Modified epithelial cell distribution in

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Modified epithelial cell distribution in chronic airways inflammation. C. Chapelin, A. Coste, L. Gilain, F. Poron, F. Verra, E. Escudier. ©ERS Journals Ltd 1996.
ABSTRACT: Although airway epithelium is known to be modified during chronic respiratory diseases, epithelial cells have rarely been precisely quantified. We therefore intended to evaluate epithelial cell distribution in inflammatory airways, using a cytological approach.

Nasal airway cells in 12 patients with nonallergic chronic rhinitis were sampled by brushing, quantified after cytocentrifugation and compared to those from eight controls. Cell populations were quantified after May-Grünwald Giemsa staining and α-tubulin immunolabelling to demonstrate ciliary differentiation.

When compared to controls, rhinitis patients exhibited lower percentages of ciliated cells (59±4 *versus* 32±2%, respectively), and higher percentages of goblet (24±3 *versus* 37±2%) and basal cells (9±1 *versus* 18±2%). After tubulin immunolabelling, positive staining was specifically detected in cells with cilia (LC+), and in the cytoplasm of some small round cells without obvious cilia (LC-). Fewer immunolabelled cells were detected in rhinitis patients than in controls (with significantly lower percentages of LC+ and higher percentages of LC-).

Nasal brushing is an effective technique for quantification of airway epithelial cells. Tubulin immunolabelling is useful to detect ciliated cells and distinguishes another cell population, possibly preciliated cells. These cytological findings suggest the presence of modifications of epithelial differentiation and proliferation, possibly related to local chronic inflammation.
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The pseudostratified epithelium lining human conducting airways is composed of three major cell types: ciliated, goblet and basal cells. Although the distribution of the various epithelial cell types varies with airway level in the normal respiratory tract, ciliated cells represent the predominant cell type [1, 2]. Similarly, there are few inflammatory cells in normal airway epithelium: in biopsies of normal nasal mucosa, mast cells are extremely rare and neutrophils scanty, whereas lymphocytes are prominent [1]. Under normal conditions, basal and goblet cells ensure permanent cell turn-over of airway epithelium [3]. Various environmental aggressions or certain airway diseases (such as asthma or chronic bronchitis) associated with local chronic inflammation can modify the distribution of the various epithelial cell types [4]. These modifications in cell proportions, possibly related to inflammatory reactions, reflect certain local pathogenic mechanisms involving epithelial cell proliferation and differentiation [5].

Although secretory hyperplasia is frequent in inflammatory respiratory diseases [4], airway epithelial cells have rarely been precisely quantified in these diseases. We therefore decided to evaluate epithelial cell distribution in the upper airways affected by mild local inflammation. We chose to study the various cell populations of nasal mucosa, using a cytological approach, in patients with nonallergic chronic rhinitis. Goblet and ciliated cells are usually identified according to their classical morphology, but it has already been suggested that tubulin immunolabelling could help to detect ciliary differentiation [6]. In the present study, cells were identified and quantified after May-Grünwald-Giemsa staining and tubulin immunolabelling.

Material and methods

Patients

Twelve patients (3 females and 9 males; mean age (±SD): 28±7 yrs) with nonallergic chronic rhinitis were included in this study. All patients complained of perennial nasal symptoms (obstruction, rhinorrhoea, sneezing) persisting for more than a year. Each patient was asked about his or her clinical history of sensitivity to common allergen and the absence of allergy was confirmed by the negativity of skin tests (Prick test) and Phadiatop (Pharmacia). An ear, nose and throat (ENT) examination, including sinus films, rhinoscopy and nasal endoscopy with cytological sampling, was performed after obtaining informed consent from the patients. Patients with clinical and radiological signs of acute or chronic infection of the paranasal sinuses or nasal polyposis were excluded from this study.

Mucosal samples from eight patients (3 females and 5 males; mean age (±SD): 39±8 yrs) undergoing aesthetic rhinoplasty were used as the control group. All eight patients were free of any nasal symptoms and mucosal abnormality on endoscopic examination.

Methods

In the absence of local infection, superficial cells of the nasal mucosa were collected by brushing the middle and posterior third of the inferior turbinate under local anaesthesia (5% lidocaine with naphazoline; Roger Bellon, France). Cells were immediately suspended in 5 mL tissue culture medium (IP 199; Eurobio, France). The total cell count was estimated using a haemocytometer (Malassez chamber). Smears were obtained from each cell suspension by cytocentrifugation (Cytospin II; Shandon, France) at 500 rpm for 10 min. One smear was stained by May-Grünwald-Giemsa (MGG) (Sigma, France), while the others were fixed in cold acetone (-20°C for 10 min) and stored at -20°C for immunocytochemistry. The cells collected by brushing included epithelial cells, but also inflammatory cells passing through the epithelium of the respiratory tract.

Three differential cell counts were performed on each cytocentrifuge preparation stained by the MGG method: a first count was performed on 200 consecutive cells in order to evaluate the proportion of epithelial and inflammatory cells; a second count was performed on 200 inflammatory cells in order to assess the predominant inflammatory cell type; a third count was performed on 200 epithelial cells in order to establish the percentages of each of the three major subtypes of epithelial cell (*i.e.* basal, goblet and ciliated cells). The subtypes of epithelial cells were determined on the basis of size and shape using classically described criteria [1]. Goblet cells (GC) were identified on the basis of their columnar appearance and the presence of secretory granules. Ciliated cells (CC) were identified on the basis of their characteristic columnar shape and the presence of cilia. Basal cells (BC) were identified on the basis of their round shape and high nucleo-to-cytoplasmic ratio [1]. Cells not corresponding to any of these three subtypes were classified as "unidentified". Results of the various cell counts were expressed as the percentage of each cell type identified.

Immunocytochemistry was performed using the modified alkaline phosphatase antialkaline phosphatase (APAAP) method [7]. All antibodies were diluted in Tris-buffered saline. Anti-human α-tubulin monoclonal mouse antibody (Amersham, France) was used, at 1:5,000 dilution, as primary antibody. Omission of primary antibody was used as negative control. Only cells with a marked red immunostaining were counted as positive. Two differential cell counts were performed on each cytocentrifuge preparation after immunostaining: a first count was performed on 200 epithelial cells in order to establish the percentage of immunolabelled cells (LC), and a second count was performed on 100 immunolabelled cells in order to determine the percentage of cells with cilia (LC+) and without cilia (LC-).

All counts were performed by two independent observers, and the mean count was calculated for each sample.

Statistical analysis

Results of the various cell counts in patients with chronic rhinitis and in controls were compared using the Mann-Whitney U-test. A p-value less than 0.05 was considered to be significant.

Results

In patients with nonallergic chronic rhinitis, nasal mucosa of the inferior turbinate was found to be red and swollen on endoscopic examination. Nasal brushings were easy to perform in every case and no bleeding occurred during cell removal, thereby allowing correct analysis of the samples. In all cases, the cellularity of samples was sufficiently rich to allow differential cell analysis. The total number of cells obtained by nasal brushings was $1.0\pm0.45 \times 10^6$ and $0.9\pm0.25 \times 10^6$ cells (mean±SEM) in the rhinitis group and in controls, respectively, without significant difference between the two groups.

After MGG staining (fig. 1), the percentages of inflammatory cells compared to epithelial cells were significantly higher in the rhinitis group than in controls
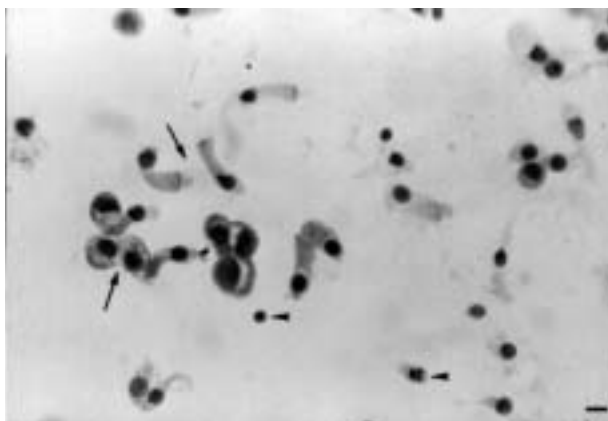

Fig. 1. – Cytocentrifuge preparation obtained by nasal brushing: epithelial (→) and inflammatory (➤) cells. (May-Grünwald-Giemsa staining; internal scale bar=10 µm).
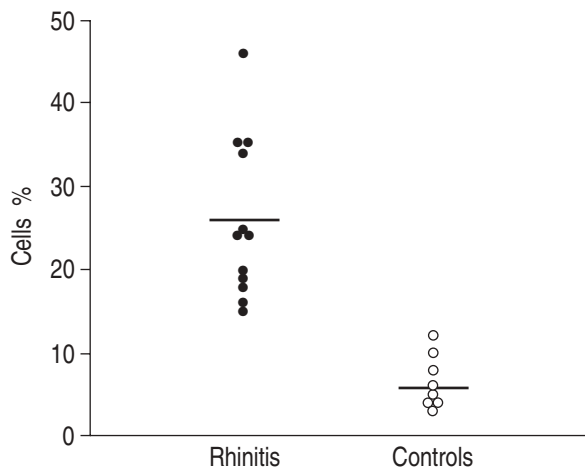

Fig. 2. – Percentages of inflammatory cells present in nasal cytological preparations from rhinitis patients (●) and controls (○). Bars indicate the means. Rhinitis patients and controls were significantly different (p<0.0001).
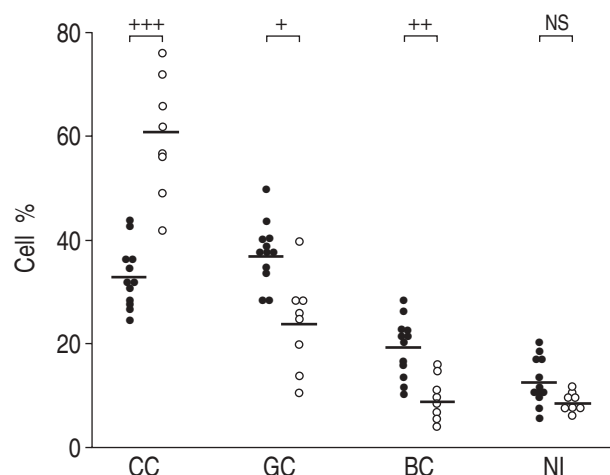
Fig. 3. – Percentages of various epithelial cell types present in nasal cytological preparations from rhinitis patients (●) and controls (○). CC: ciliated cells; GC: goblet cells; BC: basal cells; NI: cells not identified; NS: nonsignificant. Bars indicate the means. +: p<0.005, ++: p<0.001, +++: p<0.0001, significance of comparison between rhinitis patients and controls.

Fig. 5. – Percentages of α-tubulin immunolabelled cells in nasal cytological preparations from rhinitis patients (●) and controls (○). LC: labelled cells; LC+: labelled cells with cilia; LC-: labelled cells without cilia. Bars indicate the means. *: p<0.05; +++: p<0.0001, significance of comparison between rhinitis patients and controls.

(p<0.0001), as shown in figure 2. In most cases, the predominant inflammatory cells were lymphocytes (data not shown). The distributions of the various epithelial cell types in the rhinitis group and controls are shown in figure 3. Percentages of CC were significantly lower (p<0.0001), and percentages of GC and BC were significantly higher, in the rhinitis group than in controls (p<0.005 and p<0.001, respectively).
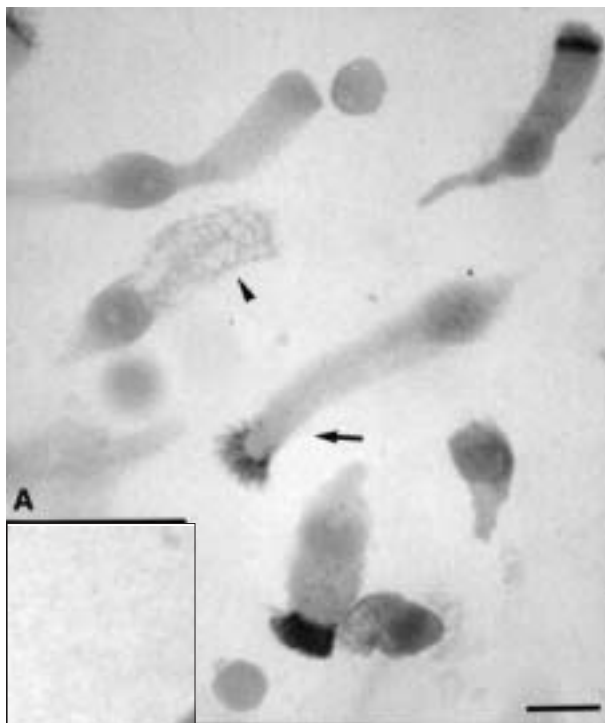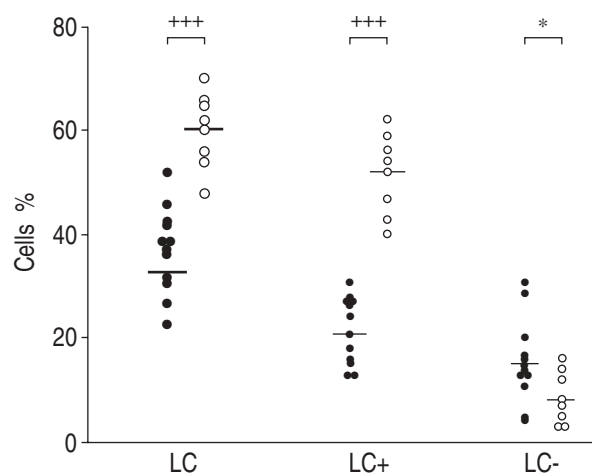
After tubulin immunolabelling, positivity was either

Fig. 4. – α-tubulin immunolabelling of airway cells in nasal cytological preparations. a) arrow (→) indicates ciliated cells with labelling localized to their cilia (LC+); note the absence of immunolabelling in goblet cells (➤). b) Small round cell with diffuse labelling of the cytoplasm (LC-). (Haematoxylin counter-stained; internal scale bar=10 µm).

localized in the cilia and basal bodies of ciliated cells (LC+) or diffuse in the cytoplasm of some small round cells (LC-) (fig. 4). The percentages of immunolabelled cells were significantly lower in the rhinitis group than in controls (p<0.0001). The percentages of LC+ were significantly lower (p<0.0001) and the percentages of LC- were significantly higher (p<0.05) in the rhinitis group than in controls (fig. 5).

Discussion

Very few studies have dealt with precise quantification of the various epithelial cell types in human airways. Most human studies are performed on biopsies, which focus on a limited area and only allow the number of each cell type to be expressed in relation to a unit area. Brushing of the respiratory tract, a noninvasive method originally proposed for ciliary studies, harvests cells in a larger area of airway mucosa than biopsy [8, 9]. Recovering superficial cells from nasal mucosa by brushing is safe and easy to perform, and allows quantification of the various cell types after cytocentrifugation [10]. In the present study, the low number of nonidentified cells in both groups confirms that nasal brushing is able to collect numerous and well-preserved dissociated cells. Epithelial and inflammatory cells were easily identified on the basis of their morphology and were, therefore, able to be precisely quantified.

In order to avoid the marked alterations of epithelial cells frequently described in severe inflammation of the respiratory tract, we chose to perform the present study in a model of mild chronic inflammation of the upper airways [11, 12]. The local appearance of nasal mucosa on endoscopy, together with the low percentages of inflammatory cells, confirm the mild degree of local inflammation in nonallergic rhinitis patients. As expected in the design of the study and in accordance with previous reports [13, 14], rhinitis patients exhibited more inflammatory cells in their nasal mucosa than controls.

In the present study, according to MGG staining analysis, the distribution of the various epithelial cell types

was modified in rhinitis compared to controls. CC, highly-differentiated cells, which are classically unable to proliferate, appeared to be decreased, while GC and BC, less-differentiated cell types, which retain the capacity to proliferate [15], appeared to be increased in chronic rhinitis. It is well-known that goblet cells increase in number in response to chronic airway insults. In different animal models, the cell primarily responsible for the proliferative response following exposure to pollutants or injury to the airways is the nonciliated columnar cell, and not the basal cell [16–18]. These cell changes suggest that, in chronic inflammation, airway epithelium may lose its highly-differentiated features, while the proliferative capacities of some epithelial cells are increased. This hypothesis is supported by the demostration, in two recent studies performed in severe inflammatory diseases of the airways [19, 20], of an increased epithelial cell proliferation. Local inflammatory reactions could be at least partly responsible for these modifications. Epithelial damage caused by inflammatory mediators [21] could require an increased turnover of epithelial cells in order to repair this damage. On the other hand, inflammatory cells infiltrating airway mucosa could modify cell differentiation and/or proliferation in the epithelium *via* local secretion of growth factors [22, 23].

As the results of standard staining suggested modifications of epithelial differentiation, we decided to define the ciliary differentiation of these cells. CC are identified according to the presence of cilia at their apical pole, but no immunohistochemical method is currently available to specifically detect human CC and their precursors. We therefore tried to identify a cytoskeletal protein, which is present in all normal mammalian cells, but which is largely expressed in CC as an important constituent of cilia [24]. It seemed useful to detect α-tubulin by immunocytochemistry, using an appropriate high dilution of a specific antibody in order to obtain distinct immunostaining of CC without staining other cells [6]. As expected, numerous labelled cells obviously presenting cilia were detected in both groups, confirming the value of the method. On the other hand, the decreased percentages of labelled cells observed in rhinitis patients are in accordance with the modifications of epithelial cell distribution found after MGG analysis.

Interestingly, although far less numerous than LC+, immunolabelled cells without identifiable cilia, *i.e.* LC, were detected in both groups. A recent study, also using α-tubulin immunodetection, reported similar positive nonciliated cells [6]. These LC- present the morphological features of epithelial cells. Their shape and size, quite similar to those of BC, do not support the hypothesis that LC- actually correspond to deciliated CC. The α-tubulin detected in LC- could correspond to a large amount of tubulin, possibly synthesized during the first steps of ciliogenesis [25, 26]. LC- could, therefore, correspond to poorly- differentiated epithelial cells at the early stages of ciliary differentiation, and could possibly overlap with preciliated cells described by some authors [1, 27]. The increased percentage of LC- in rhinitis also suggests modifications of epithelial differentiation and proliferation in human inflammatory airways.

In the present study, using cytological tools, we demonstrated that airway epithelial cell distribution is modified in nasal mucosa in the presence of chronic inflammation. Moreover, tubulin expression in epithelial cells identified an epithelial cell type, which could correspond to preciliated cells and which is increased in inflammatory mucosa. These modifications of epithelial cell distribution suggest a downward trend of epithelial differentiation, possibly associated with an upward trend of proliferation. Inflammation could be involved in these epithelial modifications according to various mechanisms. The same kind of studies could be performed in the lower airways, and should be useful in clarifying our understanding of the epithelial modifications described in asthma or chronic bronchitis.

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