

Immunocytologic analysis of nasal cells obtained by nasal lavage: a comparative study with a standard method of cell identification

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For evaluation of two methods of nasal cell identification, cell morphology and immunocytologic analysis, nasal lavage was performed in 16 healthy subjects and 29 patients suffering from rhinitis. Nasal lavage smears were stained with May-Grünwald-Giemsa (MGG), and cells were identified according to their structure as epithelial cells, neutrophils, lymphocytes, eosinophils, and metachromatic cells (basophils and mast cells). Immunocytologic analysis was performed with monoclonal antibodies by the immunoalkaline phosphatase method. The following monoclonal antibodies were used: CK1, EG2, and CD3, which identify epithelial cells, activated eosinophils, and T lymphocytes, respectively; CD15, which recognizes mature granulocytic cells; and CD14, which reacts with monocytes and macrophages. A significant difference was observed between the two methods in the number of identified epithelial cells, in both controls ($64.6 \pm 7.8\%$ with MGG, $14.2 \pm 3.5\%$ with CK1 analysis) and patients with rhinitis ($56.9 \pm 7.6\%$ with MGG, $18.2 \pm 3.7\%$ with CK1 analysis). In contrast, no significant differences were found in eosinophil and neutrophil counts when the two methods were compared. After nasal allergic provocation, a significant increase in the number of eosinophils was observed with both methods in seven patients with rhinitis. The results of this study indicate that: 1) MGG staining is a useful method to identify the cells obtained by nasal lavage, and 2) immunocytologic analysis with monoclonal antibodies accurately identifies granulocytic cells, while only a low proportion of epithelial cells are detected, probably because anticytokeratin monoclonal antibody reacts only with viable cells.

Clinical aspects

Proinflammatory cells such as eosinophils, lymphocytes, and mast cells release mediators which cause clinical symptoms in both rhinitis and bronchial asthma. Since the nose, in contrast to the bronchi, is easily accessible to repetitive studies, nasal lavage is used to investigate the role of cells and their mediators in these respiratory diseases. In the present study, we performed nasal lavages in both patients with rhinitis and healthy subjects to compare the presence of proinflammatory cells by an immunocytologic analysis and a standard method of cell identification (May-Grünwald-Giemsa stain).

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The introduction of new methods to investigate cell activity and function has increased interest in the study of cells involved in airway inflammatory diseases (1, 3, 4, 16, 18). Among these methods, nasal lavage has been shown to be a useful tool in the

study of inflammatory cells and their mediators in diseases affecting the nose (2, 13, 14, 19, 20, 23).

Some investigators have suggested that the pathophysiologic mechanisms involved in rhinitis closely resemble those implicated in asthma, whether aller-

gic or not (8). Since the nose, in contrast with the bronchi, is easily accessible for repetitive studies, several research groups are now using nasal lavage to investigate the role of cells and mediators on allergic and nonallergic airway diseases. In addition, nasal lavage has the advantages of tolerance and a lack of harmful side-effects. Different methods have been proposed to perform nasal lavage (2, 5, 6, 10, 12), and a detailed morphologic identification of obtained cells has been described (19). The analysis of cells obtained by nasal lavage presents some problems such as mucus plugs interfering in the assessment of total and differential cell counts, or broken cells which may cause misinterpretation of the results. In addition, the number of cells obtained may be low, depending on the method used (2, 3).

Differential cell count is usually performed on the basis of the structure of cells (size, number of nuclei, and presence and type of cytoplasmic granules) with various staining methods (2, 19, 20). However, the accuracy of this method of cell identification has not been compared with immunocytologic analysis by monoclonal antibodies. The objective of this study is to compare two methods of cell identification in nasal lavage cell analysis: the differential cell count in smears stained with May-Grünwald-Giemsa (MGG) and the immunocytologic analysis by monoclonal antibodies.

Material and methods

Study population

Patients. Twenty-nine patients with rhinitis (11 women and 18 men, mean age 33.2 ± 2.7 years, mean $\pm$ SD) referred to our outpatient chest clinic were studied. In all cases, diagnosis was established by clinical history. Fourteen patients were reactive to the dust-mite allergen of *Dermatophagoides pteronyssinus* (DP), as demonstrated by the skin prick test. None of the patients took medication (inhaled glucocorticoids, antihistaminics, nasal decongestant agents) in the 2 weeks before the nasal lavage. Seven patients with rhinitis underwent nasal provocation with the same batch of lyophilized DP extract (Pharmacia, Uppsala, Sweden). The allergen extract was reconstituted and diluted to 10 000 BU/ml with phosphate-buffered saline (PBS) containing 0.3 mg albumin/ml. Mite extracts were instilled in the nose with a nebulizer valve (two administrations in each nostril). Cellular analysis was performed before and after allergic provocation.

Control group. The control group was composed of 16 healthy subjects (6 women and 10 men, mean age 30.9 ± 1.4 years) recruited from the staff of our department with no clinical evidence of rhinitis or

asthma. In all of them, skin prick test was negative to common aeroallergens.

The study was performed as part of the outpatient diagnostic protocol for allergic nasal diseases. Accordingly, all patients were informed of the characteristics and nature of the procedures performed, and all of them gave full informed consent.

Nasal lavage

Nasal lavage was performed by instilling 8 ml of 0.9% sterile saline solution, 4 ml in each nostril, with the subject's head tilted 30° backwards. As reported previously (14, 15), subjects were instructed neither to breathe nor swallow for approximately 10 s during a Valsalva maneuver, and then to bend the head forward in order to collect the mixture of saline and nasal secretions.

The liquid obtained was centrifuged (500 g at 4°C for 10 min), and the cell pellet was resuspended in 0.5 ml of PBS and vigorously shaken with a pipette to dissolve mucus plugs. Total cell counts were obtained in a Neubauer's hemocytometer. From three to six cell smears were obtained by low-speed cyto-centrifugation (400 rpm, 10 min) with a Cytospin 2 (Shandon, Southern Products, Cheshire, UK), with 25×10^3 cells in 75–150 μ l. One smear was stained with MGG, and the differential cell count of 200 cells was then obtained. Cells were identified according to their structure as epithelial cells, neutrophils, eosinophils, lymphocytes, and metachromatic cells (basophils and mast cells). The remaining smears were kept at room temperature for 6–24 h and then wrapped in aluminum foil and stored at -70°C until staining with monoclonal antibodies was performed.

Immunocytologic staining

Staining by monoclonal antibodies was performed by the immunoalkaline phosphatase method previously described (24). Smears were fixed in a formaldehyde-acetone solution and incubated with the monoclonal antibody. Cell preparations were then incubated with rabbit antimouse immunoglobulins (Dakopatts, Denmark), normal human serum, with alkaline phosphatase antialkaline phosphatase immune complexes (APAAP) (Dakopatts, Denmark), and, finally, with an alkaline phosphatase substrate. For evaluation of the immunocytochemical reaction, slides were viewed under a light microscope with magnification $\times 400$ to $\times 1000$. Positive reacting cells were recognized by a continuous, bright-red staining of the membrane. In addition, eosinophils stained with EG2 monoclonal antibody showed a granular intracellular positivity. Two hundred cells of each preparation were counted, and the

percentage of positive cells was obtained. The monoclonal antibodies used in this study are detailed in Table 1. Microscopic evaluation was performed on coded slides by two different investigators.

Statistical analysis

All data are expressed as mean value $\pm$ standard deviation. For determination of the differences between both methods of cell identification, the Kruskal-Wallis and Mann-Whitney tests were used. A P value ≤ 0.05 was considered to be statistically significant.

Results

The total cell count was higher in patients with rhinitis, but without reaching statistical significance. Differential cell counts showed that epithelial cells were the main cell type identified in both patients with rhinitis ($46.9 \pm 5.5\%$) and control subjects ($70.2 \pm 7.4\%$). The percentage of eosinophils was significantly higher in patients with rhinitis ($9.4 \pm 3\%$; $P < 0.01$) than in the control group ($0.56 \pm 0.3\%$) (Table 2). The increase in the numbers of eosinophils accounted for the decreased percentage of epithelial cells observed in patients with rhinitis, as compared with controls.

Because of the low number of cells obtained, there were not enough cytocentrifuge preparations to test each monoclonal antibody in all subjects. When immunocytologic analysis was compared with differential cell counts, we observed that the number of epithelial cells identified was significantly higher in preparations stained with MGG, both in the control group and in patients with rhinitis. In the control group, the percentage of epithelial cells identified was $64.6 \pm 7.8\%$ in preparations stained with MGG and $14.2 \pm 3.5\%$ in smears stained with CK1 monoclonal antibody ($n = 10$; $P < 0.001$). In patients with rhinitis, the number of epithelial cells identified was $56.9 \pm 7.6\%$ and $18.2 \pm 3.7\%$, respectively ($n = 21$; $P < 0.001$) (Fig. 1). In contrast, no differences were found between both methods of cell identification in percentage of eosinophils when EG2 monoclonal antibody was tested, either in controls (MGG, $0.6 \pm 0.2\%$; EG2, $0.8 \pm 0.3\%$; $n = 7$) or patients with rhinitis (MGG, $11.5 \pm 4.6\%$; EG2, $10.1 \pm 3.4\%$;

Table 2. Total and differential cell counts in smears stained with May-Grünwald-Giemsa (MGG)

	Control group	Rhinitis
Subjects (n)	16	29
Total cell count ($\times 10^4$)	12.7 ± 4.1	49.8 ± 30.8
Epithelial cells (%)	70.2 ± 7.4	$46.9 \pm 5.5^*$
Neutrophils (%)	25.9 ± 7.5	37.9 ± 5
Eosinophils (%)	0.56 ± 0.3	$9.4 \pm 3^{**}$

* $P < 0.05$; ** $P < 0.01$.

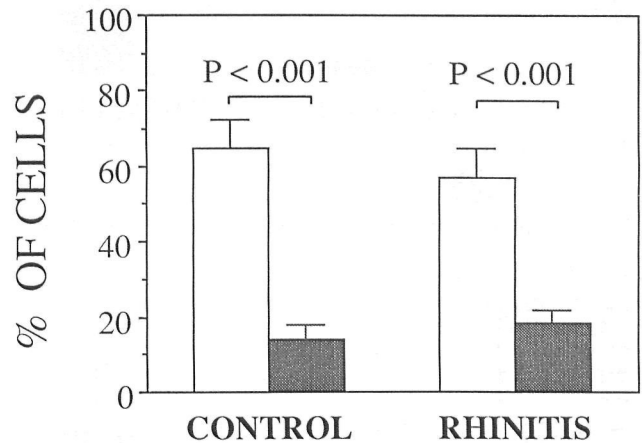


Fig. 1. Percentage of epithelial cells stained with May-Grünwald-Giemsa (open bars) and with CK1 monoclonal antibody (closed bars) for both controls ($n = 10$) and patients with rhinitis ($n = 21$).

$n = 19$) (Fig. 2A). After nasal allergic provocation, an increase in the percentage of eosinophils was observed with both methods of cell identification: MGG, $27.1 \pm 12\%$; EG2, $22.2 \pm 9.2\%$ ($n = 7$) (Fig. 2B). Similarly, we did not find differences in the number of granulocytes between differential cell counts and smears stained with CD15 monoclonal antibody in either controls (MGG, $45 \pm 10.6\%$; CD15, $44.6 \pm 10.5\%$; $n = 8$) or patients with rhinitis (MGG, $51.2 \pm 5.6\%$; CD15, $49.2 \pm 6.4\%$; $n = 24$) (Fig. 3). Only isolated cells with morphologic characteristics of lymphocytes were detected in two patients. Staining with CD3 monoclonal antibody showed only two positive cells in two patients. No monocytes or macrophages were identified with MGG stain or immunocytologic analysis by CD14 monoclonal antibody. Furthermore, we did not detect metachromatic cells by either method of cell identification.

Discussion

In the present study, we have compared two methods of cell analysis in nasal lavage fluid: differential cell counts in preparations stained with MGG, and immunocytologic analysis by a panel of monoclonal

Table 1. Panel of monoclonal antibodies used in the immunocytologic analysis

Monoclonal antibody	CD	Reactivity	Source
CK1	—	Epithelial cells	Dako
EG2	—	Eosinophil cationic protein	Sanbyo
Leu 4	CD3	T lymphocytes	Becton Dickinson
Leu M1	CD15	Mature granulocytic cells	Becton Dickinson
Leu M3	CD14	Monocytes, macrophages	Becton Dickinson

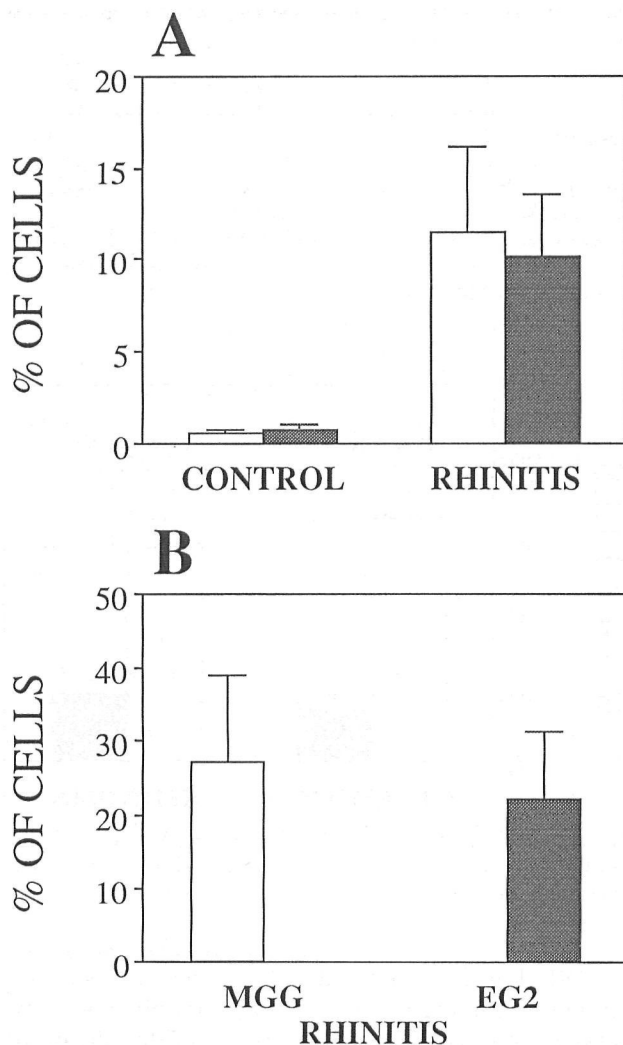


Fig. 2. A) Percentage of eosinophils stained with May-Grünwald-Giemsa (open bars) and with EG2 monoclonal antibody (closed bars) for both controls ($n = 7$) and patients with rhinitis ($n = 19$). B) Eosinophils stained with both methods after nasal allergic provocation in patients with rhinitis ($n = 7$).

antibodies. In differential cell counts using the MGG staining method, epithelial cells were the main cell type detected. A decreased percentage of these cells in patients with rhinitis with respect to controls may be explained by the increase in neutrophils and eosinophils observed in these patients. However, we found that epithelial cell counts were significantly decreased in the immunocytologic analysis. We used the anticytokeratin monoclonal antibody CK1, which reacts specifically with the human keratin intermediate filaments of epithelial cells (11). The lack of immunostaining was not related to the use of a weak antibody concentration since concentrations as high as 1/10 failed to stain cell smears. Other studies performed in our laboratory with the same antibody in epithelial cells dissociated from nasal mucosa specimens removed at surgery showed positive stain-

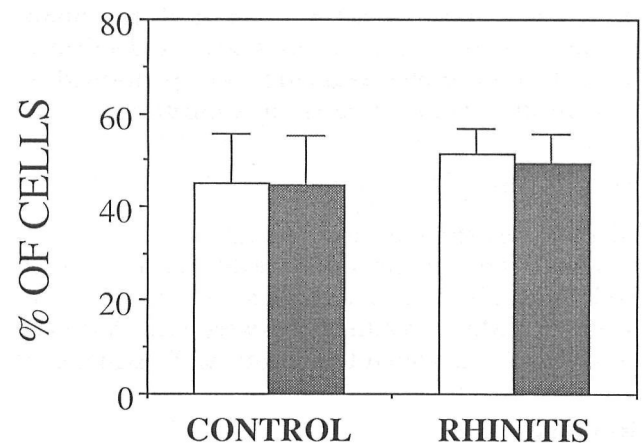


Fig. 3. Percentage of granulocytes stained with May-Grünwald-Giemsa (open bars) and with CD15 monoclonal antibody (closed bars) for both controls ($n = 8$) and patients with rhinitis ($n = 24$).

ing values close to 100% (unpublished data). The reason that epithelial cells did not react with CK1 antibody may be related to cell desquamation. Epithelial cells line the nasal mucosal surface, and many of them are desquamated and show a low viability, mainly in patients with rhinitis. Although cell viability was not determined in this study, we have observed in nasal lavage performed on both healthy subjects and patients with rhinitis that cell viability is usually low, ranging from 60% to 70%.

In contrast, we found that eosinophil counts were similar by both methods of cell identification in controls and patients with rhinitis. As expected, the percentage of eosinophils was significantly higher in patients with rhinitis than in basal values after nasal allergic provocation (3). EG2 monoclonal antibody, which binds to human eosinophil cationic protein (22), was used in our study to stain the eosinophils by immunocytologic analysis. This antibody has been widely used as a successful marker in the identification of eosinophils on bronchial mucosa in patients with asthma (1). Thus, our results indicate that staining with EG2 antibody is a useful method to identify eosinophils in nasal lavage. Similar results were obtained with CD15 monoclonal antibody, since it reacted positively with neutrophils and eosinophils observed in MGG-stained cell smears.

Although mucosal infiltration by T-cells has been demonstrated in upper airways of patients with rhinitis (9, 17), these cells were not detected in nasal lavage in the present study. These results suggest that T-cell infiltration of nasal mucosa is not reflected in nasal lavage. Recently, Poulter et al. (21) have demonstrated that bronchoalveolar lavage may be an inappropriate sampling procedure for the investigation of T-cell infiltration in the bronchial mucosa of asthmatic patients.

The present study also failed to identify mast cells

and basophils in nasal lavage. Although specific monoclonal antibodies against these cells were not used in our study, metachromatic cells are easily recognizable in smears stained with MGG (25). In addition, immunocytochemical analysis by CD15 monoclonal antibody did not stain any cell with morphologic characteristics of basophils. Thus, our results agree with previous studies dealing with nasal lavage cellular analysis in which metachromatic cells were absent or their counts were very low (3). The number of mast cells is low in histologic specimens from normal nasal mucosa, but their count increases in the nasal turbinate mucosal epithelium of patients with rhinitis (18). However, mast cells are mainly residents of the submucosal layer and stroma (7). This fact may explain the low counts or absence of mast cells in nasal lavage. Likewise, we did not detect macrophages or monocytes by either method of cell identification. As previously reported (3), these cells are not usually observed in nasal lavage.

In conclusion, our results indicate that: 1) MGG stain is a useful method to identify cells obtained by nasallavage; 2) immunocytologic analysis with monoclonal antibodies accurately identifies granulocytic cells, while only a low proportion of epithelial cells are detected, probably because anticytokeratin monoclonal antibody reacts only with viable cells.

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