Sputum induction as a research tool for sampling the airways of subjects with cystic fibrosis

N R Henig, M R Tonelli, M V Pier, J L Burns, M L Aitken

Abstract

Background—Sputum induction (SI) has proved to be a reliable non-invasive tool for sampling inflammatory airway contents in asthma, with distinct advantages over collection of expectorated sputum (ES) and bronchoalveolar lavage (BAL). A study was undertaken to evaluate the safety of SI and to assess if it might be an equally valuable outcome tool in patients with cystic fibrosis (CF).

Methods—The safety of the procedure was examined and sample volume, cell counts, cytokine concentrations, and bacterial culture results obtained by SI, spontaneous ES, and fibreoptic bronchoscopy were compared in 10 adults with CF.

Results—SI was well tolerated and was preferred to BAL by all subjects. The mean (SE) sample volume obtained by SI was significantly greater than ES (6.74 (1.46) ml v 1.85 (0.33) ml, p = 0.005). There was no significant difference in the number of cells per ml of sample collected. There was a difference in the mean (SD) percentage of non-epithelial, non-squamous cells collected (67 (28)%), 86 (21)%, and 99 (1)% for ES, SI, and BAL, respectively. These percentage counts were different between ES and both SI and BAL (p=0.03 and p=0.006, respectively). Cell differential counts (excluding squamous cells) from all collection methods were similar (mean (SD) 84 (9)%, 87 (7)%, and 88 (11)% polymorphonuclear cells for ES, SI, and BAL, respectively). The concentrations of interleukin (IL)-8 and tumour necrosis factor (TNF)-α were the same in all three samples when corrected for dilution using urea concentration. The test specific detection rate for recovery of bacteriological pathogens was 79% for SI, 76% for ES, and 73% for BAL.

Conclusion—SI offers safety advantages over BAL and may be a more representative airway outcome measurement in patients with CF.

Keywords: cystic fibrosis; sputum induction; bronchoalveolar lavage

Sputum induction (SI) is currently used as a direct non-invasive method for the evaluation of airway inflammation in diseases such as asthma and chronic obstructive pulmonary disease (COPD). The use of SI has contributed greatly to the understanding of the pathogenesis of asthma and COPD and to the effect of interventions on these conditions. SI has also been used in clinical settings to assist in diagnosis or to guide treatment. In the research setting, SI provides an alternative to collecting expectorated sputum (ES) or performing fibreoptic bronchoscopy, both of which have significant limitations. Collection of ES is often difficult in healthy subjects and oropharyngeal secretions invariably contaminate samples. In contrast, SI has been shown to permit the collection of more than 1 ml of sputum from any subject reliably, even those without lung disease or who are non-expectorators. Compared with ES, SI produces a higher proportion of viable cells, less squamous cell contamination, and better quality cytospins. Fibreoptic bronchoscopy, being invasive and costly, is poorly suited for serial sampling of airway secretions. In addition, bronchoscopy with lavage is thought primarily to sample the alveolar space rather than the airways alone. Induction of sputum offers the potential of a rapid, direct, non-invasive, and inexpensive way of collecting multiple samples of airway secretions.

In asthmatic subjects the SI procedure has been shown reliably to provide samples for the evaluation of cells and cell surface markers, cell fractions, soluble inflammatory mediators, and DNA. Although no specific procedure is universally agreed upon, sputum is generally induced by inhalation of the same (3% or 4.5%) or increasing (3%, 4%, 5%) concentrations of hypertonic saline over a set period of time (6–21 minutes with variable intervals of collection). While the concentration of hypertonic saline does not influence the cellular or biochemical markers recovered, the duration of sputum induction does.

Two different techniques have been used for processing the sputum sample once it is obtained. One technique culls out all viscid or dense portions of the sample to separate airway fluid from saliva. Alternatively, saliva can be separated from sputum at the time of collection and the resulting sample may be processed in its entirety. Both sputum processing techniques are shown to be reproducible and valid in their ability to provide differential cell counts and measurements of soluble mediators. No published comparison of the different processing techniques has been performed. Contamination with squamous epithelial cells decreases the validity of the sample and, for that reason, contamination with saliva should be minimised either during the SI procedure or by selecting out viscid plugs. Sputum may be processed with saline, trypsin, or dithiothreitol (DTT) to enhance cell dispersal from the surrounding mucus.
Like asthma, cystic fibrosis (CF) is characterised by airway inflammation. 
Although many patients with CF spontaneously expectorate sputum, many others do not, particularly early in the course of the disease. Fibreoptic bronchoscopy with bronchoalveolar lavage (BAL) has been used frequently as a research tool for evaluating airway contents in patients with CF, especially in those subjects who are symptom free and do not expectorate sputum. 
The possible advantages of SI as a research tool in this population are similar to those in asthmatic subjects—it is direct, non-invasive, inexpensive, and easy to do repeatedly. It might also serve as a research tool to study patients with CF who are unable to expectorate sputum spontaneously, including young children. This study compares SI with BAL and ES as a method for studying airway inflammation in subjects with CF. We have also compared SI with ES and BAL for the recovery of bacterial and fungal pathogens.

Methods

SUBJECTS
Subjects were recruited from the CF clinics at the University of Washington Medical Center and the Children’s Hospital and Regional Medical Center, Seattle, WA, USA. All subjects were over 18 years of age and gave written informed consent to the study which was approved by the Human Subjects Committees of the University of Washington. Subjects had initial forced expiratory volume in one second (FEV) of >50% predicted and were clinically stable. They remained on their usual medications, including any long term antibiotics, during the study period.

STUDY DESIGN
All subjects underwent bronchoscopy and sputum induction on separate days within a seven day period. The procedure order alternated from subject to subject. ES was randomly collected before either bronchoscopy or SI.

MEASUREMENT OF PULMONARY FUNCTION
On the day of sputum induction, spirometric parameters were measured at baseline, 10 minutes after inhalation of 180 µg albuterol but before SI, and 5 minutes after the completion of SI using a Cybermedic spirometer (Cybermed Inc, Boulder, CO, USA) according to American Thoracic Society (ATS) guidelines.

FIBREOPTIC BRONCHOSCOPY
Subjects fasted for a minimum of 4 hours before the procedure. Viscous lidocaine and cocaine 4% solution were used to anaesthetise the nasal septum. Conscious sedation was achieved with intravenous midazolam 1–4 mg and fentanyl 25–100 µg. Lidoine hydrochloride 2% solution was used for local anaesthesia of the airways. Flexible bronchoscopy was performed with a fibreoptic bronroscope (Pentax FB-15H, Pentax FB-19H, or Olympus BF P20D). A transnasal approach was used in eight subjects and a transoral approach was used in two subjects with nasal polyposis. During bronchoscopy no suction was applied until the tip of the bronchoscope was below the vocal cords. The bronchoscope tip was directed immediately to the right upper lobe and wedged into a subsegment. The right upper lobe was selected for sampling because this area is usually the most radiographically involved in CF. BAL was performed by instillation of two 30 ml aliquots of sterile non-bacteriostatic saline and recovered using wall suction 180 mm Hg into a sterile 70 ml specimen trap. Small volume BAL was performed in an attempt to optimise airway, rather than alveolar, sampling.

SPUTUM INDUCTION
Subjects were premedicated with albuterol 180 µg via metered dose inhaler prior to the SI procedure. Using the protocol described by Fahy et al., subjects inhaled nebulised sterile hypertonic saline (3%) solution for 12 minutes from an Ultra-Neb 99 ultrasonic nebuliser (DeVilbiss, Somerset, PA, USA). This nebuliser generates particles of a mean mass median diameter of 4.5 µm and has an output of 2.4 ml/min. After expelling saliva, subjects were encouraged to cough 2 minute intervals. The sputum was collected in a sterile plastic container for further processing.

EXPECTORATED SPUTUM (ES)
ES was collected immediately before, but on the same day as either the BAL or SI procedures. Subjects were asked spontaneously to expectorate into a sterile plastic collection container. All sputum produced over a 10–15 minute period was collected.

SAMPLE PROCESSING
All specimens were processed within 30 minutes of collection. Expectorated and induced sputum samples were processed in the same manner. The volume of sputum collected was measured and an equal volume of DTT 0.1% (Sputolyasin; Behring Diagnostics Inc, Somerville, NJ, USA) was added. The sample was then mixed gently by vortex mixer and placed in a shaking water bath at 37°C for 15 minutes. Periodically, the samples were removed from the water bath for further brief gentle mixing with a plastic transfer pipette to ensure visible homogenisation; 1 ml was removed for quantitative culture. The homogenised sputum sample was used to determine the total cell count and cytopsins were prepared and stained (Hema 3, Fisher Scientific, Santa Clara, CA, USA). The remainder of the homogenised sputum was centrifuged at 2000 rpm for 5 minutes. The supernatants were aspirated and frozen at −70°C for later analysis. Slides were batched and a blinded observer (MVP) counted 500 non-squamous epithelial cells and cell differentials per sputum preparation.

The volume of recovered BAL fluid was measured and then centrifuged at 2000 rpm for 5 minutes. The BAL supernatant was aspirated and frozen at −70°C for later analysis. The cell pellet was resuspended in 2.5 ml normal saline and an equal volume of DTT 0.1% (Sputolyasin) was added. The sample was then
mixed in the same manner as the sputum to ensure visible homogenisation and 1 ml was removed for quantitative culture; 1 ml was also removed for total and differential cell counting. Sputum for cell counts were prepared, stained, and examined as above.

QUANTITATIVE CULTURE
Quantitative microbiology was performed as previously described. The 1 ml aliquots removed for microbiological culture were plated within 2 hours of addition of DTT. Solubilised samples were serially diluted and plated directly onto the following media: MacConkey, OFPBL, DNase, streptococcal selective, mannitol salt, *Haemophilus* selective, and Mycosel agars. The MacConkey, DNA, and Mannitol salt plates were incubated in a 35°C ambient air incubator for 48 hours and the OFPBL plates were incubated in a 35°C ambient air incubator for 72 hours. Selective streptococcal and *Haemophilus* agar plates were incubated anaerobically at 35°C for 48 hours. Mycosel plates were incubated in a 30°C ambient air incubator for 5 days. Colony forming units per gram of sample were determined by counting the number of bacterial colonies of each organism growing on the selective media. The organisms were identified by standard biochemical tests.

CYTOKINE DETERMINATION
IL-8 and TNFα were measured using a quantitative “sandwich” enzyme immunoassay technique (Quantikine, R&D Systems, Minneapolis, MN, USA). Samples were run in triplicate and compared with a standard curve.

UREA DETERMINATION
There was enough remaining sample in eight of the 10 patients to determine urea concentrations in all three samples. Using the assumption that ES was an undiluted sample and that both SI and BAL samples were diluted, the correction factor varied from 0.6 to 2.5.

SUBJECT PREFERENCE
At the completion of the study subjects were asked which of the two procedures, bronchoscopy or SI, they preferred. The same investigator (NRH) asked all subjects.

DATA ANALYSIS
An ANOVA was performed for the following end points: sample volume, total and differential cell counts, and cytokine concentrations. Test specific detection rates were calculated for each method of sputum sampling based on pathogen recovery. A χ² test was used to compare proportions of overall pathogen detection. For each organism recovered kappa statistics were calculated to examine the extent of agreement beyond that which would be expected on the basis of chance alone. Kappa (κ) values of 0 were considered to represent chance agreement while values of 1 represent perfect agreement.

Results

**DEMOGRAPHIC AND SAFETY PROFILE**
Eleven subjects entered the study. One subject did not complete the study because of a severe vasovagal event associated with insertion of an intravenous line. No other adverse events occurred and there were no significant changes in forced expiratory volume in one second (FEV₁) before and after the SI procedure (fig 1). Ten subjects (8 men) completed the study. Their mean age was 27 years (range 19–38, median 28) and the mean (SE) baseline FEV₁ was 3.38 (0.29) l/min. Four subjects were prescribed rhDNase, one of whom was also on inhaled tobramycin and oral doxycycline. Three subjects were taking a single oral antibiotic—either ciprofloxacin, amoxicillin/clavulanic acid, or cephalexin.

Following bronchoscopy all patients were monitored with blood pressure, pulse oximetry, and continuous ECG tracing for 30 minutes. No adverse events occurred in that time interval. Moreover, no patient telephoned the investigators with an acute pulmonary exacerbation or pneumonia in the month following the procedure.

All subjects preferred SI to bronchoscopy. Three patients reported asymptomatic improvement after SI.

**SAMPLE VOLUMES**
The mean (SE) sample volume collected by each method was 1.85 (0.33) ml for ES, 6.74 (1.46) ml for SI, and 17.65 (2.18) ml for BAL.

**TOTAL AND DIFFERENTIAL CELL COUNTS**
The mean (SE) total cell counts/ml sample collected were 6.17 (1.66) × 10⁶ for ES, 8.00 (3.34) × 10⁶ for SI, and 4.81 (1.86) × 10⁶ for BAL (fig 2A). There was no significant difference in the total number of cells/ml of sample collected (p > 0.05 for all comparisons). There was a difference in the non-epithelial,
Table 1  Test specific detection rates* for culture of ES, SI, and BAL.

<table>
<thead>
<tr>
<th>Organism</th>
<th>ES</th>
<th>SI</th>
<th>BAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> mucoid</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> non-mucoid</td>
<td>1/4</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td><em>B. cepacia</em></td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td><em>S. maltophilia</em></td>
<td>3/4</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td><em>A. xylosoxidans</em></td>
<td>1/2</td>
<td>1/2</td>
<td>0/2</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>2/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7/7</td>
<td>6/7</td>
<td>7/7</td>
</tr>
<tr>
<td><em>Aspergillus</em></td>
<td>4/6</td>
<td>5/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

Overall sensitivity: 25/33 (76%) 26/33 (79%) 24/33 (73%)

*Number positive by specified test/number positive by any test.

Table 2  Quantification of colony counts from ES and BAL fluid compared with SI.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>±1 log₁₀</th>
<th>±2 log₁₀</th>
<th>±3 log₁₀</th>
<th>±4 log₁₀</th>
<th>≥5 log₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SI</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BAL</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Microbiology**

The presence and quantitation of a specific set of organisms was compared between SI, ES, and BAL. Mucoid and non-mucoid *P. aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Achromobacter* (formerly *Alcaligenes*) *xylosoxidans*, *Haemophilus influenzae*, and *Aspergillus* were selected because of their presumed relevance in CF lung infection, based in part on their inclusion in the Cystic Fibrosis Foundation Patient Registry. The test specific detection rate for each of the three methods (SI, ES, and BAL) was determined using the presence of any of the three cultures as an indicator of the presence of disease (table 1). The test specific detection rate of SI culture was 79% compared with 76% for ES and 73% for BAL (p = 0.68). Between the three methods of sample collection, cultures showed the following: perfect agreement (κ = 1) for recovery of mucoid *P. aeruginosa* and *B. cepacia*; high agreement (κ = 0.6–1) for recovery of *S. aureus* and *Aspergillus*; moderate to high agreement (κ = 0.4–0.7) for recovery of non-mucoid *P. aeruginosa* and *S. maltophilia* with the exception of SI v BAL where there was low agreement (κ = 0.2). *A. xylosoxidans* was detected in one subject by ES alone and in one subject by SI alone. *H. influenzae* was detected in one subject by ES alone, in one subject by BAL alone, and in three subjects by SI and ES but not BAL. Organisms isolated from SI culture that were missed by either both ES or BAL cultures included *S. maltophilia*, non-mucoid *P. aeruginosa*, and *Aspergillus*, each in a single patient.

Quantitation of organisms in each sample was also compared (table 2). A urea dilution factor was made in eight of the 10 patients in whom there was enough sample to measure urea concentrations in each sample. Colony counts ranged from 6.0 × 10^4 to 1.1 × 10^6 cfu/g specimen. For mucoid *P. aeruginosa*, *B. cepacia*, and *S. maltophilia*, the majority of SI cultures were within one log₁₀ of the ES cultures. Comparing SI with BAL, the colony counts of mucoid *P. aeruginosa* and *S. aureus* in SI cultures were within one log₁₀ in seven of the 11 specimens. For the other pathogens the BAL specimens had lower colony counts than the SI specimen. In the four instances in which organisms were detected on SI but not BAL, colony counts were ≤10^4, and <100 in two of those instances.

**Inflammatory mediators**

Two mediators of airway inflammation in CF were evaluated. IL-8 was detected in all subjects and in all samples. The concentrations with and without urea dilution correction are

Figure 2  (A) Total cell counts per ml of original sample. There was no significant difference in the number of cells per ml of sample collected (p>0.05 for all comparisons). The percentage of non-epithelial, non-squamous cells was different between expectorated sputum (ES) and both sputum induction (SI) and bronchoalveolar lavage (BAL; p=0.03 and p=0.006, respectively). (B) Differential counts of non-squamous cells. The cell populations were similar with each of the three sampling procedures.

Table 2  Quantification of colony counts from ES and BAL fluid compared with SI.

<table>
<thead>
<tr>
<th>Organism</th>
<th>±1 log₁₀</th>
<th>±2 log₁₀</th>
<th>±3 log₁₀</th>
<th>±4 log₁₀</th>
<th>≥5 log₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SI</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BAL</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ES = expectorated sputum; BAL = bronchoalveolar lavage; SI = sputum induction.

*In 8/10 patients a urea dilution correction was performed.
Table 3  Cytokine concentrations in expectorated sputum (ES), induced sputum (SI), and bronchoalveolar lavage (BAL) fluid

<table>
<thead>
<tr>
<th></th>
<th>ES</th>
<th>SI</th>
<th>BAL fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 (ng/ml)</td>
<td>18.0 (12.2)</td>
<td>12.8 (7.5)</td>
<td>4.0 (3.0)*</td>
</tr>
<tr>
<td>Urea corrected IL-8 (ng/ml) (n=8)</td>
<td>13.4 (11.0)</td>
<td>13.5 (7.1)</td>
<td>13.9 (17.9)</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>102 (101)*</td>
<td>45 (27)</td>
<td>18 (19)</td>
</tr>
<tr>
<td>Urea corrected TNFα (pg/ml) (n=8)</td>
<td>80 (71)</td>
<td>42 (42)</td>
<td>43 (42)</td>
</tr>
</tbody>
</table>

There was a difference in IL-8 concentration in SI from that in ES and BAL (p=0.01 and p=0.03, respectively). When a dilution correction was made these differences were no longer seen. There was a difference in TNFα concentration between SI and ES (p=0.03) but this was no longer seen when corrected for dilution.

shown in table 3. Without urea correction IL-8 (ng/ml) was lower in BAL fluid than in either ES or SI (p<0.05). TNFα was also detected in the three sample types. Concentrations of TNFα in SI were significantly greater than in BAL fluid (p = 0.03), but again there was no difference when a urea dilution correction was made. Urea corrected TNFα concentrations in BAL fluid and ES remained different (p=0.01).

Discussion

SI was well tolerated and preferred to BAL by all subjects. SI resulted in larger sample volumes than ES, a higher percentage of non-squamous cells than ES, higher colony counts, and tended toward higher detection rates for CF specific pathogens. Concentrations of the cytokines IL-8 and TNFα when corrected for dilution were the same.

Induction of sputum with hypertonic saline has been shown to be safe in patients with moderate to severe asthma and was associated with no adverse events in our 10 subjects with CF. In fact, three subjects reported feeling better after the procedure. Clinical improvement may be the result of an effect on airway clearance, consistent with results from a recent 2 week clinical trial of nebulised saline in CF. Hypertonic saline with cough has been shown to enhance mucociliary clearance significantly. Administration of a β2 agonist prior to SI was done for safety, but it too may have had a therapeutic effect and contributed to the subjects’ sense of improvement after SI. Neither the concentration of hypertonic saline nor pretreatment with β2 agonists alters the differential cell counts in induced sputum.

In contrast to ES, all subjects undergoing SI produced sample volumes of more than 1 ml, the minimum volume often necessary to perform routine assays and reproducible cell counts. Less squamous cell contamination occurred in the SI samples than in ES. Previous studies have shown that the method of processing CF sputum affects cell counts, often erroneously lowering counts because of poor cell dispersal. We used DTT to process our samples so that our results could be more easily compared with studies using SI in subjects with asthma; however, even higher cell counts may be achieved with methods such as enzymatic digestion. The populations of non-squamous cells seen in the cell differential counts were the same for SI, ES, and BAL, suggesting that each method was sampling primarily airway populations.

The microbiological results demonstrated a potential advantage of SI culture over both ES and BAL. Overall, the sensitivity of isolation of organisms from SI culture was slightly (although not statistically) better than both ES and BAL. SI missed a single isolation of S. aureus, but otherwise performed better overall than the traditional “gold standard” for lower airway culture, BAL. In addition, SI recovered higher colony counts, a benefit that could be an advantage in the research setting.

In children with chronic pulmonary infection oropharyngeal suction has been used to identify respiratory tract pathogens with a sensitivity of 89% compared with BAL. Since SI tended to be more sensitive than BAL for detecting CF respiratory pathogens, it may be useful both as a research tool and as a clinical method for identifying respiratory pathogens in CF.

Inflammatory mediators were found in concentrations equivalent to or greater than those previously reported in patients with CF. The presence of hypertonic saline may increase the amount of Na+ and Cl– in airway surface liquid and rehydrate the periciliary fluid. Hyperosmolarity has been shown to stimulate IL-8 production in human bronchial epithelial cells in vitro. However, we doubt that a 12 minute exposure to hypertonic saline in vivo would allow adequate time to increase intracellular IL-8 production.

Limitations of this preliminary study are that it did not look at cell viability or reproducibility of results in any given subject. Both of these outcomes have been looked at in normal subjects, asthmatic patients, and those with COPD. In other patient populations it has been shown that cell viability is enhanced with SI compared with ES, and that results from SI in these populations are reproducible.

Sputum induction has become a well validated research tool for evaluating a variety of indices of inflammation, both cellular and humoral, in non-CF diseases of the airways such as asthma. This study shows that SI is well tolerated and provides valid information regarding the markers of inflammation and the microbiology of the airways in subjects with CF. As a research tool, SI offers many advantages over bronchoscopy or the collection of spontaneously expectorated sputum. Compared with bronchoscopy, SI could obviate the need for multiple invasive procedures in appropriately chosen longitudinal studies, it is inexpensive, and is preferred by patients. Compared with BAL, the disadvantages of SI are that sequential sampling of the same subsegmental airway is not possible and the technique cannot be taught to very young children. Improving upon expectorated sputum, subjects can be well at the time of study and potential subjects, including children, who do not spontaneously expectorate need not be excluded from research protocols. Sputum induction therefore represents an alternative to standard methods of sampling the airways in patients with CF, with potential value in both the research and clinical setting.
Supported by a grant from the Firland Sheltered Workshop.


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