Phenotypic variation in hamster bronchial mucous cells induced by different airway irritants

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Summary. Chronic mucus hypersecretion (CMH), a common feature of various obstructive pulmonary diseases, is caused by a variety of airway irritants. Bronchial mucous cell metaplasia (MCM), a histological correlate of CMH, can be induced in hamster airways by a number of different irritants. Previous studies with the hamster model suggest that the secretory cell response to different agents is not stereotyped but can vary in the type of mucus glycoconjugate produced. The present ultrastructural study was conducted therefore to provide quantitative evidence of phenotypic variation in mucous cells induced independently by exposure to the metaplastic agents elastase and acid. HPA-gold lectin cytochemistry revealed an increase in N-acetyl galactosamine at the cell surface and secretory granules of mucous cells in elastase-treated vs. acid-treated animals. Although there was no quantitative difference between the acid-treated and untreated groups, a difference in the pattern of binding within granules indicated variation in the secretory product. Because mucus glycoconjugates serve as attachment sites for specific pathogens, phenotypically distinct mucous cells may promote differential microbial colonization. In humans therefore, variation in the severity and progression of CMH may be due in part to secretory cell susceptibility and response to different pathogenic stimuli.

Keywords: lectins, bronchus, neutrophil, elastase, mucus

In many patients with chronic obstructive pulmonary disease, secretory cells in the small airways accumulate excessive secretory granules, causing a lesion termed mucous cell metaplasia (MCM). MCM represents the epithelial component contributing to mucus hypersecretion which is important in the pathobiology of acute and chronic bronchitis, asthma and cystic fibrosis (Thurlbeck *et al.* 1970; Bedrossian *et al.* 1976).

To understand the pathogenesis of MCM, this lesion has been induced in many experimental animals by exposure

to various agents. These include elastase (Snider *et al.* 1984; Christensen *et al.* 1987), dilute acids (Christensen *et al.* 1988), sulphur dioxide (Greene *et al.* 1984), tobacco smoke (Lamb & Reid 1969) and many other irritants (Reid & Jones 1983; Lundgren & Shelhamer 1990).

Mucous cells that form in the hamster bronchus within three weeks after human neutrophil elastase (HNE) exposure are thought to derive by conversion of pre-existing Clara cells (Christensen *et al.* 1987). They have a different morphology as compared to the small number of normal mucous cells in the untreated airway. As quantified by lectin cytochemistry, this abnormal or altered phenotype involves an increased amount of N-

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acetylgalactosamine at the cell surface and in the stored secretory product of mucous cells in the elastase-treated animal (Jamil *et al.* 1997). The rheology of respiratory mucus is determined, in part, by the quality and quantity of oligosaccharide side chains (Puchelle *et al.* 1973). Therefore, any change induced by environmental stress or pathogenic organisms may impair mucociliary clearance and promote microbial colonization (Gump *et al.* 1976; Lopez-Vidriero & Reid 1978) which has important clinical implications.

We questioned therefore whether the mucous cells induced after different airway stresses share a common phenotype. We thought a common phenotype unlikely because light microscopic stains for mucins have yielded different results for different models of MCM. In HNE-treated animals, an equal number of both magenta (neutral) and blue (acidic) mucoglycoprotein secretory granules were detected after alcian blue-PAS staining (Snider *et al.* 1984). In contrast, acid-treated airways contained more magenta staining secretory granules than blue secretory granules (Christensen *et al.* 1988). Because paraffin sections have limited resolution and the stains are non-specific, we studied this question with ultrastructural lectin cytochemistry of airways from animals treated concurrently with either dilute acids or HNE. The gold labelled lectin, Helix pomatia agglutinin (HPA), was used to characterize the N-acetyl galactosamine content at the cell surface and within secretory granules of mucous cells in both treatment groups. We tested the hypothesis that there would be a significant difference in the binding of this lectin. This would confirm variation in response of the airway epithelium to different insults.

Materials and methods

Male Syrian hamsters were divided into three groups, each containing six animals. Under CO_2 anaesthesia, each animal in the first group received a transoral intratracheal injection of 300 μg HNE dissolved in 0.5 ml of physiological saline. The HNE was purified from purulent sputum as described previously (Snider *et al.* 1984). Each animal in the second group received an intratracheal injection of 0.5 ml of 0.1M solution of nitric acid. The acid solution was diluted with distilled water from a 0.2M stock solution, followed by the addition of sufficient NaCl to yield 0.15 molarity. The third group served as an untreated control. All animals were sacrificed at 3 weeks by exsanguination from the abdominal aorta while under deep anaesthesia with T-61 euthanasia solution (Hoechst Co., Somerville, NJ, USA). The chest cavity was exposed and the lungs perfused via the right ventricle with 4% formaldeyde-1% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3–7.4). Portions

of left lung segments containing bronchi exceeding 0.5 mm in diameter were cut in cross-section and left in the same fixative overnight. The tissue was then washed for 15 min in 0.1M cacodylate buffer. To block free aldehyde groups, the tissues were immersed for 30 min in 0.1M ammonium chloride in 0.05M cacodylate buffer (pH 7.4). They were then rinsed in 0.1M cacodylate buffer. The tissue was dehydrated in increasing concentrations of ethanol at room temperature. Embedding was done in LR White resin (Polysciences, Warrington, PA, USA) according to the manufacturer's instructions.

Ultra-thin sections of bronchial epithelium were cut and picked up on collodion coated nickel grids (200 mesh). The grids were then floated on a drop of undiluted HPA-gold conjugate (Sigma Co., Poole, Dorset), with or without the blocking sugar 0.2M N-acetylgalactosamine, in a moist and sealed petri dish at 37°C for 3 h. The sections were then rinsed with distilled water and stained with uranyl acetate for 15 min and lead citrate for 5 min. Electron photomicrographs were prepared at a final magnification of 5600x for counting HPA-gold particles associated with the cell surface or secretory granules. To analyse HPA-gold label, at least 50 mucous cells were identified by specific criteria (Christensen *et al.* 1987) and chosen randomly from each group of six hamsters. Under 10x magnification, gold particles were counted on or above the cell surface (within 100 nm of the cell membrane) at two different sites. The length of the cell membrane was measured using the Optimas image analyser computer program. The intensity of lectin binding was expressed as the number of gold particles per micron of cell surface.

Binding of HPA to secretory granules was also assessed. HPA-gold particles were counted in three randomly chosen granules per cell from 50 cells/group. The areas of the secretory granules were determined using the image analysis program. For each cell, the intensity of label was expressed as the number of gold particles per square micron of secretory granule cross sectional area.

Binding to the cell surface and to intra-cytoplasmic secretory granules was compared among the mucous cells in control and in acid-treated and elastase-treated animals. Because the data assumed a normal distribution, statistical analysis was accomplished using analysis of variance followed by the Bonferroni test to isolate differences among the groups. For all statistical tests, significance was assigned to *P*-values below 0.05.

Results

Cell surface HPA-gold binding

The degree of HPA- gold binding to the cell surface

(GP/μm) differed significantly among the mucous cells in the treated groups. Mucous cells in the elastase-treated animals showed significantly higher binding of 69 GP/μm as compared to 24 GP/μm in the acid-treated animals (Figs 1 and 2). In untreated control animals, the mucous cells showed a mean binding of 22 GP/μm, which was significantly different from elastase-treated but not acid-treated animals. Sections stained with HPA in the presence of its blocking sugar did not show any gold particle binding.

Secretory granule HPA-gold binding

The labelling density of HPA gold binding (GP/μm^2) to the secretory granules also differed significantly among the mucous cells in the treated groups. In control animals the secretory granules showed a mean binding of 38 GP/μm^2 which was significantly different from elastase-treated but not acid-treated animals. The highest density of binding observed was 154 GP/μm^2 in the elastase group which was significantly different from the 30 GP/μm^2 in the acid-treated animals (Fig. 3).

There was a variation in the pattern of gold particle binding to granules among the groups. Untreated mucous cells showed an even and diffuse distribution of gold particle binding (Fig. 4a) as compared to a clumped arrangement in the mucous cells of elastase-treated animals (Fig. 4c). The secretory granules in
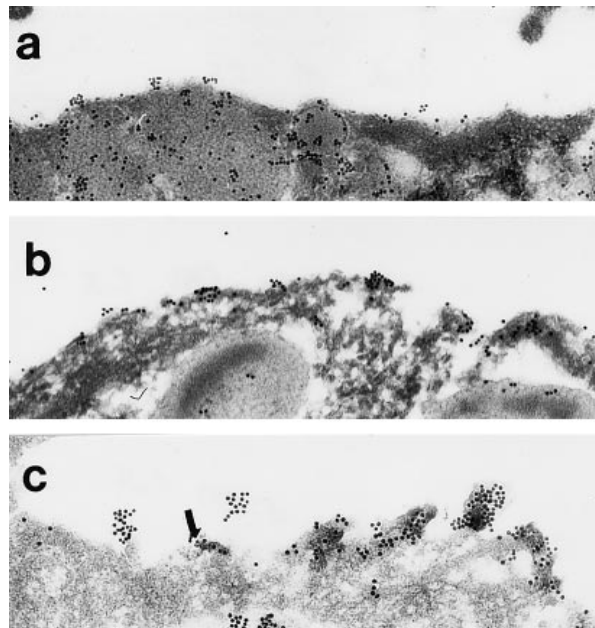

Figure 2 Electron micrographs of HPA-gold binding to hamster bronchial mucous cell surface. (a) Untreated-animal, showing relatively low amount of binding; (b) 3 week acid-treated animal, also showing relatively low amount of binding; (c) 3 week elastase-treated animal, showing higher amount of binding. Arrow indicates cell membrane. (Magnification for all approximately x44000).
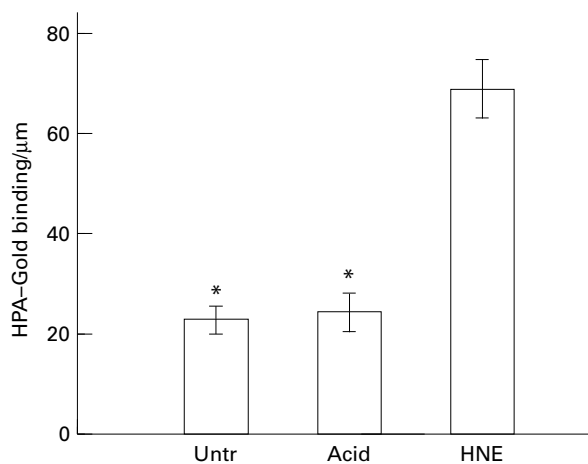

Figure 1 Comparison of HPA-gold particle binding on the luminal surface of bronchial mucous cells three weeks after exposure to 0.1N nitric acid (Acid) or human neutrophil elastase (HNE). Untreated controls (Untr) were also studied. For each group, 50 cells were analysed. Data are expressed as mean ± SEM. * = significant difference (P < 0.05) compared to HNE-treated hamsters by ANOVA/Bonferroni's tests. The acid-treated and untreated groups were not significantly different.
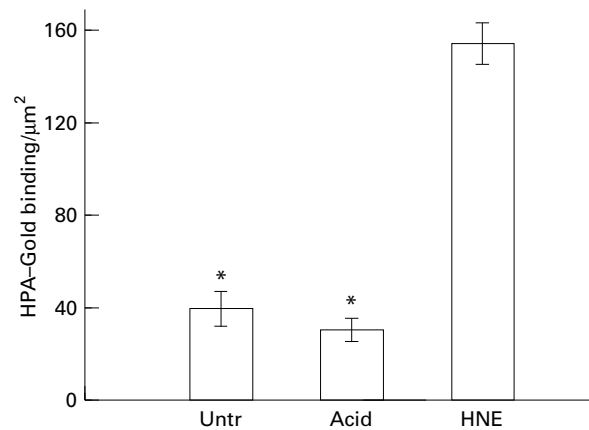

Figure 3 Comparison of the labelling density of HPA-gold particle binding to the secretory granules of bronchial mucous cells 3 weeks after exposure to 0.1N nitric acid (Acid) or human neutrophil elastase (HNE). Untreated controls (Untr) were also studied. For each group, 50 cells were studied. Data are expressed as mean ± SEM. * significant difference, (P < 0.05) compared to HNE-treated hamsters by ANOVA/Bonferroni's tests. The acid-treated and untreated groups were not significantly different.
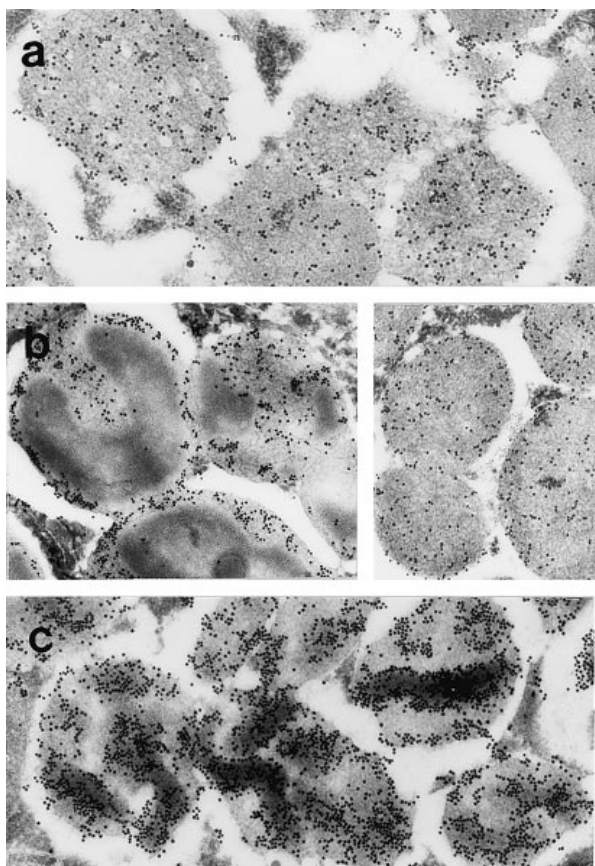
Figure 4 Electron micrographs illustrating HPA-gold binding to hamster bronchial mucous cell secretory granules. (a) Untreated-controls, showing homogeneous granules with mild diffuse binding. (b) 3 week acid-treated animal, containing (left) heterogeneous granules with an irregular dense core lacking binding adjacent to a lucent peripheral zone exhibiting greater binding and (right) homogeneous granules with diffuse binding. (c) 3 week elastase-treated animal, showing granules with clumped high magnitude binding. Magnification for all approximately ×30 000.

acid-treated animals exhibited both homogeneous and heterogeneous mottled forms. Homogeneous electron-dense granules were present in the majority of mucous cells. The heterogeneous granules, however, outnumbered the homogeneous granules and exhibited many forms. The most common form among the heterogeneous granules was one with an irregular dense core exhibiting no HPA binding surrounded by a more lucent peripheral zone exhibiting greater HPA binding (Fig. 4b).

Because sections incubated with HPA in the presence of its blocking sugar showed no gold particle binding, these findings suggest a greater occurrence of N-acetyl galactosamine in the elastase-treated animals as compared to acid-treated and untreated animals.

Discussion

This study has demonstrated that mucous cells formed within 3 weeks after elastase or acid treatment are phenotypically different. This difference is based on the occurrence of more N-acetyl galactosamine at the cell surface and within secretory granules in the elastase-treated animals as compared to the acid-treated and untreated groups. The use of only HPA, instead of a battery of lectins, limits the demonstration of possible qualitative and quantitative differences between the acid-treated and untreated animals. Nevertheless, morphological differences in the pattern of HPA binding in secretory granules suggest that acid causes an abnormality in mucous cells as does elastase (Snider *et al.* 1984; Christensen *et al.* 1988, 1990). The findings of this study therefore indicate that the upregulation of mucin granule production by secretory cells in response to different stimulants is not stereotypical.

These results are consistent with other studies reporting abnormalities in human respiratory mucus in hypersecretory states (Bhaskar *et al.* 1982; Reid & Bhaskar 1989). Mucin molecules differ in the extent of sialation and sulphation of their constituent oligosaccharides in different hypersecretory conditions (Sheehan *et al.* 1991). In cystic fibrosis (CF) patients, the concentration of sulphated mucin is higher than in non-CF individuals (Frates *et al.* 1983). There is also greater diversity of sugar groups, such as N-acetyl galactosamine and other amino sugars, in hypersecretory states such as bronchiectasis (Van-Halbeek *et al.* 1988) and cystic fibrosis (Lamblin *et al.* 1984).

In animal experiments, Dimitriadis *et al.* (1992) showed an increased amount of neutral and acidic non-sulphated and non-carboxylated polysaccharide in secretory granules in HNE-treated hamsters. Lectin cytochemistry has shown an abnormal and increased phenotypic expression of N-acetyl galactosamine at the cell surface and secretory granules of mucous cells in the elastase-treated as compared to untreated animals (Jamil *et al.* 1997). Other experimental studies using tobacco smoke (Jones & Reid 1978) and sulphur dioxide (Reid *et al.* 1983) have demonstrated qualitative and quantitative changes in airway epithelial mucus. The results of the present study lend further support to the establishment of a non-stereotypic expression of mucous cells in response to different causative agents, possibly reflecting disease-induced changes in the glycosylation of mucins.

An explanation for phenotypic variation of airway mucous cells after various agents is currently lacking. Changes in the cell surface glycoconjugates induced by elastase and perhaps other agents might alter the ability of mucous cell surface receptors to respond to extracellular signals that modulate mucin biosynthesis and secretions. Receptors have been identified on airway secretory cells for various bioactive ligands (Carstairs & Barnes 1986; Culp & Marin 1984; Sommerhoff *et al.* 1987). It is therefore possible that different perturbants may act through a variety of intracellular pathways to alter the transcription of glycosyltransferases responsible for expression of oligosaccharide side chains.

The biosynthetic mechanisms leading to disease-associated heterogeneous mucus are not clear. The presence of multiple genes coding for human mucin core protein has been reported in the gastrointestinal and respiratory mucosa (Kim *et al.* 1991; Porchet *et al.* 1991). Work on mucin protein cDNA clones indicates the presence of at least four human mucin protein genes on various chromosomes (Rose 1992; Gum 1992). Jany *et al.* (1991) showed increased transcription of a mucin gene in the airway of rats chronically exposed to sulphur dioxide. Deranged and variable expression of the mucin genes could lead to heterogeneous mucus glycoproteins. Post-translational glycosylation by Golgi- associated glycosyl-transferases is an important event in mucin biosynthesis. Shaper *et al.* (1990) have shown that glycosyltransferases are regulated at the transcriptional level. Disease-related alteration in the regulation of these enzymes could affect the oligosacharride side chains of mucins.

The clinical implications of altered secretory cell glycoconjugates are numerous. Proper rheologic properties of secreted mucins are determined, in part, by their biochemical character. Changes could impair normal clearance. Furthermore, carbohydrates on the cell surface serve as attachment sites for specific pathogenic micro-organisms (Andersson *et al.* 1983; Susuki *et al.* 1986; Ramphal *et al.* 1989). Ramphal *et al.* (1989) have shown differences in adhesion of *Pseudomonas aeruginosa* to mucin glycopeptides from sputa of patients with cystic fibrosis and chronic bronchitis. Therefore it is possible that altered and disease associated mucus results in loss of potential attachment sites leading to less entrapment and therefore diminished mucociliary clearance of *Pseudomonas* and perhaps other pathogens.

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