

Comparison of cellular composition of induced sputum analyzed with Wright staining and immunocytochemistry

Catherine Lemière, MD,^a Rame Taha, MD,^b Ron Olivenstein, MD,^b and Outayba Hamid, MD, PhD^b Montreal, Quebec, Canada

We sought to compare cell counts based on cellular morphology and obtained after Wright staining with cell counts obtained after immunocytochemistry in induced sputum. Counts of eosinophils, neutrophils, macrophages, lymphocytes, and epithelial cells identified after Wright staining were compared with the counts obtained after immunocytochemistry through use of mAbs in 25 samples. Agreement between Wright staining and immunocytochemistry was poor for lymphocytes and epithelial cells. Rather than Wright staining, immunocytochemistry should be used to accurately identify lymphocytes and epithelial cells. (*J Allergy Clin Immunol* 2001;108:521-3.)

Key words: Asthma, induced sputum, immunocytochemistry, cell count

Induced sputum is increasingly used in the investigation of airway inflammation encountered in asthma. The current gold standard for reporting a differential cell count is manual counting based on cell morphology after Wright staining. Cell counts after Wright staining are reproducible for eosinophils, neutrophils, and macrophages, but the reproducibility is low for lymphocytes and epithelial cells.^{1,2} Moreover, a discrepancy between eosinophil counts performed after 2 different staining procedures (May-Grunwald-Giemsa staining and immunocytochemistry with human major basic protein [MBP] to identify eosinophils) has been reported in bronchoalveolar lavage of rats. The number of eosinophils was underestimated by May-Grunwald-Giemsa staining in comparison with immunocytochemistry.³

In this study, we aimed to compare cell counts based on cellular morphology and obtained after Wright stain-

Abbreviation used

MBP: Human major basic protein

ing with cell counts obtained after immunocytochemistry in induced sputum of patients with asthma.

METHODS

Twenty-five samples from 9 subjects with occupational asthma were obtained; 3 samples per subject were considered. Samples were obtained before and after exposure to occupational agents. One sample was obtained at baseline, on a control day, and the other 2 samples were obtained after exposure to the occupational agents, to which the subjects were sensitized. Using this procedure, we wanted to obtain samples with a wide range of eosinophil percentages; this would reproduce the conditions that we encounter in daily life with individuals with controlled and uncontrolled asthma.

The slides were read by 2 independent observers, after Wright staining and after immunocytochemistry, to calculate the interobserver agreement for both methods. To avoid observer bias, slides were coded before analysis and read in a blinded fashion. The counts of eosinophils, neutrophils, macrophages, lymphocytes, and epithelial cells identified after Wright staining were compared with the counts obtained after immunocytochemistry.

Sputum induction and processing

Sputum was induced through use of inhalations of increasing concentrations (3%, 4%, and 5%) of hypertonic saline solution⁴ and processed as previously described.¹ Briefly, sputum was selected from the expectorate and a weighed aliquot was dispersed with 4 volumes of freshly prepared 0.1% dithiothreitol followed by 4 volumes of Dulbecco's PBS solution, after which the dispersed sample was filtered through a 48- μ m nylon mesh. The total cell count and the cell viability of the filtrate were measured through use of trypan blue exclusion, and cytopsin preparations were made (Cytopsin 3 cytocentrifuge; Shandon Southern Instruments, Sewickley, Pa).

One cytopsin was dried and then Wright-stained for a differential cell count of 400 cells, squamous cells being excluded. Cytopsin were fixed with acetone-methanol (60-40), air-dried, and frozen at -20°C for further analysis by immunocytochemistry.

Immunocytochemistry

Specific immunohistochemical markers for eosinophils (MBP), T lymphocytes (CD3), neutrophils (elastase), macrophages (CD68), and epithelial cells (cytokeratin) were used to detect the phenotype of inflammatory cells recovered by sputum induction. Immunocytochemistry was performed through use of a modified alkaline phosphatase-antialkaline phosphatase (APAAP) method, as previ-

From ^athe Department of Chest Medicine, Hôpital du Sacré-Coeur, and ^bMeakins-Christie Laboratories.

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Reprint requests: Catherine Lemière, MD, Department of Chest Medicine, Sacré-Coeur Hospital, 5400 West Gouin, Montreal, Quebec, Canada, H4J 1C5.

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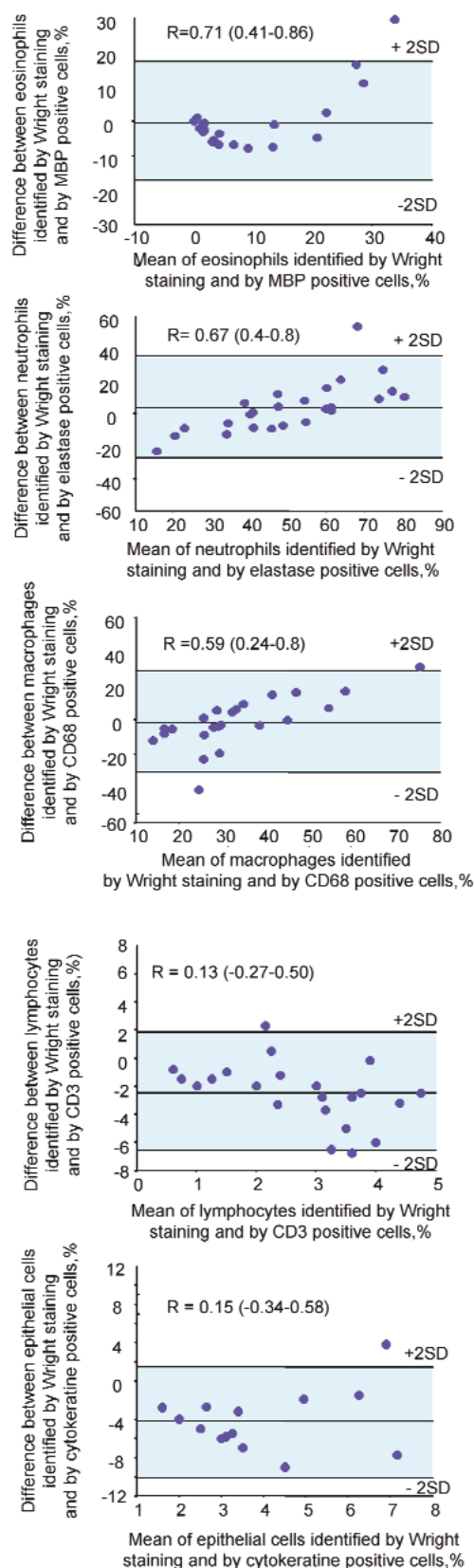


FIG 1. Agreement (%) between sputum neutrophil, macrophage, eosinophil, lymphocyte, and epithelial cell counts after Wright staining and cell counts after immunocytochemistry. *R* is the intraclass correlation coefficient, and the shaded area represents ± 2 SDs of the mean of the differences.

ously reported.⁵ Briefly, slides were incubated overnight at 4°C with the following specific antibodies: mouse anti-MBP mAb (kindly provided by R. Moqbel), mouse anti-CD3 mAb (Becton, Dickinson, Franklin Lakes, NJ), mouse anti-elastase mAb (Becton, Dickinson), mouse anti-CD-68 mAb (Dako Diagnostics, Canada, Inc, Mississauga, Ontario, Canada), and mouse anti-cytokeratin mAb (CAM-5.2) (Becton, Dickinson). After washing in TRIS-buffered saline solution, the secondary rabbit antimouse antibodies (Dako Diagnostics) were applied; the slides were then washed in TRIS-buffered saline solution and incubated with the APAAP complex. The reaction was visualized by alkaline-phosphatase substrate added to Fast Red TR (Sigma Chemical, St Louis, Mo). All of the slides were counterstained with hematoxylin and mounted before examination.

Negative control experiments were performed in a similar manner, but in the absence of the primary antibody, which was replaced by nonspecific mouse immunoglobulin or TRIS-buffered saline solution. Five hundred cells were counted per cytospin.

Statistical analysis

Sputum samples with a viability greater than 50% and containing less than 20% of contaminating squamous cells were considered suitable for analysis. Data were reported as medians and interquartile ranges, because they were non-normally distributed. Sputum slides with Wright staining and immunocytochemistry were read by 2 different observers, and an intraclass coefficient between observers was calculated. The Mann-Whitney test was used to compare cell counts obtained with Wright staining and immunocytochemistry. The intraclass correlation coefficient between Wright staining and immunocytochemistry was also calculated for each type of cells.

Agreement between the 2 techniques is expressed according to the approach proposed by Bland and Altman,⁶ and it is expected that 95% of the differences between measures are lower than 2 SDs (Fig 1). In each panel, the differences between the readings obtained through use of the 2 techniques are plotted on the vertical axis against the mean values of the 2 measurements. Significance was accepted at the level of 95%.

RESULTS

Twenty-five samples obtained from 9 subjects were suitable for analysis with Wright staining. Of these, 1 sample was not suitable for analysis with immunocytochemistry with CD68, 2 samples were not suitable for analysis with MBP, and 7 samples were not suitable for analysis with cytokeratin because the volume of sputum samples was insufficient to make enough slides (Table I).

There was a very high interobserver agreement for eosinophils, neutrophils, and macrophages for Wright staining. The agreement was lower for lymphocytes and epithelial cells (Table I).

With immunocytochemistry, there was a high interobserver agreement for each of the cell types—MBP, elastase, CD68, CD3, and cytokeratin (Table I). The agreement between the 2 methods (Wright staining and immunocytochemistry) was good for eosinophils, lower for neutrophils and macrophages, and poor for lymphocytes and epithelial cells (Table I and Fig 1).

DISCUSSION

This study shows that the agreement between Wright staining and immunocytochemistry is good for

TABLE I. Cell counts obtained after Wright staining and immunocytochemistry and intraclass correlation coefficients between the 2 methods

Cell type/staining	Cell count (%)	Intraclass correlation coefficient (95% CI)	
		Between observers	Between methods
Eosinophils			
Wright (n = 25)	1.5 (20.55)	0.98 (0.97-0.99)	0.71 (0.41-0.86)
MBP (n = 23)	6.0 (14.0)	0.95 (0.89-0.97)	
Neutrophils			
Wright (n = 25)	51.8 (32.9)	0.95 (0.90-0.98)	0.67 (0.4-0.8)
Elastase (n = 25)	47.6 (47.5)	0.80 (0.61-0.90)	
Macrophages			
Wright (n = 25)	26.8 (26.15)	0.93 (0.87-0.97)	0.59 (0.24-0.8)
CD68 (n = 24)	35.2 (16.8)	0.87 (0.74-0.94)	
Lymphocytes			
Wright (n = 25)	1.0 (2.0)	0.52 (0.16-0.75)	0.13 (-0.27-0.50)
CD3 (n = 25)	4.0 (4.0)*	0.88 (0.75-0.94)	
Epithelial cells			
Wright (n = 25)	0.5 (1.65)	0.72 (0.47-0.87)	0.15 (-0.34-0.58)
Cytokeratine (n = 18)	6.0 (2.3)*	0.85 (0.65-0.93)	

Data are given as medians (interquartile ranges).

* $P < .05$ for comparison of the cells counts between the 2 methods (Wright and immunocytochemistry).

eosinophils, lower for neutrophils and macrophages, and poor for lymphocytes and epithelial cells. The interobserver agreement with Wright staining is also lower with lymphocytes and epithelial cells than with the other types of cells, whereas the interobserver agreement with immunocytochemistry is good for all types of cells.

The discrepancy between cell counts obtained with Wright staining and immunocytochemistry might be explained in part by the fact that the recognition of cell morphology is more subjective with Wright staining than with immunocytochemistry, inasmuch as the latter method allows the staining of the relevant cells only. Repeatability of sputum cell counts obtained on 2 occasions within 6 days in patients with asthma and healthy subjects has been previously assessed¹; the authors found a good repeatability for eosinophils, neutrophils, and macrophages but a low repeatability for lymphocytes. Our study indicates that lymphocytes and epithelial cells are difficult to identify accurately with Wright staining. Indeed, lymphocytes and epithelial cell counts are almost always systematically underestimated with Wright staining in comparison with immunocytochemistry. Although the variability of these cells between slides might partially account for the discrepancy between the 2 methods, the mediocre interobserver repeatability with epithelial cells and lymphocytes compared with the other types of cells is probably related to the misidentification of these cells.

Woltman et al⁷ compared sputum epithelial cell and eosinophil counts obtained after Wright staining and after laser scanner cytometry using anticytokeratin and MBP antibodies, respectively. They found an intraclass correlation coefficient between the 2 methods of 0.7 (SD, 2.0) for epithelial cells and 0.9 (SD, 2.1) for eosinophils—higher than what we found in the present investigation. The laser scanner cytometry used by Woltman et al⁷ for counting the

slides is too different from the method that was used in our study to permit an accurate comparison of the 2 studies; however, it is of interest to note that the intraclass coefficient correlation between Wright staining and laser scanner cytometry was also lower for epithelial cells than for eosinophils, which is consistent with our findings.

We conclude that Wright staining allows an accurate recognition of eosinophils, macrophages, and neutrophils but does not allow an accurate recognition and counting of lymphocytes and epithelial cells. Immunocytochemistry should be preferred when an accurate count of these cells is needed.

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REFERENCES

- Pizzichini E, Pizzichini M, Efthimiadis A, Evans S, Morris M, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308-17.
- Spanevello A, Migliori GB, Sharara A, Ballardini L, Bridge P, Pisati P, et al. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy* 1997;27:1138-44.
- Watanabe A, Mishima H, Renzi PM, Xu LJ, Hamid Q, Martin JG. Transfer of allergic airway responses with antigen-primed CD4+ but not CD8+ T cells in brown Norway rats. *J Clin Invest* 1995;96:1303-10.
- Pin I, Gibson P, Kolendowicz F, Girgis-Gabardo A, Denburg J, Hargreave F, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992;47:25-9.
- Olivenstein R, Taha R, Minshall EM, Hamid QA. IL-4 and IL-5 mRNA expression in induced sputum of asthmatic subjects: comparison with bronchial wash. *J Allergy Clin Immunol* 1999;103:238-45.
- Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307-10.
- Woltmann G, Ward RJ, Symon FA, Rew DA, Pavord ID, Wardlaw AJ. Objective quantitative analysis of eosinophils and bronchial epithelial cells in induced sputum by laser scanning cytometry. *Thorax* 1999;54:124-30.