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Abstract

Background

In healthy children, there is a wide range in the induced sputum neutrophil differential count. To date, the reason for this is unclear. In animal models, neutrophil chemoattractant cytokines and oxidative stress are major determinants of airway neutrophilia. In this study, we therefore aimed to assess the association between interleukin-8, and the oxidative stress marker 8-oxo-7, 8-dihydro-2’-deoxyguanosine (8-oxodG), with the neutrophil count in induced sputum samples from healthy children.

Methods

Induced sputum samples were obtained from healthy children using hypertonic saline. Concentrations of interleukin-8 and 8-oxodG were determined using ELISA. Sputum differential neutrophil count determined light microscopy and absolute neutrophil count calculated. To assess repeatability, a further sputum induction was done on a subgroup of children. Spearman's rank correlation was used to assess relationship between variables.

Results

The leukocyte differential was determined in 64/114 healthy children studied. The median (interquartile range) of neutrophil differential count was 20.6 % (5.67 - 56) and absolute neutrophil count was 0.11 (0.01 – 0.77) x 10^6. The induced sputum neutrophil differential and absolute counts correlated significantly with IL-8 (Rs = 0.67, p = <0.0001 and Rs = 0.60 p = <0.0001 respectively), but not with 8-oxodG (p = 0.64). The repeatability (intraclass correlation coefficient-Ri) of the neutrophil differential count was 0.58.
Conclusions

The normal variation in the proportion of neutrophils in the lower airway of children is driven by variation in IL-8, but not oxidative stress. The neutrophil differential count in healthy children is relatively stable over several months.
**Background**

Induced sputum (IS) is an established, non-invasive method of assessing airway inflammation in children\(^1\), and normal values for the neutrophil count in IS samples in healthy children have been described\(^2,3,4\). One feature of the resulting neutrophil differential is that the normal range is very wide. For example, Cai *et al* (reference here) reported that the interquartile range for the neutrophil differential count is 12 to 88.25% (median 35%) in children. Demographic variables may be important since a study in adults has reported that the neutrophil count increases with increasing age\(^5\). However, to date no demographic variables has been reported to be associated with the neutrophil differential count in healthy children. In the diseased airway, neutrophil transmigration from the systemic circulation into the airway is mediated by the neutrophil chemoattractant cytokine Interleukin-8 (IL-8)\(^6\). Thus increased airway IL-8 concentrations are associated with sputum neutrophilia in asthma\(^7\), chronic cough\(^8\) and cystic fibrosis\(^9\). Thus variations in the spontaneous release of IL-8 by resident lung cells may therefore be a key determinant of neutrophil transmigration in the healthy paediatric lung. A further variable that may induce neutrophil transmigration is oxidative stress from both environmental and cellular sources. In a range of models, oxidative stress from increased free radical production, stimulates neutrophil chemoattractant release by lung cells via induction of Nuclear Factor-κB and Activator Protein –1, oxidant-sensitive transcription factors\(^10,11\). Oxidative stress cannot be measured directly in airway samples, but oxygen radical damage to DNA and the deoxyribonucleotide pool results in, amongst others, stable products such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), which can be measured in biological fluids\(^12\). From these data, we hypothesized that the wide range of neutrophil count in the healthy paediatric lung is a result of variations in oxidative-
stress, which is associated with variations in IL-8 production. We therefore sought to
determine the association between both IL-8 and 8-oxodG, and the neutrophil count in
induced sputum samples from healthy children.
Methods

Subjects and study design

The study was conducted in Leicester (UK), a medium-sized city. 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. The details of recruitment and subject characteristics are as described in previous paper\textsuperscript{13}. In brief, healthy children aged 8-15 years from non-smoking families were recruited. We excluded children with history of respiratory symptoms, respiratory infection in last 3 months, personal smoking and passive smoking (confirmed by salivary cotinine).

Sputum induction and processing

Lung function was recorded using a Vitalograph 2120 spirometer (Vitalograph Ltd, Buckingham England) with Vitalograph 2120 Spirotrac\textsuperscript{®} IV software (Vitalograph Ltd.) as described previously\textsuperscript{13}. 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\textsuperscript{14}. Induced sputum was processed by a standard technique\textsuperscript{15}. To assess the stability (repeatability) of the neutrophil differential count, repeat sample was obtained in a subgroup of children after six months.

Interleukin-8

Interleukin-8 in induced sputum supernatants was analyzed according to an established ELISA assay, using a BD OptEIA\textsuperscript{TM} set for human IL-8 (BD Biosciences Pharmingen, San Diego, CA, US)\textsuperscript{16}. IL-8 was expressed as ng/ml; sensitivity level of the assay was $0.8 \times 10^{-3}$ ng/ml.
**8-oxo-7,8-dihydro-2’- deoxyguanosine (8-oxodG)**

The 8-oxodG in sputum supernatant samples was analysed by 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. The addition of DTT (0.1%) in the same concentration as in the sputum samples did not interfere with the ELISA for 8-oxodG (data not shown).

**Statistics**

All data were summarised as the median and interquartile range (IQR; Q1, Q3). Data were tested for normality by Kolmogorov-Smirnov test. Spearman's rank correlation was used to assess relationship between neutrophil count i) IL-8, ii) 8-oxodG and iii) demographic factors. The 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\(^\text{17}\). Statistical analysis was done using SPSS (version 12.0.1 for Windows).
Results

Adequate samples were obtained from 64 (35 boys) /114 children meeting the inclusion criteria. The summary of demographic, lung function variables are same as previously reported for a study of the association between airway macrophage carbon content and lung function\textsuperscript{13}. The median and (IQR, Q1-Q3) of squamous cell contamination (%) was 2.6 (0 - 8.48), indicating adequate quality sampling\textsuperscript{1}.

As expected, the interquartile range of the neutrophil differential count was large (5.67 to 56, Table 1). There was no association between any of the demographic variables (age, height, weight and percent predicted FEV\textsubscript{1}) and the neutrophil absolute or differential count or with the IS IL- 8 concentration (Table 2). However, the induced sputum neutrophil differential and absolute neutrophil count correlated significantly with IL-8 (Rs = 0.67, p = <0.0001 and Rs = 0.60 p = <0.0001 respectively) (Table 2 and Figure 1). In contrast, there was no association between 8-oxodG and either IL-8 concentration or neutrophil count (Table 2). The intraclass correlation coefficient (Ri) for neutrophil % was 0.58 (Figure 2), indicating a moderate degree of repeatability (stability) in the neutrophil differential count in samples taken several months apart.
Discussion

This is the first study to examine the determinants of neutrophil count in healthy children, and the first to measure 8-oxodG in IS samples from healthy children. The median values and range for the neutrophil differential count in our study are similar to those previously reported for normal children, suggesting that these data can be generalised\textsuperscript{7,9} to all the healthy children. In contrast to one previous study in adults\textsuperscript{5}, we did not find an association between age and the differential count. Although we cannot rule out an age effect, it is not significant over the small age range of subjects recruited into the present study.

The key finding of this study is that, as hypothesised, the sputum neutrophil count is associated with the concentrations of IL-8 in the healthy paediatric airway. IL-8 is a potent neutrophil chemotactic factor\textsuperscript{18} responsible for recruitment of neutrophils in to lungs. IL-8 mediated neutrophil recruitment has been demonstrated in inflammatory conditions in children\textsuperscript{9,19} and adults\textsuperscript{20,21}, although its role in the healthy lung is unclear. In the only previous study Gibson et al\textsuperscript{7} reported an association between the neutrophil count and IL-8 in IS samples for 8 healthy adults. Our data therefore supports and extends this preliminary observation to healthy children. We have not, however, identified the source of IL-8 in paediatric IS samples. A potent pro-inflammatory resident lung cell is the alveolar macrophage, which has the capacity to both spontaneously release IL-8 and up regulate IL-8 release by epithelial cells in response to environmental stimuli\textsuperscript{22}. We therefore speculate that the normal variation in the neutrophil count results from variations in spontaneous release of IL-8 from alveolar macrophages. The putative environmental or genetic variables associated with variations in spontaneous release therefore merit further study.
We found no association between 8-oxodG and either the concentration of IL-8, or the neutrophil differential and absolute count. Thus our hypothesis that variations in IL-8 are driven by oxidative stress is not supported. Oxidative stress certainly has the capacity to induce neutrophilia through oxidant sensitive chemokines.\textsuperscript{10,11} 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 that oxidative stress, is a normal feature of the healthy paediatric airway. The origin of the 8-oxodG in IS samples remains unclear. The normal lung epithelial lining fluid contains very high levels of antioxidants, and has the capacity to rapidly neutralise free radicals.\textsuperscript{24,25} It is therefore somewhat surprising that 8-oxodG would be at a level detectable in all IS samples. We speculate therefore that this marker reflects normal intracellular oxidant generation and background level of biomolecule oxidation and repair, and hence does not represent a pathological process.

There are limitations to our study. It is perhaps unclear as to whether 8-oxodG in IS indicates systemic oxidative stress, or only in the pulmonary microenvironment. The levels of 8-oxodG in extracellular matrices (e.g. plasma and urine) are considered valid markers of oxidative stress.\textsuperscript{23} Generally these measures are thought of a reflective of ‘whole body’ stress, although in some matrices, such as cerebrospinal fluid (Olinski ref Clin Chem) such measures may be more reflective of oxidative stress in a more localised environment. We are of the opinion that, like CSF, measurement of 8-oxodG in sputum reflects oxidative stress in the lung. We cannot be certain if other oxidative stress markers may correlate with neutrophil count.
However of all the oxidative stress markers measured in extracellular matrices, 8-oxodG is perhaps the best characterised, and most studied whereas, protein and lipid oxidation appearing to be less well established (Biomarkers review). Furthermore 8-oxodG appears to be a sensitive biomarker of oxidative stress and eminently stable (Loft 2006). A longitudinal evaluation would strengthen the conclusions of this cross-sectional study. However repeated short-term sampling of healthy children will be difficult to achieve.

In conclusion, we have shown, in a large group of healthy children, that the airway neutrophil differential count is associated with IL-8. For the first time, we have demonstrated that 8-oxodG is detectable in sputum supernatant, but we found no correlation was found between neutrophil differential count and this marker of oxidative stress.
Conflict of interest statement

There are no conflicts of interest to disclose from all authors (NK, MSC, JG).

Acknowledgements

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REFERENCES


Figure Legends

**Figure 1.** The scatter plot showing the significant correlation (p < 0.0001) between sputum A) absolute neutrophil count \((x 10^6/ml)\) and B) neutrophil % and interleukin-8 (ng/ml).

**Figure 2.** Bland and Altman plot of neutrophil percentage in induced sputum performed six months apart. The limits of agreement (mean difference of two measurements [heavy solid line] ± 2SD) are represented by dotted lines. Ri is the intraclass correlation coefficient.
Figure 1.

A)

B)

[Graph showing the relationship between Neutrophil % and IL-8 ng/ml]

[Graph showing the relationship between Absolute neutrophil x 10^6 /ml and IL-8 ng/ml]
Figure 2.

Ri = 0.58

Neutrophil % difference

Neutrophil % (mean)
Table 1. Summary of sputum differential, IL-8 and 8-oxodG. The data are summarised as median and inter quartile range (IQR). IL-8 is interleukin-8, 8-oxodG is 8-oxo-7, 8-dihydro-2’deoxyguanosine.

<table>
<thead>
<tr>
<th>Analysis parameter</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count (n = 53) (x 10⁶/ml)</td>
<td>0.52 (0.18 – 1.35)</td>
</tr>
<tr>
<td>Absolute neutrophil count (n = 53) (x 10⁶/ml)</td>
<td>0.12 (0.02 –0.78)</td>
</tr>
<tr>
<td>Sputum differential (n = 64)</td>
<td></td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>20.63(5.67 - 56)</td>
</tr>
<tr>
<td>Eosinophils %</td>
<td>0.25 (0 – 0.75)</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>78.63 (41.38 – 93.25)</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>0 (0 – 0.25)</td>
</tr>
<tr>
<td>Epithelial cells %</td>
<td>0.25 (0 – 0.50)</td>
</tr>
<tr>
<td>IL -8 (ng/mL) (n = 63)</td>
<td>6.26 (0.96 - 12.06)</td>
</tr>
<tr>
<td>8-oxodG (ng/mL) (n = 40)</td>
<td>9.59 (7.46 - 13.13)</td>
</tr>
</tbody>
</table>
Table 2. Relationship between neutrophil %, IL-8 and 8-oxodG to demographic factors and lung function. Rs is Spearman’s correlation coefficient and IL-8 is Interleukin-8, and 8-oxodG is 8-oxo-7, 8-dihydro-2'deoxyguanosine and is measured as ng/mL of supernatant collected by standard method.

<table>
<thead>
<tr>
<th></th>
<th>Absolute neutrophil count (x 10⁶/ml)</th>
<th>Neutrophil (%)</th>
<th>II-8 (ng/mL)</th>
<th>8-oxodG (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rs</td>
<td>P</td>
<td>Rs</td>
<td>P</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.027</td>
<td>0.850</td>
<td>-0.159</td>
<td>0.21</td>
</tr>
<tr>
<td>Weight (kgs)</td>
<td>-0.060</td>
<td>0.671</td>
<td>-0.241</td>
<td>0.055</td>
</tr>
<tr>
<td>Height (cms)</td>
<td>0.032</td>
<td>0.820</td>
<td>-0.128</td>
<td>0.31</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>-0.139</td>
<td>0.319</td>
<td>-0.173</td>
<td>0.172</td>
</tr>
<tr>
<td>IL-8 (ng/ml)</td>
<td>0.59</td>
<td>&lt;0.0001</td>
<td>0.671</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>8-oxodG (ng/ml) (n = 40)</td>
<td>0.142</td>
<td>0.396</td>
<td>-0.076</td>
<td>0.64</td>
</tr>
</tbody>
</table>