Cigarette smoke-induced ‘chronic bronchitis’: a study in situ of laryngo–tracheal hypersecretion in the rat

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Summary

1. Mucous secretion and mucosal permeability by the larynx and trachea, isolated in situ, was investigated in normal rats and in those in which ‘chronic bronchitis’ was induced by daily exposure to cigarette smoke for 2 weeks. Fucose was used as a specific marker for the secretion of mucus glycoprotein, and hexose and protein were markers both for mucus and plasma-type glycoproteins present in tissue fluid transudate. Albumin was used as an indicator of the contribution of serum to the secretions.

2. After equilibration, the mean basal secretion of fucose (µg/30 min collection) was significantly higher in ‘bronchitic’ rats than in the controls (P<0.01). Mean values for controls were: fucose 3 (SEM 1; n=9), hexose 41 (SEM 9; n=8), protein 1082 (SEM 385; n=8), albumin <2 (n=8); for ‘bronchitic’ rats the values were: 24 (SEM 6; n=7), 101 (SEM 26; n=8), 2000 (SEM 520; n=8) and <2 µg (n=7) respectively.

3. In control and bronchitic animals acute administrations of cigarette smoke, blown directly through the laryngo–tracheal segment after equilibration, caused significant (P<0.05) transient increases in the secretion of fucose, hexose and protein, but not of albumin.

Key words: chronic bronchitis, cigarette smoke, mucus, tobacco smoke.

Abbreviations: CS, cigarette smoke; PG, prostaglandin.

Introduction

Hypersecretion of airways’ mucus is characteristic of a number of bronchial diseases in man including chronic bronchitis [1, 2], asthma [3] and cystic fibrosis [4, 5]. Inhalation of cigarette smoke (CS) is one of the most important factors in the aetiology of chronic bronchitis [6, 7]. The most obvious histological correlates of the clinical manifestations of chronic bronchitis are the increase in number (i.e. hyperplasia) of mucus-secreting cells in the epithelium throughout the airways, and hypertrophy of submucosal glands present in cartilagenous airways [1, 8]. Experimentally, administration of CS to rats for periods of between 2 and 6 weeks (i.e. ‘sub-acute’ exposure) has been found to produce ‘bronchitic’ changes in the airways which can be quantified by using light and electron microscopy [9, 10]. Over a number of years, a system which consistently produces airways’ epithelial secretory cell hyperplasia and epithelial thickening has been established [10–12]. However, although Reid [13] has reported extensive mucus in the bronchial lumen of rats chronically exposed to sulphur dioxide, no study has measured secretion in rats rendered bronchitic by sub-acute exposure to CS.

The present study, therefore, set out to measure, by chemical means, the amount of secretion, as indicated by specific components, produced by the larynx and trachea of normal rats and those made ‘bronchitic’ by sub-acute exposure to CS. An intact
preparation was used in situ in the anaesthetized animal to investigate: (i) the change in basal secretion associated with 'chronic bronchitis', (ii) the response to acute administrations of diluted CS, and (iii) in each case, the proportion of secretion which is mucus and that which is transudate. The technique has been reported previously [14].

Materials and methods

Male, albino, outbred COBS Wistar, 13 week old rats (Charles River U.K. Ltd) were used. The rats were specific pathogen-free (SPF) with a mean body weight of 346 g (SEM 6; n = 29) on the first day of the experiment. They were housed in laminar flow cabinets (Forth Tech Services Ltd, Scotland) to preserve lung cleanliness [15]. Water and dry pellet food (Heygate and Sons Ltd, Northampton, U.K.) were freely available except during the CS-exposure period, when they were withdrawn from all animals.

Cigarette smoke exposure

The cigarette smoke exposure system and measurement of carbon monoxide have already been described in detail [12] and only a brief outline is given here. Smoke, from 25 'middle to high tar' cigarettes yielding 25 mg of tar, 2.4 mg of nicotine and 14 mg of carbon monoxide per cigarette [16], was generated over a 4 h period each day for 14 consecutive days by automatic smoking machines [17] and blown into cabinets in which the rats were housed. Temperature in the cabinets was between 21°C and 22°C throughout the exposure period, and relative humidity rose from 68 to a maximum of 97% over the 4 h period of exposure to CS. Concentrations of carbon monoxide in the cabinet rose from 2.5 p.p.m. to 163 p.p.m. during the same period.

Laryngo–tracheal secretion

Between 2 and 4 days after the exposure period laryngeal–tracheal secretion in normal and 'bronchitic' rats (mean body weight now 418 g, SEM 11; n = 20) was studied in situ. Rats were anaesthetized by intraperitoneal injection of pentobarbital sodium B.P. (60–80 mg/kg body weight); anaesthesia was maintained by subsequent intraperitoneal injections. Body temperature (rectal) was maintained at between 36°C and 38°C by keeping the animals on a heated blanket (Electrophysiological Instruments Ltd, U.K.). A small animal ventilator (BioScience, U.K.) was used to assist breathing where necessary. The method, originally developed in rat trachea [18], whose secretions derive mainly from surface secretory cells, was modified to allow also collection of laryngeal secretions in which there would be a contribution of glycoproteins from submucosal glands (Fig. 1). The laryngo–tracheal segment and perfusion circuit were filled with 1.5 ml of physiological saline solution (mmol/l concentrations: Na+ 145.0, Cl− 126.0, K+ 5.9, HCO3− 26.0, Ca2+ 2.5, H2PO4− 1.2, Mg2+ 1.2, SO4− 1.2) at 37°C, pregassed with O2 + CO2 (95:5, v/v). The saline solution was circulated by peristaltic pumps (Scientific Furnishings, U.K.) at a rate of 1 ml/min during ten 30 min periods. At the end of each 30 min period (by which time the temperature of the saline solution had fallen to 27°C) the sample was collected and the circuit refilled. Acute administrations of CS or air were made after collections nos. 4 and 7. The circuit was emptied and tracheal cannulae in the circuit were disconnected from their tubing. Smoke (from 'middle tar' cigarettes yielding 17 mg of tar, 1.3 mg of nicotine and 11 mg of carbon monoxide per cigarette), diluted 1:3 with air, was blown through the tracheal segment at 16 ml/min for 5 min. Acute administrations of air were made similarly and, in both, the segment was perfused with saline solution for the remaining 25 min of the collection period. At the end of the 5 h study period, collected samples were stored at −20°C. During the study period the breathing rate of rats was determined and physiological responses were noted: defaecation, urination and arteriovenous blood flow in the dermis of the ears.

Sample preparation

Frozen samples were freeze-dried for 16–24 h (Edwards Modulyo, U.K.). Each dried sample was suspended in 1 ml of distilled water and, to each, 9 ml of 95% ethanol was added to precipitate proteins [19]. Samples were centrifuged at 2500 g for 15 min and the supernatants discarded. Each sample pellet was allowed to dry at room temperature (20°C) for 15 min, after which it was reconstituted in 850 μl of 0.1 mol/l sodium hydroxide solution and dispersed by sonication on ice (Soniprobe type 1130A, Dawe Instruments Ltd, U.K.) at 2 A, for 1 min. Each sample was divided into four portions for measurement of fucose, hexose, protein and albumin.

Analytical methods

Four hundred microlitres of each sample were assayed for fucose by the method of Gibbons [20] with α-L-(−)fucose (Sigma Chemical Co Ltd, U.K.) used as the standard; 200 μl was assayed for hexose [21] with α-glucose (BDH Chemicals Ltd, U.K.) as the standard and 200 μl was assayed for protein
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FIG. 1. Preparation for collection of airway secretions \textit{in situ}. The extra-thoracic trachea and larynx were exposed and three cannulae inserted: one, held in place by a clip, was inserted into the rostral end of the larynx so as to locate between the vocal folds, and two were at the other end of the trachea, low in the neck and just rostral to the sternum, one pointing cranially, the other pointing caudally. The lower two cannulae fitted snugly without ligatures to minimize damage to local nerves and blood vessels. The exposed tissue was covered with damp gauze to reduce water loss. The animal breathed spontaneously. Physiological saline solution was circulated through the laryngo–tracheal segment, which discharged secretions into it. Every 30 min the saline (containing secretions) was collected.

[22] with bovine serum albumin (Cohn fraction V; Sigma) as the standard. Absorbances were read on a CE 373 linear readout grating spectrophotometer (Cecil Instruments Ltd, U.K.): 400 minus 430 nm for fucose, 430 nm for hexose and 670 nm for protein. The last wavelength was chosen as most appropriate for the protein concentrations of the present study after construction of standard curves at 490, 545 and 670 nm. The presence or absence of serum albumin in portions taken from 50 µl of the collected secretions was determined by radial immunodiffusion [23]. Immunoglobulin (Ig) G goat anti-rat albumin (Dynatech Laboratories, U.K.) was used as the antibody in the dilution range 1:50–1:90 in 3% PEG 6000 with 0.1% sodium azide in the gel. Rat serum albumin (Nordic Immunological Laboratories Ltd, U.K.) was used as the standard. The mean coefficient of variation [24] of the assay was less than 10%; the mean error of repeat measurement was 3% and the mean intra- and inter-plate variation was between 5 and 6% for each. The mean coefficient of variation for sample values determined 4 months apart was less than 6% and the minimum detectable concentration was of the order of 2 µg/ml. Values of zero were confirmed by re-testing samples in 6 mol/l urea (BDH Chemicals, U.K.). The mean coefficient of variation for samples containing albumin in 6 mol/l urea was less than 10%.

\textit{Statistical analyses}

Statistical analyses were computed with Interstat software (Serendipity Systems Inc., New York, U.S.A.), checked for statistical accuracy by Statistical Advisory Services (Richmond-upon-Thames, Surrey, U.K.).

The results obtained for the secretion, in µg of each marker, were normally distributed but showed positive skewness and a two-tailed Mann–Whitney \textit{U}-test was used for comparison of median values between collections or groups. The responses to acute administrations of air or diluted CS were analysed by using a two-tailed Wilcoxon matched-pairs signed-ranks test [24]. \( P \) values equal to or less than 0.05 were taken as significant. For ease of presentation, data have been summarized as means and \( \text{SEM} \).

\textit{Results}

During the pretreatment period, control and CS-exposed rats remained healthy. Control and CS-exposed rats both gained about 2% in body weight.
over the period of exposure. Two control rats and seven CS-exposed rats died after the initial dose of anaesthetic or after breathing difficulties associated with excessive pulmonary secretions, visible in the lung cannula. Before the end of the 5 h study period, sampling was stopped in one CS-exposed and seven control rats either because the animal died or because of difficulties in maintaining the integrity of the tubing circuit. These problems most often occurred after acute administration of CS, which were associated with inhalation of visibly excessive pulmonary secretions or blockage of the trachea and its cannulae with blood or mucoid plugs. Samples collected during or after operational difficulties were discarded and are not included in the results.

The breathing rate was often altered (increased or decreased by up to 6 breaths/min) in response to acute administrations of CS. Acute administration of CS was consistently followed by a combination of ear vasodilatation, defaecation or urination; the responses were more common in ‘bronchitic’ than control rats.

Baseline secretion

The time taken to reach a stable baseline of secretion as measured by the four variables was assessed in six normal animals: mean values for collections obtained at each of 10 sample times are shown in Fig. 2. For all but albumin, there was an initially high secretion which fell to a stable base. Amounts of each marker measured at collection no. 4 were taken as representing ‘basal’ secretion because they were not significantly different from mean values at collections nos. 2 and 3 or from those at subsequent collections. Albumin was detected in 18 samples, randomly distributed between five animals, in the range 7–522 pg per collection. Movement of cannulae had been noted for collection times which gave detectable albumin.

Hypersecretion

Table 1 shows the mean secretion of fucose, hexose, protein and albumin in normal and bronchitic animals which also received acute administrations of dilute CS. Collections nos. 1, 2 and 3 represent the fall to basal values at collection no. 4; collections nos. 5–10 represent the responses to, and recovery after, two acute administrations of CS given directly into the isolated segment after collections nos. 4 and 7. The secretion of fucose was significantly ($P<0.05$) higher at each collection in bronchitic than in normal rats (Fig. 3). Hexose secretion was not significantly higher than normal except for the first two collections from bronchitic rats ($P<0.05$) (Table 1). There were no significant differences between normal and bronchitic rats in the secretions of either protein or albumin. Albumin was detected in only 19 samples from four rats (i.e. 19 out of a total of 134 samples from all rats). Its presence in the sample was most often associated with movement of the cannulae rather than with the administration of smoke: the range of concentrations was 56–430 µg per collection.

Effects of acute cigarette smoke and air

Table 1 also shows the effect of acute administrations of dilute CS (given during the first 5 min of collections nos. 5 and 8) on the mean values for
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TABLE 1. Mean secretion of fucose, hexose, protein and albumin (µg per collection)

Half-hourly samples were collected from cannulated and perfused tracheas in situ in normal and bronchitic rats. Dilute cigarette smoke (CS) was blown acutely through the tracheas on two occasions. Normal vs bronchitic rats: *P < 0.05, **P < 0.01 (Mann-Whitney U-test, two-tailed), n = 5–9. SEM values are given in parentheses. †SEM not given because only one or two rats out of seven had detectable albumin.

<table>
<thead>
<tr>
<th>Collection no.</th>
<th>Fucose</th>
<th>Hexose</th>
<th>Protein</th>
<th>Albumin</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Bronchitic</td>
<td>Normal</td>
<td>Bronchitic</td>
</tr>
<tr>
<td>1</td>
<td>24.3</td>
<td>85.6*</td>
<td>126.2</td>
<td>276.7*</td>
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<td></td>
<td>(7.3)</td>
<td>(37.6)</td>
<td>(28.4)</td>
<td>(42.4)</td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
<td>47.4**</td>
<td>53.6</td>
<td>140.2**</td>
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<td>(1.2)</td>
<td>(30.2)</td>
<td>(10.1)</td>
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<td>3</td>
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<td>54.5</td>
<td>124.9</td>
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<td>(25.2)</td>
<td>(16.7)</td>
<td>(38.0)</td>
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<tr>
<td>4</td>
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<td>23.9**</td>
<td>41.2</td>
<td>101.4</td>
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<td>(1.1)</td>
<td>(6.2)</td>
<td>(8.1)</td>
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<td>100.8</td>
<td>147.8</td>
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<tr>
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<td>2482.7</td>
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<td>(33.1)</td>
<td>(517.7)</td>
<td>(386.3)</td>
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<td>1244.7</td>
<td>1744.2</td>
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<tr>
<td></td>
<td>(598.7)</td>
<td>(446.1)</td>
<td>(410.9)</td>
<td>(361.7)</td>
</tr>
</tbody>
</table>

Fig. 3. Mean values (µg) for secretion of fucose in each of 10 half-hourly collections of secretions from the laryngo–tracheal lumen of normal (○) and bronchitic (●) rats. Bars represent SEM values. At every collection the secretion of fucose differed significantly between normal and bronchitic rats: *P < 0.05, **P < 0.01 (Mann-Whitney U-test, two-tailed), n = 5–9. Diluted cigarette smoke (CS) was blown through the lumen where indicated.
secretion of fucose, hexose, protein and albumin. In normal rats acute CS caused significant increases in fucose \((P < 0.05)\) (Fig. 3), hexose \((P < 0.01)\) and protein \((P < 0.05)\) but not albumin. But for protein, the second administration showed a similar trend which did not reach statistical significance. In bronchitic rats, although the first administration of CS had no statistically significant effect, the second caused significant increases in fucose \((P < 0.02)\) (Fig. 3), hexose \((P < 0.02)\) and protein \((P < 0.01)\) but not albumin. The most consistent responses were seen for hexose in normal rats and for fucose and protein in bronchitic rats: Fig. 4 gives, as an example of the consistency, the response for hexose in normal rats. In each case the responses to acute CS lasted only for the duration of one collection period: i.e. the raised secretion of fucose, hexose and protein fell significantly \((P < 0.05)\) to mean values which were not significantly different from basal levels of secretion (for example, see Fig. 4).

In three rats, neither of the two administrations of air had any significant effect on secretion of fucose or hexose. Protein and albumin were only available for measurement in one animal when protein rose after the first but not the second administration of air and albumin was undetected.

**Discussion**

The specific pathogen-free rat has been used previously to measure the histological, histochemical and ultrastructural changes of experimental chronic bronchitis induced by 'sub-acute' (i.e. 2–6 weeks) exposure to inhaled cigarette smoke [9–11]. The secretory cell hyperplasia of mucus-secreting cells of the surface epithelium has been extensively characterized in terms of the proliferative response [25, 26] and the effects of anti-inflammatory drugs and of the muco-regulatory agent N-acetylcysteine have been determined [12, 27–29]. Although there is evidence that acute administrations of CS cause discharge of radiolabelled mucins from an intact tracheal segment isolated *in situ* in normal cats [30, 31], we have found no previous experimental studies *in situ* which examine the release of mucus from animals made bronchitic by sub-acute exposure to CS. Preparations *in situ* have a major advantage over techniques *in vitro* in that they allow secretion to be studied in an airway segment with its nerve and blood supply intact and thus the proportions of serum and epithelial components contributing to the airway fluid can be determined by using specific markers for each.

**Effect of chronic exposure to CS**

Organ culture studies of human airway *in vitro* have established that, in chronic bronchitis, both the hypertrophied submucosal glands and increased numbers of surface secretory cells have increased basal rates of secretion and that submucosal glands are less responsive to inhibition by atropine than normal [32–34]. Similar studies on rat tracheal/laryngeal submucosal glands, taken from rats previously exposed to CS (25 cigarettes/day for 6 weeks), have demonstrated an increased rate of synthesis as determined by the rate at which radioactive precursors of mucin are incorporated into intracellular macromolecules before passing through each cell to its apex to be discharged [35].

In the present study *in situ* we have used fucose to monitor, chemically, the component of glycoprotein-rich secretions produced by the epithelial mucus-secreting cells. Epithelial glycoproteins have
large amounts of fucose (e.g. fucomucins contain a high (40-70%) carbohydrate content and contain fucose in mannose and contain little or no globulins), but in contrast plasma-type glycoproteins are rich in mannose and contain little or no mucus and epithelial glycoproteins has been used to study sputum in various respiratory diseases [40, 41]. The fucose content has been studied in sputum and serum [42]. In human studies in vivo, directed towards an analysis of 'normal' secretions, fucose concentration in prostaglandin (PG) F₂α-stimulated 'sputum' production by healthy subjects was 2.8 mol/ml compared with 5.3 mol/ml in sputum from patients with chronic bronchitis, indicative of increased glycoprotein secretion [43]. In our experimental studies chronic exposure to CS resulted in an eight-fold increase in mean basal secretion of fucose, which was maintained at a high level over the 5 h period of study. The results indicate that CS induces epithelial hypersecretion predominantly from submucosal glands of the larynx and/or surface epithelium of both larynx and trachea. Further studies in which laryngeal and tracheal secretions are collected separately for comparison will be useful in determining the separate contributions of each to airway fluid. The functional change parallels the secretory cell hyperplasia shown histologically in previous studies with the same CS exposure regimen [12, 28, 29]. The relatively mild increase in mucous cell number, seen histologically, in the trachea is likely to be due to an increased rate of discharge of intracellular mucin, which is thus not available for detection by specific stains for mucin [29].

Albumin is a major serum protein present in sputum and can be used as a 'marker' for the transudate component in various lung diseases [44, 45]. Its concentration is relatively easy to measure by radial immunodiffusion [23]. It has been shown to be a significant component of the sol phase of mucoid sputum from bronchitic patients (i.e. 30 mg/100 ml of sputum). Prostaglandin F₂α-stimulated sputum (considered to represent material which approximates to that found in normal man) is almost free of serum glycoprotein, as determined by concentrations of mannose, yet albumin is present in the range 4-40 mg/100 ml [36]. In our experimental studies of the normal rat, albumin was only rarely detected and although its presence was more often detected in the 'bronchitic' animals the difference was not statistically significant. It was particularly associated with accidental movement of the indwelling cannulae. The absence or low levels of albumin in the airway mucus collected in our tracheal segment could be explained in terms of anatomical differences: sputum represents secretion from the tracheobronchial tree and bronchio-alveolar lavage includes, in addition, alveolar liquid.

Experimental and freeze fracture ultrastructural studies of guinea pig tracheal epithelium have shown that although permeability to horseradish peroxidase (mol. wt. 40 000) is increased after 100 'puffs' of CS the epithelial 'tight junction' retains its normal structural integrity [46]. Such an intact junction may well preclude passage of molecules the size of albumin (mol. wt. 69 000) or greater.

**Effect of acute exposure to CS**

CS given acutely, directly to the airway segment in normal rats, stimulates the release of secretions rich in fucose, hexose and protein with a particularly consistent response in the case of hexose. Administration of air alone did not appear to alter fucose or hexose secretion in the three animals studied; further animals require testing before responses with acute exposure to CS can be directly compared with the response to air alone. The concentration of smoke used is likely to have been greater than that inhaled by human smokers as relatively marked physiological responses were seen.

**Origin of secretions**

The origin of the hexose- and protein-rich secretions is unknown. Presumably they are mainly epithelial, as albumin was not consistently raised after sub-acute or acute CS. After acute exposure glycosaminoglycans may be considered a component as they are hexose- and uronic acid-rich secretions characteristic of the surfaces of normal dog and human airway epithelium [3, 47]. Our earlier studies have shown, however, that rat tracheal secretions are normally free of uronic acid and it is unlikely that the hexose component of the secretions, seen in the present study, represents release of surface epithelial glycosaminoglycans. The most likely explanation is that, in response to an acute dose of CS, glycoproteins of higher molecular weight and glycosylation than normal are released, probably preferentially from submucosal glands located in the infra-glottal region of the larynx, which were included in the airway segment under investigation.

**Mechanism of short-term response**

Recent studies in the cat indicate that the release of glycoproteins, in response to acute CS, is
mediated by nicotine which is absorbed through the tracheal wall (either directly or via the blood), where it stimulates nicotinic receptors on autonomic ganglia whose axons innervate airway submucosal glands. In the present study a nervous, rather than inflammatory, mechanism is favoured for the acute effects of CS, as in both the normal and bronchitic animal the secretory response is of short duration (i.e. is restored within 30 min). The susceptibility of the response to autonomic antagonists will need to be determined before conclusions can be drawn.

In summary, we have used cigarette smoke in an animal model of bronchitis in which there is hyperplasia of mucus-secreting elements in the surface epithelium and mild hypertrophy of submucosal glands, and shown that there is an associated hypersecretion of fucose-rich material which is likely to be a glycoprotein of epithelial origin. In contrast, a fucose secretion which becomes enriched with hexose and protein is characteristic of the response to acute administration of CS. The preparation in situ we describe for the anaesthetized normal and bronchitic rat can be used also to examine differences which may exist in 'bronchitis' in terms of the possible prophylactic or therapeutic effects of selected drugs, the time course of recovery after the hypersecretory state (i.e. after cessation of CS exposure) and pharmacological responsiveness.

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