

THE aim of this study was to examine glycoconjugate secretion in human airways with and without an epithelium. Glycoconjugate release in supernatants derived from human airways *in vitro* was determined using an ELISA assay with an anti-human mucin monoclonal antibody (MAB 3D3). This monoclonal antibody reacted strongly with Le^b antigen but also recognized *in vitro* Le^a and Le^y determinants. In 11 of the 34 different lung samples (32%) studied the glycoconjugate levels were below the threshold of detection for this assay. The mean basal secretion of glycoconjugates in human airways *in vitro* was 100 ± 28 $\mu\text{g/g}$ tissue (Period I; $n = 23$ different lung samples). The amount of glycoconjugate measured in the medium derived from human isolated bronchial ring preparations did not change under control conditions during the course of the experimental procedure (Period I; 128 ± 46 $\mu\text{g/g}$ tissue and Period II; 159 ± 48 $\mu\text{g/g}$ tissue; $n = 13$ paired lung samples). In the supernatants of airway preparations with an intact epithelium the amount of glycoconjugates detected was 90 ± 38 $\mu\text{g/g}$ tissue (Period I; $n = 12$ different lung samples) and removal of the epithelium did not alter this basal glycoconjugate release (94 ± 60 $\mu\text{g/g}$ tissue; Period I, $n = 8$ different lung samples). The absence of the epithelial layer was confirmed by histological evaluation. Methacholine (100 μM) induced a 10- and four-fold increase in glycoconjugate release from airways with and without an epithelium, respectively. In contrast, in preparations with an epithelium, LTD₄ (10 μM) and anti-IgE (dilution: 1/1000) did not cause an increase of glycoconjugate release. The methacholine difference between airways with and without an epithelium was not significantly different ($P > 0.10$). However, a treatment with atropine (100 μM) prevented the increase of glycoconjugate release in preparations with an epithelium. These data derived from a limited number of experiments suggest that the epithelium may not regulate the basal or stimulated release of glycoconjugates from isolated human airways.

Key words: Human airways, Glycoconjugate, Monoclonal antibody, Epithelium, Le^b antigen, Secretion

Glycoconjugate secretion in human airways *in vitro*: effects of epithelium removal

H. Sossé-Alaoui,² C. Labat,² I. Gorenne,² V. Thomas de Montpreville,¹ J. Bara³ and C. Brink^{2,CA}

¹Laboratoire d'Anatomopathologie and
²CNRS ERS 566, Hôpital Marie-Lannelongue,
133, ave de la Résistance, 92350 Plessis-Robinson,
France; ³INSERM U-55, Hôpital Saint-Antoine,
184, rue du Faubourg Saint-Antoine, 75012 Paris,
France

^{CA}Corresponding Author

Tel: (+33) 1 4094 28 00

Fax: (+33) 1 4630 12 08

Email: brink@pratique.fr

Introduction

Early studies^{1,2} showed that the constituents of mucus are derived from goblet cells and the submucosal glands. Coles and coworkers³ using ¹⁴C-glucosamine, measured radiolabelled glycoprotein in the tissue medium from airway preparations and demonstrated that the epithelium did not contribute to the basal secretion in dog respiratory tract. However, removal of the epithelium significantly decreased the secretion of sulphated glycoprotein² suggesting that some secretory constituents may originate from the goblet cells in the epithelial layer. In contrast, Sasaki and coworkers⁴ demon-

strated that in cat airways the epithelium modulated the secretory activity of the submucosal glands. Since the epithelium decreased the methacholine induced glandular secretion, these authors suggested the liberation of an epithelium inhibitory factor which modulated the mucin release. In contrast, Sherman and coworkers⁵ using methacholine demonstrated a significant release of mucus glycoproteins in the absence of the epithelium. These latter results would suggest that the epithelium does not contribute significantly to the glycoproteins secreted. While some of these differences may be related to the species in which the experiments were performed, there is little information available

on the influence of the epithelium in modulating the secretory activity in human airways.

While the principal constituents of mucus are the mucins, a variety of proteins, glycoconjugates (serum type glycoproteins and proteoglycans) and glycolipids are also produced and secreted.⁶⁻⁸ In an attempt to characterize those factors which constitute respiratory secretion, Saint George and coworkers⁹ showed that a number of monoclonal antibodies reacted with material in goblet cells and/or submucosal mucus gland cells, data which have been confirmed by other investigators.¹⁰ Recently, Emery and coworkers¹¹ have prepared an anti-human mucin MAb against purified human respiratory mucins. These authors indicated that this anti-human MAb 3D3 was not specific for respiratory mucin but reacted, in fact, strongly with the Lewis b (Le^b) antigen, but also recognized Le^a and Le^y determinants using synthetic oligosaccharides. These investigators also demonstrated that in human airways the MAb 3D3 stained goblet cells and mucous cells of the submucosal glands as well as human epithelial cells in culture.¹² These observations suggested that this monoclonal may be a useful tool to monitor secretory activity in human airways.

The aim of this study was to examine glycoconjugate secretion in human airways *in vitro* under basal conditions and subsequent to methacholine stimulation. In order to evaluate the role of the epithelium, these experiments were performed in human bronchial ring preparations with an intact epithelium or in bronchial rings in which the epithelium had been removed.

Materials and Methods

Tissues

Human lung tissues was obtained from patients ($n = 34$) who had undergone surgery for lung carcinoma. Subsequent to the resection of lung, the subsegmental bronchi were dissected free from parenchymal tissue and washed with Tyrode's solution. The composition of the Tyrode's solution was (mM): NaCl 139.2, KCl 2.7, CaCl₂ 1.8, MgCl₂ 0.49, NaHCO₃ 11.9, NaH₂PO₄ 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). Bronchial preparations with and without epithelium were examined. The epithelium was removed by gently rubbing the luminal surface of the bronchial preparations with a moistened cotton swab.

Histological studies

Bronchial rings were fixed in formalin for 24 h, dehydrated, embedded in paraffin and sections (10 μ m) were cut and stained with haematoxylin and eosin (2.5%) to evaluate general morphology.

The method for peroxidase staining was performed as previously described,¹³ using Dako Kit LSAB-2. Briefly, tissue sections were deparaffinized with toluene, rehydrated with ethanol and washed with PBS. Endogenous peroxidases were eliminated with H₂O₂ (3% for 5 min). Tissue sections were treated with blocking serum (casein 0.25% in PBS; Dako) for inhibition of cross immunological reactions. The tissues were incubated (10 min) with anti-mucin 3D3 antibody and washed with PBS. Thereafter, the sections were incubated (10 min) with peroxidase coupled to streptavidine and peroxidase activity was developed using diaminobenzidine/H₂O₂. Tissues were counter-stained with Harris haematoxylin.

Functional studies

The preparations were set up in a microtitre plate (24 wells) containing Tyrode's solution (1 ml) and allowed to equilibrate for 1 h in a humidified incubator (37°C, 5% CO₂/air). At the end of this equilibration period, the medium was exchanged and fresh Tyrode's solution was added. The tissues were then maintained for 1 hour (Period I: basal release). The medium was collected and replaced with Tyrode's solution or Tyrode's solution containing atropine (100 μ M). After 30 min (treatment), the medium was exchanged with Tyrode's solution or Tyrode's solution containing either atropine (100 μ M) and/or methacholine (100 μ M). In some experiments, subsequent to the incubation in Tyrode's solution (30 min) the bronchial preparations were stimulated with either LTD₄ (10 μ M) or anti-IgE (dilution 1/1000). Following a 1 h exposure to these agents (Period II: post-treatment), the supernatants were again collected. The supernatants from Period I and Period II were stored at -20°C. Prior to determination of glycoconjugates (ELISA), dithiothreitol (DTT) 0.1% in PBS solution was added to these samples for 15 min at 37°C¹⁴ to permit solubilization of mucus particulates present in the supernatants.

Glycoconjugate determination

The antigen and MAb 3D3 were a gift from Bayer plc (UK) and details concerning purification and preparation have recently been published.¹¹

The glycoproteins secreted in supernatants of human bronchial rings were detected by ELISA using the MAb 3D3. Microplates (Nunc-Immuno-plate Maxisorp) were coated with a solution of MAb (dilution: 1/5000 in PBS) and incubated overnight at 4°C. The plates were washed twice with 200 μ l of PBS-Tween 20 (dilution: 1/1000) employing a microplate washer, unbound sites were blocked with (30 mg/ml) BSA diluted in PBS for 1 h at 37°C. The plates were washed with a dilution of purified human 3D3 antigen standard in PBS-Tween-20 (1-500 ng/50 μ l) and the

supernatants of the bronchial ring preparations were distributed in the wells and incubated for 1 h at 37°C. Following, three washes with Tris–Tween-20, the MAb 3D3 mixed with normal mouse serum and anti-mouse immunoglobulin IgG conjugated with alkaline phosphatase (1/500 in Tris–polyethyleneglycol (PEG at 4%) was added to wells and incubated for 1 h at 37°C. The plates were washed twice with Tris–Tween-20 and developed using a solution of p-nitrophenylphosphate (pNpp) diluted in 0.2 M Tris buffer pH > 8 to give a final concentration (1 mg/ml) substrate. The reaction was terminated (15–20 min) when the optical densities (OD) correspond to maximal concentration from 3D3 antigen standard attained 0.8 OD units. Optical densities were measured with a Titertek-Multiskan (Labsystems) microplate reader using a wavelength of 405 nm. The amount of glycoconjugate released was interpolated from the 3D3 antigen standard curve. A standard curve with dithiothreitol was carried with different amount of 3D3 antigen (1–250 ng/50 µl), the dithiothreitol (0.1%) was added to the maximal concentration of antigen and serial dilutions were subsequently performed. Since the monoclonal antibody 3D3 is directed specifically against the Le^b antigen only the supernatants of Le^b individuals in which measurable levels of glycoconjugate were observed, are presented in the results section. In lung samples from 11 of the 34 different patients examined (approximately 30%), the Le^b antigen was not detected. The percentage of different lung samples in which the Le^b antigen was detected was similar to the population estimate for Le^b antigen carrying subjects.

Source of products

Plates (maxi-sorp Nunc), Tris (Prolabo), polyethylene glycol 6000 (Merck), Kit LSAB-2 for immunolabelling containing diaminobenzidine (Dako Laboratories). All the remaining products; methacholine, atropine, mouse serum, anti-mouse immunoglobulin coupled with alkaline phosphatase, bovine serum albumin, dithiothreitol (DTT), Tween-20, p-nitrophenylphosphate (pNpp) were from Sigma Chemical Co., USA. The LTD₄ was a gift from Dr P. J. Gardiner (Bayer plc). The sheep antiserum to human IgE (anti-IgE) was obtained from Nordic Immunological Laboratories, Tilberg, the Netherlands.

Calculation

The data are represented as (µg/g tissue) or (% of basal level, that is, Period II/Period I × 100). All results are means ± SEM and *n* indicates the number of lung samples. Statistical analysis was performed using Student's *t*-test or Mann–Whitney rank sum test. A value (*P* < 0.05) was taken as an indication of significance.

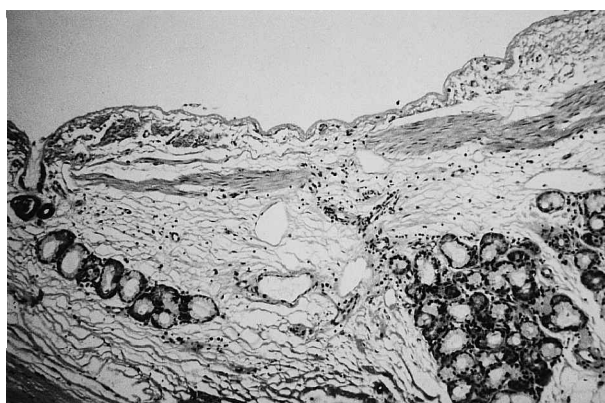
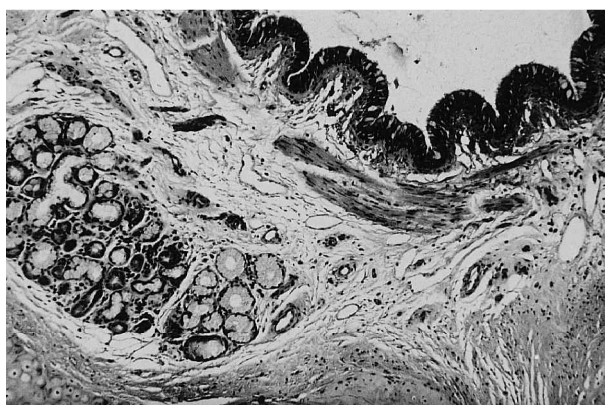


FIG. 1. Top: Morphological aspects of human bronchial preparation with an intact epithelium. The presence of a pseudostratified epithelium with goblet cells and ciliated cells was observed (magnification × 100). Bottom: Human bronchial preparations without epithelium. Tissues were stained with haematoxylin and eosin (magnification × 50).

Results

Histological data

Mechanical removal of the epithelium by rubbing the luminal surface of human isolated bronchial ring preparations led to the complete obliteration of the epithelium while the basement membrane was left intact (Fig. 1). In human bronchial ring preparations after treatment with MAb 3D3, staining showed that this MAb 3D3 antibody was associated with two specific tissue regions, namely, the epithelial layer and submucosal glands (Fig. 2).

Functional data

The DTT did not alter the 3D3 antigen standard curve (Fig. 3.). In 11 of the 34 different lung samples studied in this assay (32%), the glycoconjugate levels were below the threshold level of detection. The mean basal secretion of glycoconjugates in human airways *in vitro* was 100 ± 28 µg/g tissue (Period I; *n* = 23 different lung samples). The amount of glycoconjugate measured in the medium, derived from human isolated bronchial ring prepara-

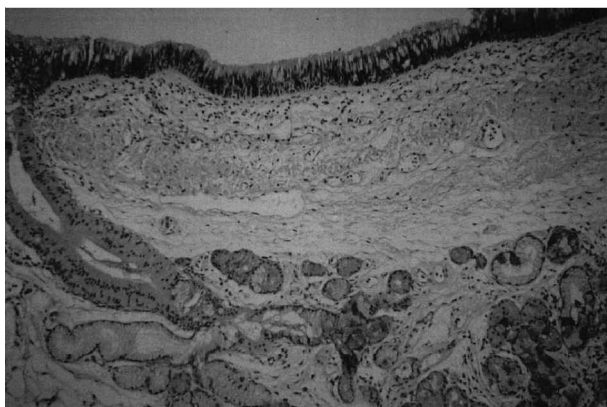


FIG. 2. Immunohistochemical labelling of human bronchus with MAb 3D3. The MAb 3D3 labelled both the epithelium including mucus goblet cells and mucus cells of the submucosal glands (magnification $\times 40$).

tions, did not change under control conditions during the course of the experimental procedure (Period I; $128 \pm 46 \mu\text{g/g}$ tissue and Period II; $159 \pm 48 \mu\text{g/g}$ tissue; $n = 13$ paired lung samples). In the supernatants of airway preparations with an intact epithelium the amount of glycoconjugates detected was $90 \pm 38 \mu\text{g/g}$ tissue (Period I; $n = 12$ different lung samples) and removal of the epithelium did not alter this basal glycoconjugate release ($94 \pm 60 \mu\text{g/g}$ tissue; Period I, $n = 8$ different lung samples). The absence of the epithelial layer was confirmed by histological evaluation.

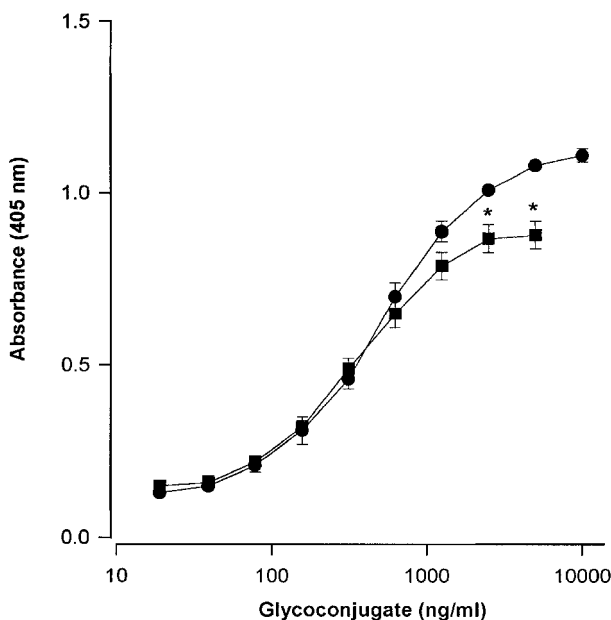


FIG. 3. Effects of DTT on 3D3 antigen standard curve (ELISA). Standard curve with (■; $n = 6$) or without (●; $n = 3$) DTT. The DTT (0.1%) was added to the standard maximal concentration of 3D3 antigen and serial dilutions were performed. Results are presented as glycoconjugate (ng/ml). Values are means \pm SEM and * indicates $P < 0.05$ (Student's *t*-test).

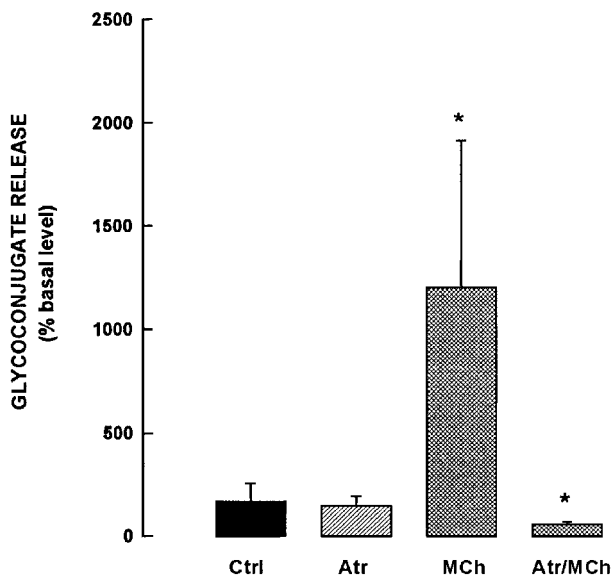


FIG. 4. The effects of muscarinic agents on glycoconjugate release from human bronchial preparations with intact epithelium. Unstimulated tissues (Ctrl; $n = 7$) and preparations treated with either atropine (Atr, $100 \mu\text{M}$; $n = 4$), methacholine (MCh, $100 \mu\text{M}$; $n = 7$) or atropine and methacholine (Atr/MCh, $100 \mu\text{M}$; $n = 4$). Results are represented as % basal release. Values are means \pm SEM and (n) indicates number of different lung samples. (*) indicates $P < 0.05$ (Mann-Whitney rank sum test).

In human isolated bronchial ring preparations with an intact epithelium methacholine ($100 \mu\text{M}$) produced a significant increase in the amount of glycoconjugate detected in the medium (Fig. 4). In contrast, no significant change was observed in human bronchial preparations stimulated with either LTD₄ ($10 \mu\text{M}$; $n = 3$, $48 \pm 24\%$) or anti-IgE (dilution 1/1000; $n = 5$, $76 \pm 17\%$). The 10-fold increase in glycoconjugate release induced by methacholine was attenuated by atropine ($100 \mu\text{M}$). Results derived from similar experiments performed in bronchial rings where the epithelium had been removed are presented in Fig. 5. These data demonstrate that removal of the epithelium did not significantly alter either the basal or stimulated levels of glycoconjugate in the tissue medium.

Discussion

MAb 3D3, a marker of secretory glycoconjugates^{11,12} was used to determine glycoconjugate release in human airways *in vitro*. Histochemical analysis revealed that the glycoconjugates which reacted with MAb 3D3 were associated with both the epithelium and the glandular mucus cells of the human bronchus. Methacholine appeared to be the most effective agent to stimulate glycoconjugate release in this model. In addition, the amounts of glycoconjugates detected (basal and stimulated levels) as measured with the MAb 3D3 were not dependent on the presence of the

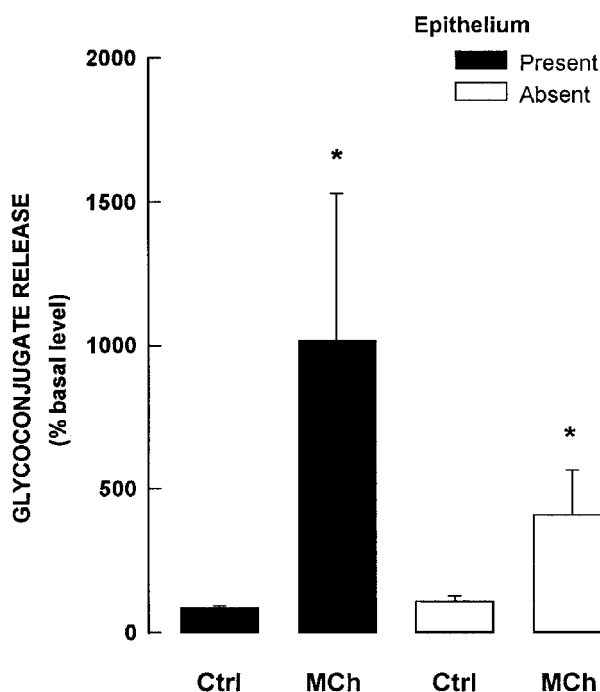


FIG. 5. Glycoconjugate release from human bronchial preparations either with (■) or without (□) epithelium. The preparations were unstimulated (Ctrl) or stimulated (MCh, 100 μ M). Values are means \pm SEM from 5–12 lung samples. (*) indicates $P < 0.05$ (Mann–Whitney rank sum test).

epithelium since removal of this layer did not significantly alter either the basal or methacholine induced release.

Stahl and Ellis² published a biochemical study showing that removal of the epithelium in dog airways permitted the detection of several glycoproteins thereby assigning the release of different substances to the epithelium or the mucosal glands of the respiratory tract of this species. Removal of the epithelium produced a significant decrease in the secretion of sulphated glycoproteins suggesting that goblet cells in the epithelium were responsible for these constituents. In addition, these authors also showed that while the epithelial layer and the mucosal glands secrete different macromolecules, they also interacted in the formation of the larger glycoconjugates which are released into the airway lumen.

Previous reports in human airway tissues^{6,10,14} have demonstrated that there was little or no change in basal secretion under control conditions, however, methacholine induced significant increases in secretory activity. The results (present report) confirm these initial observations and are in agreement with data derived from other species, such as the cat. In contrast, in this model (present report) LTD₄ and anti-IgE did not induce glycoconjugate release. These results are different from previous reports where secretory activity was assessed using radioactive

markers. Marom and coworkers¹⁵ showed a small increase in release from human airways *in vitro* (40% above control levels) using either LTC₄ or LTD₄. These data were also supported by the investigation of Coles and coworkers¹⁶ who demonstrated a similar leukotriene-induced secretory release from human airways. However, radioactive elements may be incorporated into several cells and mark a variety of molecules,¹⁷ these differences in results may be explained in part by the specificity of the markers employed to determine secretory activity.

However, removal of the epithelium did not significantly modify either the basal secretion or that induced by methacholine stimulation (present report) when the MAb 3D3 was used to evaluate glycoconjugate release from human airways. Therefore species differences may also exist since Sasaki and coworkers⁴ showed that in the respiratory tract of the cat the epithelium modified the secretory activity of the submucosal glands. The data from human airways (present report) would also suggest that the methacholine is acting principally at the glandular level. The lack of cholinergic response at the goblet cell level may be due to the absence of neuronal inputs¹⁸ at the surface epithelium. In addition, Mak and Barnes¹⁹ using an autoradiographic technique in human airways demonstrated that muscarinic receptors were not detected in the epithelial layer, since no radiolabel was observed, although label was detected in the region of the glands. Such results would suggest that muscarinic receptors may not be present in the epithelium in human airways. However, a recent publication has demonstrated that muscarinic receptor mRNA is detected in the human respiratory epithelium.²⁰ While the autoradiographic technique may not provide adequate sensitivity to detect muscarinic receptors, their location and function in the epithelium has not been adequately evaluated.

While initial reports showed that epithelial cells in culture fail to secrete mucin and/or mucin-like glycoproteins,² recent studies using human epithelial cells in culture have demonstrated that a number of substances including mucins could be detected after several weeks in culture.¹² While these investigators demonstrated that epithelial cells in culture were stained with the MAb 3D3, no indication as to the function of these epithelial cells was examined. The data from intact human airways *in vitro* (present report) extend the work of Emery and coworkers¹¹ and demonstrate that the MAb 3D3 may also be used to detect basal and methacholine induced secretory activity. However, the functional studies (present report) suggest that while the epithelial layer may fix the MAb 3D3, there was little or no secretory activity detected by MAb 3D3 from this layer. In addition, removal of the epithelium did not modulate either the basal or stimulated release of glycoconjugates in human airway preparations.

In human airways, the MAb 3D3 may be used as a marker of glycoconjugate release and this secretory activity is principally associated with the submucosal glandular level.

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