Induced Sputum Evaluation in Microwave Popcorn Production Workers
Muge Akpinar-Elci, Kimberly J. Stemple, Paul L. Enright, John V. Fahy, Toni A. Bledsoe, Kathleen Kreiss and David N. Weissman
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Objective: Severe airways obstruction and bronchiolitis obliterans have been reported in microwave popcorn production workers and attributed to inhalation of flavoring agents. We investigated whether exposure to flavoring agents is associated with airways inflammation in popcorn production workers.

Methods: Fifty-nine workers with high exposures and 22 patients with low exposures to flavoring vapors completed a questionnaire, spirometry, and sputum induction. Sputum cell counts were categorized as “high” if greater than (and “low” if less than or equal to) the median cell counts of a healthy external control group (n = 24). We compared high- and low-exposure groups as well as all workers with control subjects.

Results: Neutrophil concentrations in nonsmoking workers were significantly higher than those of the healthy nonsmoking control group (p < 0.05). The smoking-adjusted odds ratio for high neutrophil count (> 1.63 × 10⁶/mL) was 3.8 (95% confidence interval, 1.3 to 11.5) in the high-exposure group compared with the low-exposure group. Sputum interleukin-8 and eosinophil cationic protein levels were higher in high-exposure workers than in low-exposure workers (p < 0.05). For the worker group, mean values of FEV₁, percentage of predicted and FEV₁/FVC percentage of predicted were > 95%. There were no relationships between sputum characteristics and the presence of airways obstruction.

Conclusions: High exposure to popcorn flavoring agents is associated with neutrophilic airway inflammation in popcorn production workers. These data provide further evidence that popcorn production workers face a significant occupational hazard through exposure to flavoring agents.

Key words: airway inflammation; bronchiolitis obliterans; cytokines; diacetyl; flavoring; occupation

Abbreviations: BOS = bronchiolitis obliterans syndrome; ECP = eosinophil cationic protein; IL = interleukin; NIOSH = National Institute for Occupational Safety and Health; OR = odds ratio

The history of popcorn dates back many centuries. It is believed that popcorn originated in Mexico; the oldest ears of corn ever found in Mexico were about 6,000 years old. In the late 1800s, popcorn was sold all across America by street vendors using steam- or gas-powered poppers. In 1907, electric-powered corn poppers made their catalog debut. Some of the popcorn flavorings used during this era...
included orange and lemon juice, rose, peppermint, honey, vanilla, molasses, and sugar. Microwave heating was first used in the 1940s. Today, contemporary products such as microwave popcorn are made using a variety of chemicals as flavoring agents, to which popcorn production workers can be exposed.

The National Institute for Occupational Safety and Health (NIOSH) recently reported on a microwave popcorn production plant where nine former workers showed severe fixed airways obstruction. Two of the former workers underwent biopsies and had findings compatible with bronchiolitis obliterans. Current workers reported excessive rates of respiratory symptoms and had high rates of airways obstruction. The popcorn production plant produces both flavored microwave popcorn and unflavored kernel popcorn. Inhalation exposure to a flavoring mixture was implicated as an etiologic agent. Diacetyl (2, 3-butanedione), a ketone with butter flavor characteristics, was the predominant compound.

Since exposure to a flavoring mixture was associated with severe airways obstruction, we hypothesized that exposure would also be associated with airway inflammation. To test this hypothesis, we measured airway inflammation by assessment of induced sputum obtained from workers in the microwave popcorn production plant. Induced sputum analysis is a reproducible, valid, and noninvasive method for studying airway inflammation. Use of induced sputum has been studied extensively in asthma and to a somewhat lesser degree in COPD. It has been shown that induced sputum can be used to study interstitial lung diseases such as pneumoconioses, sarcoidosis, and nonspecific interstitial lung diseases. Induced sputum has also been studied in occupational lung diseases by using this technique, we measured inflammatory cells, interleukin (IL)-8, and eosinophil cationic protein (ECP), and we tested for an association between these sputum characteristics and exposure to flavorings, the presence of respiratory symptoms, and abnormal lung function.

**Materials and Methods**

**Study Population**

This study was conducted in a microwave popcorn plant in November 2001. Of 149 current workers, 140 participated in the survey. Of the 140 participants, 32 were excluded from sputum induction: 27 subjects had medical contraindications from β-blocker use (n = 8), cardiac problems (n = 5), recent operation (n = 2), recent pneumothorax (n = 1), pregnancy (n = 2), or postbronchodilator baseline FEV₁ < 60% of predicted (n = 9). Five subjects were excluded because they had worked at the plant for < 1 month. Common cold and other upper airways infections diseases were among exclusion criteria, but according the pretest questionnaire none of the participants had any infectious diseases before starting the induced sputum procedure. Of the 108 participants undergoing sputum induction, analyzable induced sputum samples were obtained from 81 subjects. Sixteen workers failed to produce sputum, 8 subjects produced inadequate samples, and 3 workers experienced a decline in FEV₁ during induction sufficient to halt the procedure. The mean age (± SD) of participant workers with analyzable sputum samples was 33.8 ± 11.9 years. Forty-nine workers were male, and 38 workers reported current smoking. There were 59 workers in the high-exposure group, with a history of high flavoring exposure in mixing, microwave packaging, maintenance, and quality control. A low-exposure group included 22 workers from office, polyethylene packaging, warehouse, and outside sections of the plant. Diacetyl exposure over the preceding year for the two exposure groups differed by up to two orders of magnitude (Fig 1). Normal control values for sputum cell counts were obtained from a previously characterized group of 24 healthy, never-smoking, nonasthmatic subjects (41.6% men; mean age, 33.8 ± 9.3 years) participating in airway research at the University of California, San Francisco.

**Questionnaire**

After obtaining written informed consent (approved by the NIOSH Human Studies Review Board), trained interviewers administered a standard questionnaire. Additional questions were added for irritation, systemic and respiratory symptoms, and occupational information. Data were collected on demography, allergic and respiratory symptoms, family and personal history of respiratory and allergic diseases, smoking, and occupational history including previous work history.

**Spirometry**

Trained NIOSH technicians followed American Thoracic Society recommendations for spirometry prior to inducing sputum. The test results were compared to the lower limit of normal values from the National Health and Nutrition Examination Survey III reference values to identify workers with abnormal spirometry results. Airways obstruction was defined to be the combination of FEV₁ and FEV₁/FVC percentage of predicted below the lower limit of normal. We calculated FEV₁ slopes if workers had more than two spirometry measurements between November 2000 and August 2003. During this period of 2.75 years, there were a total of eight spirometry tests offered to these workers.

**Sputum Induction**

Participants inhaled 3% saline solution for a total of 12 min from an ultrasonic nebulizer (model 65; DeVilbiss; Somerset, PA). This nebulizer generates particles with a mean mass median diameter of < 4 μm, with an output of 6 mL/min. To reduce salivary contamination of induced sputum samples, we had the subjects spit out saliva into separate containers just prior to attempts at coughing up sputum. Subjects began quiet breathing on the mouthpiece and every 2 min were asked to take the mouthpiece out, spit all saliva into a saliva cup, replace the mouthpiece, take a full breath, hold their breath, remove the mouthpiece, and cough the full breath out along with any sputum into the sputum cup. Halfway through the test (after 6 min on the nebulizer), FEV₁ was measured and compared to baseline. If FEV₁ fell below 80% of the baseline FEV₁, another FEV₁ maneuver was obtained to confirm the drop. If the FEV₁ remained below 80%, the test was stopped and the participant was administered an inhaled bronchodilator.
Sputum Processing

Sputum samples were collected, stored on ice, and processed within 2 h of collection. A standardized method for processing whole sputum was used. After collection, sputum volume was determined. Sputum was liquefied by addition of an equal volume of 0.1% dithiothreitol in saline solution (10% Sputolysin; Behring Diagnostics; Somerville, NJ) followed by incubation on a shaking water bath at 37°C for 15 min. Cell concentration in liquefied sputum was determined by mixing equal volumes of diluted homogenized sputum and Turks solution and counting cells in a hemocytometer. To determine sputum differential cell counts, homogenized sputum was diluted in saline and cytospins prepared using a cytocentrifuge (Cytospin II; Shandon Scientific; Sewickley, PA). Cytospins were fixed in alcohol and stained (Diff-Quik; Baxter Scientific Products; Miami, FL). For cell differentiation, 500 cells per sample were examined. Cell differentials exclusive of squamous cells were determined by microscopic examination of stained cytospins. Concentrations of various cell types (cell counts) were calculated as the product of total cell concentration times percentage cell type. Remaining homogenized samples were centrifuged at 1,037 g for 5 min. The supernatant was aspirated and stored in aliquots at –70°C for later analysis of selected soluble constituents.

Biochemical Assays

IL-8 concentrations in homogenized sputum supernatants were determined using a commercially available fluorometric bead array assay (Upstate Biotechnology; Lake Placid, NY) read on a Luminex-100 system (Luminex Corporation; Austin, TX). ECP concentrations in homogenized sputum supernatants were determined using a commercially available fluorescence enzyme immunoassay (Pharmacia ImmunoCAP; Pharmacia Diagnostics; Kalamazoo, MI).

Industrial Hygiene Evaluation

From November 2000 through November 2001, NIOSH performed four industrial hygiene surveys consisting of inspection of the entire microwave popcorn production process and air sampling for butter-flavoring chemicals. Quantitative area sampling and personal air sampling were performed for diacetyl. Diacetyl was collected on carbon molecular sieve tubes at a flow rate ranging from 30 to 150 mL/min and was analyzed quantitatively by gas chromatography according to NIOSH method 2557.

Statistics

Since sputum cell counts from the healthy control group were not normally distributed, we determined median values (neutrophils, 1.63 ± 10^5/mL; eosinophils, 0.0/mL; macrophages, 3.88 ± 10^5/mL; and lymphocytes, 0.11 ± 10^5/mL) and used these values as cutoff points to categorize cell counts into two groups: high and low. Since current smoking is known to affect cell count, we adjusted our analysis for associations between exposures, symptoms, or lung function and airways inflammation for smoking status using the Mantel-Haenszel approach. We did not adjust for age and gender, since preliminary analyses showed that these factors were not associated with the results. We compared the IL-8 and ECP results stratified by gender, symptoms, spirometry, current smoking status, and exposure group using Mann-Whitney U tests. Differences were considered significant at a level of p < 0.05. Analyses were performed using statistical software (version 11.01; SPSS; Chicago, IL).

Results

Among popcorn production workers mean FEV1 percentage of predicted and mean FEV1/FVC percentage of predicted were both > 95% (Table 1). Obstruction was present in 4 of 59 high-exposure workers and 2 of 22 low-exposure workers (Table 2). There were no significant differences between the high-exposure and low-exposure groups for spirometric values. The proportion of individuals with obstruction was not significantly increased in smokers.
There was no significant difference on sputum sampling volume between groups. Overall percentages of sputum cell types among popcorn workers were as follows: neutrophils, 69.6/11006 23.1; eosinophils, 0.4/11006 0.8; macrophages, 24.5/11006 20.2; and lymphocytes, 0.4/11006 0.5. When we evaluated the distribution of sputum absolute cell counts as shown in Figure 2, we observed a significantly higher neutrophil concentration in nonsmoking workers compared with the healthy nonsmoking control group (p < 0.05).

We calculated smoking-adjusted odds ratios (ORs) to assess relationships between various symptoms or work characteristics and high sputum cell counts (Table 3). The high-exposure group had an increased risk for a high neutrophil count (OR, 3.8; 95% confidence interval [CI], 1.3 to 11.5). However, the presence of phlegm, chest tightness, wheezing attack, fever, chills, skin, nasal or eye irritation symptoms, physician-diagnosed asthma and COPD, or obstructive spirometry results were not associated with elevated sputum cell counts. Although we did not find any significant relationship between FEV1 slopes and sputum inflammatory markers, workers with the high neutrophil count and low neutrophilic count showed mean 40 mL/yr and 25 mL/yr decreases in mean FEV1 slopes, respectively (p > 0.05).

In this report, we analyzed absolute cell counts since they were most closely associated with cytokine levels. We observed significant correlations between IL-8 and absolute neutrophil count (Pearson r = 0.33, p = 0.003) and between ECP and absolute eosinophil count (Pearson r = 0.80, p < 0.001).

Sputum IL-8 and ECP were significantly higher in the high-exposure group of workers when compared to the low-exposure group (p < 0.05) [Table 4]. Since we observed a gender difference in IL-8 levels, we compared IL-8 levels in high-exposure and low-exposure groups of workers by adjusting with gender in general linear models. The difference between high-exposure workers (estimated marginal mean IL-8, 2,726.8; 95% CI, 2,092.9 to 3,444.3) and low-exposure workers (estimated marginal mean IL-8, 1,277.7; 95% CI, 627.1 to 2,157.6) remained significant (F = 6.755, p = 0.011). Elevated levels of

### Table 1—Demographic Characteristics of Workers and Healthy External Control Subjects*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Workers</th>
<th>Healthy External Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-Exposure Group (n = 59)</td>
<td>Low-Exposure Group (n = 22)</td>
</tr>
<tr>
<td>Age, yr†</td>
<td>31.3 ± 11.5</td>
<td>40.6 ± 10.4</td>
</tr>
<tr>
<td>Male gender</td>
<td>54.2</td>
<td>77.3</td>
</tr>
<tr>
<td>Current smoker</td>
<td>52.5</td>
<td>31.8</td>
</tr>
<tr>
<td>Tenure, yr†</td>
<td>2.9 ± 4.0</td>
<td>7.7 ± 5.7</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>95.9 ± 13.0</td>
<td>95.0 ± 14.6</td>
</tr>
<tr>
<td>FEV1/FVC % predicted</td>
<td>96.6 ± 9.9</td>
<td>96.0 ± 9.5</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD or %. †Significant difference between high-exposure and low-exposure groups (p < 0.01).

### Table 2—Median Percentage of Sputum Cell Types of Popcorn Production Workers and External Control Subjects by Smoking Status and Spirometric Obstruction*

<table>
<thead>
<tr>
<th>Variables</th>
<th>High-Exposure Group (n = 59)</th>
<th>Low-Exposure Group (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Obstruction</td>
<td>Without Obstruction</td>
</tr>
<tr>
<td>Smokers</td>
<td>n = 1</td>
<td>n = 30</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>85.6</td>
<td>66.7</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Macrophages</td>
<td>12.6</td>
<td>25.9</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>n = 3</td>
<td>n = 25</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>31.9</td>
<td>83.9</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>32.4</td>
<td>13.1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Data are presented as %. Cell differentials do not add up to 100% because epithelial cells are not shown.
sputum IL-8 were associated with symptomatic complaints of phlegm and fever (data not shown). We did not observe any relationship between respiratory or other symptoms and ECP. There were no significant differences between nonsmoking and smoking workers in sputum levels of IL-8 or ECP.

**DISCUSSION**

Previous publications\(^2\,^3\) showed that inhalation exposure to an artificial flavoring mixture used in producing microwave popcorn was implicated as causing disease. In this study, we evaluated if airway inflammation was associated with flavoring exposures among popcorn production workers by using induced sputum, and we found an association between flavoring exposures and neutrophilic inflammation.

Our analysis showed that high exposure to popcorn flavoring agents is significantly associated with neutrophilic airway inflammation in popcorn production workers. Absolute neutrophil counts were higher in nonsmoking popcorn production workers than in a nonsmoking healthy external control group. Even after controlling for smoking, the relationship between high exposure to flavoring agents and neutrophilic inflammation remained significant. This further analysis supported the evidence that popcorn production workers are prone to a significant occupational hazard through exposure to flavoring agents. Even though exposure levels were lowered by engi-

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**Table 3—Smoking-Adjusted Mantel-Haenszel ORs and 95% CIs for Selected Symptoms and Other Variables With High Cell Counts per Milliliter in 81 Popcorn Production Workers**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Prevalence, %</th>
<th>High Neutrophils, OR (95% CI)</th>
<th>High Eosinophils, OR (95% CI)</th>
<th>High Macrophages, OR (95% CI)</th>
<th>High Lymphocytes, OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cough</td>
<td>35.8</td>
<td>1.2 (0.4–3.89)</td>
<td>1.8 (0.7–4.7)</td>
<td>1.4 (0.4–4.6)</td>
<td>0.5 (0.1–1.7)</td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>35.8</td>
<td>1.0 (0.3–2.8)</td>
<td>2.6 (1.0–6.8)</td>
<td>1.1 (0.3–3.4)</td>
<td>0.4 (0.1–1.4)</td>
</tr>
<tr>
<td>Wheezing</td>
<td>25.9</td>
<td>1.3 (0.4–4.5)</td>
<td>2.4 (0.8–6.9)</td>
<td>1.6 (0.5–5.4)</td>
<td>0.2 (0.0–1.3)</td>
</tr>
<tr>
<td>Flu-like aches</td>
<td>44.4</td>
<td>1.0 (0.4–2.8)</td>
<td>1.9 (0.7–4.7)</td>
<td>1.2 (0.4–3.8)</td>
<td>1.7 (0.5–5.2)</td>
</tr>
<tr>
<td>High-exposure group</td>
<td>72.8</td>
<td>3.8 (1.3–11.5)</td>
<td>1.8 (0.6–5.0)</td>
<td>5.4 (0.6–45.3)</td>
<td>2.4 (0.5–11.9)</td>
</tr>
<tr>
<td>Airways obstruction</td>
<td>7.4</td>
<td>1.8 (0.2–17.5)</td>
<td>1.5 (0.3–8.2)</td>
<td>1.0 (0.1–9.0)</td>
<td>1.2 (0.1–10.8)</td>
</tr>
</tbody>
</table>

*Higher than median cell count of external healthy control subjects.

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**Figure 2.** Mean sputum absolute cell counts and SEs are shown with an error bar of popcorn production workers by smoking status compared to an external healthy control group. *Statistically significant (p < 0.01) difference compared with the healthy control group.
neering controls in April 2001, the chronic effect of previous exposures might have continued.

Neutrophilic airways inflammation is a potentially important underlying factor in development of the bronchiolitis obliterans syndrome (BOS) previously documented in workers at this plant. Increased BAL neutrophils have been associated with the BOS occurring after lung transplantation. Elevated BAL fluid neutrophil percentages as well as levels of the granulocyte activation markers myeloperoxidase and ECP appear to be early signs of development of BOS in lung transplant recipients. Beeh et al reported that total sputum cell counts and percentage of neutrophils were increased in chronic transplant rejection patients, and concluded that sputum neutrophilia reflects an underlying inflammatory process involved in the pathogenesis of chronic rejection, promoting inflammation, tissue damage, and airway wall remodeling.

An unexpected finding in our study was the lack of a significant relationship between neutrophilic inflammatory markers in sputum and spirometric airways obstruction. Several factors might have accounted for this finding. For medical safety reasons, we excluded the nine most severe obstructive cases from undergoing induced sputum collection. This is the major limitation of field studies. Although we keep basic medical emergency and ambulatory needs handy, it is not possible to provide intensive medical care in the field. This prevented us from including severe obstructive cases in the study. Three additional participants did not complete sputum collection due to >20% decreases in FEV₁. After these exclusions, there were only six obstructive cases in our study group, which might have been insufficient to show a significant relationship between inflammation and airways obstruction. In addition, our study was cross-sectional and so would not detect a relationship between airways inflammation and obstruction if they occur at different times in the pathophysiologic process of the disease. This conclusion is consistent with our finding that workers with elevated neutrophil counts showed a somewhat higher FEV₁ decline in the slope analysis than the low-neutrophil count group. Finally, it is possible that obstruction in popcorn workers is at least in part due to processes other than neutrophilic airways inflammation.

Among popcorn production workers, sputum IL-8 and ECP concentrations were higher in the high-exposure group than the low-exposure group. IL-8 was recognized as a key chemokine to induce neutrophil recruitment and activation. The sputum concentration of IL-8 appeared to be closely associated with the degree of airflow obstruction in patients with COPD. Although ECP has been regarded as an eosinophil-specific protein, current evidence suggests that it is not a unique marker for eosinophils. Proteins immunohistochemically indistinguishable from ECP are present in peripheral blood neutrophils. High levels of sputum ECP have been observed in subjects without associated sputum eosinophilia. Finally, there is a direct correlation between sputum ECP levels and sputum neutrophils in normal individuals. Thus, elevated sputum ECP levels in our high-exposure workers are consistent with neutrophilic airways inflammation.

In conclusion, analysis of induced sputum showed that high exposure to artificial flavorings in this plant was associated with neutrophilic airways inflammation. This observation provided further support for previous reports suggesting an occupational hazard through exposure to flavoring agents in popcorn production workers.

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