EGF level gets too high in the cultures it induces apoptosis and cell death (Gray et al 1996).

Engelhardt: I was interested in the importance of ROS. ROS have been shown to be important in many cellular processes, such as apoptosis, not only inside the cell but also outside. Have you thought about whether the pathway might not be as ordered as you think? That is, extracellular ROS may be the irritating signal which promotes dimerization. Have you tried adding catalase or SOD to the medium before you perform the media reconstitution experiment, to see whether you lose this effect?

Basbaum: Yes, there is no inhibition in response to catalase, indicating that ROS are formed intracellularly in our model. *Invivo* in the smoker's lung, however, there are likely to be both intra- and extracellular sources of ROS.

Engelhardt: The reason I suggest this is that there could be multiple pathways leading to dimerization and activation. If antioxidants as well as inhibitors of the metalloprotease family have a synergistic effect, this could be very important to potential therapeutic applications.

Basbaum: Antioxidants could be a good way to go, as would metalloproteinase inhibitors. We are now concentrating on the mechanism of activation of the metalloproteinases themselves. ROS seem to be important in the case of smoke, but not in the case of Gram-positive bacteria. SRC is also a player. Between the generation of ROS and the activation of the metalloproteinase, SRC gets turned on. In addition, Goldkorn et al (1998) have shown that ROS directly activate EGFR, albeit slightly abnormally.

Salathe: The ADAM17 antisense experiments speak against direct activation of the EGF receptor by ROS.

Basbaum: That is true in this case.

Salathe: What cells are you using? There is currently no available study that shows expression of NADPH oxidase in any of the cells of the airway epithelium other than neuroepithelial cells. These cells are rare and radicals must be produced in other cells as well, but it is not clear exactly how. On another note, can you comment on the expression of the different ADAM isoforms in airway epithelial cells?

Basbaum: The appropriate studies have not been done, to my knowledge. My associate, Hassan Lemjabbar has done RT-PCR for ADAMs 5, 9, 10, 15 and 17. He has catalogued them in each of the cells that we use. NCIH292, which is our workhorse, contains almost all of them. But, as you might expect, there is some variability among cell lines.

Rogers: I was fascinated that your bacteria utilize the PAF receptor as a mimic. As I understand it PAF receptors are involved in bronchoconstriction and microvascular permeability — physiological events that are below the epithelium. This is not necessarily going to be where the bacteria make first contact with the airway. Of many possible epithelial receptors, why would the bacteria choose the PAF receptor, unless it is highly expressed in the epithelium?

Basbaum: Bacterial LTA and human PAF contain regions of homology, allowing for molecular mimicry.

Tesfaigzi: If I understand it correctly, the specificity of the response to tobacco smoke or bacteria depends on the ADAM family of proteins and not on the EGFR. Do you feel the ADAM family of proteins would be a good therapeutic target?

Basbaum: Yes. After the ADAM cleaves the ligand, the rest is just downhill.

Rose: Have you been able to demonstrate this in the primary cells at the air–liquid interface?

Basbaum: No. It is not that we haven't been able to; we haven't tried. We have instead confirmed the in vitro results in mice. We have placed mice in a smoke exposure chamber. In tracheas removed 10 min later, we observe EGFR phosphorylation. This is inhibited by GM6001, the metalloproteinase inhibitor that we used *in vitro*.

Rose: Do you see goblet cells induced by the tobacco smoke?

Basbaum: We don't smoke-expose the mice for long enough to see this. In the *in vitro* studies we expose cells to smoke for only 4 h.

Rose: Would you expect to see the EGFR and ADAM 10 on goblet cells, if these molecules are going to be regulating mucin gene expression?

Basbaum: Yes.

Nadel: If there are pro-ligands in airway epithelial cells, first of all there has to be gene expression in those cells, and then they have to migrate to the surface. Here they are fixed, apparently to an extracellular domain where they reside until they are activated. What up-regulates the expression of these pro-ligands? And what cleaves them? Though the metalloproteinases may exist in the cells, they may not be up-regulated and activated. One has to consider any protease that can cleave the extracellular domain of these pro-ligands. One of the most potent is neutrophil elastase. Many metalloproteinases are metallo-elastases.

Jackson: I have a comment with regard to ADAMs as being useful as specific targets. If you are going to recommend an ADAM as a specific target, wouldn't you have to know exactly what the stimulus for EGFR activation is in a complex disease situation? I would have thought there was a high chance of redundancy if you become too specific in your approach. You might be better off going directly for the EGFR as long as the side effects were tolerable.

Basbaum: That's a possibility.

Jeffery: All this depends on the presence of the EGFR in the first place. My understanding from Jay Nadel's work is that EGFR is not highly expressed constitutively. It needs to be induced, perhaps by TGFα, TNFα or another inflammatory mediator. Perhaps controlling the presence or absence of the EGFR is the easier mechanism to target.

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Non-allergic models of mucous cell metaplasia and mucus hypersecretion in rat nasal and pulmonary airways

Jack R. Harkema and James G. Wagner

College of Veterinary Medicine, Laboratory for Experimental and Toxicologic Pathology, Michigan State University, East Lansing, MI 48824, USA

Abstract. Mucous cell proliferation and hypersecretion of airway mucus are important pathological features of human respiratory disorders such as asthma and chronic bronchitis. In addition to airborne allergens and infectious agents, inhaled chemical irritants such as ozone and cigarette smoke have been demonstrated to induce changes in airway mucus production. Cellular and molecular mechanisms involved in non-allergic, toxicant-induced mucous cell metaplasia (MCM; transformation of airway epithelium, normally devoid of mucous cells, to secretory epithelium containing numerous mucus-secreting cells) are still unclear. We have used two experimental models of toxicant-induced MCM in the airways of rats to study the epithelial and inflammatory factors involved in the pathogenesis of MCM. Mucin-specific gene expression and MCM are induced in the nasal transitional epithelium (NTE), but not in the bronchiolar epithelium of F344 rats acutely exposed to ozone, an important air pollutant of photochemical smog. Inhalation of endotoxin, a lipopolysaccharide-protein molecule of Gram-negative bacteria, induces MCM in the bronchiolar epithelium, but not in the NTE, of rats. Both ozone- and endotoxin-induced MCM are dependent on neutrophilic inflammation. Interestingly, each toxicant enhances the MCM induced by the other toxicant. These synergistic effects elicited by coexposure to ozone and endotoxin are also mediated, in part, by neutrophils.

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Metaplasia is defined as 'the abnormal transformation of an adult, fully differentiated tissue of one kind into a differentiated tissue of another kind'. The term is derived from the Greek word *metaplasis*, which means transformation. Metaplasia is an acquired condition, in contrast to heteroplasia, which is a developmental condition. Patients with chronic bronchitis, asthma, cystic fibrosis and other chronic airway diseases have abnormally high numbers of mucous cells in their surface epithelium and underlying submucosal glands that line their respiratory airways. This phenotypic change has been referred to as

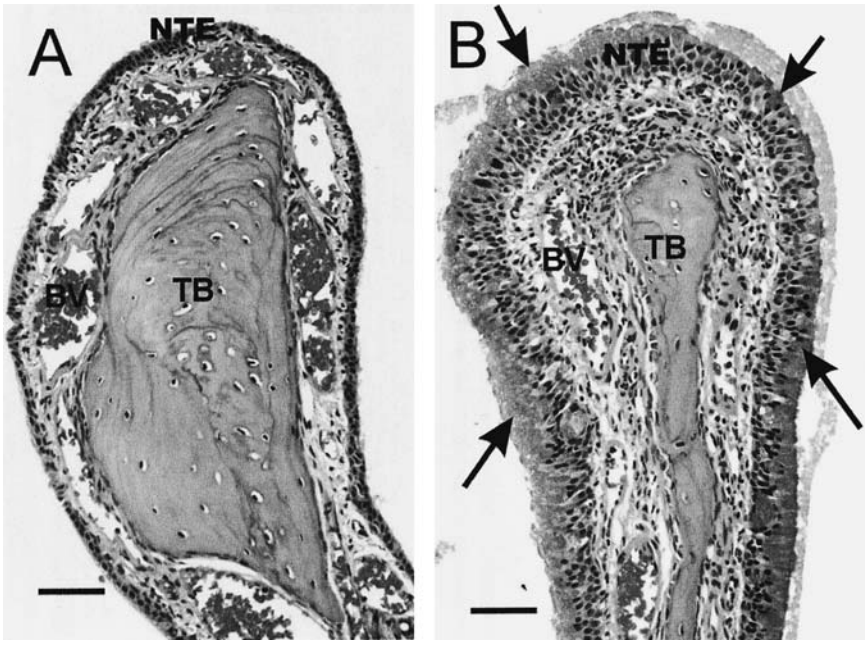


FIG. 1. Light photomicrographs of maxilloturbinates (A, B) from the proximal nasal passages of rats exposed to 0 ppm ozone (filtered-air control; A) or 0.5 ppm ozone (B) for 13 weeks. Note the thick hyperplastic epithelium with mucous cell metaplasia in the nasal transitional epithelium (NTE) lining the maxilloturbinate of the ozone-exposed rat (B). No mucous cells are evident in the normal NTE lining the maxilloturbinate of the rat exposed to filtered air alone (0 ppm ozone; A). Arrows, mucous (goblet) cells; TB, turbinate bone; BV, blood vessel in the lamina propria. Haematoxylin and alcian blue (pH= 2.5) stain; Bars= 50 μm (Harkema et al 1999).

mucous cell metaplasia (MCM). This alteration in the airway mucosa can be induced in laboratory animals by sensitization and inhalation challenge with allergenic substances (e.g. ovalbumin, dust mite antigen) or the inhalation of certain non-allergenic substances (e.g. tobacco smoke, sulfur dioxide, ozone, bacterial endotoxin, neutrophil elastase). Though considerable progress has been made in recent years in understanding the cellular and molecular mechanisms underlying MCM, the full pathogenesis of this common airway lesion is still unclear.

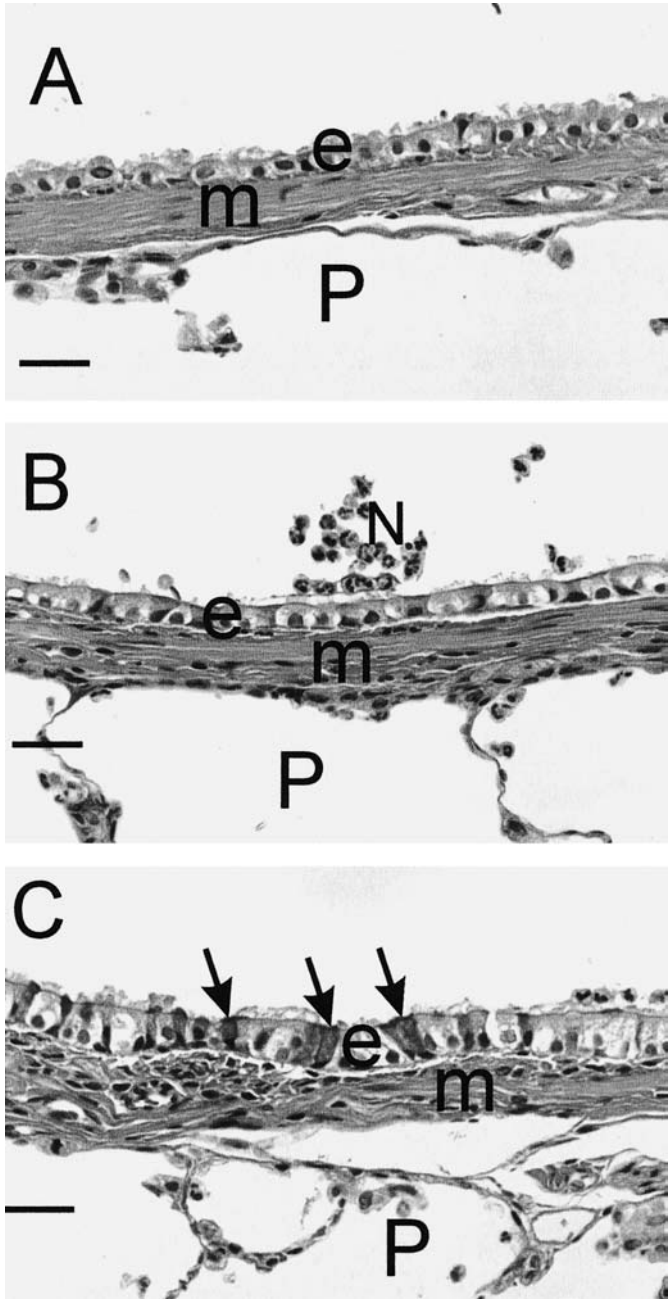
In our laboratory, we have used two non-allergic experimental models of MCM in rat upper and lower airways to better understand the cellular mechanisms involved in toxicant-induced injury, adaptation and repair. This article describes some of our work designed to understand (1) the induction of MCM in the upper and lower respiratory airways of rats exposed to ozone, the major oxidizing

component in photochemical smog, or bacterial endotoxin, a toxic lipopolysaccharide-protein molecule in the outer wall of Gram-negative bacteria, and (2) the exacerbation of either ozone- or endotoxin-MCM by toxicant co-exposures. Some of these controlled single-toxicant exposure or two-toxicant co-exposure studies were also designed to better understand the role of the airway inflammatory response, especially the influx of neutrophils, in the development or exacerbation of MCM.

Ozone induces MCM in the nasal transitional epithelium (NTE; i.e. a non-ciliated surface epithelium in the proximal nasal airways that is normally devoid of mucous cells) after short- (days) or long- (weeks or months) term exposures to near ambient concentrations (Harkema et al 1989, Harkema et al 1999) (Fig. 1). Conversely, instilled endotoxin induces MCM rapidly (within 48 h) in the tracheobronchial airways (i.e. a respiratory surface epithelium normally containing serous, but not mucous, secretory cells) of rats after a single or repeated treatments (Fig. 2). Interestingly, the metaplasia that is induced by ozone exposures appears to be restricted to the nasal airways of these rodents. Ozone does not cause MCM in the epithelium lining the pulmonary airways of rats. In contrast, mucous cell metaplasia induced by endotoxin is restricted to the tracheobronchial epithelium. Endotoxin instillation does not cause MCM in the NTE like that observed in rats exposed to ozone. Using these two novel models of MCM, we have performed a series of studies to examine the effects of (1) endotoxin instillation on ozone-induced MCM, and (2) ozone exposure on endotoxin-induced MCM. Our overall goal was to characterize morphometrically the potential interactions of ozone and endotoxin exposure on airway inflammatory and epithelial cell responses. Our specific aims were to test the hypotheses that (1) endotoxin would enhance the epithelial and inflammatory responses induced by ozone in the NTE of rats, and (2) that ozone exposure would enhance the epithelial and inflammatory responses induced by endotoxin in pulmonary airways.

Ozone-induced MCM in rat nasal epithelium

In 1989, we first reported that Fischer rats (F344/N) exposed for 7 days to 0.8 ppm ozone, 6 h/day, developed a conspicuous MCM in the NTE lining the maxilloturbinates, lateral wall, and lateral aspects of the nasoturbinates in their proximal nasal passages (Harkema et al 1989). The ozone-induced lesions in the F344/N resembled those that we previously observed in the nasal cavity of bonnet monkeys repeatedly exposed to 0.15 or 0.3 ppm ozone for 6 or 90 days (Harkema et al 1987). We further demonstrated that the cellular population in the ozone-exposed rat NTE was markedly hyperplastic and metaplastic with approximately 15% of the cell population consisting of mucous cells compared to



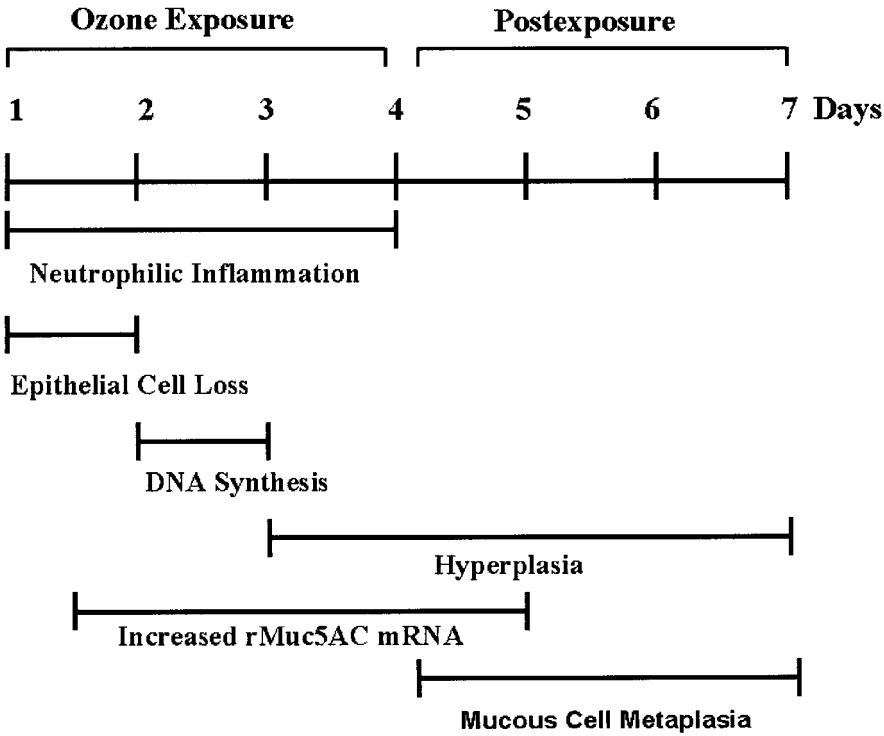


FIG. 3. Temporal sequence of inflammatory and epithelial cell responses in the rat nasal mucosa after 4 days of ozone exposure (0.5 ppm, 8 h /day)(Cho et al 1999).

a normal mucous cell density of 0–1% in the NTE of control rats exposed to filtered air (Harkema et al 1989, Harkema & Hotchkiss 1994).

In 1991, we reported that ozone-induced MCM and epithelial hyperplasia in the NTE of rats can be induced with only three consecutive, 6 h/day exposures to 0.5 ppm ozone (Hotchkiss et al 1991). Seven days after the start of the exposures, rats exposed to ozone for 3 days had MCM that was indistinguishable from that in rats exposed to the same concentration of ozone for 7 consecutive days. Thus, once initiated the development of ozone-induced phenotypic changes within the

FIG. 2. Light photomicrographs of the airway epithelium (e) lining the main axial airway (airway generation 5) from rats intratracheally instilled with saline alone (A) or saline containing 50 μg of bacterial endotoxin (*Pseudomonas aeruginosa*, serotype 10) and sacrificed 24 (B) or 48 h (A, C) post-instillation. Note the presence of mucous (goblet) cells (arrows) in the bronchiolar epithelium lining the airway in C, but not in A or B. P, alveolar parenchyma; m, smooth muscle in airway wall; N, neutrophils. Haematoxylin and alcian blue (pH= 2.5) stain; Bars= 50 μm (Hotchkiss & Harkema 1994)

epithelium are not dependent on additional ozone exposure. As depicted in Fig. 3, the proliferation of mucous cells in the NTE is preceded by a cellular inflammatory response (i.e. neutrophilic influx), an initial loss of sensitive nasal epithelial cells and subsequent proliferation of resistant epithelial cells, and mucin gene over-expression (Hotchkiss et al 1997, Cho et al 1999).

Neutrophil depletion of ozone-exposed animals

Exposure of humans to ozone causes neutrophil influx into nasal airways (Graham & Koren 1990). Although epithelial lesions have not been characterized during human ozone inhalation studies, inflammatory responses and nasal epithelial alterations similar to those induced in ozone-exposed rats are common in people living in areas with high ambient ozone concentrations (Calderon-Garciduenas et al 1995). We recently performed studies to determine the role of neutrophilic inflammation on the development of ozone-induced mucous cell metaplasia in rats exposed to ozone (0.5 ppm, 8 h /day) for three consecutive days. We depleted animals of circulating neutrophils with a rat-specific neutrophil antibody to assess the ozone-induced epithelial alterations in the absence of neutrophilic inflammation. Circulating neutrophils were approximately 1% of normal levels throughout the ozone exposure, and nasal lesions were examined 2 h or 4 days after the last ozone exposure. Ozone exposure of rats caused the infiltration of neutrophils into the NTE and underlying lamina propria that cover the proximal aspects of the maxilloturbinates. The significant increase in mucosal neutrophils present 2 h after the last ozone exposure in neutrophil-sufficient rats was attenuated in neutrophil-depleted rats. We estimated the degree of mucous cell metaplasia by counting the number of mucous cells in the NTE overlying maxilloturbinates, and by measuring the volume density of intraepithelial mucosubstances in nasal sections stained with AB/PAS that reacts with acidic and neutral mucosubstances. By 4 days after the last ozone exposure, the number of mucous cells and volume density of intraepithelial mucosubstances was significantly increased in rats exposed to ozone. In neutrophil-depleted animals, metaplastic responses were only 40% of those observed in ozone-exposed, neutrophil sufficient rats. Over-expression of the mucin gene encoding for rat mucin 5AC (*rMuc5AC*) occurred during the first day of ozone exposure and stayed elevated during the postexposure period (Cho et al 1999). In rats depleted of circulating neutrophils, ozone induced over-expression of *rMuc5AC* was similar to that measured in neutrophil-sufficient animals. These findings suggest that ozone-induced mucous cell metaplasia is in part neutrophil-dependent, whereas the increase in mucin-specific mRNA is independent of the ozone-induced neutrophil influx into nasal tissues. These results are consistent with those of our previous work in which ozone-induced MCM in the NTE of rats

was attenuated by treatment with a topical steroid (Hotchkiss et al 1998). In that study, neutrophilic inflammation was markedly decreased in steroid-treated animals. Because the effects of steroids are broad and affect several cell types other than neutrophils, including possibly epithelial cells, it was unclear from those studies if inhibition of metaplastic responses was due to the steroid-induced blockade of neutrophilic influx. Thus, our studies using neutrophil-depleting antibodies provides stronger evidence that neutrophils specifically play a critical role in ozone-induced mucous cell metaplasia in rat nasal epithelium.

Enhancement of ozone-induced mucous cell metaplasia by bacterial endotoxin

As stated previously, bacterial endotoxins are lipopolysaccharide-protein compounds derived from the cell wall of Gram-negative bacteria (e.g. *Escherichia coli*, *P. aeruginosa*). Endotoxins are the principal aetiological agents responsible for the acute inflammation in pneumonia and sepsis caused by infections of these bacterial organisms. Inhalation exposure can occur occupationally (endotoxin-contaminated organic dusts in waste treatment plants, textile mills, swine or poultry confinement buildings and grain silos) and domestically (inadvertent bacterial contamination of aerosols produced by ultrasonic humidifiers and evaporative cooler-type air conditioners). Human exposure to endotoxin has been implicated as the principal pathogenic agent in several occupational diseases including byssinosis (Rylander & Nordstrand 1974), mill fever (Pernis et al 1961), bagassosis (Salvaggio et al 1966), and asthma-like or bronchitis-like diseases induced by exposure to machining fluid aerosols (Jarvholm et al 1982, Oxhoj et al 1982).

Instillation of endotoxin into the airways of laboratory rodents causes a similar inflammatory response to those observed in humans, including neutrophil infiltration and cytokine production. We have further documented some structural and cellular changes in the airways of laboratory rodents elicited by intranasal instillation (Harkema & Hotchkiss 1991, 1992, Steiger et al 1995) and aerosolized endotoxin (Gordon & Harkema 1994). Among these are epithelial cytotoxicity, hyperplasia, and increased synthesis, storage and secretion of products by airway secretory cells.

A robust migration of neutrophils is an early response elicited by instilled and aerosolized endotoxin, and the oxidant and proteolytic potential of activated neutrophils have been implicated in epithelial cell alterations in endotoxin-treated airways (Davreux et al 1997, Kawabata et al 2000a). Furthermore we have used endotoxin-instilled rats to document epithelial cell changes such as epithelial hyperplasia and MCM in the bronchiolar epithelium, which normally consists of only serous secretory cells in these laboratory rodents (Harkema & Hotchkiss

1992, Steiger et al 1995). As mentioned previously, endotoxin does not cause mucous cell metaplasia in rat NTE.

Because airway endotoxin elicits a significant infiltration of neutrophils into airways, we exposed rats to both ozone and endotoxin to determine (1) the effects of augmented neutrophilic inflammation on the pathogenesis of ozone-induced MCM that occurs in the NTE, and (2) the interaction of two common air pollutants on the development of nasal epithelial cell alterations. In these studies, rats were first exposed to ozone for three consecutive days, and then some animals were instilled intranasally with endotoxin (100 μg) for two more days after ozone exposures (Fanucchi et al 1998). We evaluated epithelial and inflammatory responses at both 6 h and 3 days after the last endotoxin instillation. Endotoxin instillation caused a significant neutrophilic inflammation in the mucosa underlying the NTE in both ozone and air exposed-animals 6 h after. By 3 days post instillation the ozone-induced increases in stored mucosubstances were increased fivefold when animals were also exposed to endotoxin. Despite increasing tissue neutrophils in air-exposed animals, endotoxin treatment alone did not cause any metaplastic lesions in the NTE. These results suggest that neutrophilic inflammation alone is not sufficient to produce metaplasia, and that additional components (e.g. ozone exposure, neutrophil activation) are necessary for MCM to develop in the NTE.

Neutrophil depletion and endotoxin/ozone coexposure

To determine the role of neutrophilic inflammation in the potentiation by endotoxin of ozone-induced MCM, we depleted rats of circulating neutrophils after ozone exposures, but during the time of endotoxin-induced inflammation (Wagner et al 2001). Neutrophilic inflammation elicited by endotoxin instillation was completely inhibited in neutrophil-depleted rats (Fig. 4A). Moreover, the augmentation by endotoxin of ozone-induced increases in stored mucosubstances in the NTE was blocked completely in neutrophil-depleted rats (Fig. 4B). Thus, endotoxin-induced neutrophilic inflammation is required for endotoxin-induced enhancement of ozone-initiated metaplastic responses. However, neutrophils do not appear to be involved in the endotoxin-induced over-expression of mucin mRNA in ozone-exposed animals. Over-expression of *rMuc5AC* after endotoxin instillation was similar in both neutrophil-sufficient and -deficient rats that were exposed to ozone (Fig. 4C). We interpreted these results to mean that neutrophils mediate metaplastic responses by a mechanism(s) other than just turning on the mucin gene. These results are reminiscent of the results of neutrophil depletion studies of ozone-induced MCM described above, where mucin gene over-expression caused by ozone exposure was unaffected by neutrophil depletion (Cho et al 2000).

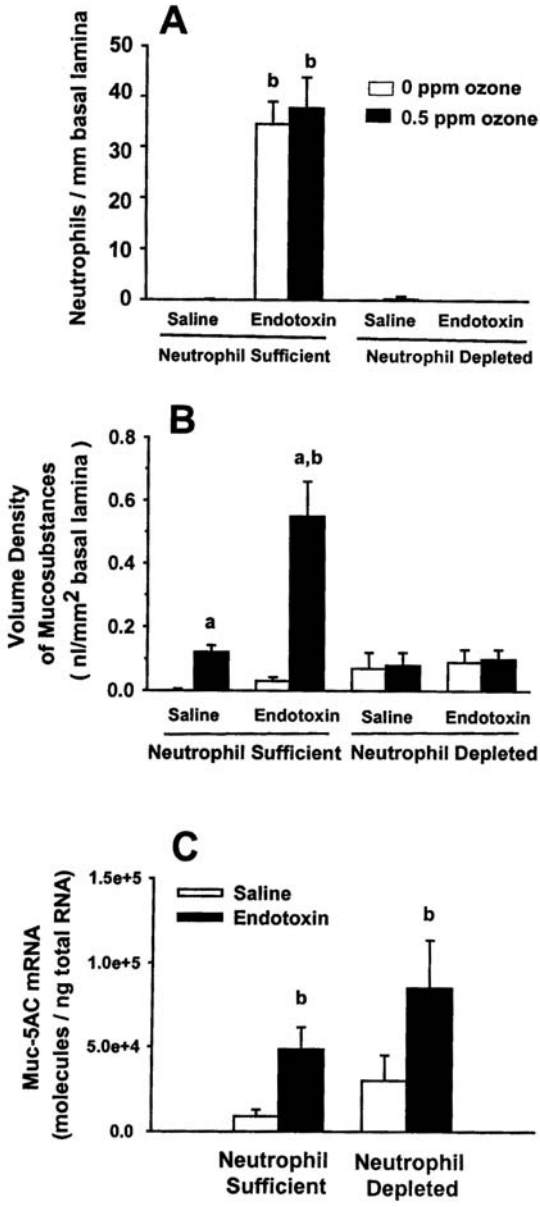


FIG. 4. Effect of neutrophil-depletion on ozone/endotoxin-induced changes in neutrophilic inflammation (A), intraepithelial mucosubstances (B), and mucin gene expression (C) in rat maxilloturbinates. Bars represent group mean± SEM (n= 6 animals/group). a, significantly different from respective group exposed to air. b, significantly different from respective group given intranasal saline (Wagner et al 2001).

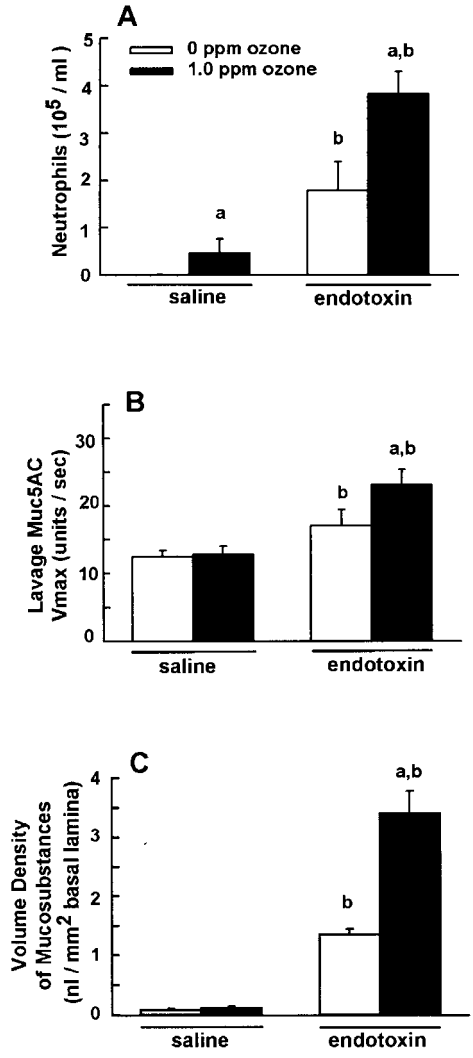


FIG. 5. Effect of ozone inhalation on endotoxin-induced increase in lavaged neutrophils (A), mucus hypersecretion (B) and increase in intraepithelial mucosubstances (C) in pulmonary airways. Bars represent group mean± SEM (n= 6 animals/group). a, significantly different from respective group exposed to air. b, significantly different from respective group given intranasal saline.

Enhancement of endotoxin-induced mucous cell metaplasia by ozone

In 1992, we demonstrated that intranasal instillation of endotoxin causes significant alterations in the mucous apparatus lining axial pulmonary airways of rats (Harkema & Hotchkiss 1992). Specifically, increases in stored mucosubstances and MCM occurred in the respiratory epithelium of airways that normally consist only of ciliated and serous cells. This is in contrast to ozone-induced MCM that occurs in the NTE that contains no secretory cells. Furthermore, unlike endotoxin, ozone exposure has no effect on the mucous apparatus in axial pulmonary airways of rats.

To test the effects of ozone exposure on endotoxin-induced MCM, we treated rats with intranasal endotoxin for two consecutive days and then exposed these rats to ozone (1 ppm, 8 h/day) for two days. Endotoxin caused an increase in the number of neutrophils recovered in lavage fluid that was further increased after exposure to ozone (Fig 5A). Instillation of endotoxin also increased the amount of secreted mucins in lavaged airway fluids compared to those from rats instilled only with saline (Fig. 5B). Ozone alone did not cause mucous hypersecretion, but the combination of ozone exposure with endotoxin enhanced the endotoxin-induced secretion of mucin protein (Muc5AC) in airway fluid. Lastly, endotoxin caused an increase in the amounts of stored intraepithelial mucosubstances in axial airways that were further increased in rats subsequently exposed to ozone (Fig. 5C). Thus, ozone exposure enhances both the secretion and storage of mucin glycoprotein induced by endotoxin instillation.

We have previously shown that endotoxin-induced MCM in the pulmonary airways is partially dependent on neutrophils (Hotchkiss & Harkema 1994). Thus, ozone may augment the metaplasia initiated by endotoxin by increasing the neutrophilic inflammation as suggested by the increase in lavage neutrophils. In the future, neutrophil-depleted rats will be co-exposed to ozone and endotoxin in order to test this hypothesis.

Discussion

On the basis of our experimental results, it is clear that ozone and endotoxin elicit MCM in different locations of the respiratory tract (Fig. 6). Interestingly, each toxicant acts to enhance the metaplastic lesion that is induced by the other toxicant. Specifically, endotoxin enhances ozone-induced metaplasia in the NTE, but does not itself cause metaplasia in the NTE. Conversely, ozone does not cause metaplasia in the respiratory epithelium in pulmonary airways, but it does augment endotoxin-induced MCM in that tissue. This unique, reciprocal potentiation of epithelial cell alterations by ozone and endotoxin extends the toxicological profile of each agent beyond what is described in studies where each toxicant is

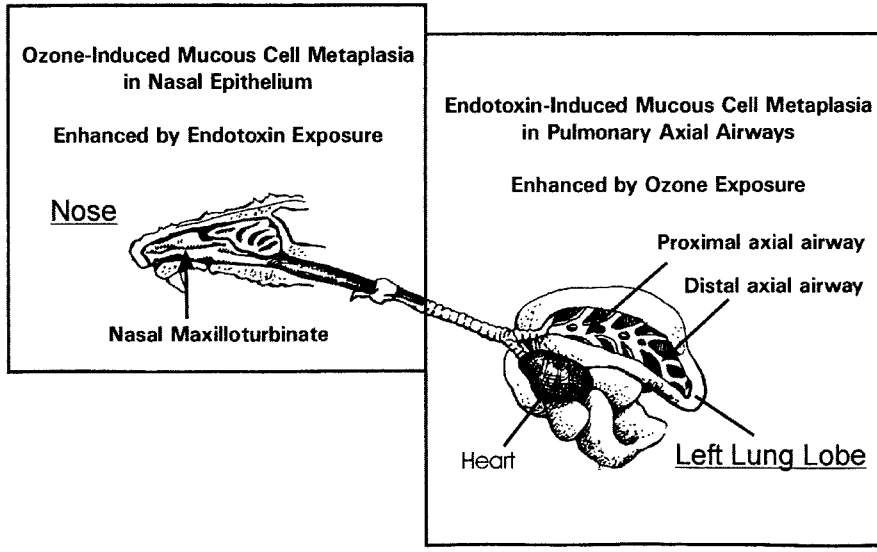


FIG. 6. The reciprocal potentiation by ozone and endotoxin of mucous cell metaplasia in the NTE of the upper airways and respiratory epithelium in pulmonary conducting airways.

used by itself. As such, regulatory standards that are derived primarily from studies that employ a single pollutant may underestimate the risk of adverse health effects when more than one co-pollutant is present. It is likely that both ozone and endotoxin will exacerbate the respiratory responses to other inhaled criteria air pollutants and biogenic substances.

Our results clearly implicate the importance of neutrophils in the development of MCM in both the respiratory epithelium of pulmonary airways and in the NTE lining the proximal nasal airways. Importantly, our results also suggest that neutrophils are not entirely responsible for metaplasia initiated by either ozone in the NTE (only a 60% reduction in metaplasia in neutrophil-depleted animals) (Cho et al 2000), or by endotoxin in the respiratory epithelium (only 50% reduction in metaplasia in neutrophil-depleted animals) (Hotchkiss & Harkema 1994). These results suggest that ozone or endotoxin may be having direct effects on epithelial cells, or that other inflammatory mediators (cellular or soluble) account for the remainder of the metaplastic response. We have recently used an *in vitro* tissue culture system to show that endotoxin, in the absence of neutrophils, increases mucin gene expression in pre-existing secretory cells (Fanucchi et al 1999). This result is consistent with our *in vivo* findings of both ozone- and endotoxin-induced MCM, that indicate that neutrophils are required for some (increased storage of mucosubstances) but not all (hyperplasia and mucin gene over-expression) of events leading to the development of metaplastic lesions.

Whereas ozone- and endotoxin-induced MCM is partially dependent on neutrophilic inflammation, endotoxin enhancement of ozone-induced metaplasia is blocked completely in neutrophil-depleted animals. This difference may be due to the type of epithelium where the metaplasia occurs. Potentiation by endotoxin of ozone-induced metaplasia occurs in the NTE, whereas MCM that is elicited by endotoxin treatment alone occurs in pre-existing secretory cells of the respiratory epithelium. We have yet to examine the contribution made by neutrophilic inflammation to ozone enhancement of endotoxin-induced metaplasia in respiratory epithelium. It may be that the potentiation pathways elicited by both toxicants are entirely mediated by neutrophils.

It is interesting that, in all our studies where neutrophil depletion inhibited MCM, the mucin gene over-expression, that is normally associated with this metaplasia, was unaffected. Thus, the up-regulation of mucin mRNA alone is insufficient for the full phenotypic development of MCM induced by ozone or endotoxin. The protein product of mucin gene translation undergoes considerable modification by glycosyltransferases, which catalyse the addition of fucose and sialic acid among other saccharide groups, and sulfotransferases which add sulfur-containing groups, to the core mucin apoprotein. It is these sugar groups and sulfated residues within mucous cell globules that react histochemically with AB/PAS stains and are used to estimate the amount of intraepithelial mucosubstances. Thus, unmodified, non-glycosylated and non-sulfated mucin apoprotein may be present in epithelial cells, but it is undetected by AB/PAS staining because it lacks reactive groups. One interpretation of these results is that neutrophils mediate the pathways responsible for the glycosylation and sulfation of mucin proteins. In the absence of neutrophils, the signals to modify the core mucin protein by glycosylation might not be present. Alternatively, mucin protein may not be translated despite the transcription of mucin genes. Either possibility requires further study.

Neutrophils are primary sources of inflammatory mediators. Proteases derived by neutrophils (e.g. cathepsins, elastase) are well-known mucous secretagogues in airway epithelial cells (Breuer et al 1993, Takeyama et al 1998). Intra-airway instillation of neutrophil elastase induces MCM in hamster airways (Breuer et al 1985, Jamil et al 1997). Elastase inhibitors can prevent MCM induced by neutrophil elastase (Breur et al 1985) and mucus hypersecretion caused by ozone (Nogami et al 2000). Furthermore, elastase has been shown to increase mucin-specific mRNA and protein expression in cultured human airway cells (Voynow et al 1999). It is unclear how neutrophil-derived proteases induce mucus secretion and mucin gene expression in epithelial cells. Elastase has been shown to bind to an extracellular site on bronchial epithelium prior to initiating metaplasia in hamster airways (Christensen & Alonso 1996). Elastase and or cathepsin G may promote cellular responses by cleaving and thereby activating a protease-activated receptor

(PAR) on airway epithelial cells (Matthews et al 1999). In this regard, mucus secretion in the rat sublingual gland is triggered by activation of a membrane-bound PAR (Kawabata et al 2000b). It is possible that PAR activation on airway epithelial cells may promote pathways that lead to the expression of mucin and glycosyltransferase genes.

A common effector pathway of proteases and inflammatory mediators to induce metaplasia in airway epithelial cells may be the epidermal growth factor receptor (EGFR) system. EGF is an important growth factor for the development of neonatal lungs and is often expressed during repair processes in adult lung (Fisher & Lakshmanan 2000, Takeyama et al 1999). Airway instillation of EGFR ligands into rat airways can lead to the development of MCM, but only after EGFR expression is induced on airway epithelium by prior treatment with tumour necrosis factor (TNF) (Takeyama et al 1999). Exposure of humans to ozone causes expression of EGFR and production of EGFR ligands in the nasal mucosa, suggesting a similar process of activation may occur during ozone-induced mucous cell metaplasia that we observe in the rat NTE (Polosa et al 1999). We have addressed the role of the EGFR system in the development of MCM by using a novel *in vitro* system of cultured nasal explants (Hotchkiss et al 2000). In these studies, neutrophil-derived products enhanced the increase in intraepithelial mucosubstances in respiratory epithelium initiated by treatment of cultures with endotoxin. When treated with kinase inhibitors that block pathways of EGFR signal transduction, we observed an attenuation of the metaplastic response elicited by the neutrophil-derived products. Other recent studies have implicated the EGFR system in MCM in models of allergic airway disease (Shim et al 2000) and in response to cigarette smoke (Takeyama et al 2000). We are presently performing studies to further elucidate the role of the EGFR system in the neutrophil-mediated and toxicant-induced MCM in nasal and pulmonary airways.

Taken together, our results illustrate a unique interaction between two airborne toxicants to alter airway epithelium that would not have been predicted from the known toxicological profile of either pollutant given alone. The importance of these findings is twofold. First, they provide a biological rationale from which to better evaluate the risk of coexposure to ozone and endotoxin. In addition, our results with ozone and endotoxin might be extended to predict the potential airway responses to exposures to other oxidant gases and biogenic substances. Secondly, the mechanism of toxicity in this model of MCM implicates a major role for neutrophilic inflammation. Therapeutic approaches that target neutrophils, their recruitment, or their products (i.e. proteases) may protect from toxicant-induced alterations in the airway mucous apparatus. Increased research efforts are needed to clarify the true risk of exposures to multiple airborne pollutants, and to determine the most effective

interventions to prevent or reverse the overproduction and hypersecretion of mucus in human airways.

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DISCUSSION

Basbaum: What proportion of the metaplastic response is neutrophil independent?

Harkema: At least one-third to one-half the response. In the ozone-treated animals it appears to be less than that. In the endotoxin experiments it looks like it is about one half. The response to ozone looks like it is dominated by the neutrophil influx.

Basbaum: In the case of endotoxin, do you have any idea as to what contributes to the neutrophil-independent induction of MCM?

Harkema: I am just speculating, but using the EGFR blockers we were able to block this in culture. It was interesting that when we put the neutrophils together with the endotoxin in this explant system the amount of enhancement almost brought it up to what we see *in vivo*. We speculated that somehow it may be going through EGFR.

Basbaum: The neutrophil-dependent stimulus may be going through EGFR.

Harkema: I know of your data on this, but I can't explain why the inhibitors also brought down the stored product which is the endotoxin alone.

Basbaum: What about Toll receptors? Have you looked at these?

Harkema: We have just started to look at this. The Toll receptor looks like a promising pathway, especially in the mouse.

Vargaftig: We have contrasting results. We have depleted granulocytes in the murine model in which either LPS or allergen is used. With allergen, whatever we deplete (and we had different procedures not only with antibody but also with vinblastine, because with the antibody we probably have complement depletion, a complicating factor), we completely suppress the eosinophils and MCM persists as before. With LPS we suppress completely different things. I think the granulocytes must be looked at one by one in the different systems. I was very interested in the differences between the Fischer and Brown Norway rats.

Harkema: The Brown Norway rat is much more like the mouse. The Fischer 344 was like your Th1 animal. We find similar results to yours with the Brown Norway rat. The Brown Norway rats already have quite a bit of mucus in their airways

already. If you give them endotoxin, there is no increase in the stored or secreted products in the bronchoalveolar lavage (BAL). But if you give ovalbumin, there is a tremendous increase in those animals. If you combine the two, the neutrophils will go up in the Brown Norway but the eosinophil response is suppressed, as is the amount of stored product. We are not sure whether this is related to secretion or production.

Nettesheim: It would be interesting to look at the recovery after a relatively short exposure to either one of your two agents. What happens after cessation of exposure to the inflammation and the mucus hyperplasia?

Harkema: We know what happens after long-term exposure. We exposed animals for three months, and we waited for three months after this. The first phenotypic change to go away is the inflammation, and the constricted blood vessels enlarge. This happens within a week or so. Surprisingly, bone comes back very quickly. The last thing to come back (and I don't think it ever does completely), is the phenotype of the surface epithelium. We then gave these animals ozone again to see whether they went through this process once more, and interestingly the epithelial cells started making mucus within 48 h, without going through a phase of cell necrosis. This suggests that there is some type of cellular 'memory' of the previous exposure.

Basbaum: On first exposure, how long does it take to get mucin overexpression?

Harkema: With the short exposure, the RNA increases just after one exposure, which is 6 h per day. You can start to see the mucin gene expressed after 24 h. With repeat exposures it stays up. But the stored mucus product appears histologically after 4 d. The question is, are those cells making mucus earlier, but secreting it rapidly without much cytoplasmic storage? During exposure the ozone or the neutrophilic influx may be causing hypersecretion of the newly made mucus. However, our electron micrographs of the epithelium during the ozone exposure do not suggest that secretory granules are being produced at those early times. What was missing in that study was that we didn't do the immunohistochemistry for MUC5AC. This would have been interesting. We would like to go back and see when this is expressed. Perhaps this mucin core protein could be detected in the epithelium prior to the fully glycosylated mucus.

Levitt: This is in the rat, and you'd have a hard time finding a reagent to do that so you could be sure you were looking at the protein.

Davis: Is MUC5B expressed in rat nasal epithelium?

Harkema: We don't know. There are large glands in the nasal mucosa that no one has really looked at with appropriate mucin-specific antibodies.

Davis: It might also be in the surface epithelium.

Randell: The controversy about Toll2 and Toll4 has caused great focus on commercial LPS preparations. At these doses you are probably looking at contaminants that are Toll2 agonists as well as LPS.

Harkema: We have conducted exposures as low as low as 10 ng of endotoxin and we still see the metaplastic response in the pulmonary airways of F344 rats.

Randell: The neutrophil is a great foot soldier, but we think of the macrophage as a smarter, higher-level organizer of the response. Have you looked for macrophages in the progression and resolution of these lesions?

Harkema: We haven't, other than selecting airways that we feel are pretty much free of macrophages. This doesn't mean that they couldn't be involved in the metaplastic process in more distal airways. The nasal septum has lots of mucous goblet cells. If we put endotoxin in the nose, within 4–6 h there is an influx of neutrophils that causes hypersecretion and a depletion of stored mucous products within the epithelium. By 24 h post-exposure the neutrophilic inflammation resolves and the stored muco-substances are restored in the nasal epithelium. We believe that neutrophil products, like elastase, are driving the endotoxin-induced hypersecretion.

Basbaum: While we are on the subject of Tolls, what is the best way of examining Toll involvement in animals?

Randell: There are two naturally occurring Toll4-deficient mice. Dr Akira in Japan has developed Toll2 knockouts. These have greatly facilitated studies in mice. In human cells and tissues, it is more difficult. But there are good inhibitory antibodies for Toll2.

Basbaum: Are those commercially available?

Randell: Generally not.

Fahy: I find the effects of neutrophils on goblet cells a little confusing. It seems that there is a mucin secretagogue effect, presumably driven by elastase. Then there is some other neutrophil-associated effect that causes the metaplasia. What do you think about this? Do you think a neutrophil elastase inhibitor would prevent ozone-induced goblet cell hyperplasia? Do you think neutrophils in some instances cause metaplasia and don't cause secretion?

Harkema: I think the confusion is caused by the different types of epithelium. The epithelium I showed you to start with, where we depleted the neutrophils and exposed to ozone, was devoid of mucous cells to begin with. The neutrophil has to be involved somehow, because when you deplete neutrophils this almost wipes out the whole mucous cell metaplasia. In contrast, the rat nasal septum normally contains many mucous cells with copious amounts of stored mucosubstances. The stored product is markedly reduced concomitantly with the influx of neutrophils after endotoxin exposure. In the short term it definitely contributes to the secretion. I think this is because of the elastases or other proteases that are released from the neutrophils that cause mucus hypersecretion. Therefore the response of the epithelium to endotoxin is dependent on the time post exposure and the type of epithelium.

Basbaum: Mary Rose, what about your work on elastase and MUC5?

Rose: This is Judith Voynow's work, in which we collaborated on the first study (Voynow et al 1999). She has shown that neutrophil elastase increases both *MUC5AC* and *MUC4* (J. A. Voynow, personal communication) steady-state mRNA expression and that MUC5AC is regulated at the post-transcriptional level (Voynow et al 1999). TNFα also regulates *MUC5AC* mRNA expression post-transcriptionally (Borchers et al 1999), as does IL8 (M. V. Bautista, Y. Chen and M. C. Rose, unpublished results) and these inflammatory mediators increase the stability of mucin mRNA transcripts. The question to answer now is what these mediators are doing to RNA-binding proteins and to determine whether these proteins bind to *cis*-sequences in the 3′ UTR of the *MUC5AC* gene.

Harkema: These data support what we saw.

Rose: It is pretty well accepted that neutrophil elastase increases mucin secretion. This is a short-term effect. Having some inflammatory mediators around for a longer time may increase mucin gene expression and thus mucin production. Then you have to ask what they are doing to cells that may be proliferating and will ultimately differentiate into goblet cells.

Harkema: In the ozone model we see epithelial proliferation, and eliminating the neutrophils does not alter this response. If you count the number of epithelial cells you see a hyperplastic response, but they won't differentiate into mucous cells without the influx of neutrophils.

Disse: Is it possible that the lower airways react differently from the nose? If we consider the clinical work of Magnussen's group on acute and repeated ozone exposure, they saw a pronounced decrease in FEV_1 (forced expiratory volume in 1 s) and neutrophil influx response in BAL (Jorres et al 2000). If the exposure was repeated on four consecutive days then there was complete tachyphalaxis to both effects.

Harkema: With ozone, the response depends on the concentration and duration of exposure. If you expose rats to a high concentration for long enough, you will see the neutrophil influx in the lung as well as the nose. Using the ozone exposure regime I described, we didn't induce MCM or inflammation in the pulmonary airways. However, we have recently observed that similar ozone exposures will enhance the endotoxin-induced MCM in the pulmonary airways of rats.

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Cytokine regulation of mucus production in a model of allergic asthma

Lauren Cohn*, Laurie Whittaker*, Naiqian Niu* and Robert J. Homer†‡

**Section of Pulmonary and Critical Care Medicine and †Department of Pathology, Yale University School of Medicine, New Haven, CT 06520 and ‡Pathology and Laboratory Medicine Service, V. A. Connecticut Health Care System, West Haven, CT 06516, USA*

Abstract. Mucus hyperproduction in asthma results from airway inflammation and contributes to clinical symptoms, airway obstruction and mortality. Th2 lymphocytes and eosinophils dominate the airway inflammatory infiltrate. We investigated the role of different lymphocyte subsets and their cytokines in the stimulation of mucus production using a system in which T cell receptor (TCR) transgenic $CD4^+$ Th cells were generated *in vitro*, transferred into recipient mice and activated in the respiratory tract with inhaled antigen. Th2 cells induced mucus production and eosinophilic inflammation, while mice that received Th1 cells exhibited airway inflammation without mucus. Th1 cells failed to stimulate mucus due to the inhibitory effects of interferon (IFN)γ. Mucus was induced by Th2 cells in the absence of interleukin (IL)4, IL5, eosinophils and mast cells, but not without IL4Rα signalling. Th2 cells lacking IL13 could not stimulate mucus production, despite the presence of airway inflammation. IL9 also stimulates mucus through an IL13-mediated pathway. Using bone marrow chimeras we show that IL13 acts on structural cells in the lung, most likely by direct stimulation of epithelial cells, and not through intermediate inflammatory cells. In asthma, airway inflammation with $CD4^+$ Th2 cells stimulates mucus production by a single pathway mediated by IL13.

2002 Mucus hypersecretion in respiratory disease. Wiley, Chichester (Novartis Foundation Symposium 248) p 201–220

Asthma is a chronic inflammatory disease of the bronchial airways defined by intermittent episodes of airway obstruction. In patients with asthma, excess mucus production leads to wheezing, coughing and contributes significantly to airway obstruction (Moreno et al 1986). Inflammation in the bronchial airways of asthmatics is believed to stimulate mucus production. Airway biopsies in asthmatics show infiltration of the mucosa and submucosa with lymphocytes, eosinophils, mast cells, and hyperplasia of goblet cells and submucosal glands (Ollerenshaw & Woolcock 1992). In autopsy specimens from patients who died in status

asthmaticus, obstructing plugs of mucus and cellular debris have been identified in the small airways.

T lymphocytes appear to have an important function in asthma and might account for the persistence of inflammation as they regulate other cells that are believed to be important in inflammation of the airways. Virtually all adaptive immune responses require the action of antigen activated $CD4^+$ T cells. Evidence from many different approaches suggests that activated T cells can differentiate into effector cells with different functional properties (Cher & Mosmann 1987). $CD4^+$ Th1 cells are specialized to activate macrophages, which they accomplish by secreting the potent macrophage activating cytokine, interferon (IFN)γ. These immune responses are highly effective against microbial invasion, particularly intracellular organisms. $CD4^+$ Th2 cells make a panel of cytokines including interleukin (IL)4, IL5, IL9, IL10 and IL13. Th2 cells are particularly potent in activating B cells to secrete antibody, and are essential for IgE production (Finkelman et al 1986). IL5 influences eosinophil differentiation, maturation and activation (Resnick & Weller 1993). The obvious associations in asthma with high IgE levels and airway eosinophilia have implicated the Th2 cell and its cytokines in the chronic inflammatory process.

Bronchial biopsies and bronchoalveolar lavage (BAL) indicate that both Th1 and Th2 cells are present in the lungs of asthmatics (Cembrzynska-Nowak et al 1993, Robinson et al 1992). But, Th2 cells are represented at an increased frequency and are activated locally after specific antigen challenge (Bentley et al 1993). Furthermore, the presence of cells secreting IL4 and IL5 correlate with airway obstruction and the number of eosinophils in the sputum correlates with its expectorated volume (Bradley et al 1991, Tanizaki et al 1993). It appears that the inflammatory response in many asthmatics is skewed towards Th2 and the presence of Th2 cells is associated with disease severity. In contrast, it has been hypothesized that Th1 cells, if activated in the lung by infection with mycobacterium or measles viruses, may reduce the incidence of asthma and atopy (von Mutius 2001).

Animal models have confirmed the importance of $CD4^+$ T cells in the development of allergic inflammation and airway hyperresponsiveness (AHR) (Wills-Karp 1999). Our own studies in mice have shown that Th2 cells can induce the histological and physiological changes associated with asthma, including mucus hypersecretion (Cohn et al 1997, 1998). Herein we review experiments that reveal the mechanisms by which $CD4^+$ Th cells regulate mucus production.

Results and discussion

We developed a system to test the role of $CD4^+$ Th1 or Th2 cells when activated in the respiratory tract of mice. Antigen-specific $CD4^+$ T cells were generated

from mice transgenic for a T cell receptor recognizing ovalbumin peptide 323–339, as previously described (Cohn et al 1997). $CD4^+$ T cells from these mice were induced to differentiate into either Th1 or Th2 cells by *in vitro* culture with cytokines known to induce selective differentiation. These cells were transferred into syngeneic recipient mice, which were then exposed to inhaled ovalbumin (OVA). Upon transfer of polarized Th1 or Th2 cells, the transferred cells retained their cytokine profiles and when exposed to inhaled antigen, the transferred transgenic $CD4^+$ T cells were specifically recruited to the respiratory tract. There was marked inflammation in the respiratory tract of mice that received either Th1 or Th2 cells. The characteristics of the inflammatory processes in the two groups of mice were quite different, as determined by lung histology and differential analysis of BAL cells (Table 1). Despite similar degrees of inflammation, mice that received Th1 cells and inhaled OVA had inflammation with neutrophils and lymphocytes, while mice that received Th2 cells had eosinophilic and lymphocytic inflammation. Th2 cells, but not Th1 cells, induced mucus hypersecretion in the bronchial epithelium (Fig. 1). Increased mucus staining of the airway epithelium was shown in histochemical staining with DPAS, alcian blue and mucicarmine, indicating an increase in acid and neutral mucins. Epithelial cells were markedly hypertrophied in mice that received Th2, but not Th1, cells and inhaled OVA. Mucins in the BAL fluid collected from mice that received

TABLE 1 Effects of Th1 and Th2 cells *in vivo*

	Type of $CD4^+$ T cell transferred		
Analysis	*Th1*	*Th2*	*Th1 + Th2 (1:1)*
Cytokines			
Produced by transferred cells	IFNγ	IL4, IL5, IL13	IFNγ IL4, IL5, IL13
Airway Inflammation			
BAL			
Neutrophils	+++	−	+++
Eosinophils	−	+++	+
Increased mucus staining	−	+++	+

OVA trangenic $CD4^+$ Th cells were stimulated *in vitro* with cytokines known to skew towards Th1 or Th2. Th1, Th2 or Th1 + Th2 cells were transferred into recipient mice and mice were exposed to inhaled OVA. The transferred cells were recruited to the respiratory tract and the cytokines they produced and their effects are shown.

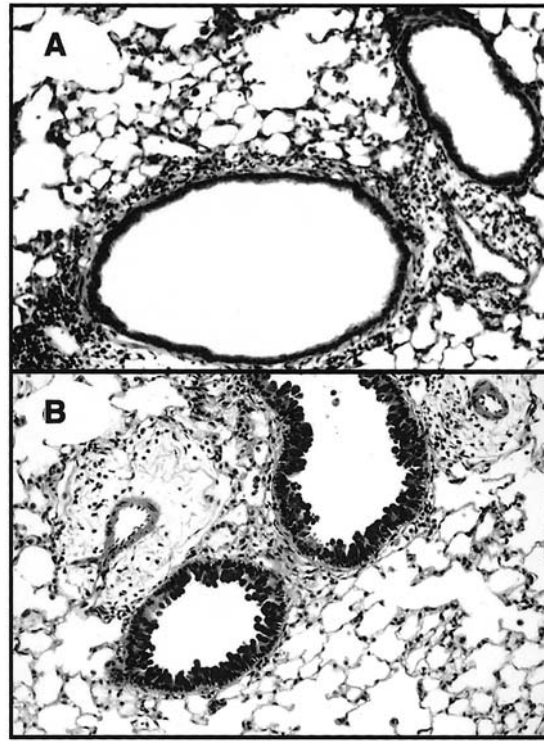

FIG. 1. Lung histology in mice that received transfer of OVA-specific Th1 or Th2 and seven days of aerosolized OVA. (A) Lungs from mice that received Th1 cells and inhaled OVA show an inflammatory infiltrate surrounding the airways and did not exhibit staining for mucus; DPAS, 100× . (B) Lungs from mice that received Th2 cells and inhaled OVA show airway epithelial hypertrophy and increased dark-staining mucus in bronchial epithelium; DPAS, 100× .

Th2 cells and inhaled OVA were increased when compared with BAL fluid from mice that received inhaled OVA and Th1 cells or no cells.

These studies from our laboratory showed that only certain inflammatory processes in the respiratory tract could stimulate mucus production. Th2 cells stimulated an inflammatory response that led to mucus production, while Th1 cell inflammation did not. There were multiple potential mechanisms of Th2-induced mucus production. Eosinophil activation in the airways has been associated with mucus production in animal and human studies. Mediators produced by eosinophils, including eosinophil cationic protein, platelet activating factor and leukotrienes have been shown to stimulate mucus secretion. Mast cells, upon activation, produce mucous secretagogues like histamine, leukotrienes

and Th2 cytokines (Galli 1997). Furthermore, Th2 cytokines themselves have been associated with increased mucus production. There was a correlation of IL4 mRNA expression with mucus production (Budhecha et al 1997). And, in transgenic mice that over-express the cytokines IL4, IL5, IL9 and IL13 selectively in the lung (Lee et al 1997, Rankin et al 1996, Temann et al 1998, Zhu et al 1999) there was excess mucus associated with airway inflammation. While it was clear that Th2 cells could stimulate mucus, it was difficult to determine if cytokines themselves were stimulating the epithelial response or if other inflammatory cells and their mediators were responsible for mucus induction.

To dissect the precise role of Th2 cells in the induction of airway mucus production, we generated Th2 cells from cytokine-deficient mice and observed their *in vivo* effects after transfer and exposure to inhaled antigen (Cohn et al 1999a). IL5 is essential for augmenting eosinophil recruitment to sites of inflammation. To determine whether eosinophils were required for mucus induction by Th2 cells, we generated IL5-deficient Th2 cells *in vitro*, transferred them into IL5-deficient recipient mice and exposed those mice to inhaled antigen. These Th2 cells produced high levels of IL4 and IL13, but no IL5 (Fig. 2A). BAL eosinophilia was abolished in the absence of IL5, yet mucus staining was induced at similar levels in mice that received IL5-producing or IL5-deficient Th2 cells (Fig. 2B). To determine whether Th2-induced mucus production was a result of mast cell activation, we transferred Th2 cells into wild-type and mast-cell-deficient (W/W^v) mice. After exposure to inhaled OVA, both mast-cell-deficient and wild-type mice exhibited equivalent mucus staining in the bronchial epithelium. Thus, these experiments showed that Th2 cells could stimulate mucus independent of eosinophils, IL5 and mast cells.

To test whether IL-4 was critical for mucus induction by Th2 cells, we generated IL4-deficient Th2 cells which produced no IL4, but secreted high levels of IL5 and IL13. Mucus was induced by IL4-deficient Th2 cells at similar levels when compared to mice that received IL4-producing Th2 cells. Thus, IL4 was not essential for Th2-induced mucus. We next transferred Th2 cells into IL4Rα-deficient mice. IL4Rα is the common receptor chain that binds both IL4 and IL13 and signals by a common transduction mechanism through Stat6. Mucus was not induced by Th2 cells in IL4Rα-deficient mice, despite the presence of activated, cytokine-producing Th2 cells in the respiratory tract (Table 2). By blocking the effects of IL4 and IL13, we inhibited mucus induction.

These studies suggested that IL13 could stimulate mucus in the absence of IL4. Yet, it was not clear whether IL4 could compensate for IL13 in this process. IL4 and IL13 have many overlapping functions due to their common signalling mechanism and both cytokines play a role in Th2 cell generation, IgE production and most functions in host immunity to parasites. IL13 has been shown to have a unique function to control gastrointestinal expulsion of the nematode,

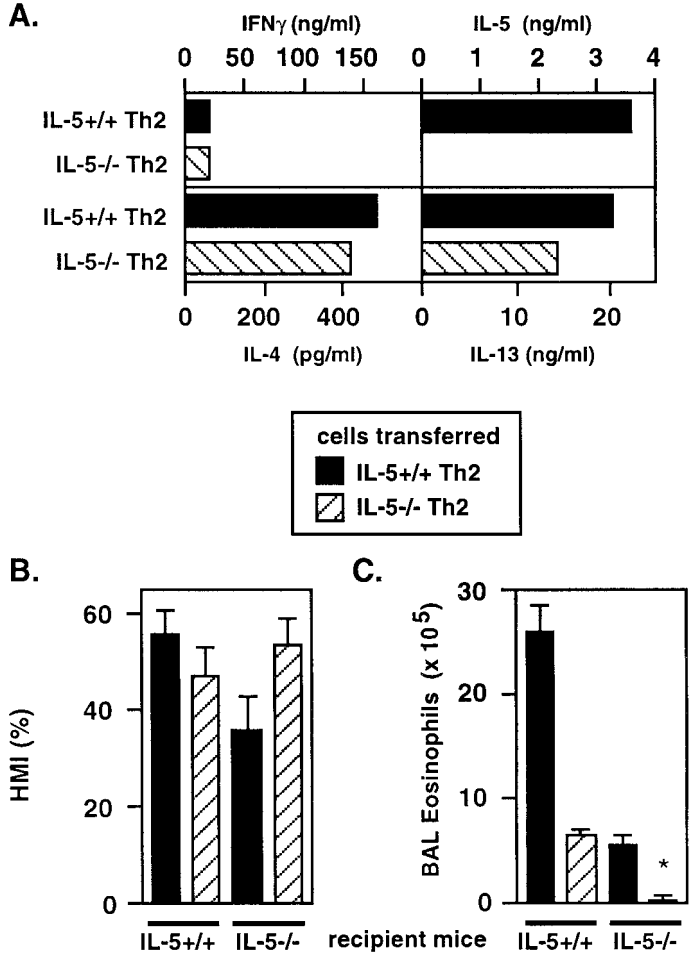

FIG. 2. Effects of IL5-deficient Th2 cells on mucus staining. $IL5^{+/+}$ or $IL5^{-/-}$ Th2 cells were transferred into $IL5^{+/+}$ or $IL5^{-/-}$ recipient mice and mice were exposed to inhaled OVA. (A) Cytokine production by OVA-specific $IL5^{+/+}$ and $IL5^{-/-}$ Th2 cells. At the time of transfer into recipient mice, *in vitro* generated $IL5^{+/+}$ and $IL5^{-/-}$ Th2 cells were cultured with antigen-presenting cells (APCs) and OVA. Supernatants were collected after 48 h and cytokine ELISAs were performed. (B) A histological mucus index (HMI) was performed on lung sections stained with PAS. The HMI is equivalent to the linear percentage of epithelium positive for mucus (Cohn et al 1999a). Mean HMI (± SEM) is shown. (C) BAL eosinophils recovered from mice after exposure to inhaled OVA. Differential counts were performed on cytospins of cells recovered from BAL of individual mice. Mean cell counts (SEM) are shown (n= 5 mice per group). One experiment is shown and is representative of three experiments. Statistical significance was determined by unpaired Student's t-test. *P< 0.03 for $IL5^{-/-}$ Th2/$IL5^{-/-}$ recipient compared to other groups. Reproduced from Cohn et al (1999a), with permission from the American Association of Immunologists.

TABLE 2 Effects of Th cells and cytokines on mucus production

*Cells transferred**	*Recipient mice*	*BAL eosinophilia*	*Mucus staining*
Th1	Wild-type	–	–
Th2	Wild-type	+	+
IL5$^{-/-}$ Th2	IL5$^{-/-}$	–	+
IL4$^{-/-}$ Th2	IL4$^{-/-}$	↓	+
Th2	IL4Rα$^{-/-}$	–	–
IL13$^{-/-}$ Th2	IL13$^{-/-}$	↓	–

*In these experiments, cytokine-deficient mice were used to generate OVA-specific Th2 cells lacking one key Th2 cytokine. Once generated, CD4^{+} Th cells were transferred into recipient mice, mice were exposed to inhaled OVA and the transferred cells were recruited to the respiratory tract. The effects in the respiratory tract of activation of these transferred cells are shown.

Nippostrongylus braziliensis. A number of studies suggest that like IL13, IL4 can stimulate mucus, but in those studies, IL13-producing cells, including mast cells, eosinophils and lymphocytes, were still present in the animals and might have contributed to the cytokine milieu. In IL4 transgenic mice, mucus production was increased, but these animals had marked inflammation in the airways with lymphocytes and eosinophils (Rankin et al 1996). Mucus and eosinophils were induced when IL4 was instilled into the airways of lymphocyte deficient mice (Grunig et al 1998). To test whether IL4 and IL13 had reciprocal functions in the induction of mucus, we generated Th2 cells from IL13 deficient mice, transferred them into IL13-deficient mice and exposed those mice to inhaled antigen. In these mice, mucus was not induced by Th2 cells that did not produce IL13, despite their production of IL4 and IL5 (Table 2). Thus, Th2 cells appeared to stimulate mucus by a unique, IL13-mediated pathway.

IL9 is another Th2 cytokine that has been shown to influence mucus production. IL9 may play an important role in asthma pathogenesis because of its chromosomal location in humans and its association with airway hyperresponsiveness in mice (Nicolaides et al 1997). IL9 stimulates T cell growth, mast cell growth and differentiation and IgE production (Renauld et al 1995). IL9 transgenic mice have marked mucus metaplasia and airway inflammation with increased numbers of Th2 cells, eosinophils and mast cells in the respiratory tract (Temann et al 1998). Because IL9 also stimulated a marked inflammatory infiltrate, it was not clear whether IL9 directly influenced mucus production. To determine whether IL9 acts independently of IL13 in the induction of mucus, we bred the IL9 transgenic mice to IL4Rα-deficient mice. There was no mucus staining in the airways of these animals (Table 2). Thus, IL9 stimulates mucus by an IL4Rα-mediated pathway. To confirm these findings, we administered an IL13

inhibitor to IL9 transgenic mice and showed that mucus induction was blocked. These studies show definitively that IL9 stimulates mucus by increasing the production of IL13. One possible explanation is that IL9 stimulates mast cells to produce IL13.

In summary, Th2 cells are believed to orchestrate the inflammatory cascade that results in asthma. Th2 cells have been shown to stimulate airway epithelial mucus production in mice by a single IL13-mediated pathway. Mucus can be induced in the absence of IL4, IL5 and IL9, mast cells and eosinophils, but not in the absence of IL13.

We next wanted to investigate how IL13 stimulated mucus. IL13 might act directly on airway epithelial cells or might activate another inflammatory pathway to stimulate the epithelium. We generated bone marrow chimeras using IL4Rα deficient mice. Irradiated wild-type or IL4Rα-deficient host mice were transplanted with wild-type or IL4Rα-deficient donor bone marrow. Mucus was induced only when IL4Rα was present on host cells, including structural cells in the lung. If IL4Rα was present on donor bone marrow-derived cells, but not on the structural host cells, mucus was not induced. Thus, IL13 most likely acts by binding its receptor on the epithelium itself, and not by influencing an inflammatory cell intermediate.

Th1 and Th2 have both been identified in the airways of asthmatics (Cembrzynska-Nowak et al 1993, Robinson et al 1992). Epidemiological studies and animal models of asthma suggest that Th1 immune responses protect against asthma (Erb et al 1998, von Mutius 2001). To study how Th1 cells regulate Th2-induced inflammation we co-transferred Th1 and Th2 cells into mice and exposed those mice to inhaled antigen (Cohn et al 1999b). As previously shown, mucus staining of the airway epithelium was increased after Th2 cell activation in the lung, but not after transfer of Th1 cells. When Th1 and Th2 cells were transferred together mucus production was reduced (Fig. 3). This inhibitory effect on mucus production was due to Th1 production of IFNγ. When Th1 cells were transferred into IFNγR-deficient mice, which could not respond to IFNγ, mucus was induced. Thus, the inability of Th1 cells to produce mucus is due to the inhibitory effect of IFNγ, since Th1 cells were capable of stimulating mucus when IFNγ effects were blocked.

Having established that Th1 cells, by producing IFNγ, inhibit Th2-induced mucus production, we next assessed the mechanism of action of IFNγ. It was possible that IFNγ blocked Th2 cell activity by inhibiting their migration to or activation in the lung. To determine whether Th2 cells were present and active in the lungs after co-transfer of Th1 and Th2 cells, we measured cytokines in the BAL fluid recovered after transfer of cells and exposure to inhaled OVA. BAL fluid contained IL4, IL5 and IL13 protein at levels comparable to that seen in mice exposed to OVA after transfer of only Th2 cells. IFNγ was also present in the

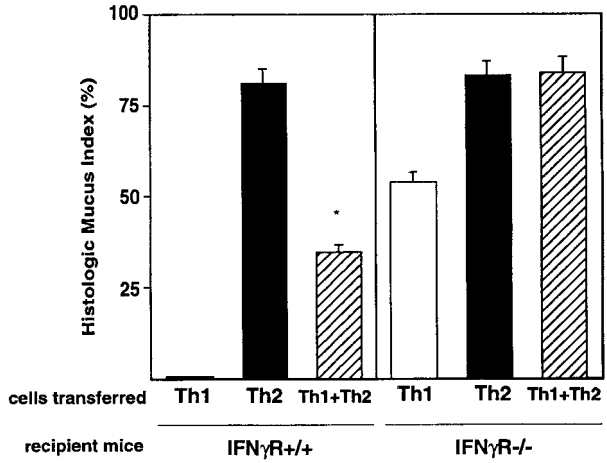

FIG. 3. Airway mucus production after co-transfer of Th1 and Th2 cells. Th1, Th2 or Th1+ Th2 cells (2.5×10^6 cells from each population) were transferred into IFNγR$^{+/+}$ (BALB/c) or IFNγR$^{-/-}$ recipient mice and mice were exposed to inhaled OVA. Mucus staining was assessed on PAS-stained lung sections. Data represents mean HMI (± SEM) (n= 4 mice per group). One experiment is shown and is representative of two experiments. Statistical significance was determined by unpaired Student's t-test. *P< 0.0004 Th2 vs. Th1+ Th2. Reproduced from Cohn et al (1999b), with permission from The Rockefeller University Press.

BAL fluid of these mice at levels similar to BAL from mice that received only Th1 cells and inhaled OVA. These data suggested that both Th1 and Th2 cells were present and actively secreting cytokines in the airways in mice that received Th1+ Th2 cells and inhaled OVA. Thus, Th1 cells do not inhibit the migration or cytokine production of Th2 cells. In IFNγR-deficient mice, the transferred DO11.10 Th2 cells expressed wild-type IFNγR and therefore, were able to respond to IFNγ secreted by Th1 cells. If Th1 cells were blocking Th2 cell function directly, inhibition of eosinophilia and mucus production would still be seen in IFNγR deficient mice. Yet, the inhibitory effects of Th1 cells on Th2 cell induced mucus production were abolished in IFNγR$^{-/-}$ mice. These studies indicate definitively that Th1 cells inhibit Th2-induced eosinophilia and mucus production through the action of IFNγ on target tissues in recipient mice and not by direct inhibition of Th2 cell activity.

IFNγ has been shown to inhibit some epithelial cell functions. In cultured gut epithelial cells, mucus and Cl^- secretion were decreased after IFNγ administration (Holmgren et al 1989, Takahashi et al 1998). Yet, the effects of IFNγ on airway mucus had not been previously shown. Mucus production is not a feature of Th1-mediated pulmonary diseases in humans. In *Mycobacterium tuberculosis*

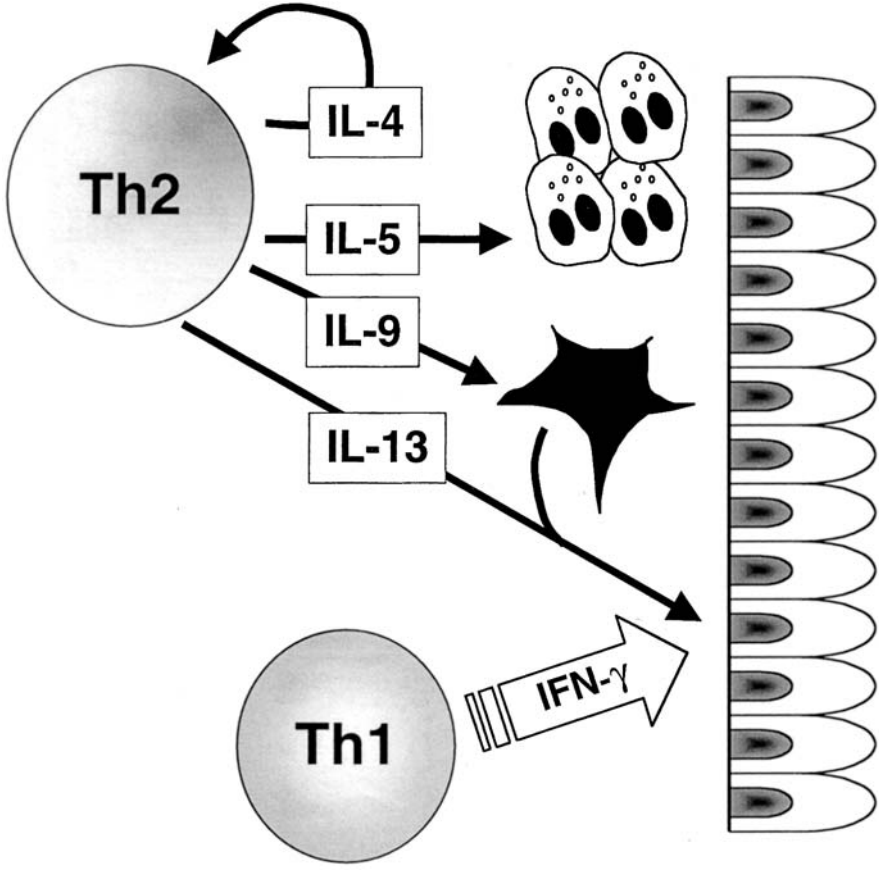

FIG. 4. $CD4^+$ Th cell regulation of mucus production. Th2 cells stimulate mucus through the production of IL13. Other Th2 cytokines, including IL4, IL5 and IL9 play important roles in regulating the inflammatory response in asthma. IL4 stimulates IgE production and promotes further Th2 cell development. IL5 stimulates eosinophil maturation, recruitment and activation. IL9 promotes mast cell growth and differentiation. IFNγ, produced by Th1 cells, inhibits mucus production by acting on the target tissue, presumably through effects on the epithelium.

infection and sarcoidosis, IFNγ-producing $CD4^+$ T cells have been identified in lung biopsies and in BAL (Bergeron et al 1997). Lymphocytic infiltrates in both conditions sometimes involve the airways, but are more common in the parenchyma. Although the lack of mucus production in these diseases may relate to the location of these infiltrates, it is possible that IFNγ is suppressing mucus production.

Th1 cells were capable of stimulating mucus when the inhibitory effects of IFNγ were blocked. This finding raised the possibility that other $CD4^+$ Th-induced inflammatory pathways could stimulate mucus production, possibly mimicking other diseases with mucus hypersecretion, like chronic bronchitis or cystic fibrosis. Alternatively, IL13 was responsible for stimulating mucus in the absence of IFNγ. Th1 cells in these experiments produced minimal IL13; in fact Th1 cells produced 100-fold less IL13 than Th2 cells. To investigate the mechanism of mucus induction by Th1 cells, we administered an IL13 inhibitor to IFNγR-deficient mice after transfer of Th1 cells. Mucus was no longer induced in these mice, showing that Th1 cell production of IL13 is responsible for the induction of mucus. These experiments highlight the ability of very low levels of IL13 to stimulate mucus.

Through secretion of cytokines, each CD4 Th cell population exerts its effects on goblet cells in the airway. Th2 cells through the production of IL13 stimulate mucus, most likely by binding IL4Rα on epithelial cells. IL13 is highly potent in its effect, suggesting that pharmacological intervention might require total inhibition of IL13. Th1 cells inhibit mucus production through the secretion of IFNγ (Fig. 4). The precise molecular pathways by which these cytokines regulate mucin genes are yet unknown. Further understanding of these pathways may help to design new therapies to suppress mucus production, thereby reducing airway obstruction in asthma.

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DISCUSSION

Basbaum: If I understand correctly, in the mouse there is an absolute requirement for IL13. Of the two types of receptor — IL4Rα dimerized with γC, versus IL4Rα dimerized with IL13Rα1 — it is the second one that is responsible for the induction of mucus by goblet cells in airways.

Cohn: IL4 and IL13 have so many overlapping functions. This mucus response is one of a couple of unique IL13 responses that have been shown. The question is, how does this receptor, its major signalling component being shared between IL4 and IL13, signal differentially in response to IL13. The differential signalling isn't well defined, but it makes sense, because there are many cytokine receptors that share a single chain and have vastly different functions. Both the IL4 and IL13 receptors have been shown to be present on the epithelium. I don't know whether human and mouse are the same regarding epithelial responses to cytokines, specifically whether the human, but not the mouse, responds to IL4 and IL9. Roy Levitt looked at primary bronchial epithelial cells from humans cultured in IL9 and showed that mucus was induced. One would assume that there are very few, if any, lymphoid cells present in these preparations, suggesting that IL9 can directly stimulate mucus. Similar findings were obtained by Dr Basbaum using BAL fluid from asthmatic dogs and humans, and stimulating a human bronchial epithelial cell line. Our studies show that IL9 cannot stimulate mucus directly and must work by inducing the secretion of IL13.

Levitt: This is a puzzling result. Jamila Louahed did primary human epithelial culture. In this case IL9 stimulation produced an up-regulation consistent with the results Carol Basbaum has found. This suggests that IL13 is not needed unless those epithelial cells are making their own IL13.

Cohn: John Fahy, didn't you identify some IL13 in human epithelial cells?

Fahy: In some preliminary experiments we have found IL13 gene expression in epithelial cells. However, I can't rule out that there aren't some inflammatory cells mixed in.

Levitt: We haven't tried inhibiting with neutralizing antibody IL13 to see whether we can eliminate the IL9 response.

Cohn: The experiment we would need to do to show this is to take epithelial cell cultures and give enough IL13 blocker or antibody, and then give the IL9.

Levitt: You could use IL13 as your control and show that it (IL13 blocker or IL13 neutralizing antibody) blocked IL13 but not IL9. It is doable.

Verdugo: What is the mucus index that you use, and how do you validate it?

Cohn: My pathologist colleague, Dr Robert Homer, counts the number of PAS-stained cells along a certain distance of linear epithelium. He always includes a certain number of small airways and large airways. If the sections haven't been cut centrally, he asks me for a central section. I'm not sure how we could validate it: can you suggest a way?

Verdugo: You could validate it in terms of mucus production. What you are looking at is the storage of mucus in granules inside the goblet cells.

Cohn: Validating these systems is a problem. People have mentioned to us before that the method we use is not precise. There are some more complex ways of looking at the volume of PAS-staining material, but then how do we look through the whole lung section? It is very complicated.

Davis: Aren't you coming from a background of zero? In a normal mouse lung there are virtually no PAS-positive cells.

Cohn: Yes. It is pretty obvious when there is mucus induction and when there isn't in our systems. We usually have enough mice in each experimental group such that the results separate out nicely.

Nadel: In your system, the IL4 receptor is important. IL13 seems to be a critical mediator. Is the EGF receptor involved in the cascade? It is in all the models that I've studied. IL13 induces IL8 or IL8-like molecules in airway epithelium. Is this a significant part of the response? The reason I ask is because in the rat ovalbumin-induced mucus hypersecretion is completely blocked by an IL8 antibody.

Cohn: I don't believe that IL8 is involved, although we've never tested it. When we block the function of IL13, it doesn't matter which cytokines are present, nothing has an effect. I would be surprised if IL8 was not present in these mouse lungs, since there is so much inflammation and there is such redundancy in induction of inflammatory mediators. Another question concerns whether this is the same signalling pathway through which IL8 is operating. We have only used *in vivo* systems and haven't addressed these issues *in vitro*.

Nadel: This is *invivo*: I'm asking whether if you gave an IL8 antibody this would block mucus production. Or if you give an EGF tyrosine kinase inhibitor, would this block mucus production?

Cohn: I have no idea. My gut feeling is that the EGF receptor is probably not involved. I only say this for one reason. In our studies with the Th2 cell transfers, when we eliminate NF-κB signalling in recipient mice by using a p50 knockout mouse, we get perfectly normal mucus production in those mice. I think that we are therefore working through a different pathway. IL13 doesn't seem to go down the common EGF pathway.

Rose: Perhaps IL13 leads to goblet cell production in the airways, in that it activates genes and pathways that lead to *trans*-differentiation of cells in the murine airway epithelium or to differentiation of progenitor cells, which ultimately arise from stem cells, into goblet cells. It doesn't appear from our data (Rose et al 2000) or those of others that IL13 actually up-regulates *MUC5AC* mucin gene expression *in vitro*. We need to distinguish between IL13 affecting the appearance of goblet cells, which endogenously express MUC5AC, and IL13 directly regulating *MUC5AC* mucin gene expression.

Levitt: In human primary cell cultures over a relatively short period we did see the cytokine up-regulation of mucin gene transcripts.

Vargaftig: The IL4Rα knockouts cannot transduce a signal for both IL4 and IL13, when the shared receptor is considered. Nevertheless, there is another IL13 receptor which is separate for IL4. If you administer IL13 directly to those IL4Rα knockout animals, will you get an effect? If so, this would have to be accounted for by a non-IL4-related receptor.

Cohn: It appears that IL13 acts to induce mucus only using this classical IL13R since in addition to my laboratory, other groups have shown that antigen-immunized IL4Rα knockout mice don't produce mucus.

Vargaftig: What about other effects? We find that one-half of the eosinophilia persists in those animals, but not in the bronchoalveolar lavage.

Cohn: We don't see any eosinophils in the lung. Perhaps our different findings are related to the fact that we use a different system to activate Th2 cells. We transfer *in vitro*-generated Th2 cells rather than immunizing mice with antigen.

Harkema: Have you looked at any other epithelia in these animals? I have always been troubled that murine pulmonary airways have Clara cells rather than mucous cells, and for some reason they are explicitly sensitive to these interleukin/Th2 responses. If you went higher up into the nasal pharynx or nose, which normally have some mucous cells, would you see the same increase in mucus? This may be more like the human airway.

Cohn: We could do that. They are obviously getting the antigen in their nose. I don't know whether they have mucus to start with.

Tesfaigzi: We have looked at endogenous mucous cells in areas where there are mucous cells already. These numbers don't increase. There seems to be a difference between existing mucous cells and ones that develop in areas that normally lack mucous cells. Do the different populations of secretory cells respond differently?

Jeffery: When we started our research, the idea at the time was that the goblet cell was a fully differentiated end-stage cell, and that it couldn't divide. The electron microscopy and experimental work that we did showed that this was totally wrong. We could find dividing goblet cells. In our rat population, in response to tobacco smoke, 27% of the dividing cell population was made up of serous cells, which are secretory cells. When these became goblet cells by transformation, they continued to divide. Thus, there is a population of cells in the existing population of goblet cells that may be increasing by cell division. Then there is also the metaplastic aspect.

I have a question for Jack Harkema: in your interesting model with LPS, you had about half the population responding that probably appeared due to division (because the BrdU experiment demonstrates DNA synthesis), and the rest were probably metaplastic. You used an inhibitor of EGFR, which reduced this response. Did this inhibition of the EGFR also reduce BrdU labelling? Was there any evidence that it altered the mitotic response associated with goblet cell hyperplasia?

Harkema: We didn't address this. The way to do this experiment would be in the whole animal system, but you could also do it in this explant system.

Jeffery: It might have helped to separate out the two different mechanisms that may be differentially controlled and differentially responsive to various irritant agents.

Harkema: There's a big difference here between our work and Lauren Cohn's in that she hasn't done BrdU labelling.

Levitt: Jeff Tepper did evaluate cytokine-related epithelial responses with BrdU labelling in his lung instillation model. This is the closest we can get currently to answering that question. He came to the conclusion that it was mostly metaplasia. This may speak to the issue of whether we are looking at something different. The cytokines may induce this phenomenon, and it is different to others in terms of division. Then the curious thing is if you are starting with so few cells, how is it possible to come up with this profound production of mucus? There wasn't a lot of turn over and differentiation.

Harkema: By turning a different gene on.

Levitt: I think that is the only answer.

Basbaum: From a pharmaceutical company's point of view it makes a big difference as to whether you are trying to inhibit cell proliferation versus inhibiting gene expression.

Nettesheim: With respect to the EGFR story, it won't quite work. If the EGFR mediates both cell proliferation and mucin gene expression, it is not possible to separate these two, and the knockout mice won't settle this question.

Jeffery: I think it is important to try to separate these effects and pathways, and to think of experiments that might do this.

Nettesheim: Years ago I borrowed some slides from Jack Harkema. These slides were from the tobacco smoke experiments in the peripheral lung. I saw that the increase in the mucous cells occurred before the labelling index rose. I concluded that there was actually a conversion of non-mucous cells to mucous cells before cell proliferation occurred.

Basbaum: Lynne Reid also noted this in response to sulfur dioxide in the 1960s.

Levitt: From a practical perspective, we are looking at cell cultures, and we are looking at either confluent cells and/or dividing cells. The results that we get may be very different depending on the cell culture conditions we are using.

Lauren Cohn, were you able to address whether IL9 could signal through your IL4Rα, specifically in terms of the strains you had? Strain background is very important: this has been emphasized to us by some work that we have done. We found that IL9 wasn't expressed to any extent in certain strains of mice. The backgrounds on which these mice are made sometimes include C57BL/6 mice that are IL9 deficient. In your experiments involving adoptive transfer of T cells, if they are derived from a strain of mice where there is no IL9 produced, then you can't say much about the role of IL9 in those systems. If there were IL9 there, could it signal through the IL13/IL4 receptor?

Cohn: IL9 has been shown to signal via its own receptor, which does not utilize any component of the IL13R. The IL9R is made up of the IL9Rα chain and the common γ chain, which is used by many cytokines, including the IL4R. IL9 transgenic mice produce high levels of IL9 and have increased mucus production. When we cross that mouse to the IL4Rα knockout mouse, mucus is absent. This shows that IL9 effects are mediated by IL4Rα.

Levitt: If IL9 signals through that same complex, you won't see it. Have you looked at this?

Cohn: We haven't looked at it directly, but the following results indicate IL9 doesn't signal through IL4Rα, but does so by inducing IL13. We administered an IL13 inhibitor to IL9 transgenic mice and by blocking IL13 eliminated mucus staining. Thus, if IL9 were somehow using IL4Rα, blocking IL13 should not have had an effect.

Davis: Again, in the normal mouse airway there are very few, or virtually no goblet cells, but they have perfectly good mucociliary clearance. Where is the mucus, or other gel, coming from? This may be relevant to the human small airway.

Plopper: It is the density of goblet cells that is low, but they are there. My concern is that Lauren Cohn is just using PAS-positive staining as a marker of mucus-secreting cells, because we can stimulate mouse Clara cells and they will become full of glycogen and stain by PAS, but this is not mucus.

Basbaum: What about lectin staining of Clara cells?

Plopper: There is some in them, but not much. There are sugars present.

Basbaum: If there are sugars, they are likely to be on epithelial glycoproteins.

Plopper: They could well be. There is an active exocytotic process taking place that has something to do with the granules.

Sheehan: Even in a conference like this, people confuse the terms mucin and mucus. It is a terrible confusion, because we talk of mucous cells and mucus-secreting cells, but in my opinion there are no such thing as mucus-secreting cells. When you talk about mucus-secreting cells, you are generally talking about mucin-secreting cells. Mucin should never be confused with mucus. There is a lot of evidence that you may make a mucus-like substance which contains very little mucin. This takes us to another point. We have no technology or methodology that identifies constitutive mucin secretion in any cell. We are only using measures of goblets that are present as evidence that the cell is secreting mucin. This is the point that Bill Davis is making here. There may be plenty of mucins in those fluids that are coming across at a great rate in the nose. But we need to do biochemical experiments to tell us what is present. If mucin production is being up-regulated, does this mean that populations of cells are created that show goblets? If this is the case, decoding goblet cells and mucin secretion becomes an important issue. Perhaps many cells could go over to a goblet-looking phenotype, on the basis of up-regulation of the mucins. These issues could be a matter of language and about how we think. It is time now to begin being rather more careful with our descriptions. There is a large and growing number of molecules that we call mucins, and a lot of them are found in the airway. They are largely unaccounted for. For example, we lack good antibodies for MUC1, MUC4 and MUC13 and probably half a dozen others. Nor do we know the lifestyle or the contributions that these mucins make to mucus. We should be a little more careful. It could be that we are focusing on a small number of easily detected gene products and are associating the properties of mucus with just them.

Levitt: I strongly agree. In 292 cells, there is no up-regulation of PAS, but you can see an up-regulation of *MUC5AC* gene and protein expression in certain cases. The big problem is that we lack the reagents to address these issues properly for other mucins or PAS-staining materials. The only mucin we have decent reagents for is MUC5AC, to my knowledge. In the human, I wasn't aware there were any peptide-specific (non-carbohydrate specific) antibodies that could be used to detect mucins other than MUC5AC.

Harkema: I don't know whether this answers Bill Davis' question, but in the serous cells, when LPS is added, you can measure a depletion of the PAS-staining stored granules. It is possible to stimulate and measure the small amount of granules that one gets with these cells as well, so I imagine that they are pumping out all kinds of stuff to the surface.

Davis: This is from a serous cell, so it might be MUC7.

Basbaum: No, you can't generalize between serous cells of the glands and those of the surface epithelium.

Sheehan: What you are saying is that if there is a constitutive secretion of a large molecule such as MUC5AC, you wouldn't be aware of it. You are not doing EM histology here, but optical microscopy.

Harkema: I was referring to the EM-level studies. In the old days we did seven foot long montages of electron micrographs covering a millimetre of tissue. We counted all the cells. I did this for a long time and ran out of money.

Basbaum: Did you learn anything from that?

Harkema: Yes, a lot. The serous cells lose those granules.

Basbaum: So what this means, in answer to Bill Davis' question, is that there are no goblet cells *per se* in these healthy rodent airways, yet there is mucociliary clearance. Serous (Clara) cell granules are ill-defined biochemically, but behave like mucous granules in the sense that when you expose them to a noxious stimulus, they are secreted. It seems likely that this fluid on the surface of the small rodent airway consists of water plus the undefined contents of these small granules. We can't yet conclude whether or not there is mucin present.

Rubin: If we are talking about pharmaceutical targets, most of these are going to be used in patients with established disease. We have mentioned CF, COPD and asthma. In established disease — at least where we will be testing these drugs initially — there is an awful lot going on in the airway lumen, far beyond just the mucins. If you are using specific inhibitors for mucins, you may solve only part of the problem. The fear is that the silver bullet is more likely to shoot the pharmaceutical company rather than the airway. There may be effective blockade once the disease is established, but no significant clinical effect because of all the other processes taking place.

Nadel: Under many circumstances there is probably a discontinuous blanket coating the airways. This will mean that some areas are covered by mucin, which can interact with the cilia to promote clearance. In other areas without mucins, foreign particles might be 'shipwrecked'. Then something turns on a system in that other area and they are no longer shipwrecked. Ciliary clearance in that area begins to occur. This could be a discontinuous process throughout the airways.

Basbaum: That may be true. Most investigators show low magnification pictures, because these are such conspicuous responses to endotoxin and ovalbumin. But there are microvesicles in ciliated cells which would be invisible at the

magnifications usually shown that could well contain mucin. These may be contributing to the function that Bill Davis is wondering about.

Fahy: Degraded collagens in the periphery of the lung and alveolae can be proinflammatory. Could broken down mucins also be proinflammatory?

Reference

Rose MC, Piazza FM, Chen YA et al 2000 Model systems for investigating mucin gene expression in airway diseases. J Aerosol Med 13:245–261

The role of apoptotic regulators in metaplastic mucous cells

Yohannes Tesfaigzi

Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, USA

Abstract. Exposure of airways to environmental toxins or allergens induces proliferation of epithelial cells. Depending on the type of exposure, existing and newly formed cells can differentiate into mucus-producing cells resulting in mucous cell metaplasia (MCM). During recovery, the epithelium reduces the number of epithelial cells to return to the original state. Understanding the mechanisms involved in this resolution could be useful in deleting mucous cells and, thereby, mucous secretions. We have found that metaplastic mucous cells induced by exposure to ozone, endotoxin, cigarette smoke or allergens in epithelia of various regions of the airways express Bcl-2, a regulator of apoptosis, and neutrophils appear to be involved in its expression. The percentage of Bcl-2-positive mucous cells is decreased prior to the resolution of MCM. Furthermore, targeted reduction of Bcl-2 expression causes a dose-dependent reduction of epithelial mucous cells, suggesting that Bcl-2 is involved in maintaining metaplastic mucous cells. Horses with recurrent airway obstruction show an increased percentage of Bcl-2-positive mucous cells compared to their normal counterparts. These studies suggest that down-regulation of Bcl-2 expression may be useful to reduce mucous secretions in diseased subjects. The role of Bax in the reduction of MCM during prolonged exposure to allergen is also discussed.

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The normal tracheobronchial epithelium contains ciliated, basal, and secretory cells, which are maintained at fixed ratios by homeostatic mechanisms. The proportion of these cell types is perturbed following various inflammatory responses or mechanical injury to the epithelium due to proliferation (Basbaum & Jany 1990). Various studies have shown that non-ciliated columnar cells are the main cell type that is recruited to the cell cycle in larger numbers (Wells 1970). Following cessation of allergen exposure, airway epithelia return to the original proportion of cell types (Blyth et al 1998). Therefore, various mechanisms must exist that regulate the numbers of mucous cells to be adjusted to the original state following proliferation and mucous differentiation of epithelial cells. These processes resemble those of normal wound healing and inflammatory responses

that appear in asthma and chronic obstructive pulmonary disease (COPD), but the processes persist in these diseases and are believed to cause the observed dramatic changes observed in airways including mucous cell metaplasia (MCM).

Existing and newly proliferating airway epithelial cells can differentiate into mucous cells following exposure to ozone, lipopolysaccharide (LPS), cigarette smoke, or allergens. The airway epithelia recover when exposures are terminated, suggesting that mechanisms must exist that reduce MCM. Various mechanisms may be responsible for the reduction of MCM, including the fact that inflammatory mediators responsible for mucin synthesis are no longer present. However, full recovery of the epithelium necessitates the reduction of epithelial cell numbers to the original state, and we hypothesized that these mechanisms may be useful to reduce metaplastic mucous cells.

Bcl-2 expression in LPS-, ozone- and allergen-induced metaplastic mucous cells

Exposure of rats to LPS, a cell wall component of Gram-negative bacteria, is characterized by infiltration of the alveolar and bronchiolar air spaces by neutrophils (Stolk et al 1992, Tesfaigzi et al 1996, Harkema et al 1990, Michel et al 1992) and induction of MCM in pulmonary airways (Harkema & Hotchkiss 1992). Three days post-instillation, the numbers of epithelial cells per mm basal lamina (BL) in control rats that were not instilled or were instilled with saline ranged from 124–135, while the number reached 180 in rats instilled with 1000 μg LPS. Following a 16 day recovery period, these epithelial cell numbers decreased to 135/mm BL, which is similar to that observed in control rats. These results show that approximately 25% of cells must be eliminated for the epithelium to revert to the original condition (Fig. 1).

To investigate the involvement of cell death programs in this recovery process, we tested expression of apoptotic regulators from the Bcl-2 family members, Bax, Bcl-2 and Bcl-x_L, in these airway epithelia. Bcl-2 is expressed in approximately 20–30% of these metaplastic mucous cells induced by exposure to endotoxin or allergen in airway epithelia (Tesfaigzi et al 2000). Bcl-2 is also expressed in metaplastic mucous cells induced in pulmonary airways of immunized Brown Norway rats by exposure to allergen (Tesfaigzi et al 2000). Interestingly, adjacent metaplastic mucous cells in these epithelia are heterogeneous in their expression of Bcl-2; some cells express high levels, whereas others express low levels or no Bcl-2 (Fig. 2). The percentages of cells to be eliminated in these respiratory epithelia and the percentage of mucous cells that express Bcl-2 are strikingly similar, and may indicate that Bcl-2 expression is associated with the recovery process.

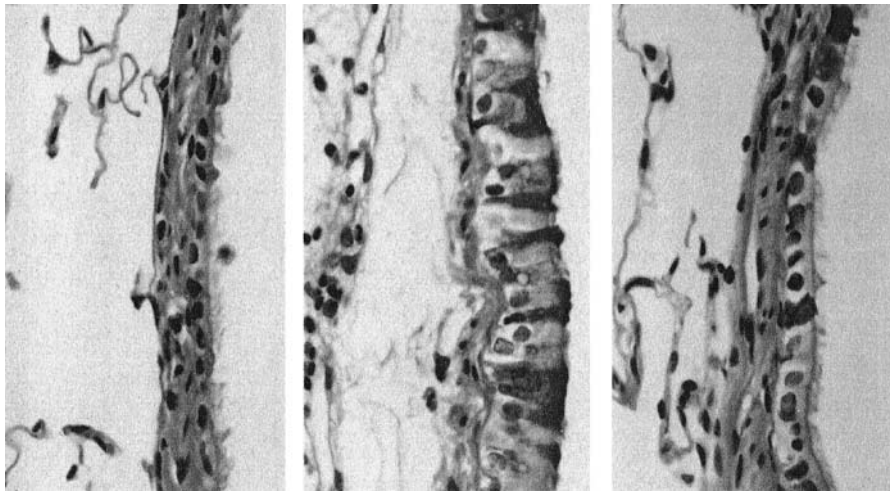

FIG. 1. A representative light photomicrograph of epithelia lining the axial airway at generation 5 in the left lung of rats following intratracheal instillation with LPS at 0, 3 and 16 days. Tissues were stained with alcian blue, and haematoxylin and eosin, and the mucous cells appear dark coloured. Very few mucous cells were detected in a naïve rat, MCM is observed at 3 days, and the epithelium recovered following 16 days post LPS instillation.

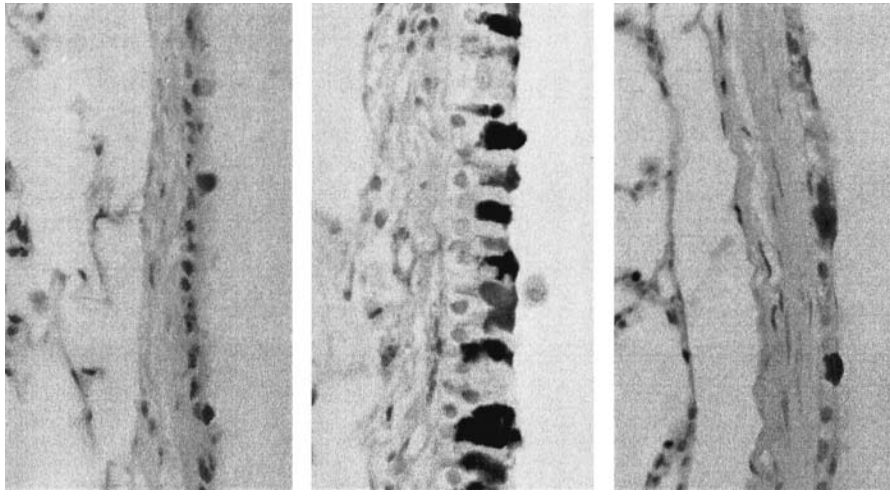

FIG. 2. Representative light photomicrographs as shown in Fig. 1. Immunoreaction of Bcl-2 antibody was detected with diaminobenzidine and is darkly coloured. Tissue sections were also stained with alcian blue to identify mucous cells.

While exposure of rats to LPS induces primarily MCM in pulmonary airways, exposure of rats to ozone induces MCM in nasal airways (Hotchkiss et al 1991, Wagner et al 2001, Harkema et al 1997). In an unexposed F344/N rat, the respiratory epithelium of the mid-septum contains many mucous cells. The number of these endogenous mucous cells did not change following exposure to ozone, and the percentage of Bcl-2-positive cells was only increased from 7%–14% after a one- or six-month exposure (Tesfaigzi et al 1998). However, the number of mucous cells in transitional epithelia lining the lateral wall and the nasal and maxillary turbinates increased from 0 to approximately 200 after ozone exposure. After a one-month exposure period, 33–55% of these mucous cells expressed Bcl-2 and 10–18% after a three- or six-month exposure period (Fig. 3). The percentage of Bcl-2-positive cells was decreased to 0–8% following a recovery period of 13 weeks (Tesfaigzi et al 1998). These results show that Bcl-2 is absent in endogenous mucous cells, but is expressed in a high percentage of metaplastic mucous cells induced by ozone in nasal epithelia.

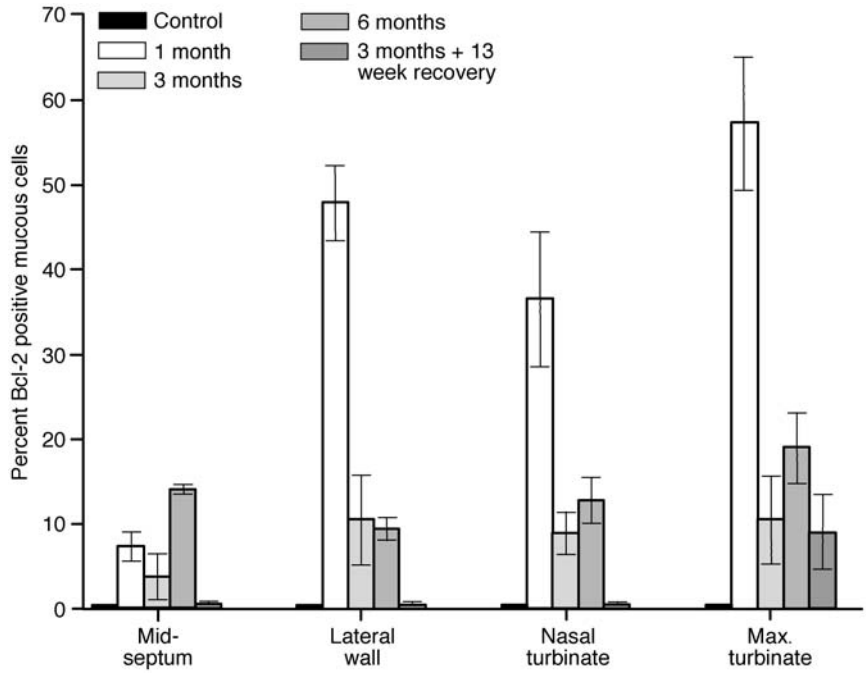

FIG. 3. The percentage of Bcl-2-positive mucous cells in nasal epithelia of rats exposed to air or to ozone for one, three and six months and in rats maintained in filtered air for a recovery period of 13 weeks after a three-month ozone exposure. Error bars denote the standard deviation from the mean.

Inflammatory responses caused by LPS instillation and by immunization and challenge with ovalbumin are characterized primarily by neutrophils or eosinophils, respectively. Regardless of the type of inflammatory response, Bcl-2 was expressed in 20–30% of the resulting metaplastic mucous cells (Tesfaigzi et al 2000). These results suggest Bcl-2 expression is induced by inflammatory factors found in both types of inflammatory responses. Furthermore, expression of Bcl-2 independent of airway location in both airway and nasal epithelia and independent of inflammatory response may indicate that Bcl-2 is inherently an important regulator of cell numbers in rat airway epithelia.

Inflammation and Bcl-2 expression

To determine whether inflammatory mediators are responsible for the expression of Bcl-2, the LPS-induced inflammation was modulated by injecting rats with bezafibrate, an inducer of cytochrome P 450 (CYP-450). Several studies have demonstrated that inducers of CYP-450 provoke a substantial drop in body temperature and inflammation (Kozak et al 1998). Injection of bezafibrate significantly attenuated symptoms of LPS-induced sickness behaviour, including a drop in body temperature, motor activity and food consumption, and a twofold decrease in MCM. In addition, the percentage of Bcl-2-positive mucous cells was decreased threefold, suggesting that certain inflammatory mediators cause expression of Bcl-2 in mucous cells (Tesfaigzi et al 2001).

To further investigate which inflammatory cell type may be associated with Bcl-2 expression, rats were intratracheally instilled with varying doses of LPS. The inflammatory cells infiltrating the lung air spaces following a one-time LPS instillation of 50–1000 μg LPS consist primarily of macrophages and neutrophils (Tesfaigzi et al 1996). Determination of the cell differentials three days after LPS instillation showed that the number of macrophages increased in a dose-dependent manner from 50–500 μg LPS and decreased by 50% at 1000 μg LPS. In contrast, the number of neutrophils increased continuously from 50–1000 μg LPS and reached 1.5 times the number of macrophages. Both the number of mucous cells and the volume of stored mucosubstances increased significantly at 100 μg LPS compared to saline-instilled rats and reached maximum levels at 1000 μg LPS. Approximately 10–20% of mucous cells expressed Bcl-2 in rats instilled with 500 or 1000 μg LPS, while the percentage of Bcl-2-positive mucous cells was not significantly increased at lower LPS doses. These data show that MCM is not always associated with the appearance of Bcl-2 and that MCM and Bcl-2 are not induced by the same dose of LPS, but may be caused by different inflammatory mediators. Neutrophils are the predominant inflammatory cell type in rats at 1000 μg LPS when the maximum percentage of mucous cells express Bcl-2, implicating this cell type or factors secreted from this cell in causing Bcl-2 expression.

Neutrophils are increased in the submucosa during exacerbation of chronic bronchitis with severe airflow limitation (Saetta et al 1994, Di Stefano et al 1998). Progression of chronic bronchitis is associated with neutrophils increasing in the subepithelium (Saetta et al 2000, Maestrelli et al 2001). Therefore, Bcl-2 may also be expressed in metaplastic mucous cells of humans with COPD or cystic fibrosis. These studies and investigations on the role of neutrophils in inducing Bcl-2 expression in metaplastic mucous cells are ongoing.

Proliferating cells and Bcl-2 expression

Previous reports have demonstrated that Bcl-2 can inhibit apoptosis as well as cell cycle progression (Mazel et al 1996, O'Reilly et al 1997, Huang et al 1997, Knudson et al 2001). To investigate the role of Bcl-2 expression in epithelial cell proliferation following LPS instillation, we continuously delivered bromodeoxy uridine (BrdU) from an osmotic pump that was surgically implanted in the subcutaneous tissue of the back of the rat. Rats were sacrificed 48 hours after intratracheal instillation of 1000 μg LPS. The lungs were processed for histology and immunohistochemistry, and each tissue section was stained for Bcl-2, BrdU and alcian blue to enable the morphometric quantification of mucous cells expressing Bcl-2 and to determine whether they had undergone a cell division cycle. Very few (< 3 cells/mm BL) BrdU-positive mucous cells were detected in non-instilled control rats or 24 hours post LPS instillation. However, approximately 20 BrdU-positive mucous cells/mm BL were present at 48 hours post LPS instillation. These results show that the first cycle of proliferation occurred after 24 hours of LPS instillation. Furthermore, approximately 35% of the mucous cells that were Bcl-2-negative were BrdU-negative, and 45% showed BrdU positivity. From the total numbers of mucous cells, 10% were Bcl-2- and BrdU-positive, while 9% were Bcl-2-postive but BrdU-negative. These data demonstrate that only about half of the Bcl-2-positive mucous cells had incorporated BrdU, representing newly formed cells, while the other Bcl-2-positive cells were BrdU-negative and, therefore, must have been existing mucous cells that were present before LPS injury. These data also indicate that the presence of Bcl-2 is not required for mucous cells to proliferate and that the entry into the cell cycle is not associated with inducing its expression. The mechanisms underlying the Bcl-2-expression in a selected number of mucous cells are under investigation.

Bcl-2 sustains MCM

Several lines of evidence suggest that Bcl-2 sustains metaplastic mucous cells. Brown Norway rats were instilled with 1000 μg LPS, and the MCM and percentage of Bcl-2 positivity were analysed over a period of 14 days. Compared with

those in non-instilled rats, the number of mucous cells was increased fivefold 2, 3, and 4 days post LPS instillation and decreased to levels observed in non- or saline-instilled control rats after 7 and 14 days. None of the mucous cells in control animals expressed Bcl-2, whereas 20–30% of mucous cells were Bcl-2-positive 1 and 2 days post-LPS instillation (Tesfaigzi et al 2000). These data show that the number of Bcl-2-expressing mucous cells decreased to background levels at 3 days; the number of mucous cells was still elevated 4 days post LPS instillation. The decrease of mucous cell numbers at least 2 days after the percentage of Bcl-2 positivity decreased supported the hypothesis that Bcl-2 as an inhibitor of apoptosis must be down-regulated before mucous cell numbers can be reduced presumably by cell death mechanisms.

Further evidence for the role of Bcl-2 in sustaining mucous cells comes from recent data showing that direct inhibition of Bcl-2 expression reduced mucous cell numbers. Several antisense oligonucleotides (ODNs) that were designed to hybridize to Bcl-2 mRNA and cause its degradation before translation occurs were tested using an organ culture system. The system used nasal mid-septa from rat noses that mimic the LPS-induced MCM and Bcl-2 expression *in vivo*. Several antisense ODNs down-regulated Bcl-2 mRNA levels, as shown by *in situ* hybridization. Along with this down-regulation, epithelial mucosubstances were reduced, and this reduction was dependent on the concentration of antisense ODN used, suggesting that it was specific to antisense ODN treatment. ODNs that did not affect Bcl-2 mRNA levels did not affect MCM. Reduction of MCM by direct targeting of Bcl-2 expression was also observed in live rats. When LPS-instilled rats were treated with antisense ODNs to Bcl-2 mRNA by subcutaneous injections for prolonged periods, LPS-induced MCM was reduced by approximately 30% *in vivo*. The reduction of mucous cell numbers when Bcl-2 levels are reduced directly links Bcl-2 to the sustenance of metaplastic mucous cells.

Bcl-2 expression in mucous cells of diseased subjects

Various studies show the importance of goblet cells as the major source of mucin in the tracheobronchial tree of humans. Heidsiek et al (1987) found significantly more stainable stored mucins in goblet cells in tracheobronchial epithelia than in the submucosal glands. In addition, biopsies taken from the large central airways from mild to moderate asthmatics and controls show a twofold increase in goblet cells in subjects with asthma compared to controls and a threefold increase in stored mucins than in control subjects (Ordonez et al 2001). Thus, it is conceivable that acute degranulation of goblet cells may represent an important mechanism of airway obstruction during asthma exacerbations, and chronic degranulation may be a mechanism for chronic airway narrowing in more severe forms of

asthma. Targeted reduction of metaplastic mucous cells, therefore, may be a useful therapy to prevent airway obstruction in diseased subjects.

Wound healing processes, and therefore MCM, may persist for several reasons in these diseases. Repeated injury of the epithelium due to repeated exposure to allergen, cigarette smoke, or environmental and/or occupational pollutants; chronic infection; or interactions of these various factors could maintain the inflammatory response and, thereby MCM. It is also possible that there is a deficiency in the epithelium to reverse these changes during the wound-healing process. While the inflammatory response may have subsided, the genetic make-up of the epithelium leads to the inability of the epithelium to heal itself, causing MCM to persist. Our hypothesis is that there is a deficiency in molecular mechanisms to down-regulate the normally transient expression of Bcl-2 leading to persistence of metaplastic mucous cells.

To test this hypothesis, we studied whether Bcl-2 expression is found in a higher percentage of mucous cells in subjects with diseases associated with increased mucous secretions. Horses with recurrent airway obstruction (RAO), an asthma-like condition, have increased airway secretions of mucous glycoproteins in their airways (Jefcoat et al 2001). The lung lobes along the main axial airway (generation 5), and peripheral airway segments at generations 10 and 16 of two pairs of horses with and without RAO were processed for immunohistochemistry with Bcl-2 antibodies. High levels of Bcl-2 protein were detected in mucous cells of the airway epithelia of horses with RAO, and alcian blue staining of the tissue sections revealed that only the mucous-containing cells expressed Bcl-2. In both control horses, only 10–20% of mucous cells showed Bcl-2 positivity, while horses with RAO showed 40–55% of mucous cells with Bcl-2 immunostaining in the bronchial epithelia. These studies indicate that Bcl-2 and inflammatory mediators that cause its sustained expression in metaplastic mucous cells may be a useful target to reduce metaplastic mucous cells and thereby reduce severe forms of airway obstructions during acute degranulation of goblet cells.

Bax in mucous cells

In mice, extensive MCM develops after 5 d of allergen exposure, but is reduced when allergen challenge is continued for 15 d. This reduction of mucous cell metaplasia is mediated by IFNγ through the Stat1 pathway (Shi et al 2002). The percentage of Bax-positive mucous cells was increased during the prolonged exposure period. Both Stat1- and Bax-deficient mice showed elevated levels of MCM compared to wild-type mice following prolonged exposure to allergen, suggesting that Stat1 and Bax play a role in the reduction of MCM. These studies further support the general findings that the Bcl-2 family of proteins are involved in restoring the normal proportions of various cell types in

the tracheobronchial epithelium following injury by environmental toxins or allergens.

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DISCUSSION

Basbaum: Is Bcl-2 normally up-regulated coordinately with mucin?

Tesfaigzi: In mucous cells, yes. We don't know whether the signalling for both is the same.

Basbaum: What caught my attention was the fact that mucin and Bcl-2 are not always correlated. You had some cells that express Bcl-2 and not mucin, and vice versa. The quasi correlation with proliferation is also of interest.

Tesfaigzi: In the LPS system we only see Bcl-2 expression in mucous cells. Because some BrdU-negative mucous cells express Bcl-2, the proliferation is not required for Bcl-2 expression. It is not necessarily doing the cell cycle regulation in this system.

Basbaum: Earlier, Jack Harkema told us that there were parts of the nose such as the proximal septum that endogenously have a lot of mucous cells. Presumably, they never express Bcl-2.

Tesfaigzi: Compared to the regions where the metaplasia occurs, the percentage of cells expressing Bcl-2 is very low.

Cohn: Your data on interferon fit very nicely with what we have shown with Th1 and Th2 cells. Th2 cells stimulate mucus production. When we transfer Th1 cells into mice at the same time as the Th2 cells, when high levels of interferon are present with the IL13, we get a reduction in mucus staining. If we put the Th2 cells in first so that the levels of IL13 are high to start and then transfer the Th1 cells into mice a little later, mucus is not inhibited. It seems that IL13 overrides the interferon signal if it is present prior to IFNγ.

Tesfaigzi: In fact, IL13 does inhibit IFNγ-induced apoptosis.

Cohn: I assume, like you said, that interferon has some other mechanism of action. Its inhibitory mechanism may not just be apoptosis; it might have some other role in inhibiting gene expression.

Jeffery: We have looked at a lot of tissue by electron microscopy over the years. There is no problem finding apoptotic cells as classically described by the condensation of nuclei and using electron microscopic techniques, as opposed to TUNEL, which has some limitation in terms of interpretation. We find apoptotic mast cells and neutrophils, but never eosinophils. I have never seen an epithelial cell (goblet or ciliated) which one could identify as apoptotic. Have you done any electron microscopy to back up what you would assume from your light microscopic data?

Tesfaigzi: That's a good question. We have looked for these apoptotic cells but we never find them. What I think is happening is that the epithelium discards these cells before we see the classic apoptotic morphology, probably by sloughing. But we do see the Bcl-2 family expression.

Jeffery: In experiments we did with LPS, within the first 12 h we noticed that there were relatively frequent intensely eosinophilic cells present in the epithelium. When we looked at these by electron microscopy they appeared to be degenerative, necrotic cells. Perhaps they were actually apoptotic. We need to revisit this. We presumed that these cells were being sloughed from the epithelium. There are interesting differential mechanisms operating here for epithelium in an apoptotic situation for clearance. By contrast, inflammatory cells such as neutrophils require engulfment by macrophages as part of the apoptotic process.

Harkema: This is an area that hasn't been well studied. In particular, we don't know much about the repair process. It is rare to see apoptotic cells. I have seen apoptotic cells in an extreme hyperplastic epithelium, but we need to do a lot of time points to see them.

Basbaum: At least we know where to look, on the basis of Yohannes Tesfaigzi's data. How long does a cell look apoptotic for before it disappears?

Tesfaigzi: In tissues it is a very short process. Within 2 h the cell disappears. To find the apoptotic morphology one has to look within a shorter time. To detect any apoptotic cells we probably need massive apoptosis.

Jeffery: We did detect them in neutrophils and mast cells. Perhaps we are looking in the wrong place. We might need to look at the sputum and in the bowel for evidence of epithelial cell apoptosis by electron microscopy.

Cohn: Do macrophages engulf bronchial epithelial cells? The rapid speed of clearance could be why we don't see apoptotic eosinophils.

Jeffery: But we would see apoptotic eosinophils engulfed by macrophages. We see neutrophils in macrophages but never eosinophils.

Tesfaigzi: In these lungs it is easy to find apoptotic neutrophils. They are within macrophages. For epithelial cells the window is small, and it is likely that they are sloughed off instead of being engulfed.

Randell: We have seen apoptotic epithelial cells by electron microscopy and TUNEL in mouse heterotopic tracheal allografts.

Harkema: Yohannes Tesfaigzi, I was going to suggest that you should look at your explants. You might see more apoptosis there. It is a little artificial, but at least the cells are captured and can be recovered from the supernatant.

Fahy: I am intrigued by the concept of endogenous goblet cells versus goblet cells that are induced acutely in response to inflammatory injury. In the normal airway phenotype, there are some 25 000–35 000 goblet cells per cubic millimetre of tissue. Asthmatic patients have three times this number, and sometimes even as many as 10 times this. Is it possible that the goblet cells that develop in response to injury might have a different mucin gene profile to endogenous goblet cells? Perhaps the newly synthesized goblet cell that goes away after a few days might have *MUC2* and *MUC5B*, whereas the standard goblet cell has *MUC5AC*. This is a simplistic idea, but is this part of your paradigm?

Tesfaigzi: Yes. When we heard about the antibodies for mucin subtypes, I was interested. In a way, although in the bigger airways there are goblet cells, in the smaller airways in humans there are no mucin cells. This is where the acute problem might be in asthmatics. The association might be with different mucin gene expression.

Jeffery: In the early work with Lynne Reid, we examined mitotic indices in the rat, comparing large airways, small airways and alveolar parenchymal tissue. We made an assumption (now shown to be incorrect) that by virtue of the mitotic

indices you could then predict turnover time for the epithelium. We calculated 45 days turnover for the large airways and 220 days in the small airways. Have you looked at different airway levels with regard to Bcl expression?

Tesfaigzi: In the rat, when we instil LPS, we see most mucous cell metaplasia in the large airways. Because there is no endogenous mucous cell in the rat, we think this represents the small airways in humans. If we sometimes get mucous cell metaplasia in the small airways, we do see Bcl-2 there. This is not where we primarily have quantified, though.

Jeffery: This raises the interesting question as to whether the Bcl-2 you are talking about is exclusively in goblet cells and not ciliated cells.

Tesfaigzi: We don't see Bcl-2 in ciliated cells.

Basbaum: I remember Lynne Reid's papers on the *de novo* appearance of goblet cells in the small airways in response to SO_2. If any of those tissue blocks were to have survived the decades, I bet you would find Bcl-2 in those cells. Those cells were persistent, as I recall.

Jeffery: Yes, they were; they lasted for at least three weeks.

Davis: If the cell doesn't have Bcl-2 expression and it's a mucous cell, is it going to be apoptotic?

Tesfaigzi: We don't know. The percentage of the Bcl-2-positive cells tracks with the percentages that have to be discarded. We have not demonstrated definitively that those Bcl-2-positive cells are the ones that die. This is an interesting question.

Davis: Are you suggesting that transient goblet cells have to express Bcl-2 to stay alive, and when they stop expressing it they die?

Tesfaigzi: That is the idea. But then in terms of transience, because it is not only proliferating cells expressing Bcl-2, I think there is another recognition that the body has to delete cells, if those Bcl-2-positive cells really are being deleted.

Engelhardt: Do you think the control of Bcl-2 expression can be determined by the neighbours of cells that they are contacting? For example, if you are a goblet cell and you happen to be in the centre of other goblet cells, you might be getting signals saying that you are a hyperproliferative metaplastic goblet cell telling you to kick off until you have contact with a ciliated cell.

Tesfaigzi: If I understand you correctly, they are not necessarily between goblet cells. They could be next to a ciliated cell and express Bcl-2. However, that is one possibility: that the signals are coming from neighbouring cells.

Jeffery: Going back to the discussion about the number of times a goblet cell secretes before it dies, you have an excellent system here: having generated goblet cells you could then go into exposure to mucin-releasing agents and look to see whether Bcl-2 expression suddenly rises.

Sheehan: Have you compared mucin-secreting cells in glands in this regard?

Tesfaigzi: We have looked at some human tissue from asthmatics. We were quantifying the percentages (we were blinded at this point). There is some expression in some submucosal glands.

Sheehan: There are a number of interesting factors that were alluded to. Ingemar Carsltedt has demonstrated a change in mucin-secreting phenotype that is associated with a change with insult of some kind. This may be reflected in your observation. Putting the antibodies onto these cells for the different mucin phenotypes might be very interesting.

Verdugo: I think the concept here is very important. The total area in the airway is occupied by different effectors, such as the ciliated cell that is not only a transporting cell but also a water and ion transporting cell across the mucosa. If you alter the ratio between the cells that produce mucus that need to be dehydrated and the ones that have to provide the water, there must be a critical point beyond which the system no longer works because there is not enough hydration and the mucus doesn't get to be transported. The control of this ratio is very important. This issue that you are addressing points directly to the control of the number of effector cells. It would be interesting to know who is in charge of controlling the number of the other type of cell that is moving water and ions across.

Danahay: There is a certain amount of evidence in the literature that the transporting epithelial cells are actually able to control the fluid secretory capacity by mediators such as IL13 and IL4 (Danahay et al 2002).

Nadel: Another way of looking at the fate of epithelial cells producing mucin is to examine cells after they degranulate. Do they become apoptotic, do they remain at 'rest' or do they regranulate? In our experiments in the rat nose, following goblet cell degranulation the epithelium regranulates completely within 48 h. What is happening to the system that you are examining during degranulation? Does degranulation change Bcl-2 expression or these other molecules? And what happens when the cells are re-forming mucins?

Tesfaigzi: Those are interesting questions.

Basbaum: That may pertain to those non-injured epithelial sheets that are just doing their housekeeping, and they don't make Bcl-2.

Tesfaigzi: That would be the preferred hypothesis.

Harkema: You could go one step further. If you damage with tobacco smoke you can change that septum to squamous metaplasia. Within two weeks it comes back to a normal-looking respiratory epithelium with mucous goblet cells.

Tesfaigzi: We have looked at squamous differentiation. We don't see Bcl-2 in those cells, but we do see it in the area that goes towards the squamous vestibule in the nose. We consistently see high levels of Bcl-2 in that region. It might be that there are precursor cells that lead to the squamous area.

Fahy: Do steroids have any effect on this system? It has been shown that steroids hasten loss of mucous cells after an allergen challenge. Do you think that steroids inhibit Bcl-2 or induce Bax?

Tesfaigzi: No, but it's a good question.

Vargaftig: Under physiological conditions IFNγ is released after IL12 is produced by APCs and related cells. Do you have any evidence that in this dichotomy you describe IL12 is involved?

Tesfaigzi: We haven't looked at IL12 in any way, but it is true that IL12 is associated with IFNγ. We haven't looked into whether IL12 itself induces apoptosis in epithelial cells.

Nadel: What is the relationship between Bcl-2 and cell confluence?

Tesfaigzi: We haven't really done controlled studies looking at the effect of confluence on Bcl-2 expression in culture. We have just looked at the airway epithelial cells *in vivo*. The only thing we know is that it has some effect that is not necessarily associated with proliferation. Existing epithelial cells that just differentiate into mucous cells seem to be expressing Bcl-2 as well as the ones that have proliferated. This doesn't seem to be a cause of Bcl-2 expression.

Nadel: I gather your prediction would be that at high confluence the cells have an increased expression of Bcl-2. When they are at high confluence, they are differentiating and are producing mucins.

Tesfaigzi: I think you are addressing another question, which is important in terms of injured epithelium. Usually, what we do is to look at NHBEs at high confluence because that is when they differentiate into mucous cells. But only injured epithelium involves an IFNγ-induced cell death programme. We don't see IFNγ affecting confluent cells greatly. We have done a lot of studies with differentiated cells cultured on transwell membranes, which have been grown in an air–liquid interface. There, IFNγ is not a major killer. The viability is only reduced by 10–15%. But IFNγ is a killer in proliferating cells. What I believe is that in the normal epithelium most cells are in G0, but following injury by exposure to allergens or LPS, there are proliferating cells. The ones transitioning in the cell cycle are the cells that are susceptible to IFNγ. It is important to do our experiments for differentiation in transwells that are confluent, but the proliferation in an injury model may better reflect an injured epithelium.

Nadel: Might it be separate from the production of the mucin by a cell? I'm not clear about the use of the word 'injury'. If a mucus cell is formed by certain mediators, do you believe that this is separate from injury?

Tesfaigzi: No. There are injury models such as wound repair models on transwell culture systems, where an injury is caused by mechanical scraping. Then the cells start to proliferate. These kinds of studies need to be done in order for us to understand this recovery issue, because these proliferating cells may be the ones susceptible to death.

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Danahay H, Atherton H, Jones G, Bridges RJ, Poll CT 2002 Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. Am J Physiol 282:L226–L236

Current and future therapies for airway mucus hypersecretion

Peter J. Barnes

National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, UK

Abstract. Mucus hypersecretion is a prominent feature of chronic inflammatory diseases of the airways, including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis, but little is known about the effects of current therapies for airway disease because of the difficulties in quantifying mucus hypersecretion in clinical studies. Anticholinergics may reduce mucus hypersecretion, whereas β_2 agonists and mucolytics have little obvious effect. Corticosteroids are highly effective in inhibiting mucus hypersecretion in asthma by suppressing the underlying inflammatory process, but are ineffective in COPD and cystic fibrosis. Novel approaches in the future may include inhibition of sensory neuropeptides by tachykinin antagonists, modulators of sensory nerves or K^+ channel openers. Inhibition of Th2 cytokines (interleukin [IL]4, IL9, IL13) may also be effective in asthma. In COPD inhibition of neutrophil-derived proteases by small molecule inhibitors or inhibiting neutrophilic inflammation in the airways by reducing neutrophil chemotaxis may also be effective strategies. Several novel targets involved in mucus hypersecretion have recently been identified, including epidermal growth factor receptors, MARCKs, Ca^{2+}-activated Cl^- channels and mitogen-activated protein kinases. However, the clinical benefits from inhibiting mucus hypersecretion are still not certain, casting some doubts on this therapeutic approach.

2002 Mucus hypersecretion in respiratory disease. Wiley, Chichester (Novartis Foundation Symposium 248) p 237–253

Although mucus hypersecretion in a prominent component of airway diseases, including asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis and cystic fibrosis, there is little known about the effects of current therapy on mucus secretion. This is largely because of the difficulties in quantifying mucus secretion. Collection of mucus and measuring volume is unreliable due to incompleteness of the collection. Subjective impressions of mucus secretion may be made using visual analogue scales or by recall of volume produced, but are also unreliable. Measuring the quality of mucus in terms of colour or viscosity is possible, but may not reflect the amounts of mucus produced. Mucociliary clearance may be quantified using radioactive tracers, such as ^{99m}technecium-labelled polystyrene particles, but this may not reflect mucus secretion and may

be more related to ciliary function. Antibodies to quantify mucus glycoprotein concentrations in sputum are not yet available.

Mucus hypersecretion is often assumed to be detrimental, as it is associated with abnormal physiology and is linked epidemiologically to airflow obstruction. But increased production of mucus in the respiratory tract is part of the normal defensive response of the airways to irritants and to pathogens. The increased mucus production may protect the delicate airway epithelium, may defend against invasion of microoganisms, serve to retain hydration in the respiratory tract and may contain antiproteases (such as secretory leukoprotease inhibitor and elafin) and antibodies (such as IgA). The increased production of mucus in cigarette smokers (chronic bronchitis) may not be detrimental *per se* but may contribute to airflow limitation in those smokers who develop COPD.

β agonists

β-adrenergic agonists increase mucociliary clearance when it is impaired in asthma and COPD (Pavia et al 1983), but this is likely to reflect an effect on ciliary beating rather than on mucus secretion. β agonists stimulate mucus secretion in isolated tracheal preparations (Phipps et al 1982), but because β receptors predominate on mucous rather than serous cells (Barnes & Basbaum 1983), this may lead to increased viscosity of mucus which is presumably detrimental. The β-receptor subtype expressed in submucosal glands is β_1 receptor, consistent with the fact that these glands are directly innervated by sympathetic nerves (Barnes & Basbaum 1983). Animal studies demonstrated that very high concentrations of systemically administered isoprenaline were associated with mucous hyperplasia and this was invoked as an explanation for the association between high doses of inhaled isoprenaline and asthma deaths (Jones & Reid 1979). However, there is no evidence that mucous hyperplasia is cause by therapeutic doses of β_2 agonists in humans.

Anticholinergics

Muscarinic receptors play an important role in neural mucus secretion and the secretory response of submucosal glands to neural stimulation is largely mediated by muscarinic receptors and blocked by atropine. The muscarinic receptor subtype responsible for mucus secretion is predominantly the M_3 receptor (Mullol et al 1992, Ramnarine et al 1996), although autoradiographic mapping studies in human airways demonstrate the presence of a small population of M_1 receptors in addition, but these do not appear to be of functional consequence. Anticholinergics should theoretically reduce mucus and liquid secretion by blocking the cholinergic vagal tone that may drive basal secretion. However, it

has been difficult to show that inhaled anticholinergics, such as ipratropium bromide and oxitropium bromide decrease mucus secretion or alter mucus viscosity (Pavia et al 1983). Oxitropium bromide is reported to reduce the amount of mucus secretion in patients with COPD, but curiously this effect occurs slowly over several weeks and its mechanism is not clear (Tamaoki et al 1994). Tiotropium bromide is a new anticholinergic that has a duration of action over 24 h that is likely to become the bronchodilator of choice in COPD (Barnes 2000). It has a kinetic selectivity for M_1 and M_3 receptors, but its effects on mucus secretion have not been reported.

Corticosteroids

It is not certain whether corticosteroids have any direct effects on mucus secretion. In asthma the increased mucus secretion is likely to result from the effects of multiple inflammatory mediators, including cytokines such as interleukin (IL)4, IL9 and IL13 (Barnes 2001). Corticosteroids are very effective in suppressing the increased expression of inflammatory genes in asthmatic airways, such as those coding for cytokines, and are therefore effective at inhibiting the mucus hypersecretion associated with asthma. Inhaled corticosteroids are highly effective in controlling asthma symptoms, including mucus secretion. In some patients with more severe asthma corticosteroids are less effective and high doses may be needed; these patients have a relative resistance to the anti-inflammatory effects of corticosteroids, including effects on mucus secretion. In patients with COPD inhaled corticosteroids are much less effective than in asthma and do not appear to be effective in reducing mucus hypersecretion. In COPD there appears to be an active resistance to the anti-inflammatory effects of corticosteroids.

Mucolytics

Several drugs were introduced that reduced mucus viscosity *invitro*, with the aim of increasing mucociliary clearance *in vivo* and thereby improving lung function in COPD and asthma. Although small clinical studies with these drugs did not show any convincing benefits, a more recent meta-analysis has demonstrated a significant benefit in terms of reducing the exacerbation rate in COPD patients (Poole & Black 2001). Most of this beneficial effect appears to be due to the effects of *N*-acetylcysteine, which acts as an antioxidant since cysteine is converted to glutathione, the predominant antioxidant in the airways. It is unlikely that this beneficial effect can be ascribed to an effect on mucus hypersecretion as antioxidants may have several other beneficial actions in COPD.

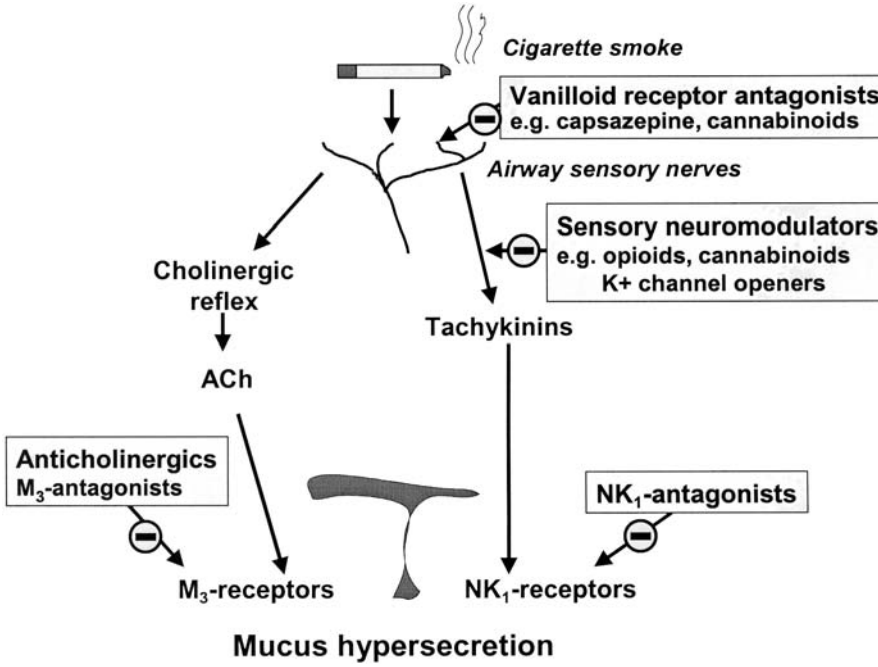

FIG. 1. Neural targets for inhibition of mucus hypersecretion include muscarinic receptors, sensory nerves activation and tachykinin release and effects.

Neuromodulators

Nerves are important modulators of mucus secretion (Rogers 2001). In addition to cholinergic nerves that increase submucosal gland secretion, non-adrenergic non-cholinergic (NANC) neural mechanisms increase the secretion of submucosal glands and goblet cells (Fig. 1). Cigarette smoke stimulates goblet cell discharge via a neural mechanism that is unaffected by the ganglion blocker hexamethonium, but is blocked by capsaicin pre-treatment to deplete neuropeptides from sensory nerve endings, indicating that a local neurogenic (axon) reflex mechanism is involved (Kuo et al 1990).

Sensory nerve inhibitors

Cigarette smoke and other irritants may activate the vanilloid receptor VR1, for which selective antagonists, such as capsazepine, have now been developed (Caterina & Julius 2001). Cannabinoids also inhibit sensory nerve activation and the endogenous cannabinoid anandamide inhibits VR1 (Pertwee 2001).

Tachykinin antagonists

The effects of cigarette smoke on mucus secretion are also blocked by tachykinin antagonists, indicating that tachykinin release from sensory nerves mediates these effects (Tokuyama et al 1990). Tachykinins are potent stimulants of mucus secretion from submucosal glands and goblet cells. Substance P stimulates secretion from human airways *in vitro* and this effect is mediated via NK_1 receptors (Rogers et al 1989). This suggests that NK_1 receptor antagonists may be useful in treating mucus hypersecretion, at least in cigarette smokers. Several non-selective and selective tachykinin antagonists are in clinical development and a preliminary report indicated that they may reduce mucus secretion in patients with COPD (Ichinose et al 1993).

Sensory neuromodulators

The release of neuropeptides from sensory nerve endings may be modulated by the activation of several types of receptor situated on the endings of sensory nerves in the airways (Barnes et al 1990). Opioids are the most effective neuromodulators and inhibit mucus secretion induced by cigarette smoke and other stimuli (Kuo et al 1992a). In human airways *in vitro* opioids inhibit mucus secretion induced by capsaicin stimulation (Rogers et al 1989). These effects of opioids are mediated by μ opioid receptors on sensory nerves, indicating that a peripherally acting opioid may be effective. Peripherally acting opioids, such as BW443c, have been developed but have not been tested in patients with mucus hypersecretion.

Several other types of agonist also reduce the release of sensory neuropeptides from airway sensory nerves and are therefore potential treatments for mucus hypersecretion. Cannabinoids are very effective in inhibiting sensory neuropeptide release from airway nerves and activate CB_2 receptors on airway sensory nerves (Patel et al 2002). This suggests that CB_2-selective agonists, such as AM1241 and SR144528, may be effective in reducing mucus hypersecretion.

Many of these prejunctional receptors on airway sensory nerves work through a common molecular mechanism that involves opening of large conductance Ca^{2+}-activated K^+ channels (K_{Ca}). Drugs which open K_{Ca} (such as NS1619) and openers of ATP-dependent K^+ channels (K_{ATP}) (such as levcromakalim), inhibit submucosal gland and goblet cell secretion induced by cigarette smoke and neural stimulation (Kuo et al 1992b, Ramnarine et al 1998). K^+ channel openers are therefore potential treatments for mucus hypersecretion, but so far no clinical studies have been reported.

TABLE 1 Inflammatory mediators that stimulate mucus secretion and their inhibitors

Mediator	*Receptor*	*Inhibitor*
Histamine	H_2	Ranitidine
Prostaglandins E_2, $F_{2\alpha}$	EP, FP	Indomethacin
Cys-Leukotrienes	Cys-LT_1	Montelukast
Platelet-activating factor	PAF	Apafant
Bradykinin	B_2	Icatibant
Endothelin 1	ET_A	Bosentan
ATP, UTP	P_{2Y2}	None
Reactive oxygen species	—	Antioxidants

Mediator antagonists

Many of the mediators that are increased in asthma may stimulate mucus secretion (Barnes et al 1998) (Table 1). However, each mediator alone is likely to have a small effect, so that antagonists of a single mediator would be no more effective in reducing mucus secretion than in controlling asthma. Different mediators are likely to be involved in COPD. Inhalation of the cyclooxygenase (COX) inhibitor indomethacin is reported to reduce mucus hypersecretion in patients with COPD (Tamaoki et al 1992), but long-term trials of COX inhibitors have not yet been undertaken. Adenosine 5′-triphosphate (ATP) and uridine triphosphate (UTP) are potent stimulants of submucosal glands and goblet cell secretion (Shimura et al 1994, Roger et al 2000). These effects are probably mediated by P_{2Y2} receptors, so that P_{2Y2} antagonists might be effective in mucus hypersecretion. Reactive oxygen and nitrogen species are potent stimulants of mucus secretion (Wright et al 1996). This suggests that antioxidants may be useful and may account for some of the clinical benefits of *N*-acetylcysteine in COPD patients. Similarly inhibitors of inducible nitric oxide synthase may also be useful.

Cytokine inhibitors

Multiple inflammatory cytokines are involved in chronic inflammatory airway diseases such as asthma and COPD. This has suggested that inhibition of these cytokines may have therapeutic potential. Some cytokines have been particularly linked to mucus hypersecretion. Tumour necrosis factor α (TNFα) causes a prolonged increase in mucus secretion and up-regulation of mucin genes (Levine et al 1995), suggesting that inhibitors of TNFα may be beneficial. However, it

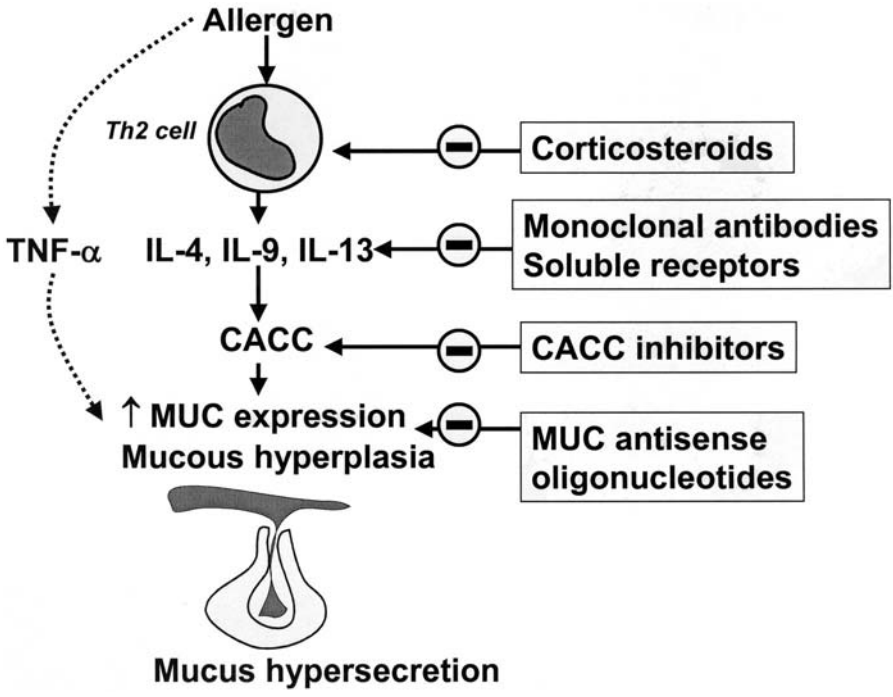

FIG. 2. Targets involved in mucus hypersecretion in asthma. CACC, Ca^{2+}-activated Cl^- channel; MUC, mucin gene; IL, interleukin; TNF, tumour necrosis factor.

seems unlikely that a single cytokine will account for the mucus hypersecretion in inflammatory airway diseases, so that antagonizing a single cytokine may not have a major therapeutic impact. The T helper 2 (Th2) lymphocyte-derived cytokines IL4, IL19 and IL13 all cause mucus hypersecretion (Dabbagh et al 1999, Temann et al 2002, Longphre et al 1999) and this may be an important mechanism for mucus hypersecretion in asthma (Fig. 2). One approach is to inhibit these individual cytokines using antibodies or soluble receptors. Another approach is to inhibit the Th2 cells which secrete these cytokines with selective immunomodulators. Suplatast tosilate is reported to be a selective inhibitor of Th2 cells which has some beneficial effect in asthma, and inhibits goblet cell metaplasia in mice (Shim et al 2000).

Epidermal growth factor (EGF) also appears to be important in mediating mucus secretion, the increased expression of mucin (*MUC*) genes and mucous hyperplasia. EGF receptors may be involved in the increased mucus secretory response to Th2 cytokines, oxidative stress and cigarette smoke (Shim et al 2001, Takeyama et al 1999, 2000, 2001; Fig. 3). Several inhibitors of EGF receptor

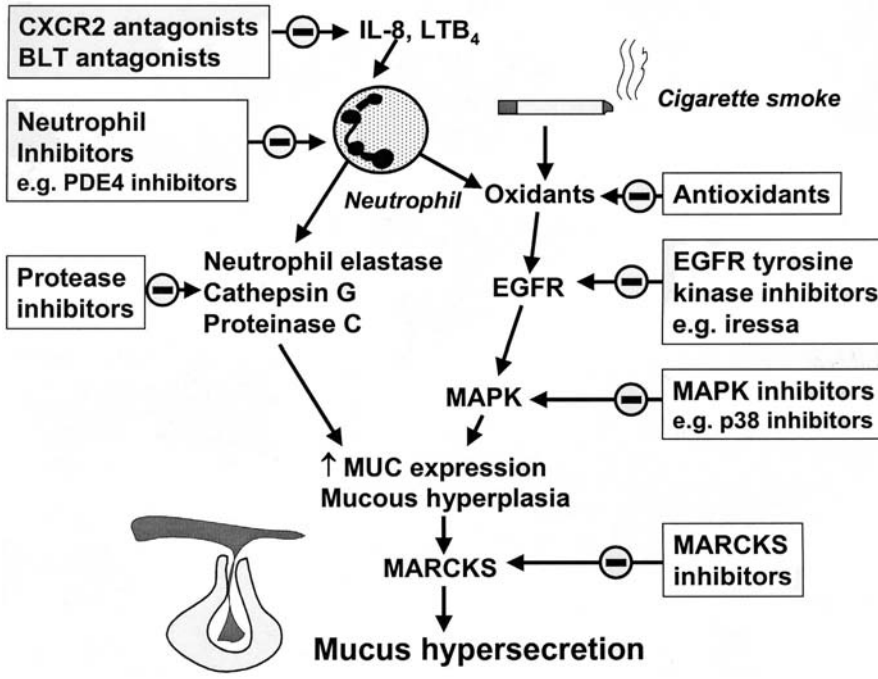

FIG. 3. Targets involved in mucus hypersecretion in chronic obstructive pulmonary disease (COPD). IL, interleukin; LTB_4, leukotriene B_4; CXCR2, CXC chemokine receptor – 2; BLT, leukotriene B_4 receptor; PDE, phosphodiesterase; EGFR, epithelial growth factor receptor; MAPK, mitogen-activated protein kinase; MARCKS, myristolated alanine-rich C-kinase substrate.

tyrosine kinase activity, such as AG1571, ZD1839 (iressa) are in clinical development for the treatment of carcinoma and may prove to be useful in mucus hypersecretion if they are safe.

Protease inhibitors

The neutrophil-derived serine proteases neutrophil elastase, cathepsin G and proteinase 3 are all potent stimulants of submucosal gland secretion and goblet cell discharge (Sommerhoff et al 1990,Witko-Sarsat et al 1999). This links the neutrophilic inflammation in the airways seen in chronic bronchitis and COPD with mucus hypersecretion and suggests that protease inhibitors (which inhibit neutrophil elastase and proteinase 3) may be effective in reducing mucus hypersecretion (Fig. 3). Several neutrophil elastase inhibitors are in clinical development, including ONO-5046 which is a small molecule inhibitor (Nogami

TABLE 2 Some novel targets and inhibitors of mucus hypersecretion

Target	*Inhibitor*
Proteases?	Macrolide antibiotics
MARCKS	MARCKS inhibitors
MAP kinases: p38	SB 203580
ERK	PD098059
Ca^{2+} activated Cl^- channels (hCLCA1)	MSI 1956
MUC genes	Antisense oligonucleotides

et al 2000). Since these proteases are derived from neutrophils another strategy is to inhibit the neutrophilic inflammation in airway diseases by blocking the influx of neutrophils in response to chemotactic factors, such as LTB_4, IL8 and GROα. Small molecule inhibitors of LTB_4 receptors (BLT_1 receptors) and of CXCR2, the chemokine receptor mediating the neutrophil chemotactic effects of CXC chemokines, are now in clinical trials. Phosphodiesterase 4 inhibitors, such as cilomilast, also inhibit neutrophil infiltration and should therefore reduce mucus hypersecretion.

Other approaches

There are several other approaches to inhibition of mucus hypersecretion that are currently being investigated, largely based on identification of new targets that are involved in mucus secretion (Table 2).

Macrolide antibiotics

Erythromycin is reported anecdotally to inhibit mucus hypersecretion and inhibits human airway mucus glycoprotein secretion *in vitro* (Goswami et al 1990). This may reflect some anti-inflammatory property of macrolide antibiotics and these drugs have recently been shown to have neutrophil elastase activity (Gorrini et al 2001).

MARCKS inhibitors

The myristolated alanine-rich C-kinase substrate (MARCKS) appears to play a key role in the intracellular pathways involved in mucus exocytosis in response to multiple stimuli and an antisense oligonucleotide directed against MARCKS

attenuates mucus hypersecretion (Li et al 2001). MARCKS may represent a novel therapeutic target for inhibiting mucus hypersecretion from multiple causes.

MAP kinase inhibitors

In chronic mucus hypersecretion there is up-regulation of *MUC* genes, particularly *MUC5AC*, in airway epithelium. This is presumably due to increased gene expression in response to inflammatory signals that activate specific signal transduction pathways, such as tyrosine kinases and mitogen-activated protein (MAP) kinases in mucus secreting cells, or cause the differentiation of non-mucus secreting into mucus-secreting cells. There is emerging evidence that p38 MAP kinase and ERK MAP kinase pathways may be involved and small molecule inhibitors of these pathways are in clinical development (Takeyama et al 2000,Wang et al 2002).

Ca^{2+}-activated Cl^- channel inhibitors

Ca^{2+}-activated Cl^- channels (CACCs) have recently been found to be important in mucus secretion from goblet cells. A novel protein termed GOB5 is expressed in airway epithelial cells of mice and is linked to airway hyper-responsiveness and mucus hypersecretion which are attenuated by an antisense oligonucleotide to this protein (Nakanishi et al 2001). The human equivalent of GOB5 is hCLCA1 which induces mucus secretion and *MUC5AC* expression and may therefore be a target for inhibition. Th2 cytokines, including IL9, may activate hCLCA1 and this may provide a mechanism to account for mucus hypersecretion in asthma (Zhou et al 2001). Small molecule inhibitors, such as MSI 1956 of CACC have now been developed (see Zhou et al 2002, this volume). However, it is not certain whether they would be effective in the mucus hypersecretion of COPD and cystic fibrosis.

MUC gene suppression

Finally, inhibition of *MUC* genes may be possible in the future. An 18-mer mucin antisense oligonucleotide is reported to suppress *MUC* gene expression induced by wood smoke in rabbit airway epithelial cells and unexpectedly to also inhibit smoke-induced metaplasia of these cells (Bhattacharyya et al 1998). It may be possible to discover small molecule inhibitors of *MUC* gene expression in the future.

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DISCUSSION

Basbaum: I was intrigued by you saying that despite the fact that *N*-acetylcysteine was taken off the market, you think it perhaps should be brought back. Have you treated patients with *N*-acetylcysteine and did they report feeling better?

Barnes: Some patients feel better, but its benefit has only become obvious with meta-analyses. The individual studies were generally negative.

Basbaum: When you last used it, years ago, did your patients feel better?

Barnes: Some patients thought that mucolytics, such as *N*-acetylcysteine and bisolvon, were very effective in reducing mucus secretions, but this was always difficult to demonstrate objectively.

Rubin: Inhaled *N*-acetylcysteine with a pH of 2.2 is incredibly irritating. Oral acetylcysteine doesn't appear to get into the airway secretions that we can measure, nor does glutathione. There is tremendous publication bias, so I would urge caution in these Cochrane analyses. The publication bias is not only that it is not particularly interesting to publish negative studies, but also that editors tend to send manuscripts out for review to other people who have already published in the field, and these earlier publications are often positive. When we did a large, randomized multicentre trial of oral acetylcysteine in patients with stable COPD, we found no significant improvement in pulmonary function, mucus properties and quality of life. We went back and found a number of abstracts showing no effect; these never made it to full publication. I contacted several of these authors and found that indeed their papers tended to be reviewed by people who had published positive studies and didn't believe the negative results.

Vargaftig: There was a paper published a few years ago showing a marked reduction of total Ig titres with *N*-acetylcysteine. This was a marked effect.

Barnes: In animal studies *N*-acetylcysteine works quite well because of the large doses that can be given. This is not possible in clinical practice. We gave *N*-acetylcysteine in recommended doses by mouth and saw no effect on exhaled markers of oxidative stress, yet in animal models *invivo* and in cells *invitro* it does have antioxidant effects. We need better antioxidants that may need to be delivered by inhalation. New antioxidants in development may be more effective.

Rubin: N-acetylcysteine has been conjugated with a basic amino acid (lysine) that also has mucolytic properties. This is Nalcystelin, which has a pseudo neutral pH

and can be delivered by aerosol. Unfortunately, studies using this agent for cystic fibrosis therapy have been small and so it is hard to interpret the results.

Disse: The problem with these kinds of drugs is that the anecdotal experience does not count, because there is a huge placebo effect. This is seen in almost every trial. Blinded trials are almost impossible with *N*-acetylcysteine because patients recognize it by the smell.

Poll: I am interested in clarifying whether you believe that any benefit that *N*-acetylcysteine may have is actually through its mucolytic properties, or through its antioxidant effects.

Barnes: This meta-analysis (Poole & Black 2001) included all the mucolytics. Most of the beneficial effect was explained by the *N*-acetylcysteine. It is not certain whether the non-antioxidant mucolytic drugs had much benefit.

Rubin: You made an important point, which is that some patients absolutely swear by some of these medications. The fact that we can't measure any response in these patients suggests that we need to develop appropriate outcome measures to determine who is the most likely to respond, and who is truly responding to these drugs. What we are measuring now doesn't seem to do the trick very well.

Basbaum: Why is breaking disulfide bonds ineffective as a treatment for hypersecretion? It sounds as though it should work.

Disse: You can't get high enough concentrations into the airways.

Sheehan: Let's say you wanted to argue that the mechanism of therapy is to break up the mucins. Then, in my experience reducing mucins in what you might term 'dirty mucus' is not a very favourable possibility. We use reductive reagents very effectively on purified mucins from which we have removed other protection mechanisms that could involve other proteins and lipids around the domains where disulfide bonds might be being maintained. These studies haven't been done systematically, but I can see why they might not be effective. Getting access to the relevant disulfide bonds may not be easy.

Rubin: There's an assumption there that reducing viscosity of the mucus will help clear the secretions. In fact, if you rely primarily on cough (airflow-dependent mechanisms), reducing secretion viscosity but retaining adhesivity actually *decreases* clearance. The analogy is a pea shooter: if I was to try to shoot out a pea, I could do a much better job with a pea than with pea soup. Reduction of viscosity on its own won't necessary improve cough clearance, although there is strong evidence that it will improve ciliary clearance.

Levitt: I don't know whether anyone has investigated trefoil factor 3. This might be a strategy for affecting cross-linking where perhaps an antibody against this might be useful.

Carlstedt: It would be fairly easy to test the lowest concentration possible to start to affect the mucins in a clean model system. We have done some studies, and we

started to see effects around 40 μM, but is it likely that this concentration could actually occur on the tracheal surface?

Basbaum: Could you envisage a compound that would somehow break up the other components of the mucus to give you access to the mucin?

Carlstedt: You could do this, but if you wanted to use such a drug orally, it may strip your stomach and you'd have ulcers instead. If you could deliver it by inhalation it might work.

Basbaum: It is important to have a rational understanding of why something doesn't work. These disulfide bond breakers, such as *N*-acetylcysteine, theoretically should work.

Barnes: But they don't get into airway secretions in sufficient concentrations.

Basbaum: Then the problem becomes how do we get into the secretions? If we can get into the mucin then they will work. So is there any way of breaking the encapsulating shell or the interfering molecules around the mucins so that we can deliver the dithiothreitol or *N*-acetylcysteine to the relevant spot?

Sheehan: I did a bioinformatics survey of the C- and N-terminal domains on mucins. They are full of interesting functional domains. We haven't talked much about the scavenging functions encapsulated in the mucus gel, and these domains could be involved in these. It is full of molecules that suck in and deal with a wide range of irritants.

Basbaum: Perhaps what you are saying would allow us to rule out the option of breaking up mucus by attacking disulfide bonds.

Sheehan: The point Ingemar Carlstedt is making (and I am repeating) is that we can do some work to estimate their effectiveness on mucins, but there are many other molecules that could be involved in the formation of the gel. Many of these we don't know about, and we don't know the involvement of disulfide bonds with those molecules either. It doesn't seem to be a promising strategy to me.

Disse: There is a fundamental problem with drugs breaking the disulfide bridges and also with antioxidants. That is, all these reagents are used up by the reaction, which means that they are oxidized themselves and have to be present in equivalent amounts to the molecules they are targeting. This would require us to provide millimolar amounts to the airways, and this is close to impossible by the oral route. If patients inhale the drugs, they would need to inhale milligrams. The highest dose among inhaled drugs that is currently used is cromoglycate, of which up to 20 mg is delivered to the airways and about 5 mg may deposit there. This is not enough for an antioxidant or disulfide bridge-breaking agent that is used up in the reaction.

Carlstedt: A drug like this could be developed. Dithiothreitol that we routinely use in the laboratory is very different from *N*-acetylcysteine in that it forms a ring structure when consumed. This ring closure is driving the reaction, which makes it much more efficient on the molar level and you could probably come down a

couple of orders of magnitude in concentration. But the important question is whether or not we want to use drugs like this.

Rubin: There are tremendous assumptions here. We can measure ciliary clearability of secretions on frog palate and the ferret trachea. We can also measure airflow-dependent clearance. But the relationships among the constituents of secretions, their biophysical properties and the clearability are still not well known. Furthermore, we don't know how these biophysical properties differ between patients with cystic fibrosis, COPD and asthma. Nor do we know how this relates to the degree of impairment in an individual patient over time, and how this would respond to therapy. The problem is these are important issues, but there isn't any enthusiasm about supporting research to try to understand this very complex interrelationship that is significant for drug development.

Fahy: Peter Barnes, I want to ask you about two points that you made. One was that steroids are all we need for asthma, and the other was that we don't have to worry about mucus hypersecretion, but instead we should concentrate on the cells. We know that steroids are effective for most asthmatics, but there is a significant unmet therapeutic need in severe asthma. Many patients with severe asthma have mucus problems that are not treatable with steroids. Mucus hypersecretion is also a big problem in acute respiratory illnesses that are not associated with chronic diseases. I think there is a large market for mucolytic drugs that can be given by the aerosolized route to help patients remove mucus from their airways. Recombinant DNAse is a successful example of this strategy. It is an expensive recombinant protein, but it works. We need to understand better the physical properties of mucus in different airway diseases so that we can have rational therapeutic strategies for treating these diseases with mucolytics. We also need easy measurements of the physical properties. If we want high throughput screens for developing mucolytic drugs, we will need easy readouts.

Basbaum: So we can't give up on mucolytics, even though it sounded from John Sheehan and Ingemar Carlstedt that it's an almost impossible approach.

Carlstedt: I said that this is possible to do from a chemical point of view, but I can't say whether patients will benefit from such a drug.

Verdugo: The point I think John Sheehan was making is that the disulfide bonds are not only in the mucins, but they might also be attaching several of the peptides that are defending the airways.

Barnes: The point I am making is that it is not the mucus hypersecretion that is the main problem in difficult asthmatics; it is the underlying disease process. If we could deal with the underlying inflammatory process then mucus hypersecretion would not be a problem. The hypersecretion is secondary to the disease process. We are not able to control every asthmatic patient with inhaled steroids, but we can control 95% of patients. In hospital clinics we tend to see the 5% of patients with

severe asthma and we clearly do need some additional treatment for these patients. It is likely that this treatment would be anti-inflammatory and would not specifically target mucus hypersecretion. Similarly, for the mucus hypersecretion of COPD, we should treat the underlying neutrophilic inflammation that probably drives the mucus hypersecretion.

Levitt: Some of those things are self limited, and we really want to treat the symptoms. Then you have the cystic fibrosis situation where it is going to be very difficult to treat the underlying process.

Barnes: Cystic fibrosis patients have lots of neutrophils in their airways that make things much worse.

Levitt: How do you get rid of them? You can't sterilize the lung.

Barnes: There are several novel approaches to neutrophilic inflammation, including p38 MAP kinase inhibitors.

Rubin: Inflammation itself is physiological, and inhibiting it may be a two-edged sword. We also have to realize that once we clear out all this garbage from the airways, you may have a better opportunity to treat the inflammation. It is not that you exclusively do one over the other: these strategies may be synergistic and it may provide more acute relief for the patient to clear the secretions out than inflammation, which may take a bit of time to show its secondary effects at reducing hypersecretion.

Basbaum: Everything that we have seen in the laboratory indicates that the pathogens and irritants themselves stimulate mucous cells to make mucus. Yes, inflammation is a big factor, but it is not everything.

Jackson: I would reinforce this. It is possible to stimulate mucus production and goblet cell metaplasia, independently of inflammatory cells, by which I specifically refer to leukocytes. The other thing I wanted to suggest was that pharmaceutical companies are already examining the possibility of interfering with inflammation. We all believe that this could influence goblet cell metaplasia and mucus production. But there is no guarantee that an individual therapy such as a p38 MAPK inhibitor is going to get to the market or be effective when it gets there. Perhaps what we need to do is look at all potential avenues, and hopefully we will get a successful therapy.

Barnes: All the clinical diseases we know that are linked to mucus hypersecretion are associated with inflammatory cell activation (usually neutrophils) in the airway lumen. I am not aware of any clinical lung disease with mucus hypersecretion that does not also have inflammation in the airways.

Reference

Poole PJ, Black PN 2001 Oral mucolytic drugs for exacerbations of chronic obstructive pulmonary disease: systematic review. Br Med J 322:1271–1274

Clinical evaluation of new therapies for treatment of mucus hypersecretion in respiratory diseases

Bernd Disse

Department of Therapeutic Area Respiratory Diseases, Boehringer Ingelheim Pharma KG, PO Box 200, 55216 Ingelheim, Germany

Abstract. In the past mucoactive drugs in airway diseases have been identified and profiled in symptom-based animal experiments and in clinical trials along related lines (cough and expectoration). Presently available drugs of this class are not generally accepted by licensing authorities worldwide and no new molecule clinically profiled as a mucoactive drug has been brought to regulatory approval in the past 20 years. Among regulatory guidelines only the CPMP 1999 'points to consider' on drug development in chronic obstructive pulmonary disease (COPD) advises for mucoactive drug development by suggesting that an indication for symptomatic treatment may be established on the basis of a symptom-related primary endpoint that should be justified as for its importance and supported by a co-primary lung function endpoint. Quality and safety of the new drug must be documented in long-term studies and the indication and use clearly described based on established or adequately profiled new primary endpoints in two pivotal studies. Published trials on mucoactive drugs have used a variety of endpoints. These include mucus hypersecretion-related symptoms by questionnaire, expectorated volume and dry weight, and mucus viscosity, elasticity and transportability. Most methods and endpoints are not validated and a positive standard of treatment is not established. New surrogate markers of efficacy for shorter term trials, e.g. induced or spontaneous sputum based assays (cellularity, mucus antigens), exhaled breath (NO), breath condensate (eicosanoids) or airway biopsy are only partially validated and the risk of false positive or negative phase II results is appreciably high. On the other hand, lung function measurements including airway hyper-reactivity assessment and typical phase III (long-term) endpoints like dyspnoea ratings, health status assessments, incidence of exacerbations and lung function decline over time are validated endpoints and offer a high likelihood of regulatory acceptance. Proof for no depression of lung mucociliary clearance is an important safety endpoint.

2002 Mucus hypersecretion in respiratory disease. Wiley, Chichester (Novartis Foundation Symposium 248) p 254–276

In the past mucoactive drugs in airway diseases have been identified and profiled in symptom-based or disease-related animal experiments, e.g. in the classical Boyd

(1972) expectorant model. Clinical trials have been performed along related lines mainly investigating sputum quantity and quality, and symptoms of patients with acute and chronic bronchitis or cystic fibrosis. The difficulty in defining the key mechanism driving clinical effectiveness of the 'classical' mucoactive drugs is reflected by the multitude of terms used to describe this class of drugs, i.e. mucolytics, secretolytics, expectorants and mucoregulators or modifiers. The correct understanding is further complicated by new mechanisms postulated to be of key importance but not proven, such as antioxidant properties of the cysteines. Drugs of this class, including *N*-acetylcysteine and ambroxol, are not generally accepted by licensing authorities worldwide because many drug licenses rely on old data. No new mucoactive drug profiled with up-to-date clinical data has been brought to regulatory approval in the past 20 years, unless the inhaled enzyme dornase alpha indicated for the management of cystic fibrosis is considered as such. Yet this drug was not clinically profiled for mucus-modifying qualities (Physicians Desk Reference 2002). In conclusion there is no recent precedent for clinical development in airway diseases of a mucoactive drug leading to regulatory approval.

Requests to the clinical developer and general overview of clinical development

The clinical developer needs to comply with the requirements of many parties within and outside of his own organization, from research and development departments requesting early feedback on the value of the mechanism to licensing authorities expecting a submission which adequately documents the quality and long-term safety and efficacy of a new drug (Fig. 1). The indication and use must be clearly described based on established or adequately profiled new primary endpoints in two pivotal studies. It is advisable to have this multitude of requirements in mind from the very beginning of designing the programme.

Any clinical development follows a standard masterplan. Figure 2 outlines the intention of the studies and Fig. 3 the dimension, the number of patients involved and the treatment duration. The standard, of course, has to be modified according to the special qualities or issues the new drug may have. Today's approaches to drug research are almost exclusively based on the molecular pharmacological mechanism and by virtue of this have been profiled with human 'systems' such as receptors. The drawback may be that the new compounds are often less well profiled in symptom- or disease-related animal models, with the disadvantage that basic pharmacokinetic properties, including distribution to the targeted site and duration of action, were less in the focus of compound selection.

At the clinical development stage, attempts may be made to try to compensate for this deficiency by the addition of measurements of pharmacological activity at

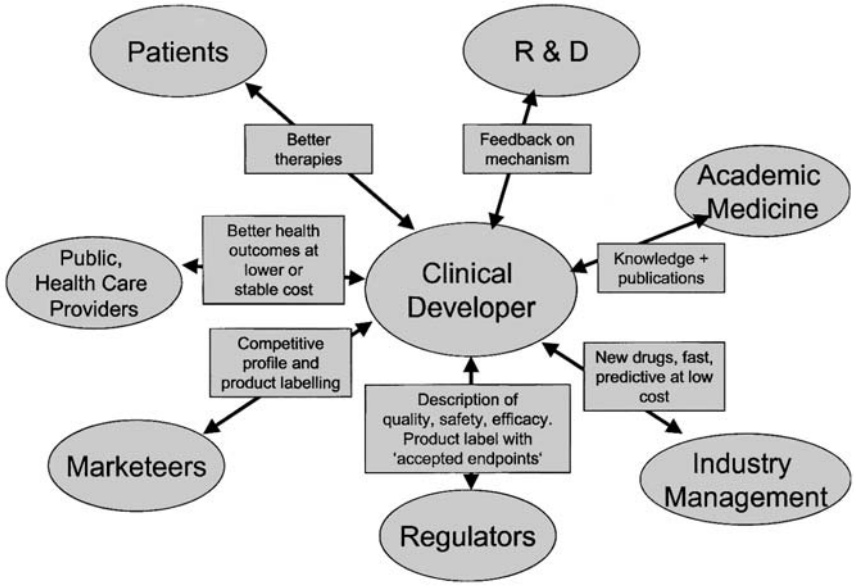


FIG. 1. Requirements for clinical development. Requests from within the pharmaceutical industry and from society which should be met by the developer of a new drug.

the target site of action to phase I single and multiple dose studies, which conventionally focus on tolerability and safety in healthy volunteers. Successful phase I and IIa studies have described the maximum tolerated dose, the safety with multiple administration to steady state and pharmacokinetics, and the safety in a small number of patients with the targeted disease. Ideally, results with surrogate markers of efficacy provide proof of concept and allow the effective dose range to be narrowed.

Phase II dose-ranging has to establish the dose–response relationship, and define a minimum effective dose as well as the plateau of effect (maximal efficacy). Such a trial may already require larger numbers of patients for prolonged treatment periods: 200 patients per study arm for three months is not unusual. Ideally these studies should already use a clinical signal as a primary endpoint that can be taken forward into the later confirmatory pivotal phase III studies.

Phase III trials have to be conducted in large and representative patient populations. They must have a pre-specified hypothesis and confirmatory statistics, and the endpoint(s) selected are the basis for formulating the indication and use section of the SPC (summary of product characteristics) of the new drug. In addition these phase III studies have to document the long-term safety in a sufficient number of patients, e.g. at least 300 for six months and 100 exposed for one year (ICH 1995).

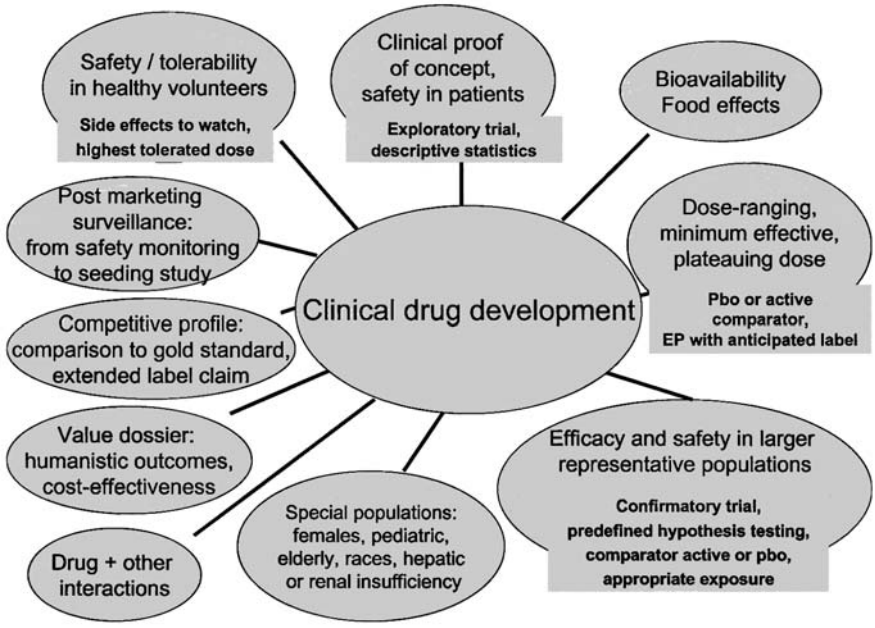


FIG. 2. Clinical masterplan: intentions and objectives of clinical studies.

Finally quite large phase IIIb studies have to evaluate the pharmacoeconomic value of the new therapy and its profile versus competitors in the field.

Clinical development guideline recommendations

Mucus hypersecretion and dyscrinia in the respiratory tract are a prominent feature of several airway diseases such as asthma, acute bronchitis, chronic (simple) bronchitis, chronic obstructive pulmonary disease (COPD), bronchiectasis and cystic fibrosis, evident from the presence of chronic or acute cough and expectoration. Among the guidelines concerning clinical drug development only the CPMP (Committee for Proprietary Medicinal Products of the European Agency for the Evaluation of Medicinal Products, London) points to consider (ptc) on clinical investigation of medicinal products in the chronic treatment of patients with COPD specifically touches on mucus disorders in the airways (CPMP 1999). The ptc have the following recommendations for inclusion of patients:

- Diagnosis of COPD following relevant guidelines of the ERS, ATS and BTS, asthma should be excluded (the first global guideline of the Global Initiative for

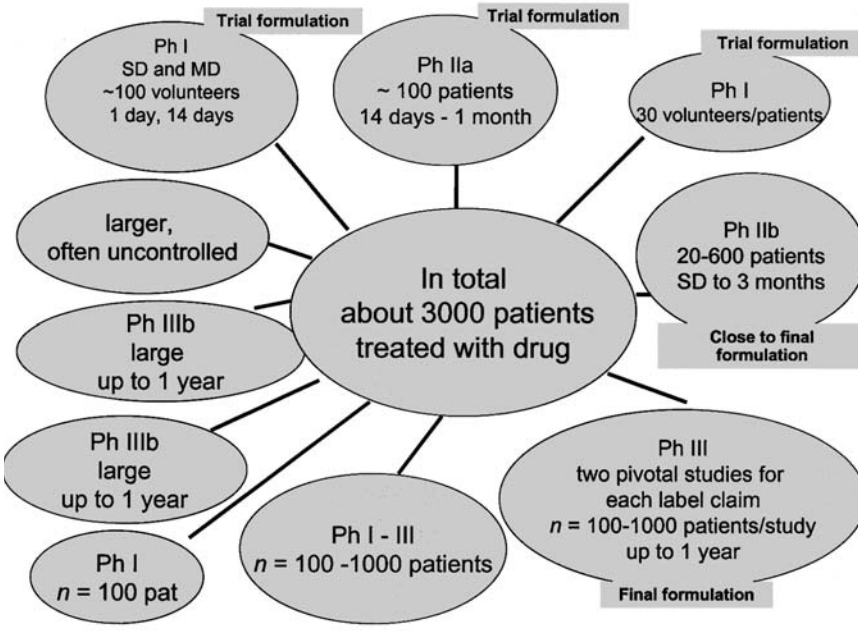


FIG. 3. Clinical masterplan: type and clinical phase of studies, number of patients and duration of treatment.

Obstructive Lung Disease, GOLD 2001, became available after the ptc document, Pauwels et al 2001).

- Characterized by reversibility of FEV_1 (forced expiratory volume in one second) to bronchodilator and glucocorticosteroids.
- Smoking status should repeatedly be recorded or even used for stratification.

Concomitant medication needs to be recorded.

Of specific value to the topic are the two development options described in the ptc document:

- A symptomatic claim may be established based on a symptom-related primary endpoint. Such a symptom-based endpoint should be justified as for its importance and supported by a co-primary spirometric measurement, FEV_1. Important secondary endpoints are other pulmonary function and gas exchange measures as well as exercise tolerance (i.e. the six-minute walk test), symptom scales, exacerbation rates and health related quality of life measures. The treatment duration required should be six months or longer.

- A claim of disease modification means to show a change in the natural history of COPD. The 'classical' measure would be to demonstrate divergence of the long-term rate of decline of FEV_1 between patient groups on the new treatment versus standard care. A treatment offset by an initial bronchodilator type of effect should be excluded. Although not mentioned in the ptc document the treatment period for such assessment is three years or longer.

Of course, any labelling claim needs to be supported by statistically significant results based on a predefined clinically meaningful improvement in two pivotal studies and the programme needs to be performed under GCP (Good Clinical Research Practice).

Are there positive standards for treatment of mucus disorders in respiratory diseases from published clinical studies?

The profiling of a new therapy is facilitated if active comparators or a 'gold standard' of therapy has been established in a particular indication. Implicitly, this would also mean that appropriate endpoints have been profiled and validated. Table 1 lists the classes of drugs used in COPD and chronic bronchitis, their general value and endpoints used in clinical studies.

Smoking cessation is the only broadly accepted treatment which improves symptoms as well as the natural history of disease. Of course, for those 20% of patients with COPD who were life-long non-smokers, this is not an option. Smoking cessation can serve as gold-standard and sets the limits for FEV_1 rate of decline changes as outlined in Fig. 4 which at best might be achieved by a new drug. **Pulmonary rehabilitation** programmes do not have an impact on mucus disorder but probably set the limit for the magnitude of health status improvement that can be achieved mid-term. **Bronchodilators** and especially the long-acting compounds tiotropium, salmeterol and formoterol set the standard for lung function improvement and improvement in dyspnea and exercise tolerance. **Anti-inflammatory treatments** such as inhaled glucocorticoids, although not licensed in many countries for COPD, are frequently used. After about 14 days of treatment they elicit a small increase in FEV_1, although they have not been shown to slow down the accelerated rate of decline in lung function in COPD. Anecdotally, it is reported that glucocorticoids reduce hypersecretion in asthma and COPD, however, such an effect is not described in the literature. To the contrary a large, double-blind, parallel group, 24 week study investigating the potential of inhaled fluticasone and salmeterol in COPD failed to show an influence on mucus hypersecretion related symptoms (FDA Advisory Committee 2002, Table 2). For this assessment, the study used an improved version of a bronchitis symptom

TABLE 1 Gold standards in COPD and chronic bronchitis therapy: guidance and active comparators for treatment of mucus disorders in the respiratory tract

Therapeutic principle	*Compound*	*Value and endpoints*
Smoking cessation	n.a.	Most effective treatment for 80% of COPD patients whose disease is smoking related. Improves *rate of decline of* FEV_1 and *symptoms* (*cough and expectoration*)[1]
Pulmonary rehabilitation	n.a.	Effective in improving general *health status*, e.g. *SGRQ* and *exercise tolerance*, benefit fades rapidly after cessation[2]
Bronchodilator	ipratropium, salbutamol, salmeterol, formoterol, tiotropium	Established first-line treatment in COPD. Improve lung function (FEV_1) and, more modestly, *exercise tolerance*. Long-acting compounds (especially tiotropium) in addition improve symptoms of *dyspnea*, and may reduce *exacerbations of COPD*. Influence on mucus hypersecretion not shown[3]
Anti-inflammatory drugs	oral glucocorticoids for exacerbations, inhaled glucocorticoids (registration)?, theophylline?, PDE4 inhibitors: cilomilast, roflumilast (Phase III)	Oral glucocorticoids reduce *hospital days* with an exacerbation and increase *time to next hospitalisation*.[4] Inhaled glucocorticoids show small increase in FEV_1, influence on rate of decline not shown, may reduce *exacerbations of COPD*.[5] PDE4 inhibitors increase FEV_1, may improve dyspnea and reduce exacerbations.[6] Influence on mucus hypersecretion not shown with any of the agents.
Mucoactive drugs, 'classical'	ambroxol, bromhexine, guaifenesin, iodinated glycerol, N-acetylcysteine, S-carboxymethylcysteine, sobrerol	Older data profile members of the class with *symptoms* (e.g. *ease of coughing up secretions*), reduction of *quantity of sputum* and improvement of *physical mucus parameters*.[7] Newer studies try to show a prophylactic influence on *frequency of exacerbations of COPD*.[8]
Mucoactive drugs, 'experimental'	oxitropium, inhaled indomethacin, recent developments not part of this review	The high-dose anticholinergic reduced *sputum volume* in patients with chronic bronchitis/panbronchiolitis and hypersecretion by 30% during an 8 week treatment. Inhaled indomethacin reduced these by 50% in 14 d. Both studies were comparatively small.[9]
Anti-infective drugs	penicillins, cephalosporins, macrolides, chinolones	Evidence of small benefit during exacerbations in patients with low baseline flow. *Dyspnoea, sputum volume* and *sputum purulence* cardinal symptoms of an infectious exacerbation.[10]

[1]Scanlon et al (2000), [2]Carone & Jones (2000), [3]Johnson & Hagan (2001), Disse & Witek (2001), Disse (2001), [4]Niewoehner et al (1999), [5]McEvoy & Niewoehner (2000), FDA Advisory Committee (2002), [6]Torphy et al (2001), [7]Germouty& Jirou-Najou (1987), [8]Poole & Black (2001), Decramer et al (2001), [9]Tamaoki et al (1992, 1994), [10]Saint et al (1995), Anthonisen et al (1987).

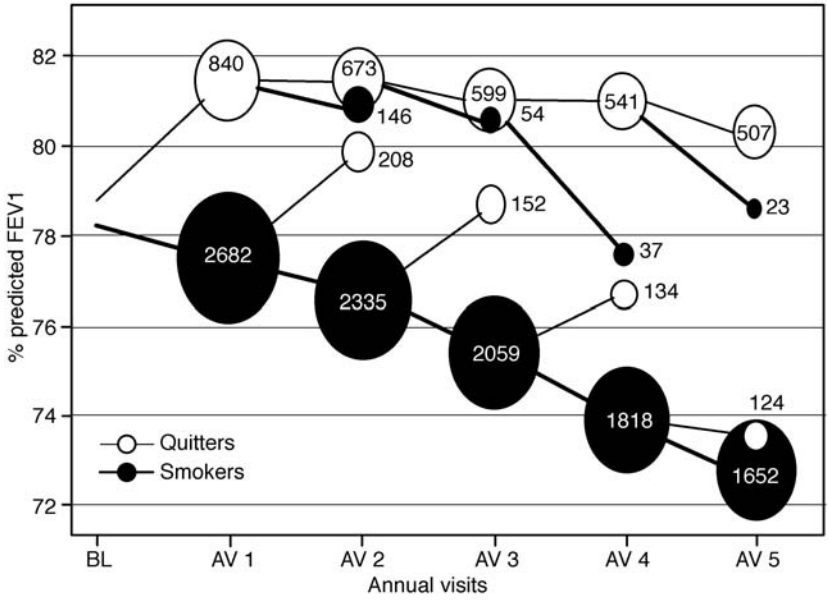


FIG. 4. Smoking cessation as a gold standard for drug therapy in COPD. The lung health study I involved 3926 smokers (with mild to moderate COPD), and the follow up tracked both continuing smokers and quitters. (Modified from Scanlon et al 2000.)

questionnaire developed by Petty (1990) and described in more detail below. A change of more than 1.4 points in total score was predefined as minimal clinically meaningful improvement. Although the group mean score dropped (improved) from baseline in this 24 week study by about two points, there was no difference between the active treatments and the placebo group. The study shows that the instrument is sensitive to changes but did not establish inhaled glucocorticoids as an active principle on mucus symptoms in COPD.

As for profiling **mucoactive drugs**, a variety of endpoints have been used in older published trials, mainly in chronic bronchitis or COPD. The study of Germouti & Jirou-Najou (1987) may be cited as a typical example. They investigated the mucolytic properties of 10 d treatment with 120 mg/day ambroxol in a double-blind, placebo-controlled parallel group study involving 60 patients. Daily sputum volume, sputum viscosity, difficulty of expectoration, cough and the peak expiratory flow rate (PEFR) were assessed by patient diary, mostly grading with 1 to 3 scoring points. The results showed an increase for 5 d, and then for the rest of the observation period a decrease of sputum volume in both groups. However, this was significantly higher from days 2–5 in the active treatment group (Fig. 4). Sputum viscosity was significantly (but only slightly) decreased, as was the difficulty of expectoration at most time points. The

TABLE 2 Summary of change from baseline in chronic bronchitis symptoms questionnaire (CBSQ) GAS[a] ITT population of study SFC 3006, sponsored by Glaxo-Smith-Kline

Time point	*Placebo (n=181)*	*SAL 50[b] (n=160)*	*FP 500[c] (n=168)*	*Sal50/ FP 500 (n=165)*
Treatment day 1 (baseline)				
n	180	159	167	164
mean	7.3	7.4	7.0	6.9
Week 12				
n	127	131	120	132
mean	5.7	5.6	5.0	4.8
mean change	1.3	1.8	2.0	2.1
Week 24				
n	112	120	100	112
mean	5.4	5.0	5.2	4.8
mean change	1.6	2.0	1.9	2.1
Endpoint				
n	172	158	161	157
mean	5.7	5.6	5.5	5.1
mean change	1.5	1.9	1.6	1.8

[a]CBSQ: the chronic bronchitis symptoms questionnaire evaluated the COPD symptoms of cough frequency and severity, chest discomfort, and sputum production on a scale of 0–4, where a rating of 0 reflects no symptoms. The domains were combined to a global assessment score (GAS). Subjects had to have a score of $\geqslant 4$ out of a possible 16 at Treatment Day 1 to qualify for the study. The minimal clinically important change of > 1.4 was predefined.
[b]Sal 50, salmeterol 50 μg b.i.d.
[c]FP 500, fluticasone 500 μg b.i.d.
Reference: FDA Advisory Committee 2002.

problem with short duration of treatment studies is that they often included patients with acute infections or infectious exacerbations, the natural course of which takes about 10 days until resolution and induces high variability. At least it can be stated that the method of sputum volume measurement proved sufficiently sensitive to record changes with time.

Petty (1990) organized a large, randomized, double-blind, placebo-controlled, parallel study including 180 patients treated with iodinated glycerol 60 mg q.i.d. versus 181 on placebo for 8 weeks. The study recruited patients with stable chronic bronchitis according to ATS criteria, complaining of cough and difficulty to bring up sputum. The instrument was an especially designed questionnaire asking the patient to assign a score for severity of symptoms from 1–5 in the categories of cough frequency, cough severity, chest discomfort and dyspnoea, which was

TABLE 3 The national mucolytic study on iodinated glycerol (Organidin): a randomized double-blind 8 week placebo-controlled parallel study[a]

Baseline characteristics	*N*	*Age*	*FEV1% predicted*	*Drop-outs n*
Organidine 60 mg q.i.d.	180	65	43.6	33
Placebo	181	66	45.5	46

Results	*Cough frequency*	*Cough severity*	*Chest discomfort*	*Dyspnea*	*Derived global*
Baseline score	3.3	3.0	2.4/2.7[b]	3.0	11.8/12.1[b]
Organidine, 8 week change (Δ8w)	−0.6	−0.5	−0.3	−0.3	−1.8
Placebo, Δ8w	−0.4	−0.3	−0.2	−0.1	−1.2
ΔOrganidine/ ΔPlacebo	1.4	1.5	1.6	2.2	1.5

Inclusion criteria: patients with stable chronic bronchitis, ATS criteria, complaining of cough and difficulties in bringing up sputum.
[a]Petty (1990).
[b]Baseline values of Organidin/placebo different.
Instrument: cough frequency, cough severity, chest discomfort and dyspnea rated 1–5, sum of scores = patient's derived global score; ease of raising sputum, patient's global and physicians' global rated 1–7 scoring points (results not shown).

finally combined to result in the patient-derived global score. In addition, the change from previous assessment for ease of raising sputum, a patients' global and a physicians' global assessment with a score from 1 to 7 was recorded. Following the evaluation of the authors, the patient-derived global score and most of the sub-scores at end of week eight were statistically significant lower (i.e. better) for active treatment (9.97 ± 0.248, mean ± SEM) compared to placebo (10.89 ± 0.252, $P = 0.0048$). However, the evaluations did not correct for differences at baseline. Table 3 demonstrates the changes from baseline and gives an impression of the change within eight weeks treatment period versus the difference of placebo and active. It seems questionable whether the difference between active and placebo of 0.6 in change of global scoring points is clinically meaningful.

Many long-term large studies have been conducted to assess the potential of mucoactive drugs to reduce or prevent exacerbations of COPD. This topic was the subject of a Cochrane systematic review. Poole & Black (2001) assessed the results of 20 selected studies which had to have a randomized, double-blind, placebo-controlled design and a treatment period with mucoactive drugs for

more than 2 months. Most of the studies showed a small favourable effect and the overall odds ratio for suffering no exacerbation was 2.2 (95% confidence interval 1.9–2.5) if the patient was on active treatment. The limitation in interpreting this result is that the authors could not provide assurance that all studies with the mentioned agents during the specified period were published and available to them, so a selection bias cannot be excluded. However, the meta-analysis confirms the high value attributed to a clinical endpoint 'reduction of exacerbations' in COPD.

With drugs of **other pharmacological classes** there are only few studies reporting consistent changes in sputum quantity and quality following treatment. *N*-quaternary anticholinergics are only occasionally reported to reduce sputum volume (Disse 2001). The higher-dosed **anticholinergic** oxitropium reduced sputum wet weight by 30% following eight weeks' t.i.d. treatment of 33 patients with chronic bronchitis or panbroncholitis in a double-blind placebo-controlled study (Tamaoki et al 1994). The **cyclooxygenase inhibitor** indomethacin showed even higher efficacy of 50% when inhaled for 14 days by 25 patients in another double blind placebo controlled study (Tamaoki et al 1992). However, the patients in the latter study had extremely high daily sputum amounts of 189 ml at baseline. Sputum dry weight showed much less change with both agents. The two studies provide evidence that 24 h sputum collection and determination of dry and wet weight is a useful instrument in clinical studies.

Endpoints in clinical studies

As described above many methods have been used as endpoints in clinical trials to assess the effects of mucoactive drugs in respiratory diseases although there are significant problems with their use. A positive standard, i.e. an approved or at least broadly used drug, is not established for most of the endpoints (Table 4). There is a huge gap between well-established validated clinical and functional endpoints in long-term clinical trials, e.g. lung function and lung function rate of decline (FEV_1), rate of exacerbations, dyspnoea (TDI by Mahler et al 1984), health status (SGRQ by Jones et al 1992) and short-term accessible surrogate markers of efficacy (Tables 4, 5). The mucus-related assessments (sputum volume and dry weight, and mucus viscosity, elasticity and transportability) as well as recently developed markers of inflammation (exhaled NO, induced sputum cellularity) have mostly not been validated and their relation to symptoms, exacerbations and the natural history of disease in chronic bronchitis are unclear. Symptom questionnaires in COPD cannot yet be regarded as validated. However, the database for symptom ratings like the Petty (1990) questionnaire is growing. This leaves the clinical investigator with the problem that while short or medium

TABLE 4 Evaluation of endpoints in clinical trials with mucoactive drugs in airway diseases: surrogate markers of efficacy

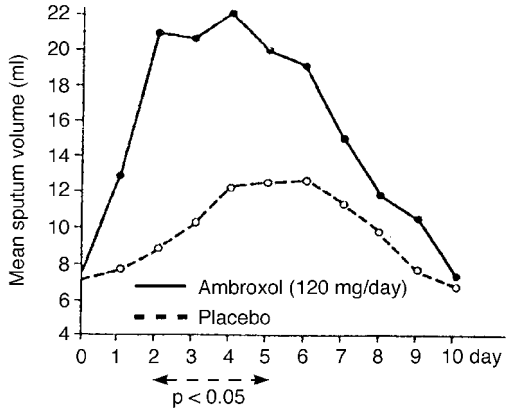
Endpoint	*Airway location and pathophysiological evidence*	*Invasive*[a]	*Validated?/ positive standard?*	*Regulatory acceptance*
Spontaneous sputum[b]	C > P, established for COPD cellularity, colour = marker of infection	No	Yes in COPD/No	SEP, co-PEP needs validation
Induced sputum[c]	C > P, established for asthma, cellularity = inflammatory marker	Y	Yes in asthma/ inhaled steroids	SEP, co-PEP
BAL	P > C, cellularity = inflammatory marker	YY	Yes interstitial lung disease/ No	SEP, co-PEP in interstitial lung disease
Sputum volume/ dry weight[d]	C > P, mucus hypersecretion, dyscrinia	No	No/No	SEP, co-PEP needs validation and MCID
Mucus Biophysics[e]	C > P, viscosity, elasticity, clearability and stickiness of mucus	No	No/COPD: *N*-acetylcysteine *in vitro*	SEP, co-PEP needs validation and MCID
Sputum MUC 5A/C, 5B, 2	C > P, mucus hypersecretion, dyscrinia	No	No/No	SEP, co-PEP needs validation
Bronchial Biopsy	C, inflammatory marker	YYY	Yes in asthma and COPD/ inhaled steroids in asthma	SEP and co-PEP
Breath NO, CO, ethane[f]	P > C, inflammatory marker, oxidative stress	No	NO: yes in asthma/ inhaled steroids in asthma	SEP and co-PEP
Breath condensate	P > C, e.g. eicosanoids as inflammatory, mucus markers?	No	No	SEP, co-PEP needs validation

[a]Invasiveness was graded No, Y (minimal), R (radiation exposure), YY (moderate), YYY (highly).
[b]Woolhouse et al (2001), Stockley et al (2000), Maestrelli et al (2001); [c]Fahy et al (1994), Maestrelli et al (2001), Pavord et al (1997); [d]Tamaoki et al (1992, 1994); [e]Rubin et al (1996); [f]Jatakanon et al (1998).
C, central airways; P, peripheral airways; PEP, primary endpoint; SEP, secondary endpoint; MCID, minimally clinically important difference; HRQol, health-related quality of life.

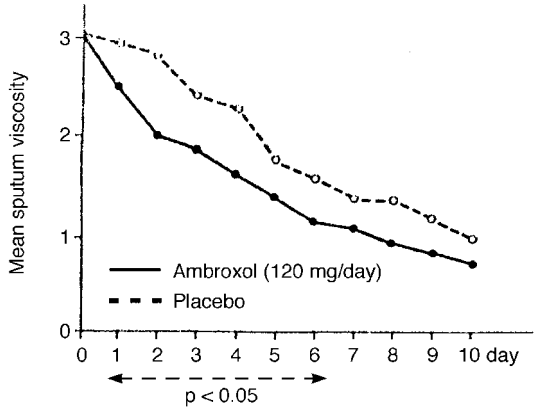
TABLE 5 Evaluation of endpoints in clinical trials with mucoactive drugs in airway diseases: clinical and functional endpoints

Endpoint	*Airway location and pathophysiological evidence*	*Invasive*[a]	*Validated?/ positive standard?*	*Regulatory acceptance*
Mucociliary clearance	C+P, clearance function	Y, R	COPD ?/No	SEP, as safety assessment mandatory for mucoactive drugs
FEV_1	C > P, airflow limitation	No	Yes(−) in asthma, Yes in COPD/ bronchodilators	PEP and co-PEP to symptoms
a.m. PEFR	C, airflow limitation	No	Yes in asthma, Yes(−) in COPD/ bronchodilators	PEP and co-PEP to symptoms
Partial Flow-Vol. Curves	C+P, airflow limitation	No	?/bronchodilators	SEP, co-PEP needs validation and MCID
FEV_1-decline	P > C, long-term lung function status	No	Yes in COPD, ? in asthma/No	PEP in COPD
CT (HRCT)	P, emphysema	Y, R	Upcoming in COPD/No	SEP, probably also as PEP after validation
Tussometry	Cough frequency and severity	No	No/antitussives	SEP/co-PEP needs validation and MCID
Symptom Questionnaire	COPD symptoms	No	Upcoming in COPD/inhaled steroids? bronchodilators	Co-PEP with lung function
Dyspnoea	Most important symptom in COPD	No	Yes/tiotropium in COPD	Co-PEP with lung function
HRQoL	Overall health status	No	Yes in asthma and COPD/inhaled steroids, bronchodilators	SEP, eventually co-PEP to lung function
Exacerbation	Stability of patient	No	Yes in asthma and COPD/inhaled steroids in asthma	PEP, MCID discussed

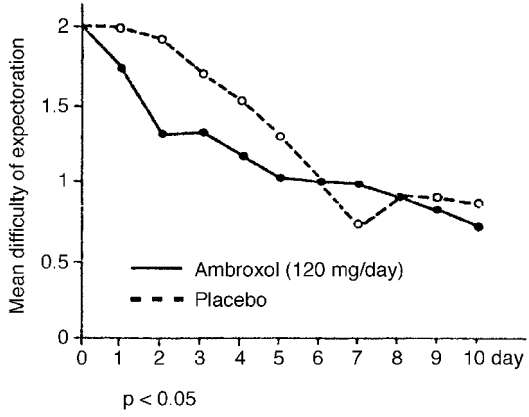
[a]Invasiveness was graded No, Y (minimal), R (radiation exposure), YY (moderate), YYY (highly)
C, central airways; P, peripheral airways; PEP, primary endpoint; SEP, secondary endpoint; MCID, minimally clinically important difference; HRQol, health-related quality of life.



Mean sputum volume



Mean sputum viscosity



Mean difficulty of expectoration

duration of treatment phase II studies can be designed using these surrogate markers to provide proof of concept and define dose and posology, the risk that long-term phase III studies based on higher value and well validated endpoints may not confirm the results is appreciable. The alternative choice is to only document safety of the new treatment in patients in phase II and to postpone proof of concept and dose-ranging to large and long lasting phase IIb/III studies. This approach involves a high financial risk to the sponsor but may avoid premature termination of a programme based on false-negative surrogate marker evidence.

Validation of endpoints takes a tremendous effort, as large and long-lasting longitudinal studies are required to show reproducibility, variability as well as selectivity and sensitivity to the wanted signal by means of correlations to established outcomes. The definition of the minimal clinically meaningful change is especially demanding. For this reason validation of new endpoints is more likely to be a side product of phase III clinical studies than an independent endeavour.

Assessment of mucus quality with physical methods (viscosity, elasticity, transportability and adhesiveness) is technically demanding but seems responsive to short term treatments, e.g. 14 days. Symptom questionnaires need longer treatment periods to respond (6 weeks to 6 months) but can be applied more easily to larger numbers of patients. As questionnaire results are subjective, they may be modified or driven by extra pulmonary effects, which is why the CPMP ptc propose using the symptom assessments as co-primary endpoints with lung function measurements in COPD (CPMP 1999). In conclusion the successful development of a mucoactive drug will have to profile effects on mucus, improvement of associated symptoms as well as long-term outcomes.

Example of a clinical development for a mucoactive drug

Single and multiple dose phase I studies will assess the tolerability, safety and pharmacokinetics. These studies should include a target-site drug exposure assessment, e.g. an estimate for receptor occupation based on airway lining fluid, sputum or airway tissue drug concentration determination (or estimate if direct

FIG. 5. Mucolytic properties of 10 d ambroxol treatment in a double-blind, placebo-controlled trial. Diary: daily sputum volume (ml), viscosity (scored 1–3), difficulty of expectoration (1–2), cough (1–3) and PEFR (l/min). Cough and expiratory flow rate are not shown because not different under treatment. (Modified from Germouty & Girou-Najou 1987.)

sampling not possible). Of higher value is the measurement of a pharmacological effect at the target site, e.g. *ex vivo* elastase inhibition by induced sputum (for an elastase inhibitor) or inhibition of a mediator challenge (e.g. capsaicin challenge for a neurokinin antagonist).

Phase IIa may be conducted either to only document safety in a number of patients or try to provide proof of concept with surrogate markers. In absence of validated markers in the field of respiratory mucus disorders a 'fishing expedition' approach may be appropriate, i.e. a range of parameters have to be measured with the prospect of identifying a sensitive endpoint influenced by the new drug. An appropriate example for such a study is the attempt to reproduce the study by Petty (1990) conducted by Rubin et al (1996). This was a 16+16 weeks, randomized double-blind crossover study in 26 outpatients with symptomatic chronic bronchitis, comparing once again 60 mg iodinated glycerol q.i.d. versus placebo. The instruments used comprised the symptom questionnaire developed by Petty (1990), supported by measurement of lung function, percentage of solids in sputum, mucus elasticity and viscosity by microrheometer, mucus spinnability by filancemeter, as well as transportability of mucus in a cough machine and on the frog palate. The contact angle on glass and the wettability served as a measure for mucus adhesiveness. The study showed a significant reduction of the percentage of solids and a significant decrease in the sum of the symptom scores with time, but no difference between active and placebo. The other parameters did not show any significant change with time or treatment. Interestingly, the spinnability of mucus correlated with the total symptom score ($r = +0.38$, $P < 0.01$). Such results form a basis for validation of surrogate endpoints. If performed today, depending on the pharmacological class under investigation, a determination of 24 h sputum volume and concentrations of mucus antigens MUC2 or MUC5A/C and B might be added and the length of the treatment period in the crossover would probably be shortened. As a proof of concept study only the highest well tolerated dose and a dose derived from, for example, pharmacological ED_{80} estimations would be included.

With proof of concept established, the most sensitive and reliable endpoint is the candidate for a phase II dose-ranging study. The co-primary endpoint should already combine functional mucus parameters and symptoms. Lung function measurement is mandatory but does not necessarily need to improve. Any mucoactive drug may negatively impact the mucociliary clearance. For this reason a study with scintigraphic assessment of the clearance of an inhaled radiotracer is mandatory. Long-term (6–12 months) confirmatory phase III trials still carry an appreciably high risk of failure, because long-term outcomes (lung function status, incidence of exacerbations, changes of health-related quality of life) cannot be predicted based on physiological mucus measurements and symptoms. The phase III co-primary endpoints would again

combine mucus functional plus symptoms and in addition the mentioned outcomes.

The minimum profile with a chance for licensing authority approval would be symptomatic improvement explained by and based on the mucus-modifying qualities of the drug.

Conclusions

Clinical development of mucoactive drugs may utilize biophysical and biochemical mucus qualifying and quantifying methods in shorter phase II proof of concept and dose-ranging studies. However, because validation and a positive standard are missing there is an appreciable risk that these surrogate marker studies are not predictive for phase III long-term outcomes. Phase III outcome methods are validated and likely acceptable for regulatory approval. If the mucus assessments find broader use in future, there is a good chance that they will be validated by correlation to clinical long-term outcomes.

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DISCUSSION

Basbaum: This is the first I have heard of the GOLD standard across the Atlantic. I have always had the idea that standards for certain drugs are different in Europe than in the USA. I know people who have friends in Europe asking them to send drugs from the USA that they can't get. Is this changing now? Is there a uniform standard world-wide?

Disse: Regulatory authorities in the USA and Europe act independently of each other. However, out of the NHLBI/WHO supported GINA initiative (Global Initiative for Asthma) originated an international panel of expert physicians in the field. They called themselves the GOLD initiative, for Global initiative for chronic Obstructive Lung Disease. They have recently published international guidelines for the diagnosis, management and prevention of COPD, which can be accessed through the internet (*http://www.goldcopd.com*) and have also been published (Pauwels et al 2001). It is a COPD initiative that attempts to reach consensus on and distribute internationally harmonised treatment guidelines for COPD.

Basbaum: Does the FDA use these guidelines?

Disse: They are of course not obliged to, but I assume they are likely to respect them. This also gives some guidance as to the importance of endpoints in clinical trials.

Rogers: You presented a fascinating list of the criteria that would have to be fulfilled for regulatory authorities. One of these was reduction of exacerbations. We heard earlier that *N*-acetylcysteine is effective in exacerbations of COPD. Consequently, we have this strange dichotomy where a drug that is generally unpopular in terms of therapy for respiratory disease actually fulfils one of your criteria.

Disse: If there were a set of sufficiently powered prospective phase III trials proving that, it would be internationally accepted. However, that is not the case. Therefore, if you want to prove this claim for your new drug, you would have to run trials versus placebo and not versus *N*-acetylcysteine. I did not intend to suggest that a claim for reduction in exacerbations is mandatory for a new mucoactive treatment, however it would have high value.

Rogers: It is just that there is this unfortunate quirk whereby *N*-acetylcysteine would fulfil this particular criterion.

Jeffery: Is there any other drug that meets that criterion in terms of exacerbation? Are they up to the standard you required here and that you outlined?

Disse: So far reduction of exacerbation of COPD has been published for ambroxol, *N*-acetylcysteine, ipratropium, Combivent (ipratropium and salbutamol), salmeterol, fluticasone, tiotropium and maybe more. However, this has always been the result of *post hoc* secondary analysis, subgroup analysis or meta-analysis of study results and not the predefined primary endpoint. Currently there is no drug for which there is official recognition of reduction of exacerbation of COPD on the label by a critical licensing authority.

Rubin: Part of the rub here is how one defines exacerbation. Patient-defined exacerbation could be very different from that defined by a physician. One thing that is being looked at is getting a uniform standard definition of exacerbation as has been done in cystic fibrosis and applying this to COPD using set criteria. Then patients who have just undergone exacerbations are treated (this is because those who have had an exacerbation are far more likely to have another exacerbation), and then a Kaplan–Meier survival analysis is used to determine time to a subsequent exacerbation. This is better defined than the number of exacerbations over a set period of time.

Levitt: Are there other lessons to be learned from cystic fibrosis in terms of how to conduct these trials in the absence of a primary standard (approved therapy) to use as a control, that is based on large pivotal trials? Pulmozyme trials were designed to get a drug approved in a situation where we had a lot to learn and similar risks. Are there other parallels we can draw from the CF trials? There could be careful measures of loss of lung function after exacerbations treated with drug versus control/placebo, similar to those in the literature.

Disse: I am not aware of creative new endpoints in these trials. Pulmozyme was approved on reduced number of respiratory tract infections in the less severe group of severity and improvement in FEV_1.

Levitt: FEV_1 over six months was less impressive. Ultimately, the question boils down to what the experts believe the most rational way forward is, in terms of symptom scores and primary endpoints. You touched briefly on the use of lung volumes. Is it a widely held belief that small airway dysfunction and specifically changes of lung volume ought to be a way to proceed, or are there other measurements that the experts here would promote as appropriate for phase II studies, taking into account the risks?

Rubin: A reasonable assumption is that mucus retention leads to trapped gas. Jeff Regnis and colleagues evaluated changes in pulmonary function (Regnis et al 1994). In this study, pulmonary gas trapping assessed by the ratio of residual volume (RV) to total lung capacity (TLC) or RV/TLC was more closely related to measurements of mucociliary clearance than spirometry (FEV_1).

Levitt: I think this is a critical issue. It is more of an objective endpoint that the FDA can accept as meaningful on a clinical basis. It has not yet been validated though.

Rubin: Patients will tell you that a drug makes them feel wonderful, but this can be difficult to measure objectively. Everyone measures FEV_1, but it's a bit like looking for your keys at night under the lamp post, not because that's where you lost them but because that is where the light is. Gas trapping may in fact be a better marker, and functional exercise capacity has an appeal to it. They are not yet validated, but it would be great if one of the things that can come from a meeting like this is support for such validation studies.

Levitt: What about the recent pulmonary hypertension experience, and the approval of new drugs there? Some of the endpoints are similar in terms of assessing these drugs. Are these lessons we can use for COPD therapy?

Rubin: These are different diseases; we can't assume that they are similar. Validation studies are needed.

Disse: The sensitivity and reliability of these methods is unproven. It is even more difficult to define the minimally clinically important improvement. I haven't seen any attempt to define this.

Barnes: Do you think a mucoregulator could be approved, if that was all it did? I am not sure the regulators would necessarily see this to be a useful therapy. However, you could argue that a treatment for COPD that included an effect on mucus would be more likely to be approved.

Disse: An efficacious mucoregulator inducing profound improvements of symptoms, but not having an effect on the natural history of disease or on exacerbations, might nevertheless get approved. But you would have to show that the compound is at least neutral with regard to mucociliary clearance, exacerbations and natural history. Ideally, the effects should be associated with improved health status shown with a validated quality of life questionnaire. Why shouldn't regulators accept symptomatic improvement? From a patient point of view this is important, even if you don't change the natural history. The problem primarily may come from another area, and that is reimbursement. The healthcare providers do not care too much about patient's symptoms; they care about costs. Reducing symptoms does not decrease costs.

Barnes: I think people should explore further how to measure mucus secretion in human respiratory diseases. We have already discussed some of the problems in this area. One possibility is to use imaging technology. Previously, tantalum bronchography was used to image the airways, and showed striking abnormalities in asthma and COPD, where airways were often completely blocked. This technique is now considered too invasive and the radiation dose is too high, but there are other imaging techniques such as high-resolution CT scanning and MRI with hyperpolarised helium that need to be explored as a way of quantifying mucus secretion.

Rubin: Even looking at the proportion of central to peripheral deposition using aerosol tags would be worth investigating. All of these need to be explored.

Nadel: In the development of drugs to treat peripheral airway plugging with mucus, identification of small plugs is problematic. Development of techniques (e.g. radiological) that can recognize these small plugs should have a high priority. A meeting like this is a good opportunity for discussing this, because it is unlikely that drug companies are going to do major work on small focal spot X-ray tubes and specialized functions to look at plugging in peripheral airways.

Basbaum: Nor is NIH going to fund this. The study section will say this is for industry; it is not basic science. You asked for support for validation studies for endpoints. I can't imagine the LBPA study section supporting this.

Rubin: It would have to go out as an RFA.

Barnes: This would be an opportunity to think about how we might do some of the things we have discussed. What is clear from this meeting is that there are major advances in basic research, but the clinical relevance of this has not been investigated because the measurements are difficult.

Fahy: If you buy into the idea that mucus hypersecretion is more than just an annoying symptom, but that it actually contributes to exacerbations and excessive decline in lung function, my belief is that a well designed drug targeting a specific mechanism (e.g. Ca^{2+}-activated Cl^- channel, or EGFR inhibitors) should result in an improvement of symptoms and a reduction in exacerbations. In phase Ib and IIa you can use symptoms; in phase III you will want to use exacerbations. For proof of concept you can go into the airway in a subgroup of these people and do invasive studies to quantify mucous cells and mucin gene expression. I am not sure that we need a lot of new outcomes here. I would like better assays for measuring mucins in induced sputum. This would be a nice outcome.

Levitt: The tissue is accessible and the gene expression is measurable. If we show a difference, is this going to be believable?

Fahy: It is believable in the context of symptoms and exacerbations, and also in proof of concept.

Levitt: It has to be put into a perspective integrated with the clinical end points. But is it going to be a meaningful secondary endpoint? If you saw some equivalent symptom scores and fewer exacerbations or a neutral effect on exacerbation, and selective down-regulation of mucin genes, would that be convincing?

Fahy: Yes. Most of these airway problems are not subtle. In cystic fibrosis and COPD there is massive change in the mucus phenotype, so you will want to see some effect on this.

Jeffery: We have just completed a double-blind placebo-controlled trial looking at Ariflo (cilomilast), a selective PDE4 inhibitor. We analysed bronchial biopsies and to my surprise there was a significant reduction in both the CD8 and CD68 population. However, the study was designed with the primary endpoint of

sputum inflammatory cell numbers, i.e. neutrophils. There was no change at all in sputum neutrophils. I was interested in your presentation regarding the regulatory authorities considering sputum outcome as secondary rather than primary. This seems rather odd. Is this well recognized by the industry?

Disse: The sponsor and investigator are of course free in their choice of primary and secondary endpoints, but regulatory authorities will advise them to take only established and validated methods as primary and the supporting evidence as secondary. If biopsy was possible in the study, from my perspective, this has a higher value than the sputum analysis.

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Final general discussion

Sheehan: I'd like us to return to the key issue of quantitation of mucins and their relationship to the properties of mucus. Dave Thornton and I have put a lot of effort into this, and I think we have defined the problem. With more work and a greater diversity of probes, we could provide a good methodology. But would it be interesting and useful to the people in this room? It is not an area where I myself would go much further, although I would be interested in collaborating with centres who were interested in quantitating mucins and had the appropriate samples and follow-ups. All the samples we get are one-offs coming to us via rather convoluted routes from interested clinicians. What we really need to take this forward are systematic studies on thousands of well-validated patients, with records of clinical outcomes, current drug treatments and smoking regimes. I'm never going to get funding to set this kind of work up, because it is not regarded as basic science.

Basbaum: So the immediate task is to correlate mucin gene expression with something that everyone agrees is disease related.

Sheehan: There is a significant underlying question here for me as a biologist. There are four distinctive large gene products that are all expressed on a cluster on chromosome 11. We clearly see changes of mucin phenotype with disease. However, I have no idea at all what the function of those distinctive mucins is. Interestingly, they are all very similar in their N- and C-termini and they vary in the repetitive tandem repeat sequences where they are glycosylated. As we look into it more, the type of glycosylation that goes onto those mucins seems to differ. Our data indicate that the glyco-phenotype is strongly maintained even in the face of inflammatory response, and this specific glycosylation presumably has functional significance. But we have no idea of the contribution of these individual mucins to different mucus secretions. Some data suggest that in the metaplasias a different kind of mucin gene product is being made, and therefore perhaps a different kind of mucus, in the context of the pathological change.

Basbaum: In this respect, John Fahy's work with laser capture and PCR of goblet cells from various human disease samples will be instructive. Some of the preliminary results show that there is a change in ratios of mucin gene expression in asthma versus cystic fibrosis versus normal and so on. For example, one of my favourite genes, *MUC2*, doesn't get expressed in the airway at all. Ingemar

Carlstedt, you once thought that it was never expressed in respiratory mucus, but presumably now you are starting to believe that it is.

Carlstedt: I believe that it is expressed; the only thing I said was that I couldn't find it in the sputum.

Basbaum: That's a fine distinction.

Rose: No it's not. It is the difference between RNA and protein expression, and it is important because RNA expression doesn't always translate into protein expression.

Sheehan: It's actually a massive distinction. There is good evidence for the turnover of genes when you can't find the secreted product, in many cases, and especially in proteins that undergo storage and regulation.

Carlstedt: In a couple of cystic fibrosis sputum samples I found a little bit of MUC2, but we are down to below 5%.

Basbaum: I wonder whether 5% of an insoluble mucin thrown into the gel like that could have a negative effect.

Sheehan: That's a very good point. But to my mind the more worrying point here is that we might not be getting the MUC2 secretion reflected back to us in the samples that we handle. There may be much more MUC2 around somewhere, but it doesn't get into our hands.

Basbaum: The laser capture that John Fahy is doing is an appealing technique. You can take frozen sections of human tissue, punch out the specific cells that you want, and do a microanalysis of the RNA they contain. It would also be possible to do protein or carbohydrate analysis on these samples.

Carlstedt: It is possible, but only if you have the energy to collect tens of thousands of cells: you need a lot more cells for proteomics than are necessary for RNA analysis. You would need even more if you want to study the carbohydrates.

Basbaum: If the question is important enough, then this could be done. John Sheehan, you are saying that the distinctive mucin polypeptides have somewhat distinctive glycosylation patterns. Pedro Verdugo, you talked about the tangled network as opposed to the cross-links. Is it not true that the identity of the terminal sugars will influence electrostatic interactions between monomers that will affect tangling and viscosity? Can we connect the dots and say that if we have MUC5AC without MUC2, and MUC5AC is fucosylated and MUC2 is sialylated, then having these different terminal sugars will affect the physical character of the polymer? Are we at this stage yet?

Verdugo: This is something I have been discussing with Ingemar Carlstedt. There are two options with the insoluble component that he has seen. One is that this material is indeed cross-linked, and it is then reprocessed once it has been secreted. There might be some building proteases inside the network that will take care of this. The alternative is that this system might be cross-linked and that you want to have a scaffold of cross-linked material together with linear

non-cross-linked polymers that are going to anneal once the material is outside the cell. There would be a cross-link that would hydrate to a certain level only. This component has a limit of swelling. At the same time there are other linear mucin polymers inside that are going to do the annealing between these scaffolds to make the mucus. These alternatives need to be tested with regard to the quaternary structure of the gel.

Basbaum: Would you expect there to be an influence on the gel?

Verdugo: Regardless of both secretions, you need to take into account not only the matrix, but also the hydration process. In this regard the way that these gels will hydrate will depend on the charge density. There are also issues of ion exchange. The mucus comes out loaded with Ca^{2+}. The Ca^{2+} needs to be wiped out by Na^{+}, and there has to be plenty of water for the swelling to occur. The whole process depends on the pH. It is a tremendously complex situation.

Basbaum: I realise that it is complicated, but let's say hypothetically that all other things being equal, would it make a difference to have a mucin that characteristically is fucosylated at its terminal sugars versus one that is sialylated? From our discussions earlier it seems well established that certain mucin gene products do have patterns of glycosylation that differ.

Verdugo: The other factor, of course, is length. A gel made of very long polymers will be different from one made of shorter polymers.

Basbaum: Is there any reason to suspect that MUC2, MUC5B and MUC5AC will be different in length?

Sheehan: When Ingemar Carlstedt and I were first isolating mucins, it turned out we were working mainly on MUC5B. We didn't know at the time that there were different mucin gene products. To our eye, most mucins were the same. We found that the generic subunit structure of all mucins is rather similar. Subsequently, it turned out that there can be different morphologies to these mucins, possibly depending on where they come from. This will be interesting to sort out. Something that does come through now is that there are different glycotypes of MUC5B. These may be strongly maintained. If you take the study of the asthma plug I mentioned earlier the material that washed out of those plugs quickly were the high-charged glycoforms. They were definitely smaller, lower molecular weight molecules. The MUC5AC molecules were long and tenuous, and washed out over a longer time. The final residual gel that was absolutely refractory to extraction was a low charged glycoform of MUC5B.

Carlstedt: Perhaps we can learn something here from 'comparative biology'. Colonic MUC2 is a 100% covalently cross-linked, fairly acidic network. Gastric surface mucus is mainly neutral MUC5AC in a tangled network. MUC5AC from the airway is a lot more acidic than in the stomach. Salivary MUC5B is about as acidic as respiratory MUC5B. The glycosylation pattern doesn't follow the gene; it follows the expression of the glycosyl transferases on the cell.

Basbaum: When you say acidic, you would say that it has more sialic acid.

Carlstedt: More sialic acid and/or sulfate.

Sheehan: There is evidence of specific glycotypes that carry more or less sialic acid and more or less sulfate. This hasn't been sorted out, but the prima-facie evidence is there.

Basbaum: When MUC2 is in the airway as opposed to the colon, is it still 100% cross-linked?

Carlstedt: It is all in what we call the insoluble phase. We didn't get enough of it to run electrophoresis, so we couldn't see whether it contains any non-reducible subunits. Pedro Verdugo, I think it is important to remember that gastric mucus must swell, even though it hasn't any charges. The charges are not mandatory for swelling.

Verdugo: I agree. It is an osmotic process. The issue is that for those species that are charged, the ion exchange process is almost mandatory in order to get this cross-linking out. On the other hand, we are putting all our focus on the mucin polymer matrix. The matrix may be important, but the rest of the processes that contribute to the swelling are also critical. We shouldn't ignore the biological processes that deal with the issues of hydration and the control of Ca^{2+}, water and pH in the surface epithelium.

Basbaum: It might be easier to make a non-viscous mucus by paying attention to these factors, than by chopping mucus up into pieces by destroying disulfide bonds.

Sheehan: The effects of EGTA on mucus rheology have not been understood. There's the definite possibility for Ca^{2+}-chelating factors that could be involved in linking up mucins together. This needs to be reevaluated.

Rose: I think that the glycosylation is under-appreciated. *O*-glycosides can be classified by their four major core types: an initial GalNAc is added to a serine or threonine, a second sugar is transferred to the C-3 of GalNAc, and the transfer of the third sugar will give a linear or a branched chain. *O*-glyocosides with all four major core types have been isolated from mixtures of bronchial mucins. We don't know what core types are present on specific MUC mucins so we don't know much about the charge/mass ratio of individual MUC mucins. Then the question of the terminal glycosylation becomes really important. Changes in sulfotransferases, sialotransferases or glucosyltransferases may reflect disease processes; they may be altering the actual mucin structure and therefore affecting mucus properties. This hasn't really been looked at and won't be until we can pull out MUC5AC and MUC5B mucins from normal individuals and people with airway diseases. This is not a trivial task.

Verdugo: To add one more factor in, the mucin is pretty much like a component that contains regions that are highly glycosylated hydrophilics, and regions that are hydrophobic. The ratio of hydrophilicity to hydrophobicity in a polymer

has critical consequences in terms of conformation. These ratios need to be investigated, in order to understand better how this polymer will relax in the solvent.

Basbaum: What does the hydrophobic part of the mucin do in the aqueous medium of the lumen?

Sheehan: We assume that these hydrophobic domains would be associated with the N- and C-termini. What Ingemar Carlstedt was talking about was the processing of these regions. Their presence and absence, and the relative amounts, become important. If I look at some of my older data, I would say that not only are there different amounts of these protein domains present, but also they may be involved with cross-linking the network in other ways, or providing binding sites for ancillary proteins that perform those cross-linking functions.

Verdugo: The hydrophobic interactions are extremely strong compared with electrostatic interactions. They could strongly limit the swelling of the material. I don't think we have the faintest idea about how these different components are operating in the gel.

Sheehan: There's so much there to follow up from Ingemar Carlstedt's observation. There is evidence that whole domains are cut away in some cases but may be present in others. The way mucins are processed in individual cells may have a crucial role in the formation of the gel.

Carlstedt: My working hypothesis here is that mucins are manufactured in a much more 'insoluble' form than we have so far appreciated. This could be a covalently cross-linked network. There is then probably an active process, most likely a protease, that turns them into a tangled network. This is how I think mucus is formed. By building in this active principle you can have different 'end points' allowing for a 'quality element'. We need probes to distinguish between the different 'end points' that could arise from this process.

Basbaum: So this processing with proteolysis is the good thing that will prevent the hydrophobic interactions. I am just trying to put this in a framework for non-specialists. So a candidate for a disease mechanism might be imperfect processing, so that there is too much retained of these hydrophobic domains that are potentially cross-linking.

Carlstedt: It might be like making a béchamel sauce. If the flour isn't dispersed properly, you get lumps. What could be happening here is that we end up with sticky lumps in the mucus.

Basbaum: This is a new candidate to explain pathophysiology that could be looked at.

Rubin: In fact, there are some opportunities to do this. The gene for α-L-fucosidase is on chromosome 1. There is an extremely rare autosomal recessive condition in which absence of α-L-fucosidase alters glycosylation of mucus. In this

disease recurrent chest infections eventually lead to death. We had the opportunity to evaluate the mucus from one of these children. These secretions had almost no viscosity and no elasticity. Because of this they were non-transportable by cough or cilia. This is a hint that glycosylation may have profound effects on secretions, and small alterations may help to separate some of the differences that we are seeing in these diseases, or with inflammation.

Index of contributors

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