Exposure to hypoxia injures tracheal epithelium (ultrastructural study)

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ABSTRACT: The ultrastructure of the tracheal epithelium in rabbits exposed for 96 hours to normobaric hypoxia was studied. In rabbits placed for 96 hours in environment with increased temperature and humidity, the first phase of common response of goblet cells to injury, represented mostly by degeneration of the exhausted cells, was revealed. The decrease in O_2 concentration highly accelerated the reaction of the goblet cells. Due to the influence of high temperature, humidity and normobaric hypoxia, the second phase of the goblet cells' reaction, massive differentiation of new secretory elements accompanied with intraepithelial mucous glands' development, was recorded. In the ciliated cells, only mild signs of pathological alteration of deeper portions of their cytoplasm were noticed. In the area of the ciliary border, significant decrease in the number of kinocilia/μm^2, increase in percentage of altered cilia and morphological signs of impairment of the vital self-cleaning ability were recorded.

Keywords: airway epithelium; goblet cells; ciliated cells; ultrastructure; rabbit

Airway epithelium performing one of the vital self-cleaning functions plays important role in the development of many disorders of the respiratory organs. It represents the first barrier to the inhaled injurants, it reacts promptly on the changes in the environment or on the therapeutic and diagnostic procedures (Konrádová, 1991). To our knowledge, only a few authors dealt with the effect of hypoxia on the structure of the epithelium lining rabbits' respiratory organs. A decrease in number of cells that belong to the diffuse neuroendocrine system was described after short exposure to environment with 13% O_2 in the tracheal epithelium of young rabbits (Echt *et al.*, 1982). In alveolar epithelium, degranulation of the type II pneumocytes was recorded after 16 hours spent in a chamber with 8% O_2 (Réffy *et al.*, 1977). We therefore decided to investigate the reaction of the lining epithelium of airways under hypoxic conditions. The experiments were performed in a chamber containing atmosphere with 10% O_2, where air humidity was 100% and temperature reached 23°C. To demonstrate the isolated effect of normobaric hypoxia, the control animals were exposed to atmosphere with only increased humidity and temperature.

MATERIAL AND METHODS

In our experiments, 9 SPF New Zealand White male rabbits (Charles River Deutschland, Sulzfeld, Germany) of body weight 1 500–3 000 g were used. Three animals served as untreated controls. Three animals (treated controls) were placed for 96 hours in a normobaric chamber containing normal atmosphere with 100% humidity and temperature 23°C. Three rabbits spent 96 hours under the same conditions, but the atmosphere in the experimental

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chamber was regulated at 10% O_2. CO_2 was less than 1.5%.

Material for electron microscopic examination was collected immediately after removal of the experimental animals from the chamber. Under general anaesthesia, using the i.m. administration of a mixture of ketamine and xylazine according to the certification of the Animals Protection Expert Commission of the Faculty, tiny fragments of the tracheal mucous membrane were removed and processed using standard methods for electron microscopy. The material was fixed for 90 min with 5% glutaraldehyde (Merck, Hohenbrunn bei München, Germany) in cacodylate buffer (pH 7.2) and then for 60 min with 2% OsO_4 (JMC, Hertfordshire, United Kingdom) in cacodylate buffer (pH 7.4), dehydrated in graded series of alcohol and embedded in a Durcupan-Epon mixture (Fluka, Buchs, Switzerland). Ultrathin sections were prepared on Ultrotome Nova (LKB, Broma, Sweden), contrasted with uranyl acetate and lead citrate and examined under the JEM 100 C electron microscope (Jeol, Tokyo, Japan).

The ciliary border and the functional state of the goblet cells were evaluated quantitatively. To evaluate the distribution of goblet cells in the epithelium, the isolated elements and the goblet cells arranged in groups were distinguished. The secretory elements were further classified into three categories: 1. mucus-filled, 2. mucus-discharging, 3. degenerated ones. Four categories of kinocilia were distinguished: 1. intact 9 + 2 cilia, 2. slightly damaged pathological cilia with local swellings of the ciliary membrane or with tiny vacuoles situated in their shafts, 3. degenerating cilia, represented by axonemes incorporated into cytoplasmic blebs or by isolated axonemes, 4. malformed cilia with either abnormal arrangement or number of microtubules in their axonemes.

In untreated controls, in animals exposed only to increased temperature and humidity and in those exposed to normobaric hypoxia 1 058 μm^2, 1 619.5 μm^2 and 1 144 μm^2 of ciliary border with 10 252, 8 077 and 11 028 kinocilia were evaluated, respectively. In those experimental groups also a total of 186, 485 and 528 goblet cells were studied, respectively. For statistical evaluation of the ultrastructural findings, relative values of individual categories of goblet cells and of cilia were evaluated by the chi-square test of homogeneity in frequency tables. To specify categories causing deviations from the hypothesis of homogeneity, adjusted standardised deviations were used. Means of cilia/μm^2 were compared by the one-way analysis of variance (ANOVA). The differences between groups were assessed by the Tukey's test or Bonferroni's method for multiple comparison. The Levene's test for equal variances was also performed. As a non-parametric analogy of the ANOVA, the Kruskal-Wallis test was used.

RESULTS

Tracheal epithelium of untreated control rabbits

In untreated control rabbits, the tracheae were lined with a pseudostratified columnar ciliated epithelium composed of ciliated, goblet, basal cells and a few differentiating elements. Isolated cells of the diffuse neuroendocrine system (DNES) were also encountered. Goblet cells were mostly scattered as isolated elements among the ciliated ones. Only 6 ± 3% of them formed tiny groups, mostly composed of two cells. The proportions of non-stimulated, mucus-discharging and degenerated goblet cells were given in Table 1. Above the epithelium, regular ciliary border was developed. The average number of cilia per 1 μm^2 was 9.7 ± 0.3. The percentage of intact and of individual types of altered cilia was demonstrated in Table 1.

Tracheal epithelium of rabbits exposed for 96 hours to environment with high temperature and humidity

Due to increase in temperature and humidity, the tracheal epithelium was altered. The intercellular spaces remained narrow and the apical junctional complexes were intact.

On the apical portions of the ciliated cells, isolated cytoplasmic blebs were observed. Axonemes of degenerating kinocilia were not encountered inside these cytoplasmic protrusions. In the deeper portions of ciliated cells' cytoplasm, a slight increase in number of small to medium-sized vacuoles and of secondary lysosomes, dilatation of the cisternae of granular endoplasmic reticulum, Golgi complex and of perinuclear cisternae were observed. Some slightly altered mitochondria were also encountered. Differentiating ciliated cells were not found in the epithelium.

Table 1.Quantitative evaluation of the tracheal goblet cells and ciliary border (CB) of rabbits after 4-day normobaric hypoxia (relative values)

	Untreated controls	Treated controls		Hypoxia
Non-stimulated goblet cells	97 ± 1%	*4 ± 1%	⟷	*80 ± 3%
Mucus-discharging goblet cells	3 ± 1%	4 ± 1%	⟷	*13 ± 2%
Degenerated goblet cells	0	*92 ± 1%	⟷	*7 ± 2%
Stimulated goblet cells (total)	3 ± 1%	*96 ± 1%	⟷	*20 ± 3%
Goblet cells arranged in groups	6 ± 3%	13 ± 1%	⟷	*60 ± 4%
Number of cilia per 1 μm^2 of CB	9.7 ± 0.3	*6.8 ± 0.4		# 7.1 ± 0.5
Intact cilia	98.8 ± 0.1%	*96.7 ± 1.3%		*97.2 ± 1.2%
Pathological cilia	0.5 ± 0.2%	*2.4 ± 1.0%		*2.1 ± 0.9%
Degenerating cilia	0.3 ± 0.1%	0.2 ± 0.1%		0.2 ± 0.2%
Malformed cilia	0.4 ± 0.2%	0.7 ± 0.3%		0.5 ± 0.1%
Altered cilia (total)	1.2 ± 0.1%	*3.3 ± 1.3%	—	*2.8 ± 1.2%

n = 3, values are expressed as mean ± SD, values designated # differ significantly (α = 0.05) from untreated controls, values designated * differ significantly (α = 0.01) from untreated controls, values connected by a line differ significantly (α = 0.05) from each other, values connected by a double arrow differ significantly (α = 0.01) from each other

13 ± 1% of goblet cells were responsible for the formation of small intraepithelial mucous glands.

4 ± 1% of goblet cells were filled with large mucous granules with tendency to fuse. Mucus was not evacuated from those secretory elements. 96 ± 1% of goblet cells were stimulated to discharge their secretion. The mucus discharging elements represented 4 ± 1% of goblet cells. Merocrine type of secretion was noticed only exceptionally. Signs of both apocrine type of secretion and of compound exocytosis were recorded frequently. The exhausted secretory elements prevailed forming 92 ± 1% of secretory elements (Table 1). They gradually degenerated, lost their connections with the basal lamina and appeared in the apical portions of the epithelium. Remnants of their condensed, various electron dense cytoplasm were frequently observed in the area of slightly altered ciliary border. Only isolated differentiating goblet cells containing small secretory granules of different electron density were discovered in the epithelium.

In the slightly impaired ciliary border, the mean number of cilia per 1 μm^2 decreased to 6.8 ± 0.4. Altered kinocilia represented 3.3 ± 1.3 %. The most numerous pathological kinocilia reached 2.4 ± 1.0%, degenerated and malformed cilia represented 0.2 ± 0.2% and 0.5 ± 0.1%, respectively (Table 1).

In the area of slightly impaired ciliary border, remnants of membranes, isolated mucous granules and portions of sloughed off degenerated goblet cells were observed. Inspissated secretion was not recorded.

Tracheal epithelium of rabbits exposed for 96 hours to environment with high temperature, 100% humidity and normobaric hypoxia

Tracheae of rabbits exposed to normobaric hypoxia were lined with an altered pseudostratified ciliated epithelium with narrow intercellular spaces and intact apical junctional complexes.

The ciliated cells revealed only mild signs of pathological alteration. On their apical portions, the process of apical blebbing was not recorded. In the deeper portions of ciliated cells' cytoplasm, mild signs of pathological alteration, similar to those observed in previous experimental group, were recorded (Figure 1). Voluminous intracytoplasmic ciliary vacuoles were frequently encountered in the basal portions of their cytoplasm. As well as in the

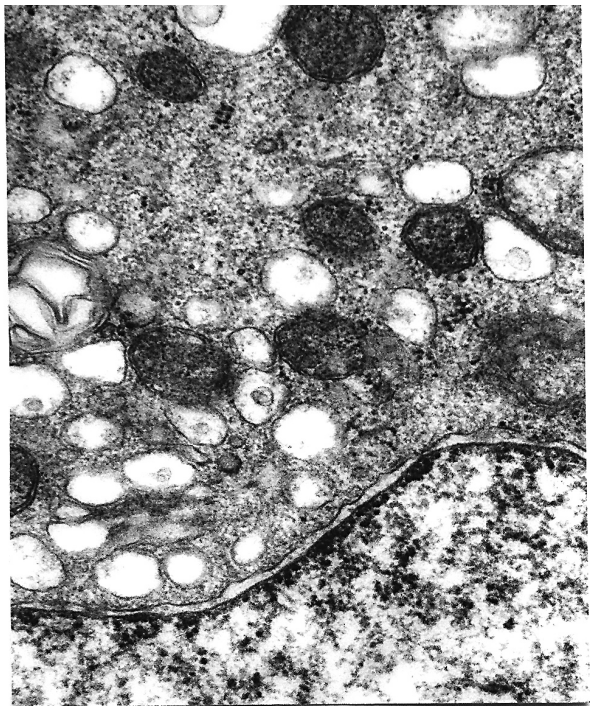

Figure 1. Tiny vacuoles, dilated cisternae of Golgi complex, small lysosome and altered mitochondria in the cytoplasm of a slightly altered ciliated cell. Rabbit – tracheal epithelium exposed for 96 hours to environment with high temperature, 100% humidity and normobaric hypoxia – 50 000×

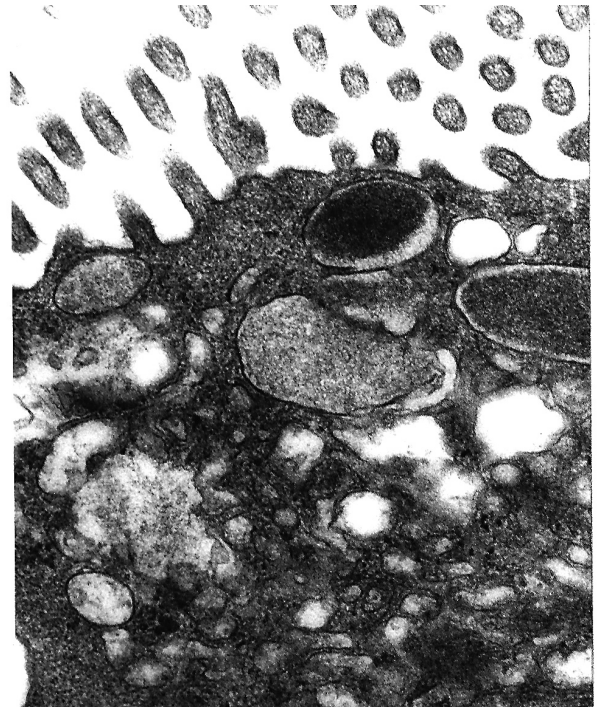

Figure 2. Apical portion of differentiating goblet cell containing isolated small secretory granules of various electron density. Rabbit – tracheal epithelium exposed for 96 hours to environment with high temperature, 100% humidity and normobaric hypoxia – 50 000×

previous experimental group, differentiating ciliated cells were not recorded.

In the epithelium, 60 ± 4% of goblet cells were arranged in voluminous groups forming thus intraepithelial mucous glands (Table 1).

79 ± 3% of goblet cells did not reveal signs of mucus evacuation. They were mostly filled with large mucous granules containing light fibrogranular matrix. The granules revealed tendency to fuse. 17% of goblet cells contained only isolated secretory granules in their cytoplasm. In some of them, the tiny granules were highly electron dense. Others contained more voluminous granules of various electron density (Figure 2). Apical portions of cells not entirely filled with mucus protruded above the level of surrounding ciliated cells. Cells filled with small mucous granules separated by voluminous cytoplasmic septa were also encountered in the epithelium.

Goblet cells discharging their secretion represented 13 ± 2% of secretory elements. Evacuation of individual apical mucous granules and detachment of whole packets of granules were often encountered. Chain exocytosis was noticed only exceptionally.

7 ± 2% of goblet cell were completely exhausted. After sloughing off, portions of their degenerated highly electron dense cytoplasm appeared above the epithelium (Figure 3).

Regular arrangement of the ciliary border was slightly impaired. The mean number of cilia per 1 μm^2 decreased to 7.1 ± 0.5. On the other hand, the number of altered elements reached 2.8 ± 1.4%. Among the altered kinocilia, the slightly altered pathological cilia were most numerous. They represented 2.1 ± 0.9% of all kinocilia. The proportions of degenerated and malformed cilia were 0.2 ± 0.2% and 0.5 ± 0.1%, respectively (Table 1).

In some places of the ciliary border, remnants of membranes, isolated mucous granules, whole apical parts of the secretory elements, portions of sloughed off degenerated goblet cells together with small clumps of inspissated mucus were noticed (Figure 4).

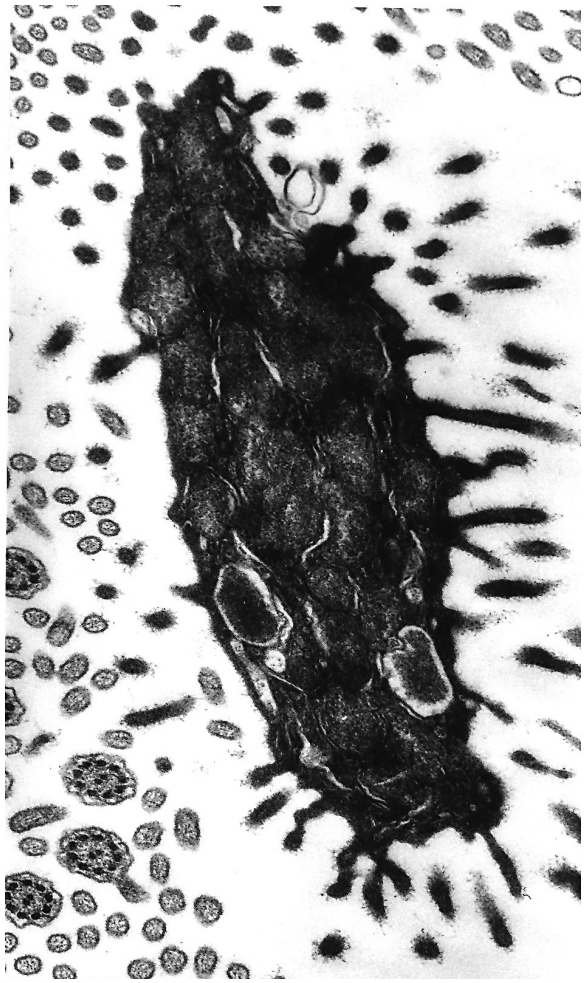

Figure 3. Portion of cytoplasm of sloughed off degenerated goblet cell in the area of ciliary border. Rabbit – tracheal epithelium exposed for 96 hours to environment with high temperature, 100% humidity and normobaric hypoxia – 37 500×

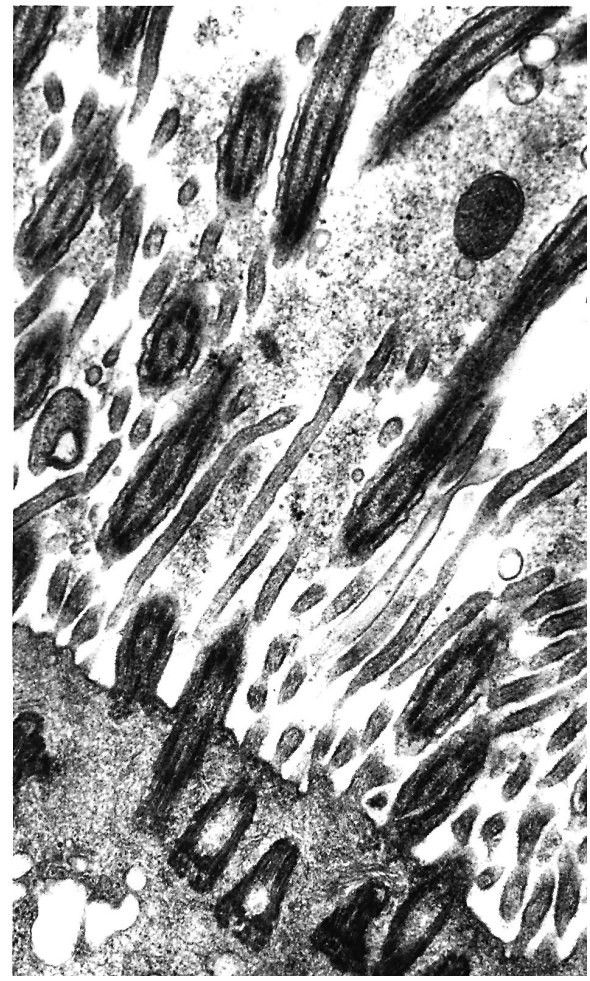

Figure 4. Layer of condensed mucus among kinocilia. Rabbit – tracheal epithelium exposed for 96 hours to environment with high temperature, 100% humidity and normobaric hypoxia – 50 000×

DISCUSSION

The target cells for the function of high temperature, 100% humidity and normobaric hypoxia were the secretory elements in the airway epithelium.

Due to the exposure to atmosphere with 100% humidity and temperature 23°C, the majority of goblet cells were overstimulated to discharge their secretory product. An increase in number of stimulated cells was highly significant ($\alpha = 0.01$) compared with untreated controls. Mechanism of secretion was accelerated. Signs of both apocrine type of secretion and of the most rapid way of mucus evacuation following compound exocytosis (Specian and Neutra, 1980; Roumagnac and Laboisse, 1987; Specian and Oliver, 1991; Konrádová *et al.*, 1996; Newman *et al.*, 1996) were frequently recorded. Merocrine type of secretion was noticed only exceptionally.

The exhausted secretory elements prevailed in the epithelium. After rapid mucus discharge, the overstimulated goblet cells mostly did not take part in further secretory cycles but they degenerated and were gradually sloughed off. Remnants of their condensed, highly electron dense cytoplasm were frequently observed among free kinocilia. Compared with controls, the proportion of the degenerated goblet cells differed significantly ($\alpha = 0.01$).

We have demonstrated that high level of stimulation of secretory cells in the airway epithelium accompanied with degeneration of about 50% of goblet cells induced a massive differentiation of

new secretory elements (Konrádová *et al.*, 1990, 1996). As the differentiating goblet cells retained the ability to divide (Becci *et al.*, 1978), the result of this process was hyperplasia of secretory elements followed by changes in their distribution in the epithelium (Konrádová *et al.*, 1990, 1996). In untreated controls, 6% of secretory elements formed small groups in the epithelium. After 96 hours of exposure to increased temperature and humidity, incipient changes in goblet cells distribution were recorded in the epithelium. The number of goblet cells arranged in groups increased. 13% of goblet cells were responsible for the formation of small intraepithelial mucous glands but those changes in the distribution of secretory elements were still not statistically significant. Only isolated differentiating goblet cells appeared in the epithelium.

The described alteration of the rabbit airway epithelium due to the high temperature and humidity had to be expected as optimum for these animals represent 15°C to 17°C and humidity 55% to 75% (Konrád, 1974).

In the tracheal epithelium of rabbits exposed to increased temperature, humidity and hypoxia, acceleration of mucus discharge was also recorded. The percentage of degenerated secretory elements significantly decreased ($\alpha = 0.01$) compared with previous experimental group, but the differentiating goblet cells in different phases of their development represented almost one fifth of secretory elements. Alteration of goblet cells' distribution in the epithelium was very significant. 60% of goblet cells participated in the formation of voluminous intraepithelial mucous glands. The percentage of goblet cells arranged in groups differed significantly ($\alpha = 0.01$) compared with findings not only in untreated controls but also in animals exposed to atmosphere with 100% humidity and temperature 23°C.

We arrived at conclusion that the decrease in O_2 concentration highly accelerated the reaction of the secretory elements in the epithelium. After exposure only to high temperature and humidity, the first phase of common response of goblet cells to injury – represented mostly by degeneration of the exhausted cells – was revealed. Due to the influence of environment with high temperature, 100% humidity and normobaric hypoxia, the second phase of the goblet cells' reaction – massive differentiation of new secretory elements accompanied with intraepithelial mucous glands' development – was recorded. We have observed similar sequence of events in our previous studies. After oral administration of salbutamol or i.v. administration of aminophylline, rapid increase in number of exhausted degenerated goblet cells was followed by an increase in number of differentiating secretory elements and changes in goblet cells' distribution in the epithelium (Konrádová *et al.*, 2000, 2001). Immediately after bronchoalveolar lavage, 99% of goblet cells degenerated, 48 hours post exposure only 6% of degenerated secretory elements were encountered, however 49% of goblet cells took part in intraepithelial mucous glands formation (Konrádová *et al.*, 1990).

In both experimental groups, the ciliated cells were less damaged than the goblet ones. The degree of alteration was similar in both experimental groups. Only mild signs of pathological alteration of deeper portions of the ciliated cells' cytoplasm were noticed.

The ciliary border was also only slightly impaired in both experimental groups. Slight, but significant decrease in the mean number of kinocilia/μm^2, was accompanied by significant ($\alpha = 0.01$) increase in percentage of altered cilia. Comparing both experimental groups, the degree of impairment of the ciliary border did not differ significantly.

Among the altered kinocilia, the slightly altered pathological cilia with local swellings of the ciliary membranes or with tiny vacuoles situated in their shafts were the most numerous. In both experimental groups, the proportions of pathological cilia differed significantly ($\alpha = 0.01$) but there were no significant differences in the numbers of degenerated and malformed cilia compared with untreated controls. Low incidence of malformed kinocilia demonstrated that neither isolated increase in temperature and humidity nor hypoxia influenced the process of ciliogenesis in the ciliated cells.

Due to the combined exposure to hypoxia and increased humidity and temperature, morphological signs of impairment of the vital self-cleaning ability of the airway epithelium were recorded (Konrádová, 1991; Stratmann *et al.*, 1991; Wanner *et al.*, 1996; Geiser *et al.*, 1997). Local disturbances in the mucus flow in airways were responsible for the appearance of small clumps of inspissated mucus in the area of the ciliary border. Isolated changes in temperature and humidity did not influence the continuous mucus flow in the airways.

Based on our study, we demonstrated that from both morphological and functional points of view, normobaric hypoxia caused more serious damage to

the airway epithelium compared with that encountered after exposure only to increased humidity and temperature.

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