Biosynthesis of Respiratory-Tract Mucins

A COMPARISON OF CANINE EPITHELIAL GOBLET-CELL AND SUBMUCOSAL-GLAND SECRETIONS

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1. A modified canine tracheal organ culture system was used to investigate differences between mucous secretions of epithelial goblet cells and the submucosal glands. 2. Denuded explants were prepared by removing goblet, ciliated and basal cells from the surface epithelium leaving an intact basement membrane and viable submucosa. 3. Denuded explants actively incorporated radioactive precursors into secreted macromolecules when cultured in medium 199 containing label. 4. Chromatography on Bio-Gel A-150m and electrophoresis on 1% agarose gels indicated that epithelial goblet cell secretions were relatively more sulphated than submucosal glandular secretions. 5. The glandular structures were shown to respond to a parasymphathomimetic agent.

The principal sources of mammalian-respiratory-tract secretions are the acinotubular glands found in the submucosa of the cartilage-containing airways together with the goblet cells of the surface epithelium (Von Hayek, 1960). These structures have been characterized physiologically and histochemically. Physiological experiments have demonstrated that the goblet cells secrete their mucus as a result of direct irritation, whereas the mucous glands are innervated and can be made to respond to a variety of parasymphathomimetic agents (Florey *et al.*, 1932; Gernez-Rieux *et al.*, 1966; Sturgess & Reid, 1972*b*). Histochemically the mucosubstance within the goblet cells appears to be a uniform periodate-reactive sulphated mucin which differs from that present in the mucous glands (McCarthy & Reid, 1964; Spicer *et al.*, 1971). On the basis of histochemical and radioautographic studies the mucous glands have been shown to contain cells staining for a variety of acid and neutral mucosubstances (McCarthy & Reid, 1964; Lamb & Reid, 1969; Spicer *et al.*, 1971). Many of these cells exhibit relatively little affinity for any of the acidophilic histological dyes and were presumably devoid of either sialic or sulphate esters (Spicer *et al.*, 1971). However, some acini are populated with cells containing abundant sulphated mucosubstances whereas others contain non-sulphated acidic mucins (McCarthy & Reid, 1964; Lamb & Reid, 1969). Although some success has been achieved in fractionating the mucins present in sputum into sulphated, neutral and sialomucins (Havez & Biserte, 1968; Roussel *et al.*, 1972), such constituents have not as yet been definitely localized to specific mucus-producing airway structures of defined histochemical or physiological characteristics.

In the preceding paper (Ellis & Stahl, 1973) a systematic attempt to characterize radioactively labelled macromolecules secreted by tracheal explants as related to those produced as respiratory tract secretions *in vivo* was presented. In the present paper we report studies of the different mucus-synthesizing structures, under controlled conditions, in a modified organ culture system (Stahl & Ellis, 1972), in which secretions originating from epithelial goblet cells are compared biochemically with those of the submucosal glands.

Experimental

Techniques for the preparation and culture of tracheal explants were previously described (Stahl & Ellis, 1972). Methacholine chloride (Methochol chloride) was obtained from Merck and Co., Rahway, N.J., U.S.A. All other materials and the assay procedures used with the explant secretions were described in the preceding paper (Ellis & Stahl, 1973).

Preparation of denuded explants

The surface epithelium was removed from canine trachea or bronchi by a modification of the technique described by Spencer (1958) for the preparation of epithelial cell suspensions. Sections of trachea or bronchi were placed in a small wide-mouth vial, such as is used for scintillation counting, and warm (35°C) medium 199 added to a depth of approximately 2 cm. The goblet and ciliated cells were freed from the basement membrane by twirling a brush with relatively stiff bristles (a regular typewriter eraser brush with 2 cm bristles is ideal) between thumb and

forefinger, while exerting slight downward pressure. After removal of the epithelium, explants were cut to size as described for intact organ cultures (Stahl & Ellis, 1972; Ellis & Stahl, 1973).

Results

Metabolism of brushed tracheal explants

The brushed explant system (Stahl & Ellis, 1972) was devised in part to provide a method for the separation of the secretions of the two mucus-producing structures of the respiratory tract. This system provides submucosal gland mucus from the denuded explants as well as goblet cell mucus from the epithelial brushings. Histological examination of brushed explants confirmed that the goblet, ciliated and the majority of the basal cells had been removed by the brushing procedure leaving the basement membrane intact and the submucosa and acinotubular glands undisturbed.

Before comparing the secretions it was necessary to show the brushed explants were functioning physiologically and biochemically. The brushed tissues, after being cut to size, were incubated in the presence of radioactive precursors for periods up to 72 h. Metabolic activity was measured by the incorporation of radioactivity into acid-precipitable material released into the medium. Tissues were viable throughout the 72 h incubation period as determined by secretion rates of labelled macromolecules/24 h period. The specific radioactivity of the macromolecules was still increasing slightly at 72 h, presumably owing to changing specific radioactivity of precursors within the tissues and the finite time for the macromolecules to be synthesized and secreted. A similar observation was made with intact tissue (Ellis & Stahl, 1973). Agarose column chromatography and gel electrophoresis confirmed that mucin-type macromolecules were secreted into the culture medium by brushed

explants. The incorporation of D-[1-¹⁴C]glucosamine, ³⁵SO₄²⁻, DL-[4,5-³H]leucine and L-[U-¹⁴C]serine into these macromolecules indicated active biosynthesis by the denuded explants.

Comparison of secretions from intact and brushed explants

The total incorporation of labelled precursors into secreted macromolecules eluted in the void volume of a Sephadex G-200 column is shown in Table 1. The greatest decrease in radioactivity in the secretions of brushed explants as compared with normal intact explants was seen with Na₂³⁵SO₄ (75%), whereas the incorporation from D-[1-¹⁴C]glucosamine, DL-[4,5-³H]leucine and L-[U-¹⁴C]serine was 10 to 25% less. These observations are consistent with a decrease in the number of structures that produce mucus as a consequence of the brushing procedure and resultant removal of epithelial goblet cells.

Fractionation of the Sephadex G-200 void-volume material on Bio-Gel A-150m columns gave two major peaks. The first peak, designated AGI, appeared near the void volume of the column. The second peak (AGII) was eluted near the total volume of the column. The AGI fraction contains mucous glycoproteins (Ellis & Stahl, 1973). The amount of radioactivity in the AGI fraction after chromatography on Bio-Gel A-150m is shown in Table 2 as the percentage of the radioactivity incorporated into secreted macromolecules from D-[1-¹⁴C]glucosamine, Na₂³⁵SO₄, DL-[4,5-³H]leucine and L-[U-¹⁴C]serine. The greatest shift in the percentage of the total macromolecular radioactivity found in the AGI fraction was seen with the ³⁵SO₄²⁻-labelled secretions where a decrease from 65% to approx. 30% was observed. This result indicates the major part of the 75% decrease in secreted labelled macromolecules occurring as a result of removal of the epithelial cells can be attributed to a loss of AGI material.

Table 1. *Macromolecular radioactivity secreted by normal and brushed canine tracheal explants incubated with either D-[1-¹⁴C]glucosamine, Na₂³⁵SO₄, DL-[4,5-³H]leucine or L-[U-¹⁴C]serine*

Explants were incubated as described in the Experimental section. Results are average values from at least two experiments with each labelled precursor and are expressed as d.p.m. incorporated into Sephadex G-200 void-volume macromolecules/initial 24 h incubation period.

Radioactive precursor	10 ⁻³ × Radioactivity of secreted macromolecules (d.p.m./g of explant)		
	Normal	Brushed	% change
D-[1- ¹⁴ C]Glucosamine	690	553	-20
Na ₂ ³⁵ SO ₄	556	132	-76
DL-[4,5- ³ H]Leucine	125	105	-16
L-[U- ¹⁴ C]Serine	277	208	-25

Table 2. *Percentage of labelled precursor found in AGI peak after fractionation of secreted macromolecules on Bio-Gel A-150m from normal and brushed explants*

The methods are described in the Experimental section. Results are expressed as percentages of the radioactivity from each precursor found in fraction AGI after chromatography on a Bio-Gel A-150m column and are means $\pm$ S.D. with the number of observations in parentheses.

Radioactive precursor	% of label found in AGI fraction of secreted macromolecules	
	Normal	Brushed
D-[1- ¹⁴ C]Glucosamine	46.5 $\pm$ 4.1 (4)	41.2 $\pm$ 4.0 (3)
Na ₂ ³⁵ SO ₄	64.8 $\pm$ 6.2 (8)	28.6 $\pm$ 5.8 (4)
DL-[4,5- ³ H]Leucine	16.6 $\pm$ 4.1 (6)	12.2 $\pm$ 3.1 (3)
L-[U- ¹⁴ C]Serine	17.0 (2)	11.0 (2)

Table 3. *Ratio of DL-[4, 5-³H]leucine/Na₂³⁵SO₄ radioactivity of labelled macromolecules secreted by normal and brushed explants incubated in vitro with DL-[4,5-³H]leucine and Na₂³⁵SO₄*

Ratios are expressed for total macromolecules (Sephadex G-200 void-volume fraction), before chromatography on Bio-Gel A-150m and for AGI and AGII Bio-Gel A-150m fractions.

Explant type	DL-[4,5- ³ H]Leucine Na ₂ ³⁵ SO ₄ radioactivity ratio		
	Total	Fraction AGI	Fraction AGII
Normal	0.42	0.17	1.19
Brushed	0.93	0.49	1.31

In an effort to further investigate the decreased SO₄²⁻ incorporation in the brushed explant secretions, two types of double-labelling experiments were performed. In the first, DL-[4,5-³H]leucine and Na₂³⁵SO₄ were used together to measure the relative decrease in amino acid incorporation as compared with SO₄²⁻ incorporation in the secreted macromolecules. The ³H/³⁵SO₄²⁻ radioactivity ratio should indicate the degree of sulphation of the macromolecules. DL-[4,5-³H]Leucine/Na₂³⁵SO₄ ratios in total secreted macromolecules and the AGI and AGII Bio-Gel A-150m fractions are shown in Table 3. The shift toward unity in the ratios found in the total secreted macromolecules (Sephadex G-200 void-volume fraction) and the AGI fraction when comparing normal with brushed explant secretions indicated a loss of significantly more SO₄²⁻ than protein in the secretions after removal of the mucus-producing goblet cells. The ratios within the AGII fractions were not significantly different. In the second type of experiment

Table 4. *Ratio of D-[6-³H]glucosamine/Na₂³⁵SO₄ radioactivity of labelled secreted macromolecules*

Ratios are expressed for total macromolecules, AGI and AGII fractions.

Explant type	D-[6- ³ H]Glucosamine Na ₂ ³⁵ SO ₄ radioactivity ratio		
	Total	Fraction AGI	Fraction AGII
Normal	0.20	0.10	0.36
Brushed	0.61	0.57	0.61

D-[6-³H]glucosamine and Na₂³⁵SO₄ were used to demonstrate the relationship of SO₄²⁻ to glucosamine in the secreted mucins. The results of this experiment are presented in Table 4 as a ratio of D-[6-³H]glucosamine/Na₂³⁵SO₄ radioactivity in both the total and chromatographically fractionated secretions. Within a given experiment, significantly more ³⁵SO₄²⁻ than D-[³H]glucosamine radioactivity was lost in the total, AGI and AGII fractions of the secretions after brushing the explants. The change in ratios of the AGII fraction, although significant, was not as great as that of the AGI fraction.

Secretions from both brushed and intact explants were compared by gel electrophoresis after reduction with dithioerythritol to determine if the loss of material could be attributed to one or more specific mucin-type glycoproteins. No pellet of mucin-type material was usually found in the secretions from brushed explants. However, supernatant macromolecules (Ellis & Stahl, 1973) and material released from epithelial cell brushings suspended in the culture medium for 16h at 37°C can be examined by gel electrophoresis (Fig. 1). The major component of the complete explant supernatant, a band migrating 15–22mm on 1%

Fig. 1. Diagram of agarose-gel-electrophoresis patterns of mucins from normal explants, brushed explants and epithelial cell brushings

The mucin fractions indicated were solubilized with dithioerythritol and subjected to electrophoresis in 1% agarose gels containing sodium dodecyl sulphate at pH 8.2. Gels were run at a constant current of 5 mA/gel for 35 min. Approx. 6 V/gel was generally required to develop the 5 mA current. The sample gel is at the top (cathode) of each gel and glycoprotein bands were located by staining for carbohydrate with periodic acid-Schiff reagent. (a) Normal explant secretion supernatant; (b) brushed explant secretion supernatant; (c) epithelial cell brushings incubated for 16 h supernatant.

agarose gels, was completely absent from the brushed explant secretion (Fig. 1b). A band, only faintly staining with the periodic acid-Schiff reagent and migrating 5–10 mm, was also seen on this gel. This component appeared to be identical with the slow-migrating band of the mucin pellet from complete explants (Fig. 2a) and was apparently of glandular origin. When the material released from the epithelial cell suspension was run on the same gels, the component missing from the brushed explant secretion was the only periodic acid-Schiff-positive material seen (Fig. 1c). Further examination of the material released from the suspended epithelial cells indicated that the cells did not synthesize mucins once they were released from the basement membrane. On incubation of the brushings in culture medium containing either D-[1- 14 C]glucosamine or $\text{Na}_2^{35}\text{SO}_4$ there was no incorporation of radioactivity into mucin-type macromolecules. The material seen by periodic acid-Schiff staining of the agarose gels (Fig. 1) was presumably synthesized before brushing and was released into the medium during incubation of the cell suspension. When intact explants were pre-incubated in the presence of $\text{Na}_2^{35}\text{SO}_4$, before removal of the epithelial cells by brushing, the material released into the medium on incubation of the

Fig. 2. Comparison of agarose-gel-electrophoresis patterns of normal explant secretions and mucin from epithelial cell brushings

Conditions were as described in Fig. 1, with gels run at a constant current of 5 mA/gel for 35 min. (a) Normal explant secretion pellet; (b) normal explant secretion supernatant; (c) epithelial cell brushings, 0–24 h incubation; (d) epithelial cell brushings, 24–48 h incubation.

brushings was labelled. On chromatography on Bio-Gel A-150m its radioactive profile was similar to that of secretions from intact explants in that the AGI fraction contained 60% of the $^{35}\text{SO}_4^{2-}$ macromolecular radioactivity. Additional gel-electrophoretic patterns of the material released from the epithelial cell suspension and those of the pelleted mucin and supernatant material from complete explants are shown in Fig. 2. Epithelial cell brushings were suspended in culture fluid for 48 h and the macromolecules released during the first 24 h were collected and compared with those released between 24 and 48 h. The profiles (Fig. 2) indicate that at least two types of periodic acid-Schiff-positive material were released from the epithelial cells. These two bands were seen separately in the pellet and supernatant material from complete explants, migrating 18–26 mm and 15–22 mm respectively, and both appeared in the initial 24 h epithelial cell sample. The disappearance of one of the components from the 24–48 h sample may be an indication of the relative amounts of the two components initially present in the epithelial cells at the time of removal from the explant.

Effect of methacholine on explant secretions

Previous attempts to radioactively label the slower-moving electrophoretic band of pellet mucin (the band showing periodic acid-Schiff reactivity at 5–10 mm in Fig. 2a), by using whole explants (Ellis & Stahl, 1973), were unsuccessful. Vagus nerve stimulation and cholinergic drugs have been shown to

Fig. 3. Effect of methacholine on incorporation of D-[1- 14 C]glucosamine into macromolecules secreted by brushed explants

Brushed explants were incubated for 24h in medium 199 containing D-[1- 14 C]glucosamine and then challenged with methacholine (0.1mg/ml) for 30min. Macromolecules secreted during the initial 24h incubation period and those released during the 30min period with methacholine were purified, solubilized with dithioerythritol and subjected to electrophoresis in 1% agarose gels at a constant current of 5mA/gel for 35min. Radioactivity in the gels was measured by scintillation counting after slicing the gels into 2mm thick discs and dissolving in water directly in the scintillation vials. —, Radioactivity present in 0–24h secretion; ----, radioactivity in secretions released during 30min incubation with methacholine.

cause an increase in tracheobronchial secretions (Florey *et al.*, 1932; Sturgess & Reid, 1972b).

We added methacholine to the explant culture fluid in an attempt to produce glandular mucus that could be identified by gel electrophoresis. Incubation of whole explants for extended time-periods in the presence of methacholine (0.1mg/ml) failed to cause the incorporation of radioactivity into the slower-moving periodic acid–Schiff-reactive band. This material, however, could be labelled under certain conditions when brushed explants were used. Brushed explants incubated for 24h in labelled medium and then challenged with methacholine secreted mucins with radioactivity in the electrophoretically slow-moving band (Fig. 3).

Discussion

The objective of the present investigation was to isolate and compare biochemically the secretions of the epithelial goblet cells with those from the sub-mucosal glands. In the preceding paper (Ellis & Stahl, 1973) techniques were described for the characterization of the macromolecules secreted by tracheal and bronchial explants *in vitro*. By using denuded ex-

plants it has now been possible to assign an epithelial or glandular origin to these macromolecules on the basis of biochemical parameters. The technique described by Spencer (1958) for the preparation of epithelial cell suspensions was successfully used to prepare viable tracheal explants from which the mucus goblet cells had been removed. The denuded explants synthesized and secreted radioactively labelled mucin-type macromolecules when incubated in culture medium containing labelled precursors. When the macromolecules secreted by the denuded explants were fractionated on Bio-Gel A-150m columns a major shift in the percentage of total macromolecular radioactivity found in the AGI (mucin) peak was seen with Na₂ 35 SO₄ (Table 2).

This large decrease is a result of removal of the surface epithelium from the explants and suggests that goblet cell secretions are highly sulphated. This observation was confirmed in double-label experiments with D-[6- 3 H]glucosamine and Na₂ 35 SO₄. Relatively more 35 SO₄²⁻ than 3 H radioactivity was lost from the AGI peak when denuded explant secretions were compared with those from whole explants.

Although goblet-cell mucosubstances with distinctive histochemical properties have been differentiated *in situ* from mucosubstances present in the mucous and serous glands (McCarthy & Reid, 1964; Spicer *et al.*, 1971) the biochemical separation on the basis of site of origin is described here for the first time.

Sturgess & Reid (1972a,b), by using radioautographic techniques, followed the movement of labelled glucose and threonine through the cells of the submucosal glandular structures in organ cultures of human bronchial explants. However, no attempt was made by these workers to collect or identify the secretions. The present biochemical characterization of labelled secretions substantiates the histochemical studies of Lamb & Reid (1969) and Spicer *et al.* (1971), which indicated that the submucosal glands contain a mixture of acidic mucins.

The successful separation of mucous glycoproteins by disc gel electrophoresis, after disulphide bond cleavage by reduction with dithioerythritol (Holden *et al.*, 1971a,b) prompted us to compare explant secretions by electrophoresis on 1% agarose gels. The presence of a single band corresponding to goblet-cell mucins from pellet and supernatant macromolecules from whole explants, whereas two bands were seen on fractionation of material released from epithelial cell brushings, suggests that one of the goblet cell components forms a complex with material originating from glandular structures in the total secretion. This possible complex formation and the necessity of prior reduction with dithioerythritol to permit the mucous secretions to penetrate the electrophoresis gels suggests that canine tracheal mucus consists of very-large-molecular-weight gel-forming macromolecules built up of smaller mucin

units joined together by interchain disulphide links. A number of mucous secretions including porcine and canine submaxillary mucins, bovine cervical mucin and pig gastric mucin have been shown to be broken down into smaller units by disulphide-bond-reducing agents (Holden *et al.*, 1971*b*; Allen & Snary, 1972). Allen & Snary (1972) have proposed a structure for pig gastric mucus where smaller glycoprotein subunits are joined by disulphide bridges to form a high-molecular-weight mucoprotein.

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References

- Allen, A. & Snary, D. (1972) *Gut* **13**, 666–672
- Ellis, D. B. & Stahl, G. H. (1973) *Biochem. J.* **136**, 837–844
- Florey, H., Carlton, H. M. & Wells, A. Q. (1932) *Brit. J. Exp. Pathol.* **13**, 269–284
- Gernez-Rieux, C., Biserte, G., Havez, R., Voisin, C., Roussel, P. & Degand, P. (1966) *Prensa Med. Argent.* **53**, 426–435
- Havez, R. & Biserte, G. (1968) in *Hypersécrétion Bronchique* (Gernez-Rieux, C. & Boulanger, P., eds.), pp. 43–67, Médic Diffuseur, Paris
- Holden, K. G., Yim, N. C. F., Griggs, L. J. & Weisbach, J. A. (1971*a*) *Biochemistry* **10**, 3105–3109
- Holden, K. G., Yim, N. C. F., Griggs, L. J. & Weisbach, J. A. (1971*b*) *Biochemistry* **10**, 3110–3113
- Lamb, D. & Reid, L. (1969) *J. Pathol.* **98**, 213–229
- McCarthy, C. & Reid, L. (1964) *Quart. J. Exp. Physiol.* **49**, 85–94
- Roussel, P., Lamblin, G., Degand, P. & Havez, R. (1972) *Clin. Chim. Acta* **36**, 315–328
- Spencer, B. (1958) *J. Histochem. Cytochem.* **6**, 105–111
- Spicer, S. S., Chakrin, L. W., Wardell, K. R. & Kendrick, W. (1971) *Lab. Invest.* **25**, 483–490
- Stahl, G. H. & Ellis, D. B. (1972) *Life Sci.* **11**, 71–75
- Sturgess, J. & Reid, L. (1972*a*) *Exp. Mol. Pathol.* **16**, 362–381
- Sturgess, J. & Reid, L. (1972*b*) *Clin. Sci.* **43**, 533–543
- Von Hayek, H. (1960) *The Human Lung*, p. 127, Hafner Publishing Co., New York