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CHANGES IN THE PROPORTION OF FUCOSYLATED GLYCOCONJUGATES IN THE AIRWAYS' GOBLET CELL SECRETION AFTER ORAL ADMINISTRATION OF AMBROXOL

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A b s t r a c t

Oral administration of ambroxol lowered only slightly the proportion of tracheal goblet cells containing sialylated glycoconjugates. Since rheological properties of the airways' mucus are mostly influenced by the proportion of fucosylated glycoconjugates, we evaluated the proportion of fucosylated-glycoconjugate containing tracheal goblet cells after the same treatment as well.

New Zealand White rabbit males were orally administered ambroxol in a dose of 7.5 mg. The material for lectin histochemistry was collected 20 min post exposure. Lectins of *Ulex europaeus* detecting α(1–2)-linked fucose and of *Aleuria aurantia* detecting α(1–3)-, α(1–4)-, and α(1–6)-linked fucose were used both individually and simultaneously.

The proportion of total goblet cells containing fucosylated glycoconjugates decreased from 44.2 ± 22.1 % in controls to 8.7 ± 1.5 % in treated animals. The proportion of goblet cells with Ulex europaeus lectin-positive content decreased from 38.9 ± 19.9 % to 5.5 ± 2.0 %. The proportion of goblet cells with Aleuria aurantia lectin-positive content decreased from 14.6 ± 7.5 % to 4.0 ± 1.9 %.

In the rabbit tracheal epithelium, the effect of the mucolytic drug ambroxol dramatically lowered the proportion of goblet cells containing fucosylated glycoconjugates. This decrease was at the expense of all the detected fucosylated glycoconjugates.

K e y w o r d s

Airways' mucin histochemistry, Fucosylation, Rabbit

A b b r e v i a t i o n s u s e d

GCCs, glycoconjugates; GC, goblet cells; AAL, *Aleuria aurantia* lectin; ULE-I, *Ulex europaeus* lectin; Fuc, Fucose; Px, peroxidase; SPF, specified pathogen-free; AB, alcian blue; PAS, periodic acid – Schiff

INTRODUCTION

Fucosylated glycoconjugates (GCCs) secreted as components of the airways' mucus contribute substantially to its viscoelastic properties as evidenced by

Finkbeiner (1999) and *Majima et al.* (1999). Fucosylated GCCs, both secreted and bound to cellular surfaces, also serve as adhesion sites for various antigens (*1*, *5*, *9*). Increased proportion of fucosylated GCCs in the airways' secretion has been described in sinusitis (*13*), chronic bronchitis (*3*, *4*), asthma (*3*), cystic fibrosis (*3*, *6*), and acute bronchiolitis in rats (*7*). *Castells et al.* (1991) revealed fucosylated GCCs in both secretory granules and cell membranes. In secretions, GCCs are usually fucosylated at α(1–2) position. In cystic fibrosis, *Glick et al.* (2001) described the shift towards fucosylation position α(1–3) at the expense of the α(1–2) position and sialylation as well.

Mucolytic drugs influence mostly rheological properties of the airways' mucus. Ambroxol is the most frequently used mucolytic agent in clinical practice. It facilitates incorporation of hydrolytic enzymes into lysosomes of the airways' secretory elements. In ultrastructural studies (*8*) on rabbits, ambroxol exhibited pronounced adverse effects on tracheal epithelium including overstimulation of goblet cells (GC) and leaving the secretory cycle due to their degeneration. Since oral administration of ambroxol lowered only slightly the proportion of sialylated-glycoconjugate containing tracheal goblet cells (*15*), we decided to evaluate the proportion of fucosylated-glycoconjugate containing goblet cells as well.

MATERIALS AND METHODS

Seven SPF New Zealand White male rabbits (Charles River, Sulzfeld, Germany) of an average body weight of 2219 ± 448 g were used. Three of them were orally administered 1 ml of Mucosolvan sol. (Boehringer Ingelheim International GmbH, Ingelheim, Germany), i.e. the dose routinely used in infants. This volume contained 7.5 mg of ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxy cyclohexyl] benzylamine). The material was collected under general (ketamine 35 mg/kg and xylazine 5 mg/kg intramuscularly) and local anaesthesia (subcutaneous infiltration of the ventral cervical field with procaine) 20 minutes post exposure. Four rabbits served as untreated healthy controls, the material was collected immediately after the induction of anaesthesia.

The middle portions of tracheae between the 15th and 20th tracheal rings were formalin-fixed, paraffin-embedded, and cut at 5–7 μm. The combined staining method of alcian blue (AB) at pH 2.5 followed by PAS-reaction according to *Mowry and Winkler* (1956) was used to reveal both total acidic and neutral glycoconjugates, i.e. to reveal the total number of GC. Thus, in each given method, we evaluated 398 goblet cells in controls and 402 goblet cells in experimental animals in total. To detect fucosylated GCCs, the methods of *in situ* lectin histochemistry were used. The legume lectin from *Ulex europaeus* (ULE-I), detecting terminal or branched fucose (Fuc) linked α(1-–2) to an oligosaccharide, and the ascomycete orange-peel mushroom *Aleuria aurantia* lectin (AAL), detecting α(1–6), α(1–3), and α(1–4) linked fucose residues, were employed (Vector Laboratories, Inc., Burlingame, USA). After dewaxing and rehydrating, the endogenous peroxidase (Px) was blocked and the sections were incubated with biotinylated ULE-I or biotinylated AAL or both lectins simultaneously at concentrations of 30 μg/ml for 60 min. Then, the sections were incubated with a solution of streptavidin-horseradish Px conjugate (Vector Laboratories, Inc., Burlingame, USA) at a concentration of 2 μg/ml for 45 minutes, followed by Sigma FAST DAB Peroxidase Substrate Tablets visualisation (Sigma-Aldrich Chemie, Deisenhofen, Germany) enhanced by $CuSO_4$. Blocking of endogenous Px was verified by omitting the first step of the method. The specific lectin binding was verified by 15-minute incubation of lectins with control substrate – 0.2 M L-fucose, preceding the incubation with sections (*2*). We evaluated only GC

containing well-developed granules with a positive reaction in each method used. The granules had to fill at least 2/3 of the height of the epithelium. Simultaneous use of both lectins enabled identification of GC containing GCCs positive for both lectins; thus the overlap of AAL- and ULE-I-positive GC could be calculated. The Venn's diagram also enabled the calculation of GC containing ULE-I-positive granules only and GC containing AAL-positive granules only, respectively.

For statistical evaluation, the relative values of the five categories of GC, revealed by individual methods (total GC, ULE-I-positive GC, AAL-positive GC, all lectin-positive GC, and lectin-positive GC with calculated overlap ULE-I-AAL) were evaluated by the chi-square test of homogeneity in frequency tables, using the Yates' correction in low frequencies when appropriate (Statistica, v.6.0 software). The significance of differences between fucosylated glycoconjugate-detecting methods was tested by the matched t-test, Spearman rank correlation, matched sign test, and Wilcoxon's paired test (BMDP New System software).

The experimental procedures were approved by the Animals Protection Expert Commission of the Faculty.

RESULTS

The tracheae of both control and treated rabbits were lined with a pseudostratified columnar ciliated epithelium composed mostly of ciliated, goblet, and basal cells. The height of the epithelium was approximately 30 μm. The distribution of secretory elements was irregular.

Using conventional histochemical methods, the secretory elements revealed typical staining patterns according to the type of GCCs they contained. The PAS-positive mucous granules were stained magenta, the alcian blue stained mucous granules, blue. Some GC exhibited various shades of violet colour; these cells were counted as containing acidic GCCs in the mixture with neutral ones.

The appearance of goblet cells reacting with the lectins used was the same in both control and treated rabbits. The positive reaction of ULE-I (*Fig. 1*) was featured with a conspicuous staining of mucous granules in the goblet cell, either in the whole volume of a granule, or as a densely contrasted ring; staining of the ciliary border was restricted to the close vicinity of apical surfaces of goblet cells. AAL-reaction depicted individual mucous granules as dark rings. The ciliary border was always densely stained, too. (*Fig. 2*).

In healthy control rabbits, we revealed 38.9 ± 19.9 % of ULE-I-positive GC and 14.6 ± 7.5 % of AAL-positive GC. The simultaneous use of both lectins revealed 44.2 ± 22.1 % of total GC (*Fig. 3*). PAS-positive GC represented 1.5 ± 2.4 % of the total GC.

Having compared the sum of percentages of GC positive for both lectins used individually with the percentages of GC positive for both lectins simultaneously using the Venn's diagram (*Fig. 4*), the calculation gave 29.6 % of GC containing ULE-I-positive granules only and 5.3 % of GC containing AAL-positive granules only, respectively. GC containing granules positive for both lectins represented 9.3 %.

Twenty minutes after the administration of 7.5 mg of ambroxol, 5.5 ± 2.0 % of GC with ULE-I-positive granules and 4.0 ± 1.9 % of GC with AAL-positive granules

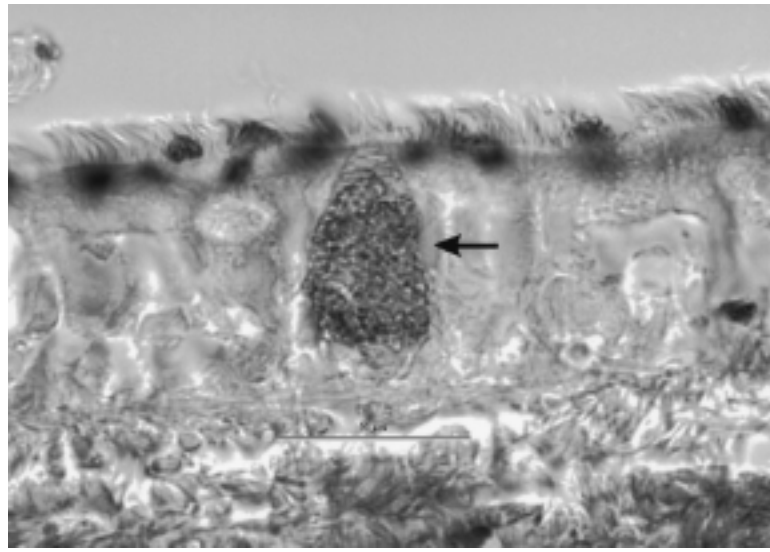

Fig. 1
Goblet cell (arrow) containing ULE-I-positive mucous granules 20 min after administration of ambroxol. Bar = 20 μm.

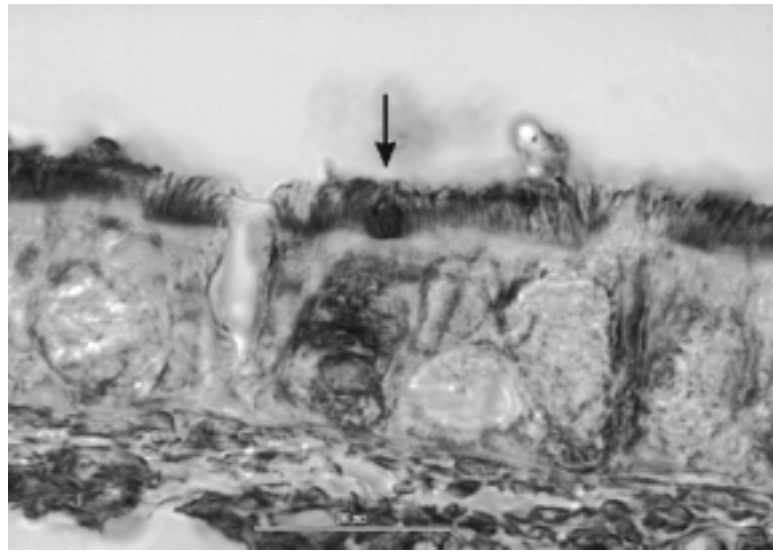

Fig. 2
Goblet cell (arrow) containing AAL-positive mucous granules 20 min after administration of ambroxol. Two goblet cells without AAL-positive reaction on both sides of the positive one. Bar = 20 μm.

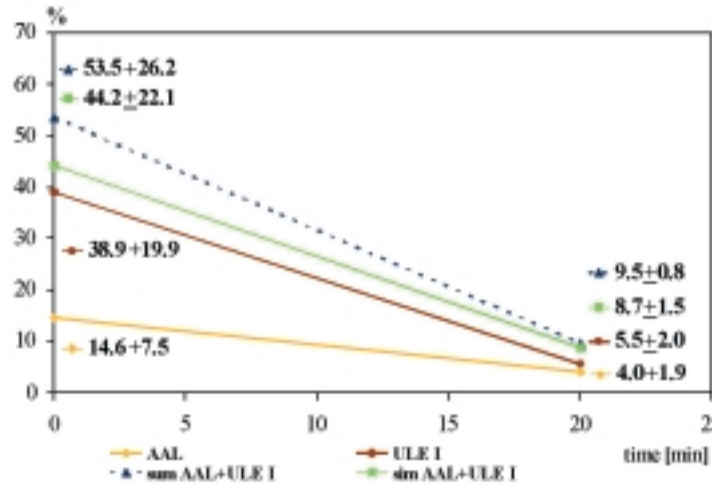

Fig. 3
Changes in percentage of goblet cells in the tracheal epithelium containing fucosylated glycoconjugates 20 min after administration of ambroxol. Lectin histochemistry. Sum = the sum of percentages of goblet cells detected individually, sim = the percentage of goblet cells detected simultaneously.

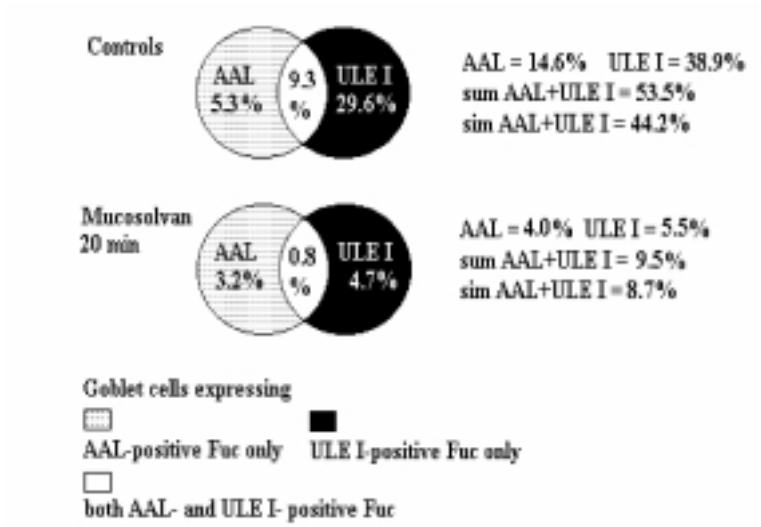

Fig. 4
Proportions of goblet cells containing AAL-positive granules only, ULE-I-positive granules only, and both lectin-positive granules. Venn's diagram. Sum = the sum of percentages of goblet cells detected individually, sim = the percentage of goblet cells detected simultaneously.

were found. The simultaneous use of both lectins revealed 8.7 ± 1.5 % of the total GC (*Fig. 3*). PAS-positive GC disappeared completely from the tracheal epithelium.

Having compared the sum of percentages of GC positive for both lectins used individually with the percentages of GC positive for both lectins simultaneously using the Venn's diagram (*Fig. 4*), the calculation gave 4.7 % of GC containing ULE-I-positive granules only and 3.2 % of GC containing AAL-positive granules only, respectively. GC containing granules positive for both lectins represented 0.8 %.

The statistical significance of differences between the individual groups of GC is given in *Fig. 3*.

DISCUSSION

The methods used allowed us to give the proportion of GC containing granules with α(1–2)-fucosylated glycoconjugates and GC containing granules with α(1–3), α(1–4)-, and α(1–6)-fucosylated glycoconjugates directly. *Robinson et al.* (1986) held the PAS-reaction as specific to fucose moieties. Since the PAS-positive GC represented only a minority population in the controls or disappeared completely in the administered animals, it should be concluded that fucosylated GCCs were components of mucous granules containing also acidic GCCs. This opinion was supported by both conventional and lectin histochemistry. The staining patterns of the lectins used corresponded with the fact that α(1–2)-fucosylated GCCs are mostly components of secreted mucins (*4, 11*); other links of fucose are more typical for membrane-bound GCCs. The shifts in the percentages of ULE-I-positive GC and AAL-positive GC reflected the same fact implicating thus that a selective release of ULE-I-positive mucous granules occurred 20 minutes after oral administration of ambroxol. *Vajner et al.* (2001) described the apparent discrepancy between ultrastructural findings and histochemical evaluations of acidic glycoconjugates that could be explained by the rapid, almost complete but proportional release of acidic mucins of the goblet cells. On the other hand, ultrastructural findings in the tracheal epithelium after the oral administration of ambroxol (*8*) indicating an overstimulation of the majority of secretory elements resulting even in their damage and degeneration could be the picture of a selective release of secretion from GC containing fucosylated GCCs. This finding also supported the premise that fucosylated GCCs-containing mucus could play a protective role against some microbes, preventing them to adhere on cell surfaces (*1*, *5*, *9*).

CONCLUSIONS

In the rabbit tracheal epithelium, the effect of the mucolytic drug ambroxol dramatically lowered the proportion of goblet cells containing fucosylated glycoconjugates. This decrease was at the expense of all the detected fucosylated glycoconjugates.

A c k n o w l e d g e m e n t s

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ZMĚNY ZASTOUPENÍ POHÁRKOVÝCH BUNĚK S OBSAHEM FUKOSYLOVANÝCH GLYKOKONJUGÁTŮ V EPITELU TRACHEY PO PERORÁLNÍM PODÁNÍ AMBROXOLU

S o u h r n

Perorální podání ambroxolu vyvolalo jen lehké snížení podílu tracheálních pohárkových buněk s obsahem sialovaných glykokonjugátů. Protože jsou však reologické vlastnosti hlenu v dýchacích cestách ovlivňovány zejména podílem fukosylovaných glykokonjugátů, studovali jsme zastoupení tracheálních pohárkových buněk s obsahem těchto glykokonjugátů ve stejně uspořádaném pokusu.

Třem samcům králíka plemene Novozélandský bílý byl perorálně podán ambroxol v dávce 7,5 mg. Dvacet minut po aplikaci jsme odebrali materiál pro vyšetření metodou lektinové histochemie. K detekci fukózy v poloze α(1–2) jsme užili lektinu hlodaše evropského (*Ulex europaeus*, ULE-I), k detekci fukózy v poloze α(1–3), α(1–4) a α(1–6) pak lektinu pomerančové plísně (*Aleuria aurantia*, AAL), a to jak samostatně, tak simultánně.

Podíl všech pohárkových buněk s obsahem fukosylovaných glykokonjugátů se oproti kontrolám (44,2 ± 22,1%) snížil na 8,7 ± 1,5% u aplikovaných zvířat. Zastoupení pohárkových buněk s ULE-I pozitivním obsahem se snížilo z 38,9 ± 19,9% na 5,5 ± 2,0%. Zastoupení pohárkových buněk s AAL pozitivním obsahem se snížilo ze 14,6 ± 7,5% na 4,0 ± 1,9%.

Účinek mukolytika ambroxolu v epitelu trachey králíků dramaticky snížil podíl pohárkových buněk s obsahem fukosylovaných glykokonjugátů. K poklesu došlo na vrub všech detekovaných fukosylovaných glykokonjugátů.

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