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Some technical factors influencing the induction of sputum for cell analysis

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ABSTRACT: Inhalation of hypertonic saline aerosol is a relatively noninvasive method to obtain sputum for examination of inflammatory processes in the airways. We investigated some technical factors which might influence the success of induction and sputum cell counts.

In total, twenty six asthmatic and 13 healthy subjects, unable to raise sputum spontaneously, inhaled nebulized saline for three 7 min intervals. In three randomized, cross-over studies we repeated sputum induction on separate days with two ultrasonic nebulizers (De Vilbiss Ultraneb 99 and Fisoneb) and one jet nebulizer (Pari LL with Master Compressor) (Study 1, n=15), with different saline concentrations (normal saline 0.9%; hypertonic saline 3% on 2 days; and hypertonic saline 3, 4 and 5%, sequentially) (Study 2, n=14) and with pretreatment with either salbutamol or placebo (Study 3, n=10). The latter two studies were double-blind. Sputum cells were dispersed with dithiothreitol, and the cell suspension was used to perform total cell counts and to prepare cytospins for differential cell counts. We compared success rate, cell counts, subject discomfort and percentage fall in forced expiratory volume in one second (FEV_1) during the procedures. All sputum examinations were performed blind to the clinical procedures.

The success rates and the cell counts of the specimens obtained with the two ultrasonic nebulizers were not different, whilst general discomfort was proportional to the saline output of the nebulizer. Induction of sputum by hypertonic saline was more successful than normal saline, but more disagreeable to the subjects. Induction with saline 3% on two days was only successful in 6 of 14 subjects. Pretreatment with salbutamol inhibited the development of significant airway constriction in asthmatic subjects. Sputum cell counts were not affected by the tonicity of the saline or by salbutamol.

We conclude that sputum induction is more likely to be successful when performed with an ultrasonic nebulizer and hypertonic saline, and that cell counts are unaffected. Pretreatment with salbutamol can inhibit bronchoconstriction and does not alter the cell count.

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The development of noninvasive methods to investigate airway inflammation can be expected to improve our understanding of the pathogenesis, pathophysiology and treatment of asthma and other lung conditions. Recent work suggests that the examination of spontaneous or induced sputum may have many advantages over invasive methods [1–10]. Sputum induction was initially developed to investigate lung cancer and respiratory infection [11–19]. More recently, the method was modified for use in asthmatics [4]. Using an ultrasonic nebulizer, a 3–5% hypertonic saline aerosol was generated and inhaled for 5–30 min. In asthmatics, pretreatment with salbutamol (200 μg) was used to inhibit potential airway constriction caused by the hypertonic saline. The reported success rate in sputum induction has varied between 76 and 100% in asthmatic subjects, [4, 5, 7–10] and is almost 100% in studies using induction of sputum for the diagnosis of *Pneumocystis carinii* pneumonia in human immunodeficiency virus (HIV) positive patients [14–19]. It is not clear whether differences in success rate are a result of subject selection or variation in the methods of induction. The success of sputum induction with an aerosol of saline might be influenced by a number of technical factors (such as nebulizer output, saline concentration, particle size, and pretreatment with beta-agonist), and by nontechnical factors (such as smoking, whether the person is healthy or has respiratory disease and the degree of asthmatic or other airway inflammation). There has been little investigation of the effect of these factors on the success of induction or sputum characteristics [9, 20].

We report the results of three studies to examine the effects of nebulizer output, saline concentration, and

pretreatment with inhaled salbutamol on success of sputum induction, total and differential sputum cell counts, subject discomfort, and safety. Stable healthy and asthmatic subjects unable to produce sputum spontaneously were selected. Nontechnical factors which might increase the production of sputum were controlled. The design of all studies was randomized, and cross-over, and studies 2 and 3 were double-blind. All examinations of sputum cell viability and counts were blind to the clinical protocols.

Subjects and methods

Subjects

Thirty nine adult volunteers who had asthma or were healthy were recruited from patients, staff and friends of the Firestone Regional Chest and Allergy Unit of St Joseph's Hospital, for three studies (table 1). In the first study, the presence of asthma was determined by a history of asthma symptoms and a previous physician diagnosis; healthy subjects had no symptoms of chest disease and normal spirometry. In the two other studies, the presence of asthma was also objectively documented by the presence of methacholine airway hyperresponsiveness. Healthy subjects had no history of asthma symptoms, forced expiratory volume in one second (FEV_1) >80% predicted, and normal methacholine airway responsiveness. All subjects were stable and the asthmatics were controlled. Medications taken by asthmatics were unchanged for 1 month and during the study. None of the subjects were able to produce sputum spontaneously, and none had symptoms of a respiratory infection within 1 month. Only one subject (in Study 3) was a current smoker. The studies were approved by the hospital research committee and all subjects gave written informed consent.

Study design

The three studies were performed in sequence. In the first study, the effects of three types of nebulizers on sputum induction were compared using both asthmatic and healthy subjects. Two ultrasonic nebulizers (DeVilbiss Ultraneb 99, DeVilbiss Co., Somerset, PA, USA and Fisoneb, Fisons Co., Bedford, MA, USA) and one jet nebulizer (Pari LL with Master Compressor, Pari Respiratory Equipment Inc., Richmond, VA, USA) were used to generate an aerosol of 3% hypertonic saline to induce sputum. Each nebulizer was used on separate days, at the same time of day within 8 days. Having used this protocol in 11 subjects, we observed that the Pari jet nebulizer was able to induce sputum in only one. The protocol was, therefore, modified and the remaining four subjects were tested only with the two ultrasonic nebulizers. In the second study, the effect of inhaled normal saline aerosol was compared with 3% hypertonic saline (on 2 days) and 3% followed by 4% and 5% hypertonic saline aerosol generated by a Fisoneb on separate days, at the same time of day within 14 days in both asthmatic and healthy subjects. In the third study, pretreatment with salbutamol 200 µg was compared with placebo given 10 min before sputum induction on separate days, at the same time of day within 1 week in asthmatics only. Sputum was induced using a Fisoneb nebulizer with 3% followed by 4% and 5% hypertonic saline. In each study, the order of procedures to be compared was randomized and was double-blind in the last two. In each study, the success rate and cell counts were examined blind to the clinical procedures.

The outcome measures of each study were: success of sputum induction, as measured by the countable cytospins and characteristics of sputum (weight of selected portion of sputum, absolute number of cells retrieved from this and cell viability), differential cell counts, subject discomfort; and safety measured by percentage fall in FEV_1.

Methods

Subject characteristics were documented by questionnaire, spirometry was performed with a Collins water spirometer, methacholine inhalation test was carried out as described by JUNIPER *et al.* [21], and allergy skin tests were performed using the modified prick technique [22] with 19 common allergen extracts. The results of the methacholine test were expressed as the provocation concentration to cause a 20% fall in FEV_1 (PC_{20}) in noncumulative units; a value of >16 $mg \cdot ml^{-1}$ was regarded as normal.

Table 1. – Characteristics of subjects

Characteristics	Study 1	Study 2	Study 3
Asthma/healthy n	7/8	9/5	10/0
Age yrs*	35 (19–58)	35 (19–53)	40 (26–54)
Sex M/F	6/9	4/10	4/6
Atopic n	13	13	10
Smoking current/ex n	0/1	0/2	1/3
FEV_1 % pred*	96 (74–115)	93 (73–114)	90 (68–106)
PC_{20} methacholine $mg \cdot ml^{-1}$ **	ND	3.68 (0.59–>16)	1.04 (0.04–12.9)
On inhaled steroid† n	4	5	5

*: mean and range in parenthesis; **: geometric mean and range in parenthesis; †: beclomethasone dipropionate or budesonide, 400–800 µg daily. Atopic means one or more positive early responses to allergy skin-prick tests. M: male; F: female; FEV_1: forced expiratory volume in one second; % pred: percentage of predicted; PC_{20}: provocative concentration producing a 20% fall in FEV_1; ND: not determined.

Sputum induction. The method was modified from that described by PIN *et al.* [4]. Subjects were informed of the purpose of the test and the procedure. Inhaled bronchodilator was withheld for 6 h. FEV_1 was measured. The asthmatics in Studies 1 and 2 then received salbutamol 200 µg (two puffs) from a pressurized inhaler, and the FEV_1 was repeated after 10 min. Then nebulized saline was inhaled for three 7 min periods, through a mouthpiece without a valve, without using noseclips. In Study 1, the DeVilbiss was connected by a tube (162 cm long and 2.2 cm in diameter) to the mouthpiece; its output, calculated by weighing the nebulizer before and after use minus the change in the weight of the tube without the subject inspiring, was 2.17 $ml \cdot min^{-1}$ and the aerodynamic mass median diameter (AMMD) was 4.14 µm. The Fisoneb had an output of 0.87 $ml \cdot min^{-1}$ and AMMD of 5.58 µm, and the Pari an output of 0.49 $ml \cdot min^{-1}$ and AMMD of 3.1 µm. After each period of inhalation, the FEV_1 was measured again, and subjects were asked to rinse their mouth with water and attempt to cough sputum into a sterile container. If there was a fall in FEV_1 of ≥20% of the presaline value, or if bothersome symptoms occurred, the inhalation of saline was discontinued and the subject was treated with inhaled salbutamol; this only occurred in four subjects in Study 3. At the end of the periods of inhalation, in Study 1, subjects were asked to grade chest discomfort (tightness, shortness of breath) on a modified Borg scale (mBs) [23]. General discomfort (salty taste, burning or irritation of the throat, gagging) was graded using the same values of the Borg scale. In studies 2 and 3, general and chest discomfort were graded on a 100 mm horizontal visual analogue scale (VAS).

Sputum examination. The collected sputum samples were examined within 2 h as described by POPOV *et al.* [24]. All portions considered to originate from the lower respiratory tract (selected portion of sputum) were selected under an inverted microscope, placed in a preweighed Eppendorf tube, and then weighed. Dithiothreitol DTT (Sputolysin, made by Calbiochem Corp., San Diego, CA, USA), freshly prepared in a dilution of one in 10 with distilled water, was added in a volume (in µl) equal to 1.5 times the weight of the sputum portion (in mg). It was mixed mechanically with the sputum by aspiration in and out of a pipette and further diluted with Dulbecco's phosphate buffered saline (D-PBS) in a volume equal to the sputum plus (DTT). The clear cell suspension was filtered through 52 µm nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) to remove debris and mucus, and was centrifuged at 450×g for 10 min. The pellet was resuspended in a volume of D-PBS equal to the sputum plus DTT volume, and a total cell count was determined using a Neubauer haemocytometer. The number of cells $\times 10^6 \cdot mg^{-1}$ of processed sputum was calculated. The cell suspension was then adjusted to achieve a concentration of 0.75–1.0 $\times 10^3 \cdot mg^{-1}$, 75 µl was placed in each cytocentrifuge cup already in place in a Shandon 3 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA) and cytospins were prepared at 450 rpm for 6 min. Separate cytospin slides were fixed by methanol and Carnoy's solution and were stained, respectively, by the Wright method for an overall differential cell count on 500 nucleated nonsquamous cells and by toluidine blue for metachromatic cell count on 1,500 cells (only in Study 3). Countable cytospins were defined by: 1) low salivary contamination defined as ≤20% squamous epithelial cells; and 2) cell viability >50%.

Statistical analysis

Data are expressed as the mean and standard errors of the mean (SEM). Non-normal distributed data were log or square root transformed before analysis. Paired t-tests and repeated measures analysis of variance (ANOVA) were used to compare the absolute number of cells retrieved from sputum samples obtained under different conditions, weight of sputum, cell viability, differential cell count, FEV_1 change and discomfort scores. McNemar test was used to compare success rates of sputum induction as measured by countable cytospins. Correlations were examined by Pearson correlation coefficient. Significance was accepted at the 95% level.

Results

Effects of different nebulizers (Study 1)

The ultrasonic nebulizers were more successful than the jet nebulizer in inducing sputum as indicated by countable cytospins, weight of selected portion of sputum and total cell counts (table 2). Countable cytospins were obtained in nine out of 15 subjects after sputum induction with DeVilbiss and Fisoneb ultrasonic nebulizers; five of these successes were shared by both devices. Only one of 11 subjects produced sputum with the Pari

Table 2. – Success rate of sputum induction

	Countable cytospins %+	Weight of sputum† mg	Total cell count $\times 10^3$	Cell viability %
Study 1 (n=15)				
De Vilbiss	60	103±48.9	115±54.3	63
Fisoneb	60	114±57.4	88±22.3	76
Pari	9*	6*	13*	79
Study 2 (n=14)				
NS 0.9%	36**	109±43.5	224±102.3	62
HS 3%	64	263±107.9	298±129.7	65
HS 3%	57	226±119.5	258±83.9	68
HS 3–5%	79	297±131.0	425±154.0	73
Study 3				
Salbutamol	80	180±50.9	572±170.5	81
Placebo	70	160±-76.2	722±489.2	80

Results are expressed as mean or mean±SEM (except Study 1, Pari where n=1). +: defined as percentage of subjects in study group producing countable cytospins. †: selected portion. NS: normal saline; HS: hypertonic saline. *: $p<0.05$ between Pari and Fisoneb or DeVilbiss; **: $p<0.05$ between NS and HS 3–5%.

Table 3. – Differential cell counts from sputum samples

	Neutro %	Eosino %	Lympho %	Macro %
Study 1				
De Vilbiss	46.6±11.4	4.6±2.9	2.3±1.0	44.7±9.9
Fisoneb	33.7±7.4	8.6±4.5	2.5±0.7	52.3±10.7
Pari	7.0	0	3.0	89.0
Study 2				
NS 0.9%	42.0±9.2	4.4±3.7	1.8±0.3	40.7±6.7
HS 3%	43.8±7.5	6.7±4.9	2.2±0.5	39.5±5.3
HS 3%	43.0±4.7	5.8±3.1	3.0±0.7	41.2±3.7
HS 3–5%	42.1±4.6	3.2±1.8	3.2±0.7	45.0±5.1
Study 3				
Salbutamol	54.9±10.2	5.4±3.9	1.3±0.4	38.3±10.6
Placebo	72.6±5.6	6.9±4.1	1.2±0.4	21.5±5.4

Data are presented as mean±SEM except for Pari where n=1. For number of samples see Table 2. NS: normal saline; HS: hypertonic saline. Neutro: neutrophils; Eosino: eosinophils; Lympho: lymphocytes; Macro: macrophages.

jet nebulizer. There was no significant difference in the percentage of viable cells from the sputum samples induced with the ultrasonic nebulizers. The differential cell count was similar for the five subjects successful with both DeVilbiss and Fisoneb (table 3).

The general discomfort experienced was proportional to the nebulizer output (fig. 1); it was significantly lower with the Pari jet nebulizer (0.9±0.01) than the DeVilbiss (2.40±0.42) or the Fisoneb (1.87±0.25 (repeated measures ANOVA: F=7.89; p=0.003)). The FEV_1 did not change significantly during the procedures (fig. 2).

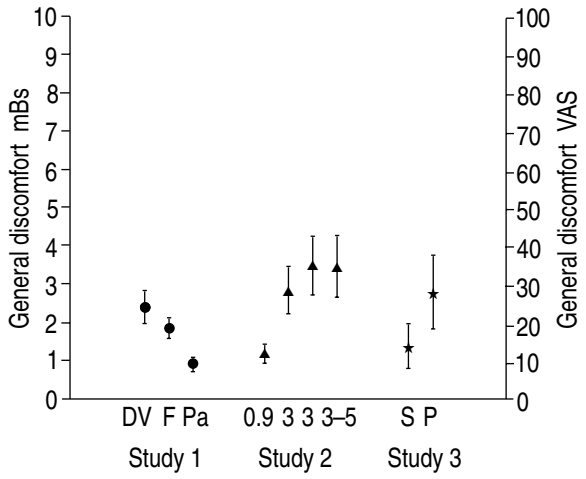

Fig. 1. – General discomfort caused by sputum induction. In Study 1, mean (±SEM) scores indicated by use of a modified Borg scale (mBs) [23] are given. DV: DeVilbiss 99 ultrasonic nebulizer; F: Fisoneb ultrasonic nebulizer; Pa: Pari LL jet nebulizer. In Studies 2 and 3, the scores are given as the mean (±SEM) scored on a horizontal 100 mm visual analogue scale (VAS). 0.9, 3, 3, 3–5%: saline concentrations inhaled; S: pretreatment with salbutamol; P: placebo. Discomfort was significantly greater ($p<0.05$) with the ultrasonic nebulizers, all concentrations of hypertonic saline, and pretreatment with placebo.

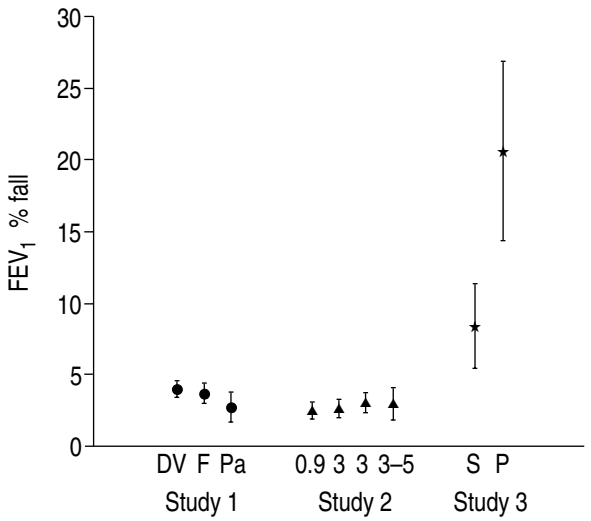

Fig. 2. – Changes in forced expiratory volume in one second (FEV_1), after sputum induction with inhaled saline, expressed as mean (±SEM) % fall from baseline in each study. Study 2: values are % saline. A significant fall ($p=0.01$) was observed only after pretreatment with placebo. For abbreviations see legend to figure 1.

Effects of different saline concentrations (Study 2)

Hypertonic saline 3–5% was more successful than normal saline in inducing sputum, as measured by countable cytospins ($p=0.003$, McNemar test). Cytospins were obtained in five subjects after 0.9% saline, in eight subjects after the first 3% saline, in nine after the second 3% saline, and in 11 after 3–5% saline (table 2). Only six out of the 14 subjects were able to produce sputum on both 3% saline days. The average weight of the selected sample of sputum for processing was higher on all three hypertonic saline days as compared to the normal saline, but the difference within subjects did not reach statistical significance (repeated measures ANOVA: $F=2.64$; $p=0.06$). The absolute number of cells retrieved from the specimens, the cell viability and the differential cell count were similar for all saline concentrations (table 2 and 3).

Less general discomfort from the procedure was experienced after the normal saline day (repeated measures ANOVA: $F=7.04$; $p<0.001$) (fig. 1). There was no difference in the percentage fall in FEV_1 after the procedures (fig. 2).

Pretreatment with salbutamol or placebo (Study 3)

The success of sputum induction after pretreatment with salbutamol or placebo was similar as indicated by countable cytospins, weight of sputum, total cell count and cell viability (table 2). Two subjects could not produce sputum after either salbutamol or placebo. One asthmatic was successful after salbutamol, but induction after placebo was discontinued due to a fall in his FEV_1 of 73% before he could produce any sputum. There was a trend towards higher percentage of neutrophils on the placebo day (table 3); but there were no differences

between eosinophils, macrophages, lymphocytes, and metachromatic cells (0.21±0.11 *vs* 0.23±0.17; p>0.9).

The procedure was more disagreeable after placebo pretreatment as indicated by chest discomfort (30.8±9.6 *vs* 12.3±5.0; p=0.02) (fig. 1). There was a similar trend for general discomfort (28.4±9.5 *vs* 14.3±5.8; p=0.07). The percentage fall in FEV_1 was significantly higher after placebo (20.7±6.2 *vs* 8.4±2.9%; p=0.01) (fig. 2). The magnitude of the fall in FEV_1 was negatively correlated with PC_{20} methacholine on the placebo day (r= -0.76; p=0.01), but not on the salbutamol day (r= -0.51; p=0.2).

Discussion

In these three studies, we have demonstrated that induction of sputum is more successful with hypertonic saline aerosol (3%, or 3–5% sequentially), if it is generated by an ultrasonic nebulizer than when normal saline or a lower output jet nebulizer are used, but is more unpleasant. Salbutamol pretreatment does not influence success but inhibits possible airway constriction caused by the hypertonic saline. None of the variables tested influence sputum cell counts. The results are relevant to the successful induction of sputum and interpretation of results.

This is the first study to investigate the effect of different nebulizers and concentrations of saline aerosols on the success of sputum induction. At the time of writing, there has been only one published abstract of the effect of different concentrations of saline aerosol on induced sputum cell counts [20], and one abstract on the effect of salbutamol pretreatment on the success of sputum induction and cell counts [9]. Our results are similar to these reports, with the exception that IREDALE and co-workers [9] observed a nonsignificant trend for salbutamol to decrease the success of sputum induction.

Our success rates are consistent with our previous studies [4, 5] but lower than those reported by others [7, 9, 10, 25]. The reason for this difference is not known; one possibility is subject selection. In our studies, we selected subjects who could not produce sputum spontaneously, so that the success could be examined; whether this was a criterion in other studies with better success was not recorded. In the present study, increasing success in Studies 1–3 is in keeping with the difference in subject selection. Studies 1 and 2 included healthy subjects (less likely to produce sputum); in Study 3 all subjects were asthmatics. Presumably, the success of sputum induction is influenced by nontechnical factors, such as level of asthma control, the presence of other inflammatory diseases and smoking, as well as technical factors, such as nebulizer output, particle size, concentration of saline aerosol, and any pretreatment to prevent airway constriction. In uncontrolled asthma, for example, there would be more airway mucosal inflammation and the production of sputum would be easier. In uncontrolled asthma, the number of eosinophils in sputum is higher [1]. The percentage of sputum eosinophils in our asthmatic subjects (as illustrated in Study 3) was lower than those reported by IREDALE and co-workers [25], who found more than 10% sputum eosinophils in 70% of their asthmatic subjects. This lack of asthma control might account for the higher success of sputum induction of 96% in the latter study.

We compared the effect of aerosol generation by the Fisoneb with the DeVilbiss 99 ultrasonic nebulizer, because these had already been used to induce sputum in other studies with apparent different results [7, 8, 17, 19], and because the apparently more successful DeVilbiss 99 had an output more than twice that of the Fisoneb. We added the Pari jet nebulizer to the comparison because the output was half that of the Fisoneb. The particle size of the two ultrasonic nebulizers was similarly large and of the jet nebulizer was smaller. We found no difference in the success between the Fisoneb and DeVilbiss, which makes it unlikely that nebulizer output was the reason for the difference in previous studies; the lower output and particle size jet nebulizer was less successful. The lack of difference in the success between with the two ultrasonic nebulizers was unexpected, because one would expect success to be related to dose. Possible reasons why we detected no difference include the method of inhalation and the definition of success. The method of inhalation (three inhalations for 7 min each) may not be the best to compare success; the method which uses the same concentration for increased doubling periods of time [25, 26] may be more discriminatory. Similarly, the method we used to select sputum from saliva and to define success on the quality of cytospins may give a lower success than examining both sputum plus saliva without consideration of cell viability and low contamination with squamous epithelial cells [7, 8]. These possibilities require further investigation.

Sputum induction with hypertonic saline was more successful than with normal saline. Hypertonic saline 3% was not more effective than 3–5% given sequentially. Individual asthmatic subjects were not similarly sensitive to the effects of induction with hypertonic saline on different days. This low repeatability of success, in subjects unable to produce sputum spontaneously, would appear disappointing if one did not consider that, after a failure, induction can be repeated and can be successful.

The mechanism of induction of sputum with hypertonic saline is not understood. Possibilities include a volume effect, an increase in mucociliary clearance, an osmotic effect - drawing more fluid into the airway, and a stimulation of glandular secretions. A volume effect is probably not the cause, because inhalation of normal saline was less effective. Mucociliary clearance has been shown to be increased by inhalation of hypertonic saline in patients with chronic bronchitis and cystic fibrosis [27, 28]. In these studies, the increased clearance could not be entirely accounted for by increased cough. Spontaneous cough was usually not caused by hypertonic saline aerosol in the present studies, also excluding this as an important factor in sputum induction. The sputum was more easily induced at higher saline concentrations (3–5%) than with normal saline. This might be the result of enhanced water influx into the airway lumen in response

to the deposition of hypertonic saline, or to an increased vascular permeability [29], or even due to the increased osmolarity in the periciliary fluid favouring mast cell degranulation and release of histamine and other mediators [30, 31].

We were unable to demonstrate any impairment in sputum production by pretreatment with inhaled salbutamol. Since salbutamol increases mucociliary clearance, an impairment would not be expected [32]. The report by IREDALE and co-workers [9] that there was a trend to inhibition was based on the finding that after salbutamol two of 12 subjects could not produce sputum. Since this trend was not statistically significant, and in Study 2 we observed that sputum induction was not always successful, inhibition of the success of induction by salbutamol is doubtful.

The effect of induction on inflammatory indices in sputum is important to consider. Cell counts were not affected by any of the variables of induction. However, we have consistently observed higher neutrophil counts with dispersed cells examined on cytospins compared with those previously reported in smears of sputum [1, 4]. The reason for this is probably the improved dispersion of the cells after dithiothreitol treatment. The increased counts may be due to less clumping of cells and better recognition of different cell types [24]. Similar results have been observed by others [33]. Whilst cell counts are unaffected by the induction procedure, other indices such as various fluid-phase measurements may be affected and this may require further investigation.

Salbutamol pretreatment is known to prevent airway constriction from hypertonic saline aerosol [34]. The protection afforded in the present studies was good. However, the degree of protection is likely to vary depending on the subjects selected (*e.g.* controlled asthmatics *vs* uncontrolled asthmatics) and the duration of saline inhalation. Sputum induction has been performed successfully and safely without salbutamol pretreatment by IREDALE and co-workers [9]. If no salbutamol pretreatment is given and constriction does occur, it can be reversed and the procedure of hypertonic saline inhalation can be continued if required.

In conclusion, sputum induction is more successful when performed with an ultrasonic nebulizer and hypertonic saline, and cell counts are unaffected. Pretreatment with salbutamol can inhibit bronchoconstriction and does not alter cells count. As a result of these studies we have continued to pretreat with inhaled salbutamol and induce sputum with the Fisoneb ultrasonic nebulizer with sequential doses of 3, 4 and 5% hypertonic saline. However, the Fisoneb is not made to operate for 7 min periods and commonly breaks down. The method of induction might be simplified by the method described by SMITH and ANDERSON [26] for measurement of airway responsiveness, which uses 4.5% saline for increasing times of inhalation. This method needs to be compared with the one described here.

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