Validity and reproducibility of morphologic analysis of nasal secretions obtained using ultrasonic nebulization of hypertonic solution

Maria T. Ventura, MD*; Olga Toungoussova, PhD†; Maria P. Foschino Barbaro, MD‡; Onofrio Resta, MD§; Giovanna E. Carpagnano, MD‡; Silvano Dragonieri, MD†; Giovanni B. Migliori, MD‖; Margherita Neri, MD¶; and Antonio Spanevello, MD†‡

Background: Collection of nasal secretions is important for the evaluation of upper airways inflammation in many nasal disorders.

Objective: To study the validity and reproducibility of nasal secretion cellularity induced by nebulization of hypertonic solution in patients with allergic rhinitis (AR), patients with nonallergic rhinitis with eosinophilia syndrome (NARES), and control subjects.

Methods: Sixty-eight individuals (29 with AR [mean ± SD age, 33.3 ± 16.9 years], 23 with NARES [mean ± SD age, 46.4 ± 16.6 years], and 16 controls [mean ± SD age, 42.1 ± 15.1 years]) underwent ultrasonic nebulization of hypertonic (4.5%) saline solution on 2 different occasions to study the validity and reproducibility of total and differential cell counts of nasal secretions.

Results: The mean ± SD percentage of eosinophils was significantly higher in samples from patients with AR (20.8% ± 23.1%) and NARES (18.7% ± 22.8%) than in samples from controls (0.6% ± 0.6%; $P < .001$ for both). There was a significant correlation between 2 samples of nasal secretions obtained on 2 different occasions for percentages of macrophages, neutrophils, eosinophils, and epithelial cells.

Conclusions: The analysis of nasal secretions obtained using ultrasonic nebulization of hypertonic solution can distinguish patients with AR and NARES from controls. The reproducibility of this technique is good for macrophages, neutrophils, eosinophils, and epithelial cells. This method could be used to detect nasal airway inflammation in clinical settings.

Ann Allergy Asthma Immunol. 2007;99:232–235.

INTRODUCTION

Collection of nasal secretions is important for the evaluation of upper airways inflammation in many nasal disorders. Evidence has been provided that inflammatory cells are present not only in the airways of patients with asthma but also in the airways of patients with seasonal allergic rhinitis (AR).[1]

Physicians and researchers are often faced with the problem of collecting adequate samples of nasal secretions. Various techniques have been used to obtain specimens from nasal mucosa and to study inflammation in the nasal cavity.[2] The most commonly used techniques include nasal lavage, nasal biopsy, nasal brush, nasal scraping, nasal mucosa collection, application of a microsuction, and direct aspiration of secretion.[3–6] Each method has several disadvantages, including being invasive procedures and the unsuitability of some samples for analysis. Furthermore, direct comparison of the validity and reproducibility of these methods in the same group of patients has never been performed.

Recently, a new noninvasive method for obtaining nasal secretion samples based on nebulization of hypertonic solution was described.[7] Samples obtained using this method are suitable for morphologic analysis. To our knowledge, no studies have analyzed and validated the results of examination of nasal secretions obtained by means of nebulization of hypertonic solution from patients with AR, patients with nonallergic rhinitis with eosinophilia syndrome (NARES), and control subjects. Thus, the aim of the present study is to assess the validity (ability to distinguish disease vs normality) of the method based on nebulization of hypertonic solution for the analysis of nasal secretions obtained from patients with AR, patients with NARES, and controls by comparing total and differential cell counts. In addition, the reproducibility of the method was examined by comparing measurements in 2 samples of nasal secretions obtained on 2 different occasions in a group of patients with AR, patients with NARES, and controls.

* Department of Internal Medicine, Immunology, and Infectious Diseases, University of Bari Medical School, Policlinico, Bari, Italy.
† Fondazione Salvatore Maugeri, Care and Research Institute, Cassano Delle Murge, Bari, Italy.
‡ Department of Respiratory Diseases, University of Foggia, Foggia, Italy.
§ Institute of Respiratory Diseases, University of Medicine, Bari, Italy.
‖ World Health Organization Collaborating Centre for TB and Lung Diseases, Fondazione Salvatore Maugeri, Care and Research Institute, Tradate, Varese, Italy.
¶ Fondazione Salvatore Maugeri, Care and Research Institute, Tradate, Varese, Italy.
Authors have nothing to disclose.
Received for publication February 23, 2007.
Received in revised form April 24, 2007.
Accepted for publication May 14, 2007.

MATERIALS AND METHODS

Patients

Sixty-eight individuals were enrolled to study the validity of the nasal secretion method: 16 controls and 52 patients with either AR (n = 29) or NARES (n = 23) (Table 1). Twenty-nine of the 68 participants did not show up for the second induction of nasal secretion or did not produce adequate sample on either the first or the second occasion. Thus, the reproducibility of the method was studied using 39 individuals (17 with AR, 12 with NARES, and 10 controls). AR was diagnosed on the basis of a positive skin test reaction to 1 or more allergens, positive allergen specific IgE antibody test results, and a history of seasonal symptoms.[8] NARES was defined as a clinical syndrome comprising symptoms consistent with AR in which an absence of atopy has been demonstrated by allergen skin testing, and nasal cytologic analysis demonstrates eosinophils.[9]

Study Design

Each participant enrolled in the study underwent ultrasonic nebulization of hypertonic (4.5%) saline solution on 2 different occasions at the same hour of the day within 1 week to study the reproducibility of total and differential cell counts of nasal secretion. The validity of the method was evaluated by comparing differential cell counts of nasal secretions obtained from patients with AR, patients with NARES, and controls. The protocol was approved by the ethics committee of Fondazione Salvatore Maugeri, and all the participants provided written informed consent.

Ultrasonic Nebulization of Hypertonic Saline Solution

Ultrasonic nebulization of hypertonic (4.5%) saline solution was generated using an ultrasonic nebulizer (Orion 2; Nova, Heyer, Germany) at maximum output power (4.4 mL/min) for 5 minutes.[7] During nebulization the participants had their necks extended to facilitate penetration of the inhaled solution on the whole surface of the nasal mucosa, including the posterior nasal cavity. The mean size of particles inhaled through the nose was 2.5 to 5.0 μm. After 5 minutes of inhalation the participants were asked to press the left nostril and to blow the right nostril forcefully into a Petri dish. The blowing procedure was repeated for the left nostril.

Sample Processing

The secretions collected were processed.[7] In brief, the secretions were immediately weighted, and dithiothreitol 0.1% reagent (Sputolysin; Calbiochem Corp, San Diego, California) freshly prepared was added to the secretions. The volume of dithiothreitol was equal to 4 times the weight of the secretions portion. The sample was placed at 37°C for 20 minutes and was vortexed every 5 minutes to ensure cell dispersion. The sample was then filtered on sterile gauze, and a small volume (20 μL) was used to evaluate total cell counts using a standard hemocytometer. The sample was then divided into 2 aliquots: 1 was diluted to obtain a final cellular concentration of 2×10^5/mL; 100 μL of this sample was cytocentrifuged at 400 rpm for 5 minutes; the slides were stained with a Romanowski-based stain (Diff-Quik; Dade Behring AG, Düdingen, Switzerland), and a differential cell count was performed on 200 cells.

Statistical Analysis

Descriptive statistics were used to summarize clinical and demographic characteristics of the participants. The results are expressed as mean ± SD for age, lung function values, and cellular composition of nasal secretions. The comparison of differential cell counts between patients with rhinitis and controls was evaluated using *t* tests. The reproducibility of measurements was examined using the Pearson correlation coefficient. $P < .05$ was considered statistically significant.

RESULTS

Cellular Composition of Nasal Secretion Samples

The percentage of eosinophils was significantly higher in samples from patients with AR (20.8% ± 23.1%) and NARES (18.7% ± 22.8%) than in samples from controls (0.6% ± 0.6%; $P < .001$ for both) (Table 2). Samples of nasal secretions collected from patients with AR (1.2% ± 1.4%) and NARES (1.2 ± 1.0) were characterized by an increased percentage of lymphocytes compared with controls (0.0% ± 0.0%; $P < .001$ for both). The percentage of neutrophils increased significantly in samples from patients with NARES vs controls (78.0% ± 21.0% vs 65.4% ± 20.7%; $P = .04$), whereas this increase was not significant in patients with AR (70.8% ± 25.9% vs 65.4% ± 20.7%; $P = .22$). The percentage of epithelial cells (AR vs controls: 5.9% ± 12.5% vs 30.1% ± 19.6%; NARES vs controls: 2.0% ± 3.8% vs 30.1% ± 19.6%; $P < .001$ for both) and macrophages (AR vs controls: 1.2% ± 6.7% vs 3.9% ± 2.8%; $P = .03$; NARES vs controls: 0.0% ± 0.0% vs 3.9% ± 2.8%; $P < .001$) decreased significantly in samples of nasal secretions

Table 1. Demographic and Clinical Characteristics of the 68 Study Participants

Characteristic	AR group (n = 29)	NARES group (n = 23)	Control group (n = 16)	Total (N = 68)
Sex, M/F, No.	13/16	9/14	6/10	28/40
Age, mean ± SD, y	33.3 ± 16.9	46.4 ± 16.6	42.1 ± 15.1	39.8 ± 17.2
FEV_1, mean ± SD, % predicted	98.4 ± 16.6	93.9 ± 16.5	105.2 ± 18.1	98.7 ± 17.2
FVC, mean ± SD, %	102.2 ± 12.8	99.7 ± 28.5	109.6 ± 16.8	104.5 ± 14.8

Abbreviations: AR, allergic rhinitis; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; NARES, nonallergic rhinitis with eosinophilia syndrome.

Table 2. Comparison of Differential Cell Counts of Nasal Secretion Samples From Patients With Rhinitis and Controls[a]

Cell count	Control group	AR group	NARES group	Total
TCC, $\times 10^6$/mL^{-1}	0.8 ± 1.1	1.0 ± 1.7	0.8 ± 1.3	0.9 ± 1.4
Viability, %	73.4 ± 7.9	69.3 ± 7.4	70.3 ± 8.0	70.5 ± 7.7
Macrophages, %	3.9 ± 2.8	1.2 ± 6.7[b]	0.0 ± 0.0[c]	1.5 ± 4.8
Neutrophils, %	65.4 ± 20.7	70.8 ± 25.9	78.0 ± 21.0[b]	72.0 ± 23.3
Eosinophils, %	0.6 ± 0.6	20.8 ± 23.1[c]	18.7 ± 22.8[c]	15.3 ± 21.5
Lymphocytes, %	0.0 ± 0.0	1.2 ± 1.4[c]	1.2 ± 1.0[c]	0.9 ± 1.2
Epithelial cells, %	30.1 ± 19.6	5.9 ± 12.5[c]	2.0 ± 3.8[c]	10.4 ± 16.9

Abbreviations: AR, allergic rhinitis; NARES, nonallergic rhinitis with eosinophilia syndrome; TCC, total cell count.
[a] Data are expressed as mean ± SD.
[b] $P < .05$.
[c] $P < .001$.

from patients with AR and NARES compared with controls. No statistically significant difference was observed in total cell counts between samples obtained from patients with AR, patients with NARES, and controls.

Reproducibility of the Method

Total and differential cell counts of 2 samples obtained from 39 participants on 2 different occasions were compared using the Pearson correlation coefficient (Table 3 and Fig 1). There was a significant correlation between the 2 samples of nasal secretions obtained on 2 different occasions for percentage of macrophages, neutrophils, eosinophils, and epithelial cells, indicating good reproducibility of the method. The correlation between the 2 samples for total cell counts and percentage of lymphocytes was at the border of significance.

DISCUSSION

According to the aim of the present study, the validity of the method based on nebulization of hypertonic solution[7] for analysis of nasal secretions obtained from 29 patients with AR, 23 patients with NARES, and 16 controls was studied by comparing total and differential cell counts. Analysis of nasal secretions collected using ultrasonic nebulization of hypertonic solution can distinguish patients with AR and NARES from controls. Furthermore, the reproducibility of the method was examined by comparing measurements in 2 samples obtained on 2 different occasions in a group of patients with AR, patients with NARES, and controls. The reproducibility of this technique was found to be good for macrophages, neutrophils, eosinophils, and epithelial cells.

Table 3. Reproducibility of Nasal Secretion Analysis for Total and Differential Cell Counts

	r	*P* value
Total cell count ($\times 10^5$)	0.32	.05
Macrophages	0.58	<.001
Neutrophils	0.64	<.001
Eosinophils	0.63	<.001
Lymphocytes	0.31	.05
Epithelial cells	0.83	<.001

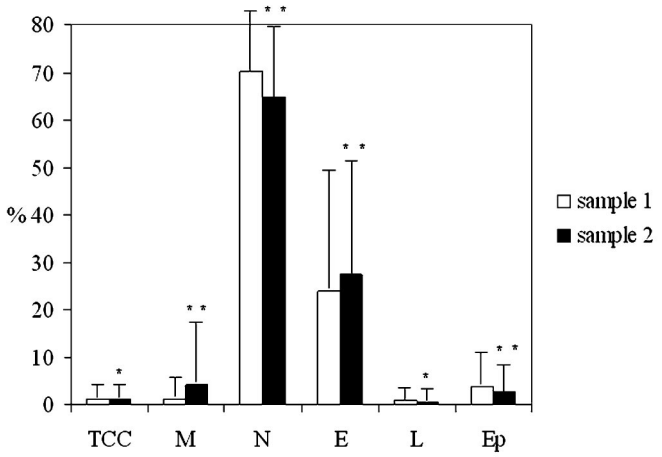

Figure 1. Total cell counts (TCCs) and differential cell counts of 2 samples obtained from 39 individuals enrolled in the present study using ultrasonic nebulization of hypertonic saline solution on 2 different occasions. E indicates eosinophils; Ep, epithelial cells; L, lymphocytes; M, macrophages; N, neutrophils. Error bars represent SD. *$P = .05$. **$P < .001$.

In this study, nasal secretion samples were obtained by stimulation of nasal mucosa using hypertonic saline solution (4.5%), which is known to be a significant nasal irritant. Commonly, isotonic saline solution has been used for stimulation of nasal mucosa and collection of samples from patients with different nasal diseases. However, recently the use of hypertonic saline solution has increased substantially owing to its ability to reduce edema and improve mucociliary clearance. The improvement in mucociliary clearance is of great importance, and it allows the collection of adequate samples from patients and controls, who usually have a limited amount of nasal mucosa secretion.[10]

The hypertonic concentration of saline solution might be expected to affect the cellular characteristics of nasal secretions. The study by Kim et al[11] identified the effect of hypotonic, isotonic, and hypertonic saline irrigation on secretory mucins and morphologic features of cultured human epithelial cells. According to this study, none of the saline concentrations significantly changed the secretion of total mucins.

Treatment with hypertonic solution produced few holes in the epithelium layer due to secretory cell exfoliation compared with treatment with isotonic solution. However, cell-to-cell integrity was maintained, and no damage of cells was observed after irrigation with hypertonic solution.[11] To our knowledge, the effect of saline concentration on the cellular characteristics of nasal secretions has not been reported. However, there are indications that stimulation of lower airways secretion by different saline concentrations does not affect total and differential cell counts in selected portions of induced sputum.[12] Thus, the results of the present study might not be affected and biased by the use of hypertonic solution for nasal secretion collection.

Total and differential cell counts of nasal secretions obtained by means of ultrasonic nebulization of hypertonic solution can be used to monitor the activity of eosinophilic inflammation in AR and NARES. Clinical implementation of this method requires establishment of a reference range of total and differential cell counts of nasal secretion samples from controls. To our knowledge, this is the first study attempting to define reference ranges for nasal secretions obtained by means of ultrasonic nebulization of hypertonic solution.[7] Controls usually have a limited amount of nasal mucosa secretion, thus making it difficult to collect a sufficient amount of secretion.[10] The present study analyzed only 16 samples of nasal secretions from controls. However, these reference ranges of nasal secretions can be used as orientation for the interpretation of data in individual patients.

Furthermore, this study demonstrates that this method[7] can discriminate between controls and patients with AR and NARES. Samples of nasal secretion from these patients are characterized by an increase in the percentage of eosinophils and lymphocytes and a decrease in the percentage of epithelial cells and macrophages. A previous study[7] demonstrated eosinophilia in patients with AR. This is the first study characterizing changes in differential cell counts in samples from patients with AR and NARES.

In the present study we examined reproducibility in 2 samples of nasal secretion obtained on 2 different occasions using the Pearson correlation coefficient. We found that the method is reproducible for macrophages, neutrophils, eosinophils, and epithelial cells. This finding is in agreement with the previous study.[7] We also found that the reproducibility of total cell counts and lymphocytes was at the border of significance. This can be due to the difficulty in the accurate recognition of this cell type or due to very low numbers of this cell in the samples.[13] In many cases, we did not count any lymphocytes in one sample, whereas there was a low number of lymphocytes in the other specimen, resulting in poor reproducibility in that patient. Furthermore, the reproducibility of lymphocytes has not previously been reported.

This study shows that the analysis of samples obtained using ultrasonic nebulization of hypertonic solution can distinguish patients with AR and NARES from controls. The reproducibility of this technique is good for macrophages, neutrophils, eosinophils, and epithelial cells. This method could be used to detect nasal airway inflammation in clinical settings.

REFERENCES

1. Foresti A, Leone C, Pelucchi A, et al. Eosinophils, mast cells, and basophils in induced sputum from patients with seasonal allergic rhinitis and perennial asthma: relationship to methacholine responsiveness. *J Allergy Clin Immunol*. 1997;100:58–64.
2. Pipkorn U, Karlsson G. Methods for obtaining specimens from the nasal mucosa for morphological and biochemical analysis. *Eur Respir J*. 1988;1:856–862.
3. Belda J, Parameswaran K, Keith PK, Hargreave FE. Repeatability and validity of cell and fluid-phase measurements in nasal fluid: a comparison of two methods of nasal lavage. *Clin Exp Allergy*. 2001;31:1111–1115.
4. Ruocco L, Fattori B, Romanelli A, et al. A new collection method for the evaluation of nasal mucus proteins. *Clin Exp Allergy*. 1998;28:881–888.
5. Chapelin C, Coste A, Gilain L, Poron F, Verra F, Escudier E. Modified epithelial cell distribution in chronic airways inflammation. *Eur Respir J*. 1996;9:2474–2478.
6. Biewenga J, Stoop AE, Baker HE, Swart SJ, Nauta JJP, Van Kramp GJ. Nasal secretions from patients with polyps and healthy individuals, collected with a new aspiration system: evaluation of total protein and immunoglobulin concentrations. *Ann Clin Biochem*. 1991;28:260–266.
7. Melillo G, Balzano G, Stefanelli F, Iorio C, De Angelis E, Melillo E. Ultrasonic nebulization of hypertonic solution: a new method for obtaining specimens from nasal mucosa for morphologic and biochemical analysis in allergic rhinitis. *Allergy*. 1998;53:794–797.
8. Quillen DM, Feller DB. Diagnosing rhinitis: allergic vs. nonallergic. *Am Fam Physician*. 2006;73:1583–1590.
9. Ellis AK, Keith PK. Nonallergic rhinitis with eosinophilia syndrome. *Curr Allergy Asthma Rep*. 2006;6:215–220.
10. Klimek L, Rasp G. Norm values for eosinophil cationic protein in nasal secretions: influence of specimen collection. *Clin Exp Allergy*. 1999;29:367–374.
11. Kim CH, Hyun Song M, Eun Ahn Y, Lee JG, Yoon JH. Effect of hypo-, iso- and hypertonic saline irrigation on secretory mucins and morphology of cultured human nasal epithelial cells. *Acta Otolaryngol*. 2005;125:1296–1300.
12. Chanez P, Holz O, Ind PW, Djukanovic R, Maestrelli P, Sterk P. Sputum induction. *Eur Respir J Suppl*. 2002;37:3s–8s.
13. Spanevello A, Migliori GB, Sharara A, et al. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy*. 1997;27:1138–1144.

Requests for reprints should be addressed to:
Antonio Spanevello, MD
Fondazione Salvatore Maugeri
Care and Research Institute
Via Per Mercadante km 2
70020 Cassano Delle Murge
Bari, Italy
E-mail: aspanevello@fsm.it