

Fractional analysis of sequential induced sputum samples during sputum induction: Evidence that different lung compartments are sampled at different time points

Neil H. Gershman, MD,^a Hong Liu, MD,^b Hofer H. Wong, BS,^b Jane T. Liu, MS,^b and John V. Fahy, MD^{a,b} San Francisco, Calif

Background: The effect of the duration of sputum induction on markers of inflammation in induced sputum is unknown, and the optimal duration of sputum induction for research purposes in airway disease is uncertain.

Objective: We sought to determine whether the duration of sputum induction influences the cellular or biochemical characteristics of induced sputum.

Methods: Induced sputum was collected sequentially at 4-minute intervals during a 20-minute sputum induction in 12 subjects with mild and moderate asthma. Each 4-minute sample was collected and analyzed separately for total and differential cell counts and for levels of eosinophil cationic protein, fibrinogen, mucin-like glycoprotein, and surfactant protein SP-A.

Results: The percentages of eosinophils and neutrophils were significantly higher at the beginning of the 20-minute sputum induction than at the end, whereas the percentage of macrophages was significantly lower at the beginning than at the end. In addition, the levels of eosinophil cationic protein and mucin-like glycoprotein were significantly higher at the beginning of the 20-minute induction than at the end, whereas the level of surfactant protein SP-A was significantly lower.

Conclusions: The duration of sputum induction significantly affects the cellular and biochemical composition of induced sputum in a manner suggesting that large airways are sampled at the beginning of sputum induction, whereas peripheral airways and alveoli are sampled at later time periods. Our data demonstrate the importance of standardizing the duration of sputum induction in clinical research studies, and on the basis of these data, we have chosen 12 minutes as the optimal duration for sputum induction in asthmatic subjects. (*J Allergy Clin Immunol* 1999;104:322-8.)

Key words: Asthma, sputum induction, induced sputum, hypertonic saline, eosinophil, eosinophil cationic protein, neutrophil, mucin, surfactant protein A, fibrinogen

Sputum induction is a useful noninvasive method for collecting airway secretions from asthmatic subjects, and analysis of induced sputum for cellular and biochemical

Abbreviation used

ECP: Eosinophil cationic protein

constituents has been found to be a valid method for monitoring airway inflammation.¹⁻⁶ An unresolved issue in the method of sputum induction is the effect of the duration of sputum induction on the cellular and biochemical characteristics of induced sputum. The duration of sputum induction is a potentially important variable because the composition of induced sputum might be expected to vary according to the duration of induction. For example, the duration of induction determines the dose of hypertonic saline delivered to the airway, and the duration of induction may also determine the compartment of the lung sampled by sputum induction. Support for these possibilities comes from the study of Holz et al,⁷ who found that neutrophil and eosinophil percentages in induced sputum from healthy and asthmatic subjects decrease during 3 consecutive 10-minute sputum inductions; in contrast, the percentage of macrophages increased in these samples.

In this study our aim was to determine whether the duration of sputum induction influences the cellular or biochemical characteristics of induced sputum. To investigate this hypothesis, we collected induced sputum at 4-minute intervals during a 20-minute sputum induction and analyzed each sample separately for cells and chemicals. Because we suspected that different lung compartments might be sampled at different time points during sputum induction, our analysis included a marker of airway secretions (mucin-like glycoprotein) and a marker of alveolar secretions (surfactant protein SP-A).

METHODS

Subjects

Twelve atopic asthmatic subjects were studied (Table I). All subjects had been on a stable therapeutic regimen for their asthma for at least 4 weeks before study entry. Inclusion criteria were a history of symptoms of asthma and bronchial hyperreactivity to methacholine (PC₂₀FEV₁ methacholine <8 mg/mL). Exclusion criteria were age greater than 65 years, a history of lung disease other than asthma, or a history of an upper respiratory infection in the preceding 4 weeks. These subjects were recruited from our asthma subject

From ^athe Department of Medicine and ^bthe Cardiovascular Research Institute, University of California, San Francisco.

Received for publication Jan 22, 1999; revised Apr 5, 1999; accepted for publication Apr 8, 1999.

Reprint requests: John V. Fahy, MD, Box 0111, University of California, San Francisco, 505 Parnassus Avenue, San Francisco, CA 94143.

Copyright © 1999 by Mosby, Inc.

0091-6749/99 \$8.00 + 0 1/1/99114

TABLE I. Clinical characteristics of the study subjects

Subject	Age (y)	Sex	Treatment	FEV ₁ (L)	PC ₂₀ FEV ₁ (% predicted)	FEV ₁ methacholine (mg/mL)
1	31	M	B	2.8	70	0.34
2	28	F	B, ICS	3.1	103	0.74
3	29	M	B	2.9	78	1
4	37	F	B	3.2	97	0.13
5	33	M	B	3.4	77	0.18
6	46	F	B, ICS	3.2	100	0.38
7	34	M	B	2.7	73	0.13
8	36	F	B, ICS	2	65	0.36
9	36	M	B	2.8	72	0.52
10	29	M	B	2.9	78	0.18
11	39	F	B	2.4	77	0.41
12	45	F	B	2.5	83	0.18
Mean	35	—	—	2.8	81	0.31*

B, β -Agonist; ICS, inhaled corticosteroid.

*Geometric mean.

database, which consists of individuals who responded to newspaper, radio, and bulletin board advertisements. The subjects signed consent forms approved by the Committee on Human Research at the University of California, San Francisco.

Protocol

The study involved a single visit to the laboratory. All subjects invited to participate had participated in other research studies in our laboratory within the previous 12 months, and all had been characterized by methacholine challenge testing and allergen skin testing. During the visit for this study, consent was obtained, and spirometry and sputum induction were performed.

Spirometry, methacholine challenge, and allergen skin testing

Spirometry, methacholine challenge, and allergen skin testing were performed by using methods previously described.⁸

Sputum induction

All subjects were pretreated with 360 μ g of albuterol administered by metered-dose inhaler, after which they were seated in an aerosol containment chamber (Nuair model #NU-810-224, Plymouth, Minn). They inhaled nebulized sterile 3% saline for 20 minutes through a mouthpiece while wearing a noseclip. The solution was nebulized by a DeVilbiss Ultraneb 99 ultrasonic nebulizer, the reservoir of which was filled with 150 mL of 3% saline; this nebulizer generates particles of a mean mass median diameter of 4.5 μ m and has an output of 6.2 mL/min. During the induction procedure, lung function was monitored at 2- to 4-minute intervals by measurements of peak expiratory flow, spirometry, or both, as previously described.⁹

Sputum collection

Subjects were instructed to spit saliva into a container labeled "saliva" before coughing sputum into a container labeled "sputum." The inhalation was interrupted every 2 minutes, at which time the subject was asked to forcefully cough up secretions. At 4-minute intervals, during the inhalation of hypertonic saline, the subject was given a new container in which to deposit sputum. Therefore the total number of induced sputum samples for each induction procedure was 5: 0 to 4 minutes, 4 to 8 minutes, 8 to 12 minutes, 12 to 16 minutes, and 16 to 20 minutes. The labels on the sputum containers

were coded, and therefore the investigators processing the sample were unaware of the time intervals during which the sputum was collected.

Processing of induced sputum and cell counts

Sputum samples collected during each time interval were processed by using methods previously described. Briefly, 250 μ L of homogenized sputum (diluted in saline to prevent cell crowding on the slide) was spun in a cytocentrifuge (model 7 cytospin; Shandon Scientific, Sewickley, Pa) and stained with Diff-Quik stain (Baxter Scientific Products, Miami, Fla). All cells were enumerated and counted, which allowed calculation of both the squamous cell percentage of the total cell sample and the epithelial, macrophage, neutrophil, eosinophil, and lymphocyte percentages of the nonsquamous cells in the sample. At least 200 nonsquamous cells were enumerated. All slides were examined by the same investigator, who was blinded to the time interval of the sample.

Our definition of an adequate induced sputum sample was one in which there were fewer than 80% squamous cells on differential cell analysis. The portion of the homogenized sputum samples not used for total and differential cell counts were centrifuged at 1037g for 5 minutes. The supernatant was aspirated and frozen at -70°C for later analysis.

Biochemical assays

Eosinophil cationic protein (ECP) concentrations were determined by using a sensitive RIA (Pharmacia Diagnostics Inc, Fairfield, NJ). Mucin-like glycoprotein and fibrinogen levels were determined by ELISA, as previously described.^{2,10} Surfactant protein SP-A was measured by using a 2 mAb sandwich ELISA. The mAbs, raised by standard techniques against recombinant human surfactant protein SP-A produced in Chinese hamster ovary cells,¹¹ recognized separate epitopes in the carbohydrate recognition domain of SP-A, as determined by competitive ELISA (Sam Hawgood, personal communication). Briefly, 96-well Nunc ELISA plates were coated overnight at 4°C with the first mAb at a concentration of 5 μ g/mL, pH 8.5. The plates were washed with PBS and 0.05% Tween-20 and blocked for 30 minutes at room temperature with 2 mg/mL ovalbumin in PBS. After removing the blocking solution, standards of human SP-A in 2-fold dilutions from 0.125 μ g/mL to 0.001 μ g/mL in PBS with 2 mmol/L CaCl_2 or serial dilu-

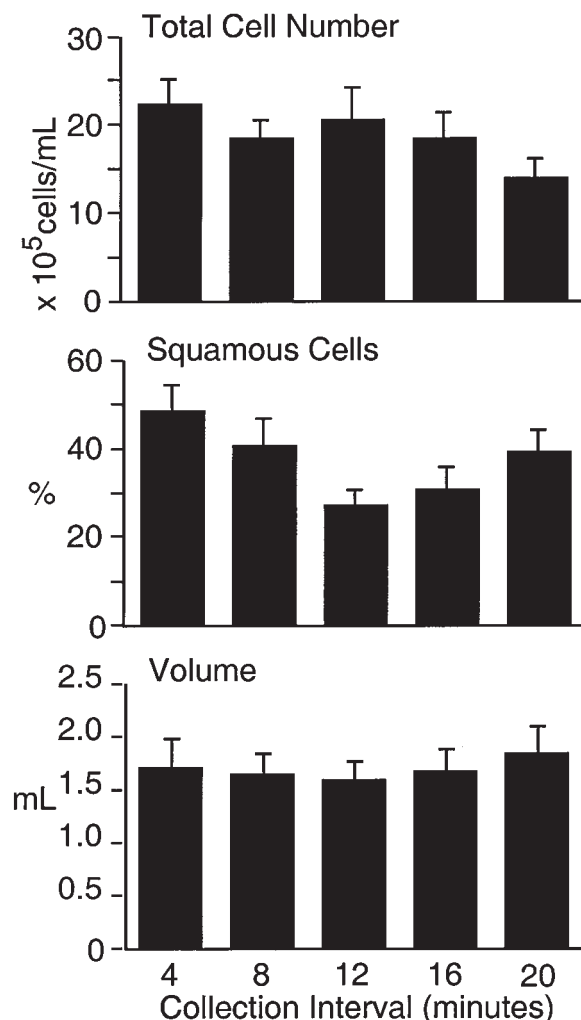


FIG 1. Total cell count, squamous cell percentage, and volume of induced sputum samples collected individually at five 4-minute intervals during a 20-minute sputum induction. None of the changes in these parameters were statistically significant.

tions of the samples in the same buffer were added and allowed to incubate for 1 hour at 37°C or overnight at 4°C. The wells were then washed in PBS/Tween and a 1:32000 dilution of the biotinylated second mAb, which was added for 1 hour. After washing 3 times, the plates were incubated with streptavidin-horseradish peroxidase diluted 1:6000 and developed with *o*-phenylenediamine. The reaction was stopped after 2 minutes with H_2SO_4 , and the color was read at 490 nm.

Statistical analysis

Data is presented as means $\pm$ SEM unless otherwise specified. The Friedman test, a nonparametric repeated-measures ANOVA, was used for comparisons of cells and chemicals in induced sputum collected during the 5 intervals during sputum induction (total of 60 samples from 12 subjects). When a significant difference was detected with the Friedman test, between-group comparisons were made by using the Student-Newman-Keuls test. For analyses of data from 2 time intervals (eg, mucin-like glycoprotein and surfactant protein A), the Wilcoxon signed-rank test was used. A *P* value of less than .05 with a 2-tailed test was considered significant.

RESULTS

The volumes of induced sputum were not significantly different for any of the 5 time periods during sputum induction (Fig 1). In addition, the total cell counts and squamous cell percentages were not significantly different for any of the 5 time periods during sputum induction (Fig 1), although there was a trend for a higher squamous cell percentage in the 0- to 4-minute time period ($P = .07$), and the squamous cell number was significantly higher at this time period than at the other 4 later time periods ($P < .05$).

The percentages of eosinophils, neutrophils, macrophages, and epithelial cells varied significantly over the time course of the sputum induction (Fig 2). Between the 0- to 4- and 16- to 20-minute intervals, the percentages of eosinophils and neutrophils decreased by 73% and 50%, respectively. In contrast, the percentages of macrophages increased by 46%, and the percentages of lymphocytes did not change significantly between these 2 same time periods. Epithelial cells were highest during the 8- to 12-minute collection period. The changes in cell numbers during sputum induction paralleled the changes in cell percentages, but only the change in epithelial cell numbers reached statistical significance ($P = .014$; Fig 2).

The levels of ECP and fibrinogen fell significantly over the time course of the sputum induction (Fig 3). Between the 0- to 4- and 16- to 20-minute intervals, the levels of ECP and fibrinogen decreased by 45% and 49%, respectively. Sample and reagent limitations permitted analysis of mucin-like glycoprotein and surfactant protein A levels for the 0- to 4- and 16- to 20-minute time periods only. We found that the levels of mucin-like glycoprotein increased significantly from 0 to 4 to 16 to 20 minutes, whereas the levels of surfactant protein A decreased significantly between these 2 time periods (Fig 4).

The results of the fractional analysis of the induced sputum suggested to us that 12 minutes might be a duration for sputum induction that would yield induced sputum enriched with airway secretions and would have the advantage of being relatively short and easier for subjects to tolerate. Accordingly, we did an analysis of the data from the individual time periods, whereby we calculated cumulative data for sputum eosinophil percentage and sputum ECP levels by using volume-weighted averages for the percentages of eosinophils and concentrations of ECP in induced sputum for the first 3 samples collected during sputum induction (12-minute sample) and for all 5 induced sputum samples collected during sputum induction (20-minute sample). We found that the percentage of eosinophils was higher for the cumulative 12-minute sample than for the cumulative 20-minute sample ($P = .055$). Similarly, we found that the levels of ECP were higher for the cumulative 12-minute sample than for the cumulative 20-minute sample ($P = .019$).

DISCUSSION

The main finding of our study is that the composition of induced sputum changes significantly during sputum

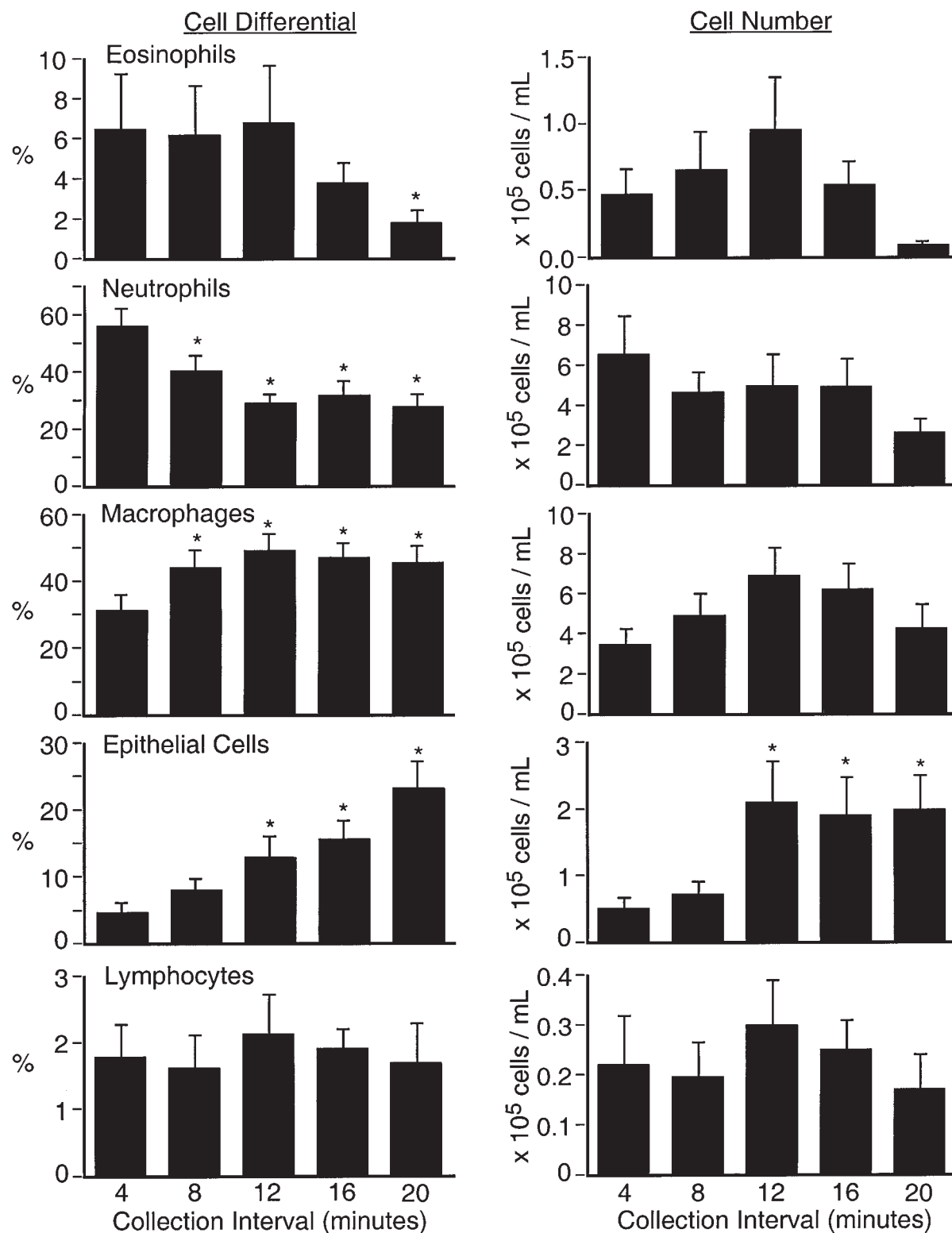


FIG 2. Changes in cell differential and numbers of nonsquamous cells in induced sputum samples collected individually at five 4-minute intervals during a 20-minute sputum induction. The 5 panels in the column on the left show the data for eosinophil, neutrophil, macrophage, epithelial cell, and lymphocyte percentages, and the 5 panels on the right show the cell numbers of these cells. *Significantly different from the 0- to 4-minute interval ($P < .05$).

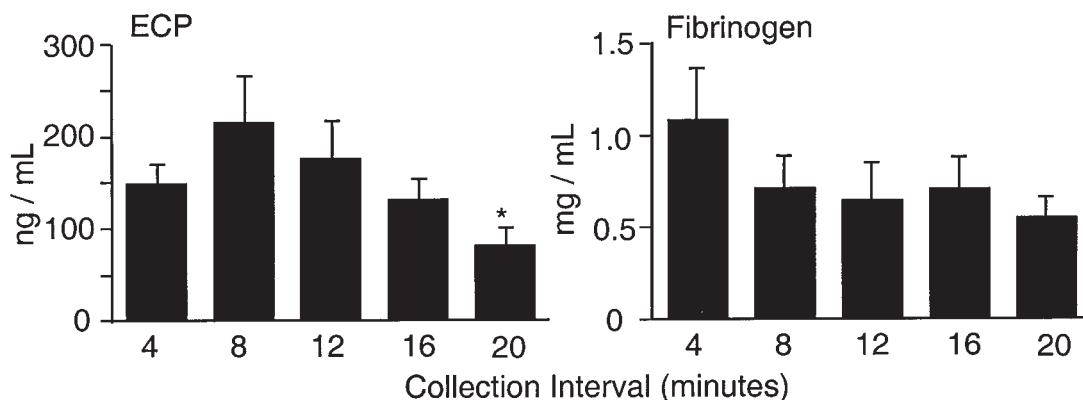


FIG 3. Changes in the levels of ECP and fibrinogen in induced sputum samples collected individually at five 4-minute intervals during a 20-minute sputum induction. *Significantly different from the 0- to 4-minute interval ($P < .05$).

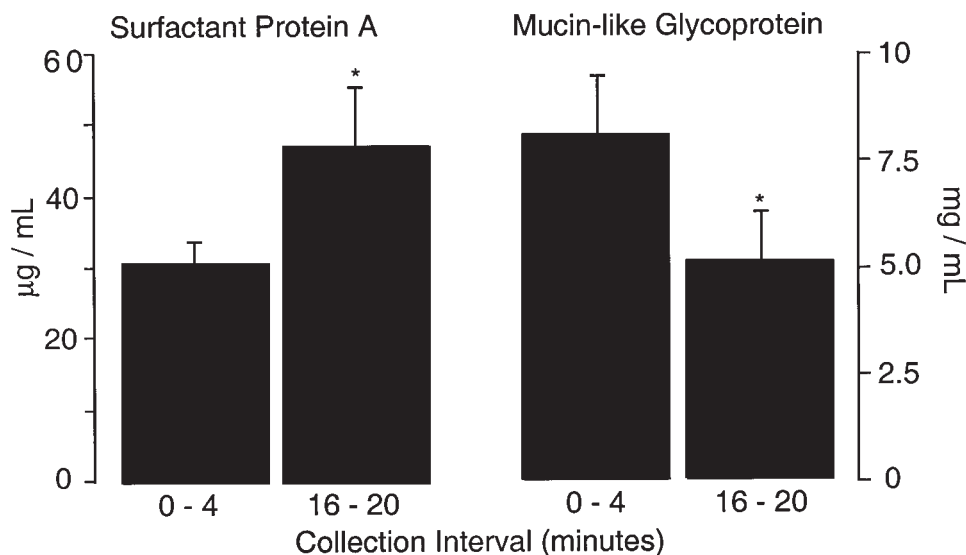


FIG 4. Changes in the levels of surfactant protein A and mucin-like glycoprotein in induced sputum samples collected individually at the beginning (0 to 4 minutes) and end (16 to 20 minutes) of a 20-minute sputum induction. *Significantly different from the 0- to 4-minute interval ($P < .05$).

induction. Cells associated with airway secretions (eosinophils and neutrophils) were prominent in samples collected early in the induction, whereas cells associated with alveolar secretions (macrophages) were prominent in samples collected later. Similarly, a protein associated with airway secretions (mucin-like glycoprotein) was prominent in samples collected early in the induction, whereas a protein associated with alveolar secretions (surfactant protein SP-A) was prominent in secretions collected later. These data underscore the need to standardize the duration of sputum induction in clinical research protocols and suggest that the large airway compartment of the lung is sampled early in sputum induction and the smaller airways and alveoli are sampled later in sputum induction.

The method of sputum induction favored by our group involves inhalation of 3% saline for a fixed time period,

and we initially chose a duration of 20 minutes for our research studies on the basis of our clinical experience with sputum induction for the purposes of diagnosing pneumonia in patients.² We prefer to analyze the entire expectorate when processing induced sputum, although for several years we have been collecting saliva separately from the rest of the expectorate and discarding it.⁹ In the study described here, we have carefully analyzed the effects of the duration of sputum induction on the cellular and biochemical constituents of induced sputum. We did this with the objective of providing data with which to choose the optimal duration of sputum induction for the study of airway disease. Our data show that by using our methods asthmatic subjects cough up approximately 1.5 mL of induced sputum in a 4-minute period; this volume does not change significantly from the beginning to the end of a 20-minute induction period. The cellular

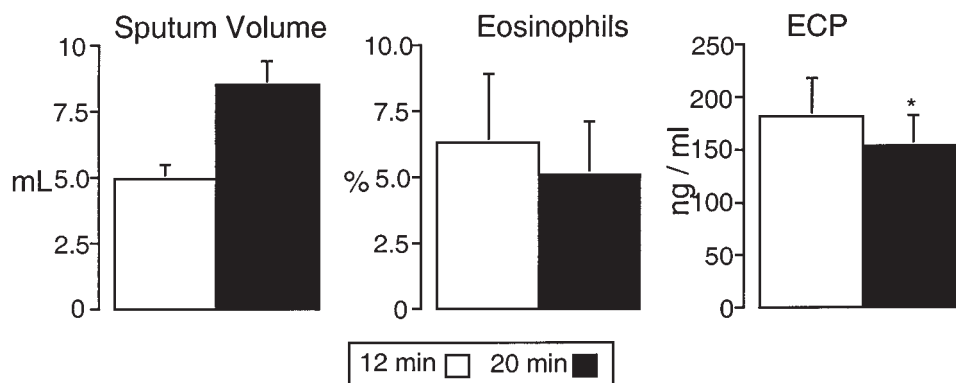


FIG 5. Cumulative data analysis from induced sputum samples collected individually at five 4-minute intervals during a 20-minute sputum induction in which volume-weighted averages were used to calculate the percentages of eosinophils and the concentrations of ECP in 12-minute versus 20-minute induced sputum samples. *Significantly different from the 12-minute sample ($P < .05$).

composition of the induced sputum does change, however. For example, the percentage of neutrophils and eosinophils is highest in the induced sputum samples collected at the beginning of the induction and lowest in the samples collected at the end of the induction. In contrast, the percentage of macrophages is lowest in the induced sputum collected at the beginning of sputum induction and highest at the end of sputum induction. On the basis of the observed changes in eosinophil, neutrophil, and macrophage percentages in induced sputum samples collected during this 20-minute sputum induction period, we speculate that induced sputum collected early in sputum induction originates primarily from larger airways, whereas induced sputum collected later in sputum induction originates primarily from smaller airways and alveoli. Arguing against this interpretation is our data on epithelial cell percentages in induced sputum, which increased sequentially during sputum induction, but other data for fluid-phase constituents of induced sputum (discussed below) support the idea.

The concentrations of most of the chemicals measured in the fluid phase of the induced sputum collected early in sputum induction were higher than in the induced sputum collected later. For example, the concentrations of ECP, fibrinogen, and mucin-like glycoprotein were all higher in the induced sputum collected at the 0- to 4-minute time point compared with the 16- to 20-minute time point. The exception was the concentration of surfactant protein SP-A in the induced sputum collected at the 16- to 20-minute time point, which was higher than in samples from the 0- to 4-minute time point. Because surfactant protein SP-A is a glycoprotein secreted by type 2 pneumocytes in the alveolar epithelium^{11,12} and is thus a marker of alveolar epithelial secretion, and because mucin-like glycoprotein is a product of large airway secretion, we argue that these data support our hypothesis that induced sputum collected early during sputum induction originates primarily from larger airways, whereas induced sputum collected later in sputum induction originates primarily from smaller airways and alveoli.

The implications of our results for the methodology of sputum induction for research purposes are clear—the duration of sputum induction must be standardized if the data from analysis of induced sputum are to be interpretable. Two examples illustrate this point. First, because asthmatic subjects are more sensitive to hypertonic saline than healthy subjects, they may be found to tolerate shorter durations of sputum induction than healthy control subjects in comparative studies. In this instance it will not be possible to ascribe any differences in the cellular or biochemical characteristics of induced sputum in the asthmatic subjects to asthma if the confounding issue of the duration of the induction procedure is not controlled for. Second, in any treatment protocol in asthmatic subjects, the treatment may make the subjects less sensitive to the bronchoconstrictor effects of hypertonic saline. Thus sputum induction may be better tolerated at the end of a treatment regimen than at the beginning, translating to a longer duration of sputum induction. In this instance the cellular and biochemical characteristic of the sputum may change (eg, the sputum eosinophil percentage may decrease). This decrease or any other change will not be interpretable if the duration of sputum induction is longer at the end of treatment than at baseline. Thus to interpret induced sputum data in studies like these, it is necessary to control for the duration of sputum induction; the easiest way to do this is to ensure that the duration of sputum induction in each subject is the same at the beginning and end of the study.

Examination of the data presented in Figs 1 to 4 has led us to shorten the duration of sputum induction in our protocol from 20 minutes to 12 minutes. We chose 12 minutes of induction for future studies because induced sputum samples collected after 12 minutes of induction in this study had lower eosinophil percentages and lower ECP levels than samples collected earlier, and markers of eosinophilic inflammation are common outcomes in our clinical studies in asthmatic subjects. In an analysis of the data from the 5 individual time periods of induction, during which cumulative data were calculated by using

volume-weighted averages for the percentages of eosinophils and the concentrations of ECP, we confirmed that the percentage of eosinophils and the levels of ECP were higher for the cumulative 12-minute sample than for the cumulative 20-minute sample. In addition, a protocol with 12 minutes of induction has the practical advantage of simplifying the protocol for staff and subjects, of decreasing the risk of bronchospasm because of delivery of a lower dose of hypertonic saline, and of yielding sufficient induced sputum for analysis (approximately 5 mL on average; Fig 5).

In summary, we have demonstrated that the cellular and biochemical characteristics of induced sputum change significantly depending on the duration of sputum induction. Our data suggest that the airway compartment sampled during sputum induction depends on the duration of the induction and clearly demonstrate the need to standardize the duration of sputum induction in clinical research protocols.

REFERENCES

1. Pin I, Freitag AP, O'Byrne PM, Girgis-Gabardo A, Watson RM, Dolovich J, et al. Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. *Am Rev Respir Dis* 1992;145:1265-9.
2. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993;147:1126-31.
3. Iredale MJ, Wanklyn SAR, Phillips IP, Krausz T, Ind PW. Non-invasive assessment of bronchial inflammation in asthma: no correlation between eosinophilia of induces sputum and bronchial responsiveness to inhaled hypertonic saline. *Clin Exp Allergy* 1994;24:940-5.
4. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumour necrosis factor alpha in induced sputum from patients with chronic obstructive pulmonary disease and asthma. *Am J Respir Crit Care Med* 1996;153:530-4.
5. Spanavello A, Beghe B, Bianchi A, Migliori GB, Ambrosetti M, Neri M, et al. Comparison of two methods of processing induced sputum: selected versus entire sputum. *Am J Respir Crit Care Med* 1997;157:665-8.
6. Diamant Z, Hiltermann JT, van Rensen EL, Callenbach PM, Veselic-Charvat M, van der Veen H, et al. The effect of inhaled leukotriene D4 and methacholine on sputum cell differentials in asthma. *Am J Respir Crit Care Med* 1997;155:1247-53.
7. Holz O, Jorres RA, Koschik S, Speckin P, Welker L, Magnussen H. Changes in sputum composition during sputum induction in healthy and asthmatic subjects. *Clin Exp Allergy* 1998;28:284-92.
8. Fahy JV, Wong HH, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *Am J Respir Crit Care Med* 1995;152:53-8.
9. Gershman NH, Wong HH, Liu JT, Mahlmeister MJ, Fahy JV. Comparison of two methods of collecting induced sputum in asthmatic subjects. *Eur Respir J* 1996;9:2448-53.
10. Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, Boushey HA. Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993;147:1132-7.
11. Haagsman HP, White RT, Schilling J, Hawgood S, Clements JA. Studies of the structure of lung surfactant protein SP-A. *Am J Physiol (Lung Cell Mol Physiol)* 1989;257:L421-9.
12. Mason RJ, Greene K, Voelker DR. Surfactant protein A and surfactant protein B in health and disease. *Am J Physiol (Lung Cell Mol Physiol)* 1998;19:L1-13.