Exhaled nitric oxide in cystic fibrosis patients with allergic bronchopulmonary aspergillosis


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Abstract
Exhaled nitric oxide (NO) is thought to be a marker of asthmatic inflammation. Levels in cystic fibrosis (CF) are generally low. This study aimed to measure exhaled NO in CF patients at high risk of developing ABPA and patients at low risk. We studied nine patients at high risk of developing ABPA and 36 at low risk. The two groups were similar in age and spirometry. All patients in the high-risk group were taking oral or inhaled glucocorticoids, compared to 56% in the low-risk group (P=0.02). The exhaled NO levels were lower in the high-risk group than in the low-risk group (2.0 vs. 3.6 ppb), mean difference (95% CI) 1.6 (0.3 to 0.4) ppb, P=0.001. On subgroup analysis of patients on oral glucocorticoids, the exhaled NO levels were significantly lower in patients with a high risk of developing ABPA (n=7) than patients with a low risk (n=8) (P=0.011). The number of patients who were on inhaled, but not oral glucocorticoids was too small to analyse usefully. Exhaled NO levels were lower in CF patients with a high risk of developing ABPA and on glucocorticoids. This may be because oral glucocorticoids exert a greater effect on exhaled NO than inhaled glucocorticoids. Alternatively, inducible nitric oxide synthase may be down-regulated by Aspergillus toxin.

INTRODUCTION
Nitric oxide (NO) is excreted in the exhaled breath of humans and other mammals (1). It is an important mediator in many organ systems. In the lung, it mediates mucosal inflammatory responses, airway smooth muscle contractility, and pulmonary vasodilatation (2). Central to the formation of NO and its reactive nitrogen products is the enzyme nitric oxide synthase (NOS) which has been characterised in almost all the cells within the airways (3). Several forms of NOS have been described and an inducible form (iNOS) can be up-regulated by inflammatory cytokines (4). As a result, NO concentrations in exhaled breath have been suggested as being useful in monitoring disease activity in inflammatory airway diseases, particularly in asthma. Exhaled NO concentrations are particularly raised in patients with atopic or allergic asthma (5–7), but are not raised in patients with cystic fibrosis (CF) or bronchiectasis (8,9).

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity reaction to Aspergillus fumigatus (A. f.), which was first described in 1952 (10). CF patients may be prone to colonisation with A. f. because of their very tenacious sputum. Untreated ABPA may lead to lung damage, including proximal bronchiectasis and segmental, lobar or whole lung collapse, and it is difficult to differentiate this damage from that due to CF itself.

Given the allergic nature of ABPA and the presence of normal levels of NO in the exhaled breath of CF patients, we hypothesised that exhaled NO may prove to be a useful marker of the presence of an allergic airway disease in this group of patients. The levels of exhaled NO were studied in CF patients at high risk of developing ABPA and compared with CF patients at low risk of developing ABPA.

SUBJECTS AND METHODS
Subjects
We studied 45 adult patients with CF (mean (±SE) age 25.8 ± 1.1 years, 22 female). The patients were recruited
from the regional adult CF unit at Birmingham Heartlands Hospital. None were cigarette smokers or had a history of asthma. All participants gave their written consent, having been fully informed of the nature, risks and potential benefits of the study. The study was observational and was approved by the East Birmingham Local Research and Ethics Committee.

Study design

Patients were interviewed at routine CF clinics or as inpatients if they were receiving in-patient treatment. Data were obtained during the interview and from the case notes. Age, sex, height, CF genotype and glucocorticoid therapy (both inhaled and oral) were recorded. Peripheral venous blood was taken for measurement of total IgE, specific IgE to timothy grass pollen, house dust mite, cat dander, mixed moulds, Cladosporium herbarum and Af. The peripheral eosinophil count and serum precipitating antibody to Af were also measured by an immuno-diffusion technique. Spirometry was performed using a dry bellows spirometer (Vitalograph, Buckingham, U.K.), using the best of three reproducible measures.

Definition OF ABPA

In this study, we categorised our patients into two groups: (1) CF patients at high risk of developing ABPA and (2) CF patients at low risk of developing ABPA. We defined patients at high risk of developing ABPA as having (i) elevated specific IgE antibodies (RAST score > 2) to Af, with (ii) an increased level of total serum IgE < 500 kU/l or a recent doubling in the level of the serum total IgE above 200 kU/l, and (iii) reduced lung function on at least one occasion responding to oral steroids, but not to intravenous antibiotics over a 5-year period. Patients who did not fulfil the above criteria were considered as low risk of developing ABPA. This was based on the suggestions that high absolute level of specific IgE to Af (11–13) and a raised serum total IgE herald the onset of ABPA (13–15).

Nitric oxide excretion measurements

All exhaled NO was measured at least 6 h after inhaled steroids. Concentrations of NO in the exhaled breath were measured using a chemi-luminescence analyser (LR2000, Logan Research, Rochester, Kent, U.K.). The analyser was calibrated daily against a known standard (99 parts per billion (ppb)), sampling at 25 Hz in real time with a sensitivity of 0.3 ppb. Concentrations of NO in the exhaled breath representative of concentrations in the lower respiratory tract were measured during three separate exhalations with the subject exhaling at 200 ml/s against resistance, using a visual feedback display to maintain constant flow. The analyser sampled from the stream using a side arm and lower respiratory tract samples were assessed when 75% of the vital capacity had been exhaled (16). Typical values with this technique in normal subjects are 5–10 ppb. The within subject co-efficient of variation is approximately 10% when single measurements are made (17,18). The mean of three measurements was therefore taken to minimise this variation.

Statistics

Demographic details are presented using summary statistics. Categorical variables were compared using chi-squared analysis or Fisher’s exact test. If data were normally distributed, unpaired t-tests were used, and if non-normal, the Mann–Whitney rank sum test was used to assess the differences in demographic details, lung function, blood results and exhaled NO concentrations between the two groups. For all tests, P < 0.05 was used to define a statistically significant result.

RESULTS

Nine CF patients with a high risk and 36 CF patients with a low risk of developing ABPA as a control group were identified. The groups’ characteristics are summarised in Table 1. All were non-smokers and none of the patients were taking antifungal treatment at the time of study. The two groups were similar in age, sex distribution, and spirometric lung function. Three of the nine (33%) patients in the high-risk group were homozygous for DF508 for the CF transmembrane conductance regulator (CFTR) mutation and another three were heterozygous for DF508. Fifteen of the 36 (42%) patients in the low-risk group were homozygous for DF508 for the CFTR and another 15 were heterozygous for DF508. The CFTR mutation was non-detected in two patients in the high-risk group and one in the low-risk group. No differences were observed in the number of patients with sputum colonised by Pseudomonas aeruginosa, Staphylococcus aureus and upper respiratory flora or no growth. Two of the nine patients with a high risk of developing ABPA had precipitating antibodies to Af, which is consistent with previous work (18). The mean peripheral eosinophil counts between the groups were similar.

Seventy-eight percent of the CF patients with a high risk of developing ABPA were sensitised to mixed moulds and 33% in the low-risk group (P = 0.02). Similarly, 78% of the patients in the high-risk group were sensitised to C. herbarum and 100% to Af (P < 0.0001 and 0.006, respectively). No statistically significant difference was noted in the number of patients sensitised to grass pollen, cat
dander and house dust mite. One hundred percent of the CF patients with a high risk of developing ABPA, but only 56% of the low risk patients, were taking either oral or inhaled glucocorticoid medication. None of the patients were taking any antifungal agent (e.g. itraconazole) at the time of this study.

The concentration of NO in the exhaled breath was lower in the high-risk group (n=9) than in the low risk group (n=36), 2.0 ppb vs. 3.6 ppb, mean difference (95% CI) 1.6 (−3.6 to 0.4) ppb, P=0.001 (Table I). On subgroup analysis of patients who were on oral glucocorticoids, the exhaled NO was significantly lower in CF patients at high risk of developing ABPA (n=7) than patients of low risk (n=8), 2.0 vs. 3.1 ppb, mean difference (95% CI) 1.1 (−1.8 to −0.5) (P=0.011) (Fig. I). For patients who were taking inhaled glucocorticoids, there was no difference in exhaled NO between the high-risk (n=2) and low risk groups (n=12) (2.0 vs. 3.4 ppb).

**DISCUSSION**

The diagnosis of ABPA in CF patients is difficult as the signs and symptoms of ABPA are similar to those of CF. Patients with ABPA usually develop the disease in childhood, but it often remains undiagnosed for many years (19). One study reported that the diagnosis was made on an average of 10 years following the first symptom of ABPA (20). The disease can progress insidiously to its later stages, while there is no characteristic clinical manifestation. An easily used, sensitive and specific test for this serious disease is needed.

The "gold standard" diagnostic criteria for ABPA according to Nelson et al. (21) are (i) pulmonary infiltration radiologically, (ii) wheezing, (iii) positive sputum culture for Af, (iv) positive immediate reaction to Af in skin prick testing, (v) increased level of serum total IgE, (vi) increased level of specific IgE to Af, and (vii) presence of precipitating antibody to Af. Greenberger and Patterson described that five criteria were considered to be essential, namely asthma, positive immediate skin test to Af, IgE of >1000 ng/ml, precipitating antibody to Af and

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**Table I.** Characteristics of CF patients with a high risk (n=9) and CF patients with a low risk of developing ABPA (n=36)

<table>
<thead>
<tr>
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<th>High-risk group (n=9)</th>
<th>Low-risk group (n=36)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Age (years): mean (range)</td>
<td>25.1 (18–39)</td>
<td>26.0 (17–45)</td>
<td>0.75</td>
</tr>
<tr>
<td>Sex: Male/Female</td>
<td>7/2</td>
<td>16/20</td>
<td>0.14</td>
</tr>
<tr>
<td>Predicted FEV1 (%)</td>
<td>43.8 (47)</td>
<td>53.6 (37)</td>
<td>0.26</td>
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<tr>
<td>Predicted FVC (%)</td>
<td>70.3 (71)</td>
<td>74.6 (42)</td>
<td>0.65</td>
</tr>
<tr>
<td>IgE (KU/l): mean (SD)</td>
<td>786.1 (1471)</td>
<td>165.4 (41.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with precipitating antibodies to Af: number (%)</td>
<td>2 (22%)</td>
<td>2 (6%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Peripheral eosinophil count (×10⁹/l): mean (range)</td>
<td>0.16 (0–0.5)</td>
<td>0.14 (0–0.6)</td>
<td>0.98</td>
</tr>
<tr>
<td>Patients receiving glucocorticoids (either inhaled or oral): number (%)</td>
<td>9 (100%)</td>
<td>20 (56%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Total prednisolone dosage per day (mg/day): median (range)</td>
<td>15.0 (5–20) (n=7)</td>
<td>12.5 (5–20) (n=8)</td>
<td>0.64</td>
</tr>
<tr>
<td>Total beclometasone dipropionate (BDP) equivalent dosage per day (mcg/day): median (range)</td>
<td>900 (800–1000) (n=2)</td>
<td>1000 (800–4000) (n=12)</td>
<td>0.30</td>
</tr>
<tr>
<td>Exhaled nitric oxide (ppb): median (range)</td>
<td>1.8 (1.4–3.2)</td>
<td>2.9 (1.2–18.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>
specific IgE to Af (II). In a later study, they found that only elevated IgG, IgE antibodies and central bronchiectasis were highly specific for ABPA (12). An earlier study by Rosenberg et al. described the case histories, diagnostic criteria, immunologic and radiographic findings of 20 patients with definite or possible ABPA. Total serum IgE above 1000 ng/ml was found in 19 of the 20 cases. The authors argued that an elevation of total IgE is not pathognomonic for ABPA, as it has been found in other conditions such as atopic dermatitis and atopic asthma. They did not include patients with CF in their study (22). A recent study of CF patients with ABPA by Skov et al. (13) showed that a total serum IgE level above 200 KU/l is associated with an 80% positive predictive value and a 100% negative predictive value for diagnosing ABPA. A total serum IgE level above 500 KU/l has also been described by Marchant et al. (14) as strongly suggestive of the diagnosis of ABPA in children with CF. Furthermore, a doubling of the total IgE was suggested by Greenberger (15) as being sufficient to signal the onset of ABPA. Consequently, we believe our diagnostic approach to diagnosis is robust and justified.

Patients with CF have a high prevalence of atopy. This has been confirmed by us (23) and others (21,24,25). This can raise the level of total serum IgE, so that a cutoff of > 1000 nm/l is neither specific nor sensitive for the diagnosis of ABPA in patients with CF. For these reasons, there is a risk of confusion and delay in making this important diagnosis in CF. Therefore, we used a combination of: raised total serum IgE above 500 KU/l or a recent doubling in the level of total serum IgE above 200 KU/l; raised specific IgE (RAST > 2) to Af; and reduced lung function on at least one occasion responding to oral steroids, but not intravenous antibiotics over a 5-year period to identify patients with a high risk of developing ABPA. In CF, pulmonary infiltrates, wheezing and bronchiectasis are common. Precipitating antibody to Af is present in CF with or without ABPA (26,27,38). It may vary with time and can disappear spontaneously (38). This may be due to technical reasons such as the potency of Af antigen and the experience of technicians (28).

Exhaled NO concentrations are particularly raised in allergic airway diseases such as atopic asthma (5), while concentrations of NO in the breath of CF patients are typically low (8). Since ABPA is a hypersensitivity lung disease induced in individuals previously exposed to Af (29), we hypothesised that concentrations of exhaled NO may be raised in CF patients with a high risk of developing ABPA. In this study, we have shown that exhaled NO levels were significantly lower in patients at high risk of developing ABPA compared to a demographically similar group of CF patients at low risk of developing ABPA.

No significant difference was observed between the high-risk and low-risk groups in their % predicted FEV₁. Although various studies have shown that within an individual exhaled NO declines with bronchoconstriction (30–32), no such effect has been noted with regard to FEV₁ between individuals. Changes in FEV₁ can therefore not explain our findings.

The high-risk group had a significantly higher prevalence of sensitisation to mixed moulds, C. herbarum and Af (78, 78 and 100%, respectively), but the exhaled NO level was significantly lower than the low-risk group. However, the use of glucocorticoids was universal in the ABPA group with only half of the control group using these agents.

There are a number of potential explanations for these findings. Firstly, there may be a decrease in production of NO due to inhibition of iNOS by Aspergillus toxin. This is supported by the inhibition of the oxidative burst in alveolar macrophages by an Af diffusate toxin (33). Secondly, NO in the exhaled breath is predominantly produced by the epithelium. Since ABPA is characterised by damage to the airway epithelium, this could also explain our findings. Thirdly, the airway epithelial cells in patients with ABPA may have a reduced expression of iNOS due to a lower functional CFTR activity than in patients without ABPA. Absence of functional CFTR may lead to a change in cell signalling events which alters the expression of iNOS (34,35). Finally, it has been demonstrated that ABPA is associated with an antigen-specific TH2 lymphocyte immune response, as indicated by excessive IL-4 and IL-5 secretion in ABPA (36). This could explain our findings, as IL-4 inhibits the induction of macrophage NO synthase (37).

Subgroup analysis on patients who were on oral glucocorticoid therapy showed that the exhaled NO levels were significantly lower in CF patients with a high risk of developing ABPA than patients with a low risk. The group of patients, who were on inhaled, but not on glucocorticoid therapy was too small to analyse usefully. All exhaled NO was measured at least 6 h after their inhaled steroids and hence this potential factor has been excluded in this study. The number of patients in the high-risk group and the low-risk group who used inhaled steroids were 22% (2/9) and 33% (12/36), respectively. No difference was observed in the total beclometasone dipropionate dosage between the two groups (F²=0.3). Therefore, the low exhaled NO level in the high-risk group cannot be attributed to the inhaled steroids.

The finding that patients with ABPA were more likely to be sensitised to mixed moulds and C. herbarum confirmed that patients with CF show a high reactivity to aeroallergens as described by Becker et al. (24). It has recently been suggested that atopy is a risk factor for the development of ABPA in CF patients (23,36), which led to the postulate that patients with CF and ABPA are more prone to sensitisation to fungal aeroallergens than patients without ABPA.
In summary, the concentration of NO in the exhaled breath was significantly lower in CF patients with a high risk of developing ABPA and on oral glucocorticoid therapy compared to those at low risk also taking oral steroids. This may be explained by oral glucocorticoids having a greater effect on exhaled NO than inhaled glucocorticoids, or by inhibition of iNOS by *Aspergillus* toxin in these individuals. The measurement of exhaled NO measurement is a non-invasive and straightforward procedure. The equipment required to perform this test is becoming more user friendly and more widely available. As the current diagnostic criteria for ABPA in patients with CF are neither specific nor sensitive, improved markers are required in order to prevent delay in making this important diagnosis in CF patients. To this end, the measurement of exhaled NO may be a useful supplementary test. A prospective study on exhaled NO levels in CF patients with a high risk of developing ABPA is required to confirm the usefulness of exhaled NO in monitoring the activities of ABPA in this group of patients.

REFERENCES


