Neutrophils in induced sputum from healthy children: Role of interleukin-8 and oxidative stress

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KEYWORDS
Induced sputum; Neutrophil differential count; Interleukin-8; Oxidant stress; Healthy children

Summary
Background: It is unclear why the neutrophil differential count in induced sputum (IS) from normal children is highly variable. Since levels of neutrophil chemoattractant cytokines and oxidative stress are determinants of airway neutrophilia in animal models, we sought to determine the association between IS neutrophils from healthy children and (i) interleukin-8 (IL-8) and (ii) the oxidative stress marker 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG).

Methods: IS was done using hypertonic saline. The proportion (differential %) and the absolute number of IS neutrophils were determined by light microscopy. IS IL-8 and 8-oxodG were determined using ELISA. Spearman’s rank correlation (Rs) was used to assess relationship between variables.

Results: Adequate IS samples for analysis were obtained from 64/114 healthy children. The median (interquartile range) neutrophil differential count (n = 64) was 20.6% (5.67–56), and absolute neutrophil count (n = 53) was 0.11 × 10⁶ (0.01–0.77). Both the % neutrophils, and the absolute neutrophil count were associated with IL-8 (Rs = 0.67, p < 0.0001 and 0.60, p < 0.0001, respectively), but there was no association between IS neutrophil variables and 8-oxodG (n = 40). The repeatability (intraclass correlation coefficient—Ri) of the neutrophil differential count was 0.58 (n = 15).

Conclusions: The variation in the proportion and number of neutrophils in the lower airway of healthy children is associated with IL-8, but not with oxidative stress. The IS neutrophil differential count in healthy children is relatively stable over several months. © 2007 Elsevier Ltd. All rights reserved.
Neutrophils in induced sputum from healthy children

Background

Induced sputum (IS) is a well established, non-invasive, method for sampling lower airway inflammatory cells from children. Several studies of children with respiratory disease, have also reported IS neutrophil variables from healthy controls. A wide range for the IS neutrophil differential count is a consistent finding of these studies. For example, Cai et al. reported that the interquartile range for the IS % neutrophils in healthy children was between 12% and 88.2% with a median of 35%. In adults, the IS neutrophil count increases with age, but to date, no demographic variable has been reported to be associated with IS neutrophils in normal children. In animal models, a major determinant of increased neutrophil transmigration into the airway is the potent neutrophil chemoattractant interleukin-8 (IL-8). Increased levels of airway IL-8 have been reported to be associated with increased IS neutrophils in a range of respiratory conditions such as asthma, chronic cough, and cystic fibrosis. From these data, we hypothesised that variation in the spontaneous release of IL-8 by resident lung cells drives the normal variation in airway neutrophils in children. Since oxidative stress stimulates IL-8 release by lung cells via oxidant-sensitive transcription factors, we further hypothesised that levels of oxidative stress are associated with increased levels of both IS IL-8 and neutrophils. In this study, we sought to test these hypotheses by assessing the association between IL-8, oxidative stress, and neutrophil variables in IS samples from healthy children. Oxidative stress cannot be measured directly in IS samples. We therefore measured 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a stable marker generated by radical damage to DNA and the deoxyribonucleotide pool.

Methods

Subjects and study design

The study was conducted in Leicester (UK). Children were recruited as part of a study into the association between exposure to particulate air pollution and lung function. The study protocol was approved by the Institutional Review Board (Leicestershire Research Ethics Committee). Parents of healthy children gave written informed consent, and children gave written assent. Subject characteristics are described previously. In brief, healthy children aged 8–15 years from non-smoking families were recruited. Children with history of respiratory symptoms, respiratory infection in last 3 months, personal smoking and passive smoking (confirmed by salivary cotinine) were excluded.

Sputum induction and processing

Lung function was recorded using a Vitalograph 2120 spirometer (Vitalograph Ltd., Buckingham, UK) with Vitalograph 2120 Spirotrac IV software (Vitalograph Ltd.) as described previously. Sputum induction was done by a standard methodology using nebulised 4.5% saline via an ultrasonic nebuliser (Sonix 2000 nebuliser, Clement Clarke International, Harlow, UK) in sequential 5 min inhalations. IS was processed by a standard technique. To assess the stability (repeatability) of the neutrophil differential count, repeat IS samples were obtained in a subgroup of children after 6 months.

Interleukin-8

IL-8 in IS supernatants was analysed according to an established ELISA assay, using a BD OptEIA™ set for human IL-8 (BD Biosciences Pharmingen, San Diego, CA, USA). IL-8 was expressed as ng/ml; sensitivity level of the assay was 0.8 × 10⁻³ ng/ml.

8-oxo-7,8-dihydro-2′-deoxyguanosine

8-oxodG in IS supernatant was analysed by a competitive ELISA according to the manufacturer’s protocol (Japan Institute for the Control of Aging, Fukuroi City, Japan). The range of the assay’s calibration curve was 0.5–200 ng/ml (1.77–706 pmol/ml). 0.1% Dithiothreitol (DTT) was added to standards and incubated as for samples to assess effect of DTT on the assay. DTT (0.1%) did not interfere with the ELISA for 8-oxodG (data not shown).

Statistics

All data are summarised as the median and interquartile range (IQR; Q1, Q3). Data were tested for normality by Kolmogorov–Smirnov test, and since most were non-normally distributed, the Spearman’s rank correlation was used to assess relationship between variables. p-Values <0.05 were considered statistically significant. The repeatability on two occasions was determined by intraclass correlation coefficient (RI) and represented graphically by plotting the difference against the mean as suggested by Bland and Altman. Statistical analysis was done using SPSS (version 12.0.1 for Windows).

Results

Adequate samples were obtained from 64/114 healthy children meeting the study inclusion criteria (35 boys). Absolute IS leucocyte counts were determined in 53 subjects, and IS differential counts (%) in all 64 children. Squamous cell contamination was low (median 2.6% (IQR 0.60–8.48)), indicating an acceptable lower airway sampling. Consistent with previous studies, there was a wide range of the IS neutrophil differential count (IQR 5.6–56, Table 1). There was no association between demographic variables (age, height, weight, and percent predicted FEV₁) and IS neutrophil variables (Table 2). There was no association between demographic variables and IS IL-8 concentration (Table 2). However, both IS neutrophil variables were significantly associated with IL-8 (%; Rs = 0.67, p < 0.0001, and absolute count; Rs = 0.60, p < 0.0001, Table 2, Fig. 1). There was no association between IS 8-oxodG and IL-8 (n = 40), or between 8-oxodG and IS neutrophil variables (n = 40, Table 2). Fifteen children had a repeat IS 6 to 8 months later. The intraclass correlation coefficient (RI) for neutrophil differential in this subgroup was 0.58 (Fig. 2),
indicating a moderate degree of repeatability (stability) in the neutrophil differential count over several months.

**Discussion**

This is the first study to examine the determinants of the neutrophil differential count and absolute IS neutrophil count in healthy children. Our data for the neutrophil differential count are similar to those previously reported for normal children, suggesting that the study population was representative. However, the age range of children in the present study was small, and an effect of age on IS neutrophil in healthy children cannot be excluded.

The major finding of our study is that, as hypothesised, IS neutrophil variables are associated with IS IL-8 levels. Since both the neutrophil % and absolute count increased with increased IL-8, the most likely explanation is that IL-8 induces increased numbers of systemic neutrophils to migrate into the airway. These data are compatible with the known function of IL-8 as a potent chemotactic cytokine. Indeed, IL-8-mediated neutrophil recruitment into the airway has been demonstrated in several inflammatory conditions in children and adults, but its role in recruiting neutrophils into the healthy lung is unclear. However, our data support the preliminary observation by Gibson et al., who found an association between the IS neutrophil count and IL-8 in 8 healthy adults. Although we have not identified the source of airway IL-8, a resident lung that has the capacity to both spontaneously release IL-8, and to increase IL-8 release by epithelial cells, is the alveolar macrophage (AM). We therefore speculate that the normal variation in the neutrophil count results from variations in spontaneous release of IL-8 from AM. The putative environmental or genetic variables associated with variations in spontaneous release, and the specific role of AMs, merit further study.

We found no association between 8-oxodG and either the concentration of IL-8, or the IS neutrophil variables. Thus, our hypothesis that variations in IL-8 are driven by oxidative stress is not supported. Oxidative stress has the capacity to induce neutrophilia through oxidant sensitive chemokines, and 8-oxodG was detected in IS samples from healthy children, using a robust and reliable ELISA technique. Furthermore, we excluded the possibility that DTT might interfere with the 8-oxodG assay. The presence of 8-oxodG in IS from all the healthy children studied suggests

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**Table 1** Induced sputum leukocyte differential, IL-8 and 8-oxodG.

<table>
<thead>
<tr>
<th>Analysis parameter</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count ($n = 53$) ($\times 10^6$/ml)</td>
<td>0.52 (0.18–1.35)</td>
</tr>
<tr>
<td>Absolute neutrophil count ($n = 53$) ($\times 10^6$/ml)</td>
<td>0.12 (0.02–0.78)</td>
</tr>
</tbody>
</table>

**Table 2** Relationship between induced sputum neutrophil %, IL-8 and 8-oxodG to demographic factors and lung function.

<table>
<thead>
<tr>
<th>Analysis parameter</th>
<th>Absolute neutrophil count ($\times 10^6$/ml)</th>
<th>Neutrophil (%)</th>
<th>IL-8 (ng/ml)</th>
<th>8-oxodG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.027 0.850</td>
<td>-0.159 0.21</td>
<td>-0.182 0.15</td>
<td>-0.119 0.46</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>-0.06 0.671</td>
<td>-0.241 0.055</td>
<td>-0.127 0.32</td>
<td>-0.119 0.46</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.032 0.820</td>
<td>-0.128 0.31</td>
<td>-0.110 0.39</td>
<td>-0.083 0.61</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>-0.139 0.319</td>
<td>-0.173 0.172</td>
<td>-0.095 0.457</td>
<td>0.237 0.14</td>
</tr>
<tr>
<td>IL-8 (ng/ml) ($n = 63$)</td>
<td>0.59 &lt;0.0001</td>
<td>0.671 &lt;0.0001</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8-oxodG (ng/ml) ($n = 40$)</td>
<td>0.142 0.396</td>
<td>-0.076 0.64</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Rs is Spearman’s correlation coefficient and IL-8 is interleukin-8, 8-oxodG is 8-oxo-7,8-dihydro-2′-deoxyguanosine and is measured as ng/ml of supernatant collected by standard method.
that oxidative stress is normal in the healthy paediatric
airway, but its origin remains unclear. The normal lung
epithelial lining fluid contains very high levels of antiox-
didants, and has the capacity to rapidly neutralise free
radicals.23,24 It is therefore surprising that we could detect
8-oxodG in all IS samples. We speculate that 8-oxodG in IS
reflects normal intracellular oxidant generation, and back-
ground level of biomolecule oxidation and repair, and not a
pathological process.

There are limitations to our study. First, it is unclear
whether 8-oxodG in IS indicates systemic oxidative stress,
and has the capacity to rapidly neutralise free radicals.23,24
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pathological process.

There are limitations to our study. First, it is unclear
whether 8-oxodG in IS indicates systemic oxidative stress, or
reflects oxidative stress limited to the pulmonary micro-
environment. Levels of 8-oxodG in extracellular matrices
such as plasma and urine are considered valid markers of
oxidative stress,25 and these measures are thought of a
reflective of ‘whole body’ stress. However in some matrices,
such as cerebrospinal fluid,26 biomarkers may be more
reflective of oxidative stress in more localised tissue
environments. Our speculation is that, like cerebrospinal
fluid, measurement of 8-oxodG in IS reflects oxidative stress
in the lung microenvironment. A second limitation is that
there may be different markers of pulmonary oxidant stress
associated with IS neutrophilia. However, of all the oxidative
stress markers measured in extracellular matrices, 8-oxodG
is perhaps the best characterised, and most studied. In
contrast, markers of protein and lipid oxidation are less well
established.27 Furthermore, 8-oxodG appears to be a
sensitive biomarker of oxidative stress and is very stable.28

In conclusion, we have shown, in a large group of healthy
children, that IS neutrophils are associated with IL-8. We
have also demonstrated, for the first time, that 8-oxodG is
detectable in IS supernatants from healthy children. In
contrast to our original hypothesis, we found no correlation
between IS neutrophils and 8-oxodG.

Conflict of interest

There are no conflicts of interest to disclose from all authors
(N.K., M.S.C., J.G.).

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