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M1/MUC5AC mucin released by human airways *in vitro*

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ABSTRACT: A series of monoclonal antibodies which bind to a mucin known as M1 (anti-M1 MAbs) have also been shown to detect the product of the human gene MUC5AC. The aim of this investigation was to determine the concentration of the M1 mucin in the surface epithelium of human bronchial preparations by means of immunohistochemistry and in the bronchial fluid derived from human airways by means of an immunoradiometric assay.

Human bronchial ring preparations from the resection material of 20 patients were challenged with methacholine, leukotriene D_4, or anti-immunoglobulin E. Experiments were performed in preparations with an intact epithelium as well as in tissues in which the epithelium had been mechanically removed.

The anti-M1 MAbs stained the goblet cells in the epithelium intensely and there was also light and less uniform staining in the submucosa. The M1/MUC5AC mucin in the fluids secreted by the bronchial preparations was not modified during either the experimental protocol or stimulation with the different secretagogues. However, in preparations in which the epithelium had been removed, there was a significant reduction in the amount of M1/MUC5AC mucin detected.

These data suggest that the M1/MUC5AC mucin detected in the biological fluids produced by human airways *in vitro* may be released constantly, and principally from the goblet cells in the epithelial layer.
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There are presently nine different human mucin genes that have been cloned and these have been designated MUC1–MUC8 (including MUC5AC and MUC5B). Some of these correspond to previously well-known mucin components. MUC1 [1] has been referred to as carbohydrate antigen (CA)-15.3, episialin, polymorphic epithelial antigen (PEM), and epithelial membrane antigen (EMA) [2], while MUC5B [3] and MUC7 refer to the high molecular weight human salivary mucin (MG1) and low molecular weight human salivary mucin (MG2) [4], and MUC5AC to gastric M1 mucin [5]. The M1/MUC5AC antigen or gene, initially described as an early oncofoetal marker of colonic carcinogenesis [6, 7] is abundantly expressed in surface gastric epithelium but is also found in goblet cells of the bronchial epithelium [6] and is a prominent mucin in respiratory secretions [8]. Previous investigations have shown that the MUC5AC gene is located on chromosome 11p15 near the MUC2, MUC5B and MUC6 genes, and recently the 5′-flanking region of this gene has been cloned [9]. In addition to these investigations, other techniques have been used to establish the constituents found in mucus.

Monoclonal antibodies (MAbs) have been used in immunohistological studies to localize and identify the different gene products associated with the secretory process in the tracheobronchial tissues of several species including man [10–14]. These techniques have also been applied to studies of epithelial cells in culture [15–17]. However, these latter studies have concentrated on the methodology involved in the culture techniques and on the immunohistochemical expression of mucus production. Relatively few functional investigations using selective MAb probes have been performed in order to study the basal and/or stimulated release of secretory products from human airways [13, 14].

There are a variety of MAb probes available, and at least two categories have been reported to be directed against mucins [6, 18]. One group of MAbs is directed against the saccharide moiety of glycoconjugates, for example the MAb 3D3 which recognizes the Lewis b blood group-related antigen and cross-reacts with the Lewis a and Lewis y determinants [19]. A second group of MAbs is directed against the overall tertiary peptide structure of the mucin, such as M1 antigens [6]. The M1 antigens are expressed in columnar mucous cells of the surface gastric epithelium [20], and have been shown to mark some goblet cells of the precancerous rat colonic mucosa [21].

Since gastric M1 mucin is encoded by the MUC5AC gene [5] and has been previously characterized using a mixture of eight MAbs [22], the aim of the present study was to use anti-M1 MAbs to detect the release of mucin antigens from human airways *in vitro* under basal conditions as well as during challenge with several potent bronchoconstrictor agents that have been reported to increase mucus production.

Materials and Methods

Tissues

Human lung tissues were obtained from 20 patients who had undergone surgery for lung carcinoma. Subsequent to

the resection of the lung, the bronchi were dissected free of parenchymal tissue and washed with Tyrode's solution. The composition of the Tyrode's solution was (mM): NaC1 139.2, KC1 2.7, $CaC1_2$ 1.8, $MgC1_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, and glucose 5.5, pH 7.4. Bronchial tissues were cut as rings (4–7 mm internal diameter and 100–400 mg wet weight). A total of 88 bronchial preparations were examined. In some experiments, the epithelium was removed by gently rubbing the luminal region of the bronchial rings with a moistened cotton swab. The epithelium was removed in order to evaluate the relative contribution of the goblet cells to the amounts of mucin detected in the biological fluids derived from human bronchial preparations.

Immunohistological studies

The method for immunoperoxidase staining was performed as previously described [23], using a DAKO LSAB-2 kit (DAKO S.A., Trappes, France). Briefly, tissue sections were deparaffinized with toluene, rehydrated with ethanol and washed with phosphate-buffered satine (PBS). Endogenous peroxidases were eliminated using hydrogen peroxide (3% for 5 min). Tissue sections were treated with blocking solution (casein 0.25% in PBS; DAKO) for inhibition of cross-immunological reactions. The tissues were incubated (10 min) with the anti-M1 MAb (1-13M1) and washed with PBS. The sections were incubated (10 min) with peroxidase coupled to streptavidin and peroxidase activity was developed using diaminobenzidine/H_2O_2. Tissues were counterstained with Harris' haematoxylin.

Functional studies

The human bronchial preparations were set up in a microtitre plate (24 wells) containing Tyrode's solution (1 mL) and allowed to equilibrate for 1 hr in a humidified incubator (37°C, 5% CO_2/air). At the end of this equilibration period, the medium was replaced with fresh Tyrode's solution. The tissues were then maintained for 1 hr (Period I: basal release). The medium was collected and replaced with fresh Tyrode's solution for 30 min (treatment). After this period, the medium was replaced with fresh Tyrode's solution containing methacholine (Meth, 100 μM), leukotriene (LT) D_4 (10 μM) or anti-human immunoglobulin (Ig)E (anti-IgE, dilution 1:1,000). Following a 1-hr exposure to these agents (Period II: post-treatment), the fluids were again collected. The samples from Period I and Period II were stored at -20°C.

Antibodies

The eight (IgG) anti-M1 antibodies used in this study were directed against seven epitopes associated with the peptide core of gastric mucin. These epitopes are conformational, partially destroyed by trypsin and completely destroyed by mercaptoethanol (except for 19M1 and 21M1). The MAbs are designated: 1-13M1, 2-11M1, 2-12M1, 9-13M1, 58M1 [6] 19M1, 21M1 and 45M1 [22]. The MAbs 19M1 and 21M1 recognized the same epitope, called M1-f [22] which is encoded by the MUC5AC gene [5]. The mixture of the eight anti-M1 MAbs is called PM_8.

Mucin determination

The presence of the M1/MUC5AC mucin in the fluids derived from human bronchial preparations was determined *via* an immunoradiometric assay using anti-M1 MAbs, as previously described by BARA *et al.* [24]. This technique involved the use of small polystyrene stars (Cis-Bio-international, Marcoule, France), which were coated with an anti-M1 MAb (1-13M1, 0.01 mg·100 mL^{-1}), rinsed three times with PBS containing 0.1% Tween 20 and subsequently incubated overnight with bovine serum albumin (BSA) (1%) in PBS. The stars were then washed and dried at 37°C. An ovarian mucin antigen (10 μg·mL^{-1}, containing, arbitrarily, 10,000 M1 U·mL^{-1}) was used as a standard and serially diluted. The supernatants derived from the bronchial preparations were diluted in a similar fashion. A fixed volume (300 μL) of the different dilutions was then added to the tubes containing the anti-M1 MAb-coated stars. The tubes were incubated for 24 h at 37°C. They were then rinsed three times with PBS containing 0.1% Tween 20 and incubated with ^{125}I-anti-M1 MAbs (PM_8, ~100,000 counts per minute·300 μL^{-1}) for 24 h at 37°C. In some bronchial fluids, the mucin was detected using only a single iodinated anti-M1 MAb, namely 21M1, which has been shown to recognize the recombinant mucin encoded by the 3′ end of the MUC-5AC gene [5].

Following this period, the tubes were washed three times with PBS containing Tween 20 and then counted using a gamma counter (Wizard Model 147005; EG&G Instruments, Evry, France). The quantities of M1/MUC5AC mucin detected in the biological fluids derived from the human bronchial preparations were estimated from the M1 antigen standard curves (fig. 1). The amounts detected were then corrected for the initial volume in which the bronchial preparations were immersed (1 mL). These estimated values were then arbitrarily expressed as M1 U·mL^{-1}·mg tissue wet $weight^{-1}$.

Source of products

The LSAB-2 kit for tissue and cell immunolabelling containing diaminobenzidine, was obtained from DAKO-Laboratories. All the remaining products, Meth, atropine,

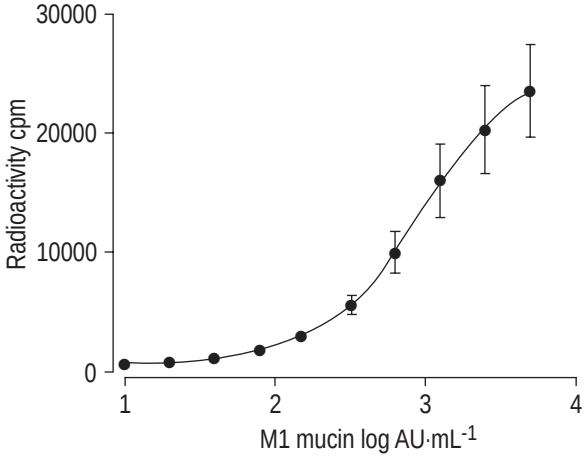

Fig. 1. – M1 mucin immunoradiometric assay standard curve. Values are mean±SEM (n=14). The data were obtained by serial dilution of a purified ovarian mucin antigen (see *Materials and methods*). cpm: counts per minute; AU: arbitrary units.

LTD_4, BSA and Tween 20 were from Sigma (Sigma-Aldrich Chimie Sarl, St. Quentin Fallavier, France). The anti-IgE was obtained from Nordic Immunological Laboratories (Tilberg, the Netherlands).

Calculation

Data are presented as M1 $U \cdot mL^{-1} \cdot mg$ tissue wet $weight^{-1}$. All results are mean±SEM and n indicates the number of different lung samples. Statistical analysis was performed using Student's t-test and a value of $p<0.05$ was taken as an indication of significance.

Results

Immunohistological studies

Examination of isolated human bronchial preparations using light microscopy revealed intense staining of the goblet cells in the epithelium of preparations which had been treated with the anti-M1 MAb (fig. 2). There was also light but less uniform staining in the submucosal layer. Periodate (5 mM) pretreatment did not alter the staining pattern in bronchial preparations exposed to anti-M1 MAb (data not shown).

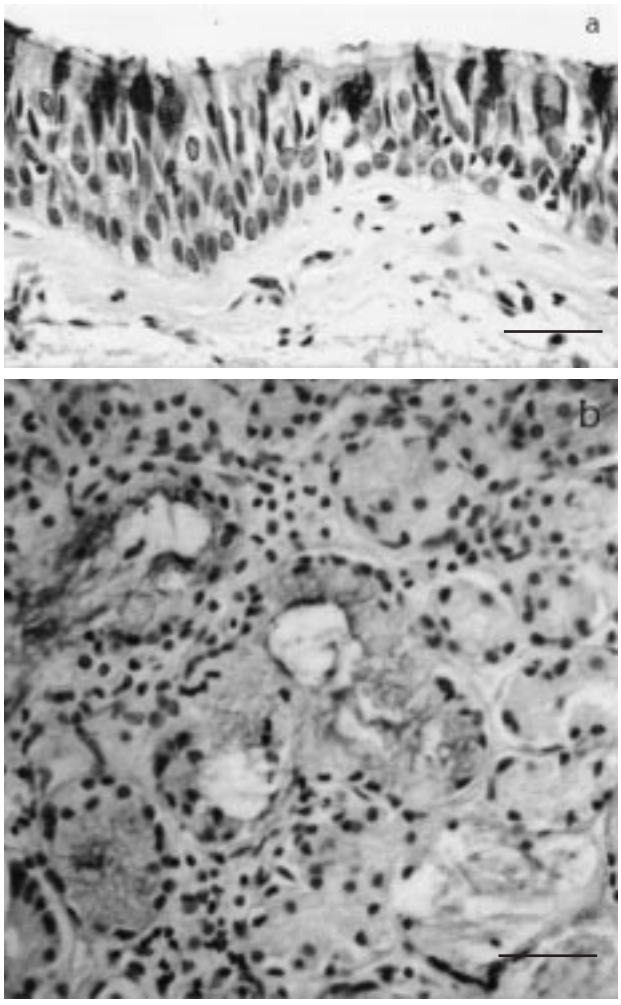

Fig. 2. – Immunohistochemical staining of isolated human bronchial preparations using an anti-M1 Mab (1–13 M1): a) goblet cells in the epithelium were intensely labelled; and b) mucous cells in the submucosal glands exhibited light labelling. Internal scale bar=10 μm.

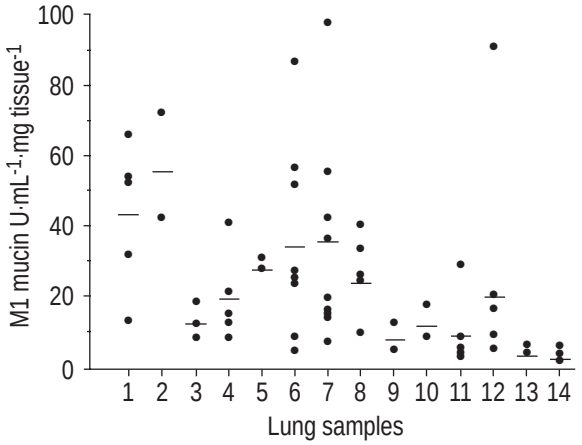

Fig. 3. – Determination of the amount of M1/MUC5AC mucin released under basal conditions (Period I) from human airways *in vitro* obtained from 14 patients using an immunoradiometric assay and anti-M1 monoclonal antibodies. The individual values for each bronchial preparation from different lung samples are shown. The horizontal bars indicate the geometric mean for each lung.

Functional studies

The amount of M1/MUC5AC mucin detected (Period I, basal release) was 29±4 M1 $U \cdot mL^{-1} \cdot mg$ tissue wet $weight^{-1}$ (73 preparations from 20 different lung samples). In bronchial preparations from the same lung, the quantities of mucin detected were variable (fig. 3). There was no change in the basal release of mucin, as measured by anti-M1 MAb in the bronchial fluids during the course of the experimental protocol (fig. 4). However, there was a significant reduction in the amounts of mucin detected when measurements were performed on samples derived from human bronchial preparations in which the epithelium had been removed (fig. 5). In bronchial preparations with an intact epithelium which were challenged with either Meth (100 μM), LTD_4 (10 μM) or anti-IgE (dilution 1:1,000), no significant modification of basal release was observed (fig. 6). In addition, in bronchial preparations (n=3) without an epithelium, Meth (100 μM) did not

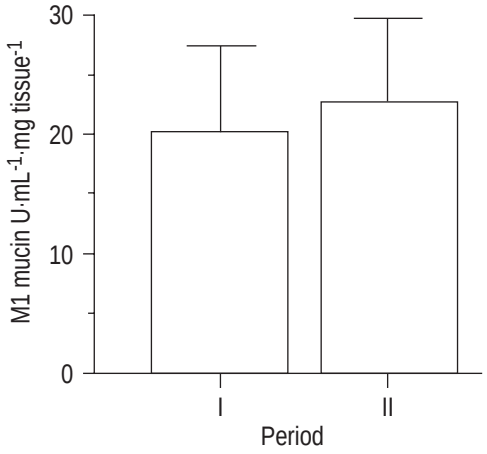

Fig. 4. – Determination of the amount of M1/MUC5AC mucin released from human airways *in vitro* with the epithelium present using an immunoradiometric assay and anti-M1 monoclonal antibodies. Data are presented as mean±SEM and are from Period I (basal) and Period II (post-treatment) and were obtained from 14 different lung samples.

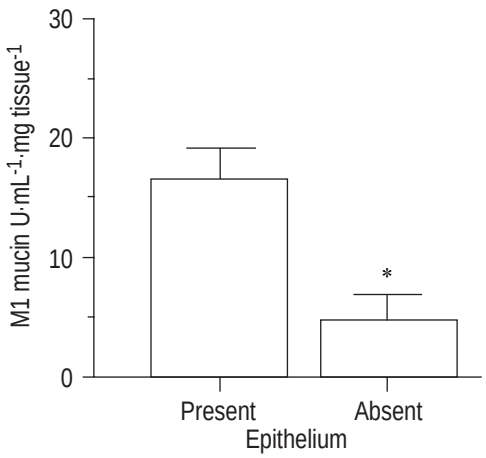

Fig. 5. – Determination of the amount of M1/MUC5AC mucin released from human airways *in vitro* in period II (post-treatment) in the presence and absence of the epithelium using an immunoradiometric assay and anti-M1 monoclonal antibodies. Data are presented as mean±SEM and were obtained in paired bronchial preparations from four different lung samples. *: $p<0.05$.

significantly modify the quantities of M1 (in $U \cdot mL^{-1} \cdot mg$ tissue wet $weight^{-1}$ detected in the medium (~20% increase above Period II levels, data not shown).

The results presented in table 1 demonstrate that the use of the eight MAbs (PM_8) facilitated the detection of mucin in the bronchial fluids and that there was an ~10-fold difference in sensitivity between PM_8 and the MAb 21M1. The concentration of mucin present in bronchial preparations without an epithelium was below the threshold level of the assay when 21M1 was used on its own.

Table 1. – Determination of the amount of M1/MUC5AC mucin released from human airways *in vitro* using an immunoradiometric assay and anti-M1 monoclonal antibodies

	M1 mucin $U \cdot mL^{-1} \cdot mg$ $tissue^{-1}$	
Epithelium	PM_8	21M1
Present	81±23	8±4
Absent	52±35	ND

Values are mean±SEM of measurements made using the bronchial fluids obtained from preparations derived from the lungs of four different patients during Period I (basal). PM_8: mixture of the eight monoclonal antibodies directed against the seven M1 epitopes associated with the peptide core of gastric mucin; 21M1: one of the eight monoclonal antibodies, recognizing the epitope called M1-f, which is encoded by the MUC5AC gene; ND: not detected (below the detection level of the assay).

Discussion

The data obtained using anti-M1 MAbs suggest that M1/MUC5AC mucin was constantly released from isolated human airways. In the absence of the epithelium, there was a significant reduction in the amount of M1/MUC5AC mucin detected in the fluids derived from bronchial preparations. Since the immunohistological data demonstrated that the anti-M1 MAb stained the goblet cells of the epithelium intensely, this mucin may be derived principally from the goblet cells. Furthermore, the basal release of mucin was not enhanced when preparations were stimulated either with Meth, LTD_4 or anti-IgE, suggesting that mucin release, as measured by anti-M1 MAbs, may not be increased by potent bronchoconstrictor agents.

Previous studies have demonstrated that basal secretion does not increase during the course of experimental protocols, suggesting that the secretory factors which were detected using ^{3}H-glucosamine [25, 26] or an MAb directed against the Le^b antigen [14] are continuously released. However, the relative contribution of goblet cells and submucosal glands to the basal and stimulated release has not been documented. Although different mucins are produced by the surface epithelium and the submucosa in the human trachea, HOVENBERG *et al.* [27] demonstrated that MUC5AC mucin was the major mucin derived from goblet cells. The data in the present report demonstrate that M1/MUC5AC mucin is also present in and released from goblet cells found in the lower human respiratory tract. However, there was a low residual secretory level detected in the bronchial fluids following epithelium removal, and this may be due to the submucosal glands,

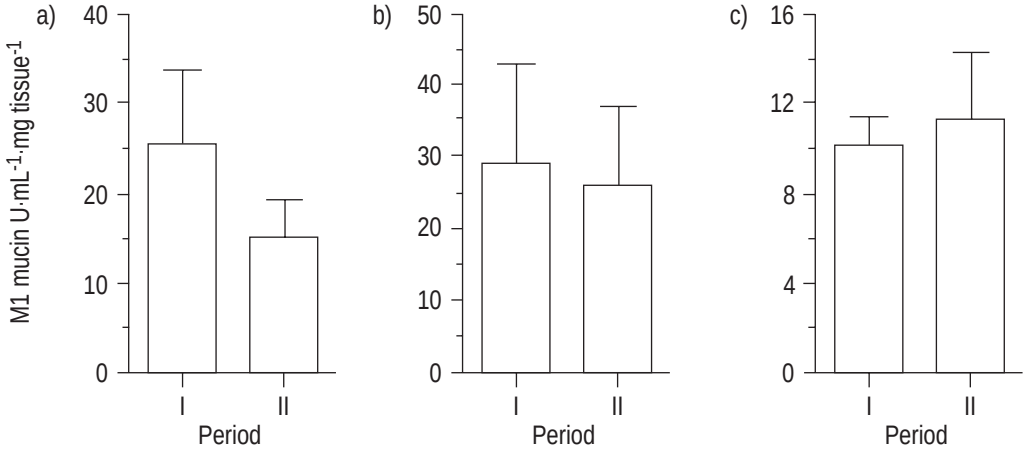

Fig. 6. – Determination of the amount of M1/MUC5AC mucin released from human airways *in vitro* with the epithelium present using an immunoradiometric assay and anti-M1 monoclonal antibodies. Data are presented as mean±SEM and are from Period I (basal) and after stimulation with different agonists (Period II): a) methacholine (Meth, 100 μM, n=12); b) leukotriene D_4 (LTD_4, 10 μM, n=6); and c) anti-human immunoglobulin E (anti-IgE, dilution 1:1,000, n=7).

since light immunohistochemical staining was observed in this region of the bronchial preparations.

The amounts of M1/MUC5AC mucin detected in the bronchial preparations were quite variable, and at present no explanation for this variation is available. The preparations used in this study were derived from the subsegmental region of the lung of patients with pulmonary carcinomas. The number and distribution of goblet cells present in these bronchial preparations may not be constant. An early report [28] showed that there are more goblet cells in tissues derived from proximal airways in which cartilage is present as compared with tissues obtained from distal parts of the human lung in which little or no cartilage is present. Thus, a quantification of goblet cell number in these preparations may be required in order to resolve the variation in the mucin determinations reported. Furthermore, the iodinated MAbs may not always have access to the epitope that they are directed against since the mucin-like glycoproteins present in biological fluids obtained from human bronchial preparations may be masked by other constituents present in the medium. Further studies are presently underway to resolve these issues.

A recent publication by Sossé-Alaoui *et al.* [14] showed, using the MAb 3D3, that, in isolated human airways, Meth markedly enhanced the release of glycoconjugates in tissues with and without an epithelium. Since this MAb is known not only to react strongly with Lewis b antigen but also to recognize Lewis a and Lewis y determinants, these data suggest that glycoconjugate release may be modulated by cholinergic agonists. In contrast, the release of M1/MUC5AC mucin from the epithelium, as detected by anti-M1 MAb (this study), appears not to be responsive to cholinergic stimulation. There is no information on the presence of muscarinic receptors on goblet cells; however, the failure to stimulate release may be related to an absence of functional muscarinic receptors on these cells. A similar explanation may be proposed for the lack of effect of both LTD_4 and anti-IgE; the latter is known to release cysteinyl-LTs (CysLTs) from human lung tissue [29] and these mediators are known to activate specific $CysLT_1$ receptors in human airway preparations [30].

In a previous study, in which glycoconjugate release was assessed using radioactive markers, Marom *et al.* [31] showed a small increase in release from human airways *in vitro* when the explants were stimulated with either LTC_4 or LTD_4 (40% above control levels). These data were further supported by the investigation of Coles *et al.* [26] who demonstrated that CysLTs caused release of ^{3}H-glucosamine from human airways, whereas they did not stimulate the release of lysozyme. However, in both of these studies, radioactive markers were used to detect the release. Since radioactive elements may be incorporated into several cells and mark a variety of constituents of mucus [32], the differences in the results obtained in the present study may, in part, be explained by the specificity of the probes employed in the measurements. In addition, the data (table 1) suggest that the M1/MUC5AC mucin detected in goblet cells may not be stimulated by potent secretagogues.

In conclusion, these observations suggest that the M1/MUC5AC mucin secreted by isolated human airways and detected using the anti-M1 monoclonal antibodies is continuously released and principally derived from the epithelial layer. Secretory activity, as detected using anti-M1 monoclonal antibodies, is not enhanced when the airways are challenged with agents that have been demonstrated to be potent secretagogues. These observations indicate that the probes used to monitor the secretory activity of human airways may help to clarify the origin and composition of the factors which are secreted from human airways.

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