Induced Sputum*

Comparison Between Isotonic and Hypertonic Saline Solution Inhalation in Patients With Asthma

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Background: Sputum induction by hypertonic saline solution inhalation is widely used to study airways secretions in patients with asthma. However, hypertonic saline solution is a potent indirect bronchoconstrictor.

Study objectives: We studied the validity of isotonic saline solution (0.9%) inhalation as a means to induce sputum by comparing it to hypertonic saline solution (4.5%) inhalation.

Patients: Sixteen patients with moderate-to-severe asthma reporting a clinical history of mucus hypersecretion.

Methods: Subjects underwent sputum induction twice at 1-week intervals. Saline solution (hypertonic or isotonic) was inhaled for three periods of 5 min. The parameters assessed in sputum samples were cell counts, sodium, eosinophil cationic protein (ECP), and albumin concentrations, osmolality, and pro-matrix metalloproteinase (MMP)-9 activity by zymography.

Results: The maximal fall in peak expiratory flow during sputum induction was greater after inhalation of hypertonic saline solution than after inhalation of isotonic saline solution (p < 0.01).

Each subject produced analyzable sputum on both visits. There were no statistically significant differences in total and differential sputum cell counts, and the reproducibility coefficients were high for eosinophils and neutrophils when comparing the two methods. Likewise, sputum levels of ECP and albumin as well as sputum pro-MMP-9 activity were not different between the two methods, and were highly reproducible as shown by high intraclass coefficients (Ri) of correlation (0.72, 0.74, and 0.77 for ECP, albumin, and pro-MMP-9, respectively). Sputum sodium concentrations and osmolality were higher after inhalation of hypertonic saline solution (p < 0.05).

Conclusion: In patients with moderate-to-severe asthma reporting a clinical history of mucus hypersecretion, inducing sputum by isotonic or hypertonic saline solution inhalation leads to comparable results in eosinophil and neutrophil cell counts and fluid phase mediators/proteins.

(CHEST 2001; 120:1815–1821)

Key words: albumin; asthma; eosinophil cationic protein; metalloproteinases; osmolality; sputum

Abbreviations: ECP = eosinophil cationic protein; MMP = matrix metalloproteinase; PBS = phosphate-buffered saline solution; PEF = peak expiratory flow

Since its first description a few years ago,1 sputum induction by inhalation of hypertonic saline solution has been validated and is now widely used to study airways secretions in patients with airway diseases such as asthma.2 This method, which is inexpensive and does not require complex instrumentation, has the advantage of being less invasive than bronchoscopy and BAL. Its safety has been studied in patients with asthma and found to be good even in patients with more severe disease.3,4 The short-term reproducibility of the method has been extensively assessed5,6 and found to be good with respect to cell counts and a number of soluble markers of inflammation.

Hypertonic saline solution inhalation is, however, a potent bronchoconstrictor stimulus, acting through mast cell activation7–9 and release of sensory neuropeptides.10 It has been hypothesized that the release of these mediators might modify vascular
tine in pulmonary vessels.11 Although sputum induction is generally safe, breakthrough bronchoconstriction may develop in some patients during the procedure despite premedication with β2-agonists.3,4 Given the potential occurrence of breakthrough bronchoconstriction and the increased sensation of breathlessness12 after hypertonic saline solution inhalation, it is important to assess the validity of isotonic saline solution inhalation as a means of inducing sputum in subjects with poor lung function. To our knowledge, there have been few studies12–15 comparing isotonic and hypertonic saline solution as sputum inducers in patients with asthma. These previous works investigated the total and differential cell counts and did not find any significant difference between the two methods of induction. However, the authors of one of these studies13 have shown an increase in methacholine responsiveness following hypertonic saline solution inhalation, thereby suggesting that hypertonic saline solution inhalation per se might cause mediator release in the airways.

In this study, we compared the cellular and biochemical composition of induced sputum in patients with moderate-to-severe asthma after inhalation either of hypertonic or isotonic saline solution. With respect to the biochemical components, eosinophil cationic protein (ECP) and albumin were chosen as markers of eosinophil activation and plasma exudation. Matrix metalloproteinase (MMP)-9 was assessed because of its potential involvement in airway remodeling occurring in patients with asthma,16 and as a marker of granulocytes degranulation.17,18 We have also compared the bronchoconstriction occurring during the procedure by assessing the fall in peak expiratory flow (PEF) rates during the procedure.

### Materials and Methods

#### Patients

Sixteen subjects were enrolled who had asthma according to the criteria established by the American Thoracic Society (Table 1).19 Asthma was diagnosed as a clinical history of recurrent cough, wheeze, breathlessness, and bronchial hyperresponsiveness. Nonspecific bronchial hyperresponsiveness was demonstrated either by reversibility of airway obstruction, with an increase in FEV1 > 15% and 200 mL after inhalation of 400 µg of salbutamol when baseline FEV1 was < 80% predicted, or by methacholine airway hyperresponsiveness as defined by a provocative concentration of methacholine causing a 20% fall in FEV1 of < 8 mg/mL when baseline FEV1 was > 70% predicted. All subjects were classified with moderate or severe persistent asthma according to the Global Initiative for Asthma guidelines.20

Subjects were excluded from the study if they had a respiratory tract infection or exacerbation of the disease during 1 month preceding the study. All subjects reported a clinical history of hypersecretion in the airways, but some patients had difficulty producing expectorate and none had evidence of bronchiectasis based on chest radiography.

All of the subjects attended the laboratory twice 1 week apart and randomly inhaled isotonic or hypertonic saline solution, and were blinded to the saline solution concentration. Sputum induction was performed on both occasions at the same time of day (± 1 h). The protocol of the study was approved by the local ethics committee, and all subjects gave their written informed consent.

#### Sputum Induction and Processing

Sputum induction was performed by inhalation of hypertonic saline (NaCl 4.5%) or isotonic saline solution (NaCl 0.9%) 15 min after premedication with 400 µg of inhaled salbutamol. Aerosols were generated by an ultrasonic nebulizer, the output of which was set at 1.5 mL/min.

The subjects inhaled saline solution aerosols for a fixed period of 15 min. A measurement of PEF was performed before the procedure and after every 5-min period. The inhalation of saline solution was discontinued if the fall in PEF was > 20% from the

### Table 1—Subject Characteristics*

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Sex</th>
<th>Age, yr</th>
<th>FEV1p, mL</th>
<th>FEV1p % Predicted</th>
<th>FEV1 After β2- Agonist, % Predicted</th>
<th>PC20 mg/mL</th>
<th>Tobacco Use</th>
<th>Atopy</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>70</td>
<td>1,904</td>
<td>68</td>
<td>90</td>
<td>ND</td>
<td>Past (5 PY)</td>
<td>Yes</td>
<td>β2-Agonist on demand</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>69</td>
<td>1,323</td>
<td>49</td>
<td>61</td>
<td>ND</td>
<td>Past (7 PY)</td>
<td>No</td>
<td>β2-Agonist on demand</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>26</td>
<td>2,170</td>
<td>76</td>
<td>ND</td>
<td>3.2</td>
<td>0</td>
<td>Yes</td>
<td>β2-Agonist on demand</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>70</td>
<td>1,799</td>
<td>64</td>
<td>78</td>
<td>ND</td>
<td>Current (9 PY)</td>
<td>Yes</td>
<td>Fluticasone, 500 µg/d</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>52</td>
<td>1,534</td>
<td>47</td>
<td>64</td>
<td>ND</td>
<td>Current (5 PY)</td>
<td>No</td>
<td>β2-Agonist on demand</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>70</td>
<td>1,790</td>
<td>73</td>
<td>ND</td>
<td>3.8</td>
<td>0</td>
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<td>7</td>
<td>M</td>
<td>70</td>
<td>1,058</td>
<td>35</td>
<td>44</td>
<td>ND</td>
<td>Past (8 PY)</td>
<td>No</td>
<td>β2-Agonist on demand</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>29</td>
<td>2,963</td>
<td>67</td>
<td>112</td>
<td>0.148</td>
<td>Past (2 PY)</td>
<td>Yes</td>
<td>Budesonide, 1,600 µg/d</td>
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<tr>
<td>9</td>
<td>F</td>
<td>56</td>
<td>953</td>
<td>41</td>
<td>62</td>
<td>ND</td>
<td>0</td>
<td>Yes</td>
<td>Prednisone, 8 mg/d</td>
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<tr>
<td>10</td>
<td>M</td>
<td>50</td>
<td>3,400</td>
<td>87</td>
<td>0.45</td>
<td>Current (7 PY)</td>
<td>Yes</td>
<td>β2-Agonist on demand</td>
<td></td>
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<tr>
<td>11</td>
<td>F</td>
<td>29</td>
<td>2,540</td>
<td>90</td>
<td>0.19</td>
<td>0</td>
<td>Yes</td>
<td>Fluticasone, 1,000 µg/d</td>
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</tr>
<tr>
<td>12</td>
<td>F</td>
<td>20</td>
<td>2,910</td>
<td>94</td>
<td>6.8</td>
<td>0</td>
<td>Yes</td>
<td>β2-Agonist on demand</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>68</td>
<td>2,222</td>
<td>72</td>
<td>2.3</td>
<td>Current (9 PY)</td>
<td>Yes</td>
<td>β2-Agonist on demand</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>56</td>
<td>2,011</td>
<td>55</td>
<td>ND</td>
<td>0</td>
<td>No</td>
<td>Fluticasone, 1,000 µg/d</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>36</td>
<td>1,990</td>
<td>54</td>
<td>70</td>
<td>ND</td>
<td>Current (5 PY)</td>
<td>Yes</td>
<td>β2-Agonist on demand</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>52</td>
<td>2,539</td>
<td>67</td>
<td>94</td>
<td>ND</td>
<td>Past (8 PY)</td>
<td>No</td>
<td>β2-Agonist on demand</td>
</tr>
</tbody>
</table>

*M = male; F = female; PC20 = provocative concentration of methacholine causing a 20% fall in FEV1; ND = not determined; PY = pack-years.
basal state. Subjects were encouraged to expectorate sputum after mouth rinsing with tap water every 5 min. Samples were collected in a plastic container and kept at 4°C until processing. Samples were diluted twice with phosphate-buffered saline solution (PBS) containing 10 mM dithiotreitol (Calbiochem; San Diego, CA) and centrifuged at 400 g for 10 min at 4°C in order to separate cellular and fluid phases. The cell pellet was resuspended in PBS containing 20 mM ethylenediaminetetra-acetic acid and 2% human serum albumin (Dade Behring; Marburg, Germany). Cell counts were performed on samples centrifuged (cytospin) and stained with Diff Quick (Dade; Brussels, Belgium), and supernatants were stored at −80°C until analysis. Cell viability was measured using the trypan blue exclusion method.

Measurement of ECP, Albumin, Sodium Concentrations, and Osmolality

ECP was measured by fluoroimmunoassay (ECP Fluoroimmunoassay Unicap System; Pharmacia; Uppsala, Sweden). The detection limit of the assay was 2 ng/mL. Albumin levels were measured in the supernatant of induced sputum by rocket immunoelectrophoresis as previously described.21 Ten microliters of sputum supernatant was mixed with the same amount of nonreducing electrophoresis buffer. Electrophoresis was carried out on a sodium dodecyl sulfate 10% polyacrylamide gel containing gelatin at a concentration of 1 mg/mL. Gels were then incubated in 2.5% (v/v) octoxynol-9 (Triton X-100) for 30 min and soaked for 16 h in an activation buffer containing 10 mM CaCl₂ and 100 mM NaCl at 37°C. The gels were rinsed and stained 30 min in Coomassie brilliant blue G250. Gelatinase activity was detected as a white lysis zone against a blue background. Quantitative evaluation of the gelatinolytic activity was then performed by scanning the gels using a Bio-Rad GS 700 Imaging Densitometer (Bio-Rad; Richmond, CA). On each gel, internal standard consisted of a graded dilution of HT1080-conditioned medium containing high amounts of gelatinases A and B.22 Samples were diluted until the optical density resulting from gelatinolysis was in the linear part of the standard curve. Results were expressed as arbitrary units of pro-MMP-9 activity.

Figure 1. Decrease of PEF expressed as percent of predicted during sputum induction by isotonic (0.9%) and hypertonic (4.5%) saline solution inhalation. PEF values were recorded before and during inhalation of isotonic (0.9%) or hypertonic (4.5%) saline solutions. Horizontal bars represent mean values. Inhalation of hypertonic saline solution caused a significantly greater decrease in PEF than isotonic saline solution (p < 0.05).
per milliliter of sputum. Molecular weights were estimated relative to the molecular weight markers “broad range” (Bio-Rad; Hercules, CA).

**Statistical Analysis**

Falls in PEF during sputum induction with isotonic or hypertonic saline solution were expressed as mean ± SEM, compared using a Student’s t test for paired data. Results of sputum cell counts and mediator measurements were expressed as median (range). Comparisons between the results of the two visits were performed using paired Wilcoxon rank test. Differences were considered to be significant at p < 0.05.

Repeatability was expressed as intraclass correlation coefficient (Ri). This was calculated by a repeated-measures analysis of variance and represented the following ratio: (variance between subjects – residual variance) / (variance between subjects + residual variance + 2 [variance between subjects]). Its value may vary between –1 and +1, and a value > 0.6 reflects substantial agreement between two different measurements. The differences in mediator levels in sputum fluid phase between the two visits were shown graphically by plotting the difference against the mean as recommended by Bland and Altman.

**Results**

**Effect of Sputum Induction on Airway Caliber**

The mean fall in PEF was greater (p < 0.05) after inhalation of hypertonic saline solution than after isotonic saline solution (Fig 1). Mean ± SEM PEF fell from 83.8 ± 5.2% to 71.4 ± 4.7% predicted during the inhalation of hypertonic saline solution (p < 0.0001), and from 81.8 ± 3.9% to 77.25 ± 4.4% predicted after the inhalation of isotonic saline solution (p < 0.01). Five of 16 subjects had a fall in PEF > 20% after inhalation of hypertonic saline solution, necessitating the discontinuation of the procedure after 10 min in three patients. However, none had severe bronchospasm requiring admission to the emergency department.

**Weight of Sputum Samples**

All subjects produced sputum at both visits. The total amount of expectorate was significantly higher when the subjects inhaled hypertonic saline solution (mean, 4.4 g; range, 1.6 to 9.9 g) than isotonic saline solution (mean, 2.5 g; range, 0.8 to 5 g) [p < 0.001; Table 2].

**Total and Differential Sputum Cell Counts**

There was no significant difference between the two methods of induction in the quality of samples as judged by the viability and the extent of squamous cell contamination (Table 2). Similarly, there were no statistically significant differences regarding the total and differential cell counts expressed either as absolute or relative values between sputum induced by hypertonic and isotonic saline solutions. Furthermore, the repeatability was good (intraclass correlation coefficient > 0.6) for total cell counts and polymorphonuclear neutrophils and eosinophils when expressed as a percentage. By contrast, the repeatability of lymphocytes and epithelial cells counts was poor (Table 2).

**Sputum Fluid Phase Mediator/Protein Levels and Gelatinolytic Activities**

There was no difference regarding the levels of ECP, albumin, and pro-MMP-9 activity between sputum induced by hypertonic or isotonic saline solutions (Fig 2; Table 2). In addition, the reproducibility of ECP, albumin, and pro-MMP-9 activity measurement was good as demonstrated by high intraclass coefficient of correlations reaching 0.72, 0.74, and 0.77 for ECP, albumin, and pro-MMP-9, respectively (Fig 2).

### Table 2—Comparison of Sputum Composition*

<table>
<thead>
<tr>
<th>Sputum Components</th>
<th>Inhalation of NaCl 4.5%</th>
<th>Inhalation of NaCl 0.9%</th>
<th>Wilcoxon Test p Value</th>
<th>Reproducibility Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>4.4 (1.6–9.9)</td>
<td>2.5 (0.8–5)</td>
<td>&lt; 0.001</td>
<td>0.06</td>
</tr>
<tr>
<td>Viability, %</td>
<td>98 (77–100)</td>
<td>96 (77–100)</td>
<td>&gt; 0.05</td>
<td>0.36</td>
</tr>
<tr>
<td>Squamous cells, %</td>
<td>7.5 (2–24)</td>
<td>13.5 (1–30)</td>
<td>&gt; 0.05</td>
<td>0.18</td>
</tr>
<tr>
<td>Total cell counts, 10⁶/g</td>
<td>2.08 (0.12–81.6)</td>
<td>2.26 (0.08–53.6)</td>
<td>&gt; 0.05</td>
<td>0.61</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>30.7 (3.6–88.8)</td>
<td>22.7 (2.6–69)</td>
<td>&gt; 0.05</td>
<td>0.41</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>3.5 (0.2–51.2)</td>
<td>3.2 (0–59.8)</td>
<td>&gt; 0.05</td>
<td>0.72</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>50 (5–94)</td>
<td>54.3 (11–97)</td>
<td>&gt; 0.05</td>
<td>0.62</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>0.8 (0–5.60)</td>
<td>0.7 (0–10.6)</td>
<td>&gt; 0.05</td>
<td>0.29</td>
</tr>
<tr>
<td>Epithelial cells, %</td>
<td>2.2 (0–18)</td>
<td>2.2 (0–11)</td>
<td>&gt; 0.05</td>
<td>0.33</td>
</tr>
<tr>
<td>ECP, μg/L</td>
<td>58.2 (6–1,230)</td>
<td>130.7 (14.2–1,297.5)</td>
<td>&gt; 0.05</td>
<td>0.72</td>
</tr>
<tr>
<td>Albumin, mg/L</td>
<td>115.2 (25–1,665)</td>
<td>113.3 (1–1,035.4)</td>
<td>&gt; 0.05</td>
<td>0.74</td>
</tr>
<tr>
<td>MMP-9, arbitrary units/mL</td>
<td>5,953 (290–45,455)</td>
<td>8,790 (290–66,667)</td>
<td>&gt; 0.05</td>
<td>0.77</td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>143 (90–175)</td>
<td>134 (85–154)</td>
<td>&lt; 0.05</td>
<td>0.43</td>
</tr>
<tr>
<td>Osmolality, mosm/kg</td>
<td>272 (193–335)</td>
<td>250 (102–309)</td>
<td>&lt; 0.01</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Results are expressed as median (range).
Osmolality and Sodium Concentration

Sputum sodium concentrations and osmolality were significantly higher in the samples obtained after inhalation of hypertonic saline solution (p < 0.05; Table 2).

Discussion

This study demonstrates that in asthmatics reporting a clinical history of mucus hypersecretion, analysis of sputum induced by inhalation of hypertonic (4.5%) saline solution and isotonic (0.9%) saline solution yields comparable and reproducible results regarding the eosinophil and neutrophil cell counts and fluid phase mediators. By contrast, and not surprisingly, both sodium concentration and osmolality are higher in samples recovered after hypertonic saline solution inhalation.

We found herein that inhalation of hypertonic saline solution in our asthmatic subjects caused a breakthrough bronchoconstriction in most of the cases despite premedication with 400 μg of salbutamol. Five of our subjects experienced a fall ≥ 20% in PEF during inhalation of hypertonic saline solution, while falls in PEF remained < 20% in all subjects after inhalation of isotonic saline solution. Popov et al.12 reported that there were no differences regarding bronchoconstriction between the two methods, but the patients studied in that study had less severe conditions than our patients.

The amounts of expectorate collected after inhalation of hypertonic saline solution were greater than those obtained after inhalation of isotonic saline solution. This is unlikely to be due to increased plasma exudation, since albumin levels were found to be very similar between the two conditions. Hypertonic saline solution inhalation has been described to enhance clearance of bronchial secretions25 and to induce cough.26 These are plausible explanations for the increased amount of sputum recovered after hypertonic saline solution inhalation. Another explanation would be that the amount of saliva produced augments in sputum, but this is not supported by the absence of significant differences in the squamous cell counts between the two methods of induction. The quality of the samples recovered was equally good between the two methods. A previous study27 comparing sputum produced spontaneously or induced by hypertonic saline solution showed a lower cell viability in the former. Our results show that cell viability was excellent and similar between the two methods.
methods of induction. This suggests that the samples induced by isotonic saline solution inhalation do not correspond to “old” airways secretions, as may be the case for spontaneously produced sputum. In addition, the percentages of squamous cells were always < 31% and not different between the two methods.

In agreement with previous studies, we did not find any statistically significant difference with respect to total and differential cell counts between sputum induced by hypertonic and isotonic saline solutions. We chose to perform the two inductions at a 7-day interval in order to avoid any influence of the first induction on the cellular composition of the second sputum sample, as has been shown for neutrophil counts. As data indicate that neutrophil counts may vary according to the duration of inhalation, we chose to keep the time of induction constant at 15 min for both isotonic and hypertonic saline solution. In accordance with previous data, and although our subjects inhaled different saline solution concentrations, we found good reproducibility for eosinophils and neutrophils expressed as a percentage of total cell counts.

The effects of hyperosmolality on inflammatory cells may lead either to activation or inhibition of exocytosis and cell adhesion by the regulation of expression of different genes. We describe herein for the first time (to our knowledge) that inhalation of 4.5% saline solution (1,541 mosm/kg) leads to a significant increase in sputum osmolality despite being probably buffered by the epithelial lining fluid and the dilution in PBS. As expected, this increase in sputum osmolality is due to a raised sodium concentration (143 mmol/L vs 134 mmol/L). Nevertheless, the osmolality of collected sputum remains within the physiologic range. We cannot rule out, however, that endobronchial osmolality during the hypertonic saline solution challenge could reach supraphysiologic levels, which might promote granulocyte degradation. Therefore, we have assessed mediators contained in granules from eosinophils and neutrophils and released in response to different stimuli. We did not find any significant difference in the levels of ECP and MMP-9 between samples collected after inhalation of hypertonic or isotonic saline solutions, suggesting that hypertonic stimulus does not induce degranulation of endobronchial granulocytes. In a rat model, inhalation of hypertonic saline solution (3.6 to 14.5%) caused a dose-related increase in vascular permeability as demonstrated by the extravasation of monastral blue. As we did not find raised sputum albumin levels after inhalation of hypertonic saline solution, we have no argument to support that inhalation of 4.5% saline solution aerosol for 15 min may cause a rise in bronchial vascular permeability in humans in vivo.

In summary, we conclude that, when compared to sputum induced by hypertonic saline solution, sputum induced by isotonic saline solution inhalation yields comparable results regarding the eosinophil and neutrophil cell counts and fluid phase mediators/proteins in subjects with moderate-to-severe asthma reporting a clinical history of bronchial hypersecretion. Furthermore, isotonic saline solution causes much less bronchoconstriction than hypertonic saline solution in those patients. Thus, in such asthmatic patients, we recommend performing sputum induction with isotonic saline solution.

ACKNOWLEDGMENT: We thank Professor Gielen for his assessment of osmolality and sodium, and J. Sele and M. Henket for technical support.

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