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Potentially pathogenic bacteria cultured from the sputum of stable asthmatics are associated with increased 8-isoprostane and airway neutrophilia

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Abstract

Potential bacterial pathogens are found in the airways in several diseases that are associated with neutrophilic inflammation. The aim of this study was to characterize subjects with stable asthma, with no symptoms of respiratory infection, to assess whether key potentially pathogenic bacteria were present in significant quantities in the airways and to correlate this with the pattern of airway inflammation and oxidative stress. Subjects with stable asthma ($n = 115$) and healthy controls ($n = 8$) underwent clinical assessment, including hypertonic saline challenge combined with sputum induction. A significant load of potentially pathogenic bacteria ($> 10^6$ cfu/mL) was cultured from the sputum of 17 (15%) subjects with stable asthma and was associated with higher total cell counts, proportion and number of neutrophils, sputum IL-8 and 8-isoprostane concentrations. The role of bacteria in potentiating neutrophilic asthma warrants further investigation. Therapies such as antibiotic and antioxidant treatment may be most effective in this sub-group of patients.

Keywords: *Airway inflammation, asthma, bacteria, IL-8, 8-isoprostane, oxidative stress*

Introduction

The lower airway is normally sterile; however, defects in local immunity can lead to the presence of bacteria in the lower airways [1,2]. Isolation of bacterial pathogens from sputum samples is a common and accepted feature of some airway diseases, including bronchiectasis [3] and chronic obstructive pulmonary disease (COPD) [4–6]. The presence of certain bacterial species is associated with an airway inflammatory response [6–8], including increased expression of the neutrophil chemoattractant IL-8 [6–9] and leads to neutrophil recruitment and activation [6,10], resulting in the excessive release of neutrophil derived proteases, which damage the airway epithelium [6,8,11]. During phagocytosis of bacteria, neutrophils also undergo the respiratory burst, releasing reactive oxygen species, which overwhelm host antioxidant defences and cause oxidative damage to the airways [12]. Oxidation products which have been detected in the airway lining fluid include 8-isoprostanes, which are produced by free radical-catalysed peroxidation of arachidonic acid [13]. Known adverse pathophysiological effects associated with bacterial isolation include mucus hypersecretion and impaired mucociliary clearance [14]. In stable COPD, isolation of potential bacterial pathogens and increased bacterial load has been associated with various adverse clinical outcomes, such as increased exacerbation frequency and severity [15], worse health status [8] and lung function decline [16].

We have previously demonstrated that defects in neutrophil function exist in asthma, that lead to a neutrophilic inflammatory sub-type. This sub-type is characterized by dysregulation of toll-like receptors, persistent neutrophilic influx and activation and an increase in sputum levels of IL-8 and IL-1β [17]. These local immune defects may lead to impaired clearance mechanisms and potentially to bacterial

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persistence within the lower airway in asthma. While respiratory infection with viruses [18] and atypical bacterial pathogens such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* [19] has been associated with asthma exacerbations, the effects of potentially pathogenic bacteria in asthma are not well understood. However, recent recognition of innate immune defects in stable asthma [17], leading to a neutrophilic inflammatory phenotype, raises the possibility that bacterial pathogens may contribute to disease pathology.

Several microbial pathogens have an established or possible role in acute exacerbations of COPD, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* [1]. *Staphylococcus aureus* and Gram Negative Enteric Baciili (GNEB) (e.g. *Escherichia coli*) can be isolated, but have an undefined role in acute exacerbation of COPD [1]. These bacteria can also be isolated with increased frequency from the respiratory tract in older people and those with obstructive airway diseases [1,8,20]. While their clinical significance in stable disease is undefined, they may be associated with an abnormal inflammatory response in the airway characterized by increased neutrophilia [1]. The role of bacterial pathogens in the neutrophilic inflammatory response that occurs in some asthma phenotypes is not known. However, both the presence of bacteria or an increase in the load of these bacteria could induce innate immune responses and neutrophilia and contribute to the pathogenesis of asthma with a neutrophilic phenotype. Alternatively, an innate immune defect in asthma may predispose to both bacterial isolation from the lower airway and asthma. People with severe asthma have been shown to be at increased risk of invasive pneumococcal disease, which suggests there may be a defect in the immune response to a common pathogenic bacteria in asthma [21]. We hypothesized that in adults with stable asthma and no symptoms of respiratory infection, the isolation of certain potentially pathogenic respiratory bacteria, at a load that leads to significant growth in the laboratory ($> 10^6$ cfu/mL), is associated with increased airway neutrophilia, oxidative stress and neutrophilic asthma.

The aim of this study was to characterize subjects with stable asthma, with no symptoms of respiratory infection, to assess whether key potentially pathogenic bacteria were isolated in significant quantities in the airways and to correlate this with the pattern of airway inflammation and oxidative stress.

Materials and Methods

Study subjects and design

Stable non-smoking subjects with asthma ($n = 115$), defined by GINA criteria [22] using a clinical diagnosis of symptomatic asthma and airway hyper-responsiveness (AHR) to hypertonic saline, were recruited for this study. All subjects were stable at the time of assessment. Subjects were excluded if they had experienced symptoms of (or antibiotic treatment for) lower respiratory tract infection in the previous 4 weeks. Subjects were also excluded if they had experienced an exacerbation of respiratory disease or changed their maintenance medications in the previous 4 weeks. Subjects with a prior diagnosis of bronchiectasis or known immunodeficiency were excluded. Asthma control was assessed using the Juniper Asthma Control Questionnaire [23]. In addition, stable, non-smoking healthy controls ($n = 8$) were recruited, from research volunteer databases. These subjects had no respiratory symptoms, had never had a doctor's diagnosis of asthma, had no airway hyper-responsiveness, had normal lung function and were steroid naïve. All subjects underwent clinical assessment, spirometry (KoKo PD Instrumentation Louisville Co, USA) and combined bronchial provocation testing and sputum induction with hypertonic saline (4.5%), as previously described [24]. The dose response slope (DRS) was assessed for all tests by dividing the percentage fall in FEV1 from pre-challenge FEV1 by the dose of hypertonic saline inhaled (mL). Subjects were recruited from the Respiratory Ambulatory Care Service at John Hunter Hospital or research databases (controls) and gave written informed consent. The Hunter New England Area Health Service and University of Newcastle Research Ethics Committees approved this study.

Methods

Sputum culture and quantitative bacterial culture. Sputum plugs were separated from saliva using forceps. An aliquot of sputum was selected using a positive displacement pipette and used for quantitative bacteriological culture [25]. Sputum samples were homogenized using dithiothreitol (DTT), diluted with PBS to 10^{-3}, 10^{-4} and 10^{-5} and used to inoculate chocolate bacitracin and blood agar plates. Plates were transported for Gram staining and identification to the Microbiology Department of the Hunter Area Pathology Service for culture and reporting. All plates were incubated at 37°C in an atmosphere of 5% CO_2 in air and examined for bacterial growth after 24 and 48 h by trained microbiologists. Biochemical testing was performed on pathogenic bacteria and confirmatory tests were performed prior to reporting. Subjects who had a bacterial load $> 10^6$ colony forming units (cfu)/mL for any individual potentially pathogenic bacteria were considered to have a significant bacterial load.

Sputum cell counts The remaining selected sputum was dispersed using dithiothreitol (DTT) as previously

described [24]. The suspension was filtered and a total cell count (TCC) of leukocytes and assessment of viability performed. Following centrifugation, supernatant was aspirated and stored at –80°C. Cytospins were prepared, stained (May-Grunwald Geimsa) and a differential cell count obtained from 400 non-squamous cells.

Sputum supernatant measurements. 8-iso-$PGF_{2\alpha}$ concentrations were determined in sputum supernatants, by enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI) by comparison with standard curves using purified 8-iso-$PGF_{2\alpha}$ in DTT solution. The assay has a 4 ng/L detection limit, uses anti-serum that has 100% cross-reactivity with 8-isoprostane and < 0.3% with prostaglandin analogues and has been previously validated for measurement of 8-iso-$PGF_{2\alpha}$ in sputum supernatant [26]. IL-8 concentrations in sputum were measured by ELISA (R&D Systems Minneapolis, MN). The assay has previously been validated for measurement of IL-8 in sputum supernatant [27].

Asthma sub-type classification. Asthma inflammatory sub-type was classified as previously described into four groups, those with eosinophilic, neutrophilic, mixed granulocytic (both increased neutrophils and eosinophils) and paucigranulocyic phenotype [26]. For the analysis of inflammatory mediators, neutrophilic asthma included subjects from both the neutrophilic and mixed groups. This was based on previous observations from our group, which have shown that neutrophilic and mixed asthma phenotypes have a very similar inflammatory profile [26].

Data analysis

Data were analysed using Stata 9 (Stata Corporation, College Station, TX), with results reported as median and interquartile range unless otherwise indicated. Analysis was performed using the two-sample Wilcoxon Rank Sum test or Kruskal-Wallis test for more than two groups with Bonferroni correction. Fishers' exact test was used to analyse categorical data. Associations between data were examined using Spearman's rank correlation. 8-isoprostane and IL-8 were log transformed for multiple regression modelling using neutrophilic asthma and bacterial load as predictors of $\log_{10}$8-isoprostane and $\log_{10}$IL-8. Results were reported as significant when $p < 0.05$.

Results

A significant load of potentially pathogenic bacteria was cultured from the sputum of 17 (15%) subjects with stable asthma (Table I). Each of these subjects were found to have a significant load of a single pathogen and these included *H. influenzae*, *S. aureus*, *M. catarrhalis*, *P. aeruginosa* and Gram Negative Enteric Bacilli (GNEB). *H. influenzae* was the most common potentially pathogenic bacteria identified. Asthmatic subjects with a significant bacterial load were significantly older than the asthmatic group without a significant bacterial load. Both groups had long-standing asthma, with the median duration of asthma, 25.3 (14.4–48.8) and 28.3 (18.2–46.0) years, respectively. There was no significant difference between the groups in frequency of atopy, smoking history or smoking intensity. GINA pattern and asthma control score were not significantly different between groups. However, the group with a significant bacterial load had a higher frequency of chronic bronchitis and were taking twice the dose of inhaled corticosteroids. This group also had reduced lung function with more severe airflow obstruction, but were less hyperresponsive, indicated by a lower dose response slope to hypertonic saline (Table II).

Table I. Potentially pathogenic bacteria cultured from sputum of 17 subjects with stable asthma.

Isolate	Number of subjects
Haemophilus influenzae	10
Staphylococcus aureus	2
Moraxella catarralis	2
Pseudomonas aeruginosa	2
Gram Negative Enteric Bacilli (GNEB)	1

None of the healthy controls had a significant load of potentially pathogenic bacteria in the respiratory tract. The age of the healthy controls was similar to subjects with stable asthma: bacterial load $< 10^6$ cfu/mL. However, the healthy controls had a lower rate of atopy and better lung function, including %FEV1, FEV1/FVC, DRS and fixed airflow obstruction (Table II).

Asthmatics with a significant bacterial load had an altered inflammatory pattern, including a significantly higher total cell count and proportion and number of neutrophils and significantly lower proportion of macrophages, lymphocytes and columnar epithelial cells compared to asthmatic with no significant bacterial load (Table III). This group also had a higher proportion of subjects with a neutrophilic and mixed inflammatory phenotype and a lower proportion of subjects with an eosinophilic phenotype (Table IV). All healthy controls had proportions of sputum neutrophils and eosinophils that were within the normal range. The median sputum inflammatory cell counts for the healthy control group were similar to the group with stable asthma: bacterial load $< 10^6$ cfu/mL. However, the healthy control group had a lower proportion of eosinophils in sputum (Table III).

Asthmatic subjects with a significant bacterial load also had increased levels of inflammatory markers in sputum compared to asthmatics without a significant

Table II. Subject characteristics.

	Stable asthma: bacterial load $< 10^6$ cfu/mL	Stable asthma: bacterial load $> 10^6$ cfu/mL	Healthy controls
n	96	17	8
Male/Female	39/57	8/9	3/5
Age, years*	57.7 (44.0–64.8)	67.0‡# (53.6–69.9)	47.1 (25.4–58.8)
Duration of asthma (years)*	28.3 (18.2–46.0)	25.3 (14.4–48.8)	NA
Atopy present, *n* (%)	72 (75)#	15 (88)#	2 (25)
Ex-smoker, *n* (%)	31 (32)	8 (47)#	0 (0)
Pack years*	6.0# (1.5–33.0)	10.3# (6.2–42.3)	0 (0–0)
Chronic bronchitis, *n* (%)	34 (41)	13 (87)‡#	0 (0)
GINA pattern, *n* (%):			
Intermittent	19 (20)	3 (18)	NA
Mild persistent	24 (25)	2 (12)	
Moderate persistent	29 (30)	4 (24)	
Severe persistent	24 (25)	8 (47)	
Asthma control score†	1.3 (0.8)	1.6 (0.8)	NA
ICS dose (beclamethasone equiv, μg/day)	1000 (800–2000)	2000‡ (2000–3625)	NA
FEV_1 % predicted†	78.3 (19.0)#	65.8 (27.1)‡#	109.6 (16.6)
FEV_1/FVC %†	68.5 (10.3)#	60.6 (10.4)‡#	84.0 (6.0)
Dose response slope (%fall/mL)	2.47# (0.84–10.05)	0.69‡ (0.19–3.70)	0.44 (0.04–0.61)
Fixed airflow obstruction, *n* (%)	54 (56)#	14 (82)#	0 (0)

*Data is median (IQR); † Data is mean (SD); ‡ $p < 0.05$ vs Stable asthma: bacterial load $< 10^6$ cfu/mL; # $p < 0.05$ vs Healthy controls.

bacterial load. This included elevated IL-8 concentrations and increased oxidative stress, indicated by increased 8-isoprostane levels (Figure 1A). Subjects with neutrophilic inflammation in the airways also had increased IL-8 and 8-isoprostane concentrations compared to subjects without neutrophilia, irrespective of the bacterial load (Figure 1B). However, multiple regression analysis of data from stable asthmatics identified that the presence of a significant bacterial load, and not neutrophilic asthma, was a significant determinant of induced sputum concentrations of both $\log_{10}$IL-8 and $\log_{10}$8-isoprostane (Table V). Analysis of subjects with a significant bacterial load indicates that there was no difference in inflammatory markers in never-smokers compared to ex-smokers (Table VI).

Discussion

In this study we demonstrate for the first time that a proportion (15%) of adults with stable asthma have a significant load of key potentially pathogenic bacteria detected in the airways, in the absence of symptoms of respiratory infection. The identified bacteria were *H. influenzae*, *S. aureus*, *M. catarrhalis*, *P. aeruginosa* and GNEB, which have been widely associated with other airway diseases with a neutrophilic component such as COPD and bronchiectasis. This study is also the first to demonstrate that the presence of a significant load of key potentially pathogenic bacteria in the airways of stable asthmatics is associated with neutrophilic asthma, increased inflammation and oxidative stress.

H. influenzae was identified in 60% of cases in our study. *H. influenzae* frequently colonizes the human respiratory mucosa and causes various respiratory tract diseases, including bronchitis and pneumonia, particularly when the host has an underlying airway disease, such as COPD or bronchiectasis [28]. *H. influenzae* has been shown to induce an inflammatory response, dominated by neutrophil recruitment to the infection site and increases in IL-8 protein and ICAM-1 gene expression in airway epithelial cells [29,30]. While our study design, which analysed

Table III. Induced sputum cell counts.

	Stable asthma: bacterial load $< 10^6$ cfu/mL	Stable asthma: bacterial load $> 10^6$ cfu/mL	Healthy controls
Total cell count ($\times 10^6$/mL)	2.52 (1.3–4.4)	8.2*‡ (5.0–17.2)	1.9 (1.6–4.4)
Neutrophils %	30.0 (13.8–45.3)	69.2*‡ (34.8–82.0)	14.3 (7.0–23.0)
Eosinophils %	1.3‡ (0.25–3.75)	0.5‡ (0–8.5)	0.0 (0–0)
Macrophages %	58.0 (41.3–74.8)	21.0*‡ (12.3–46.5)	73.8 (55.3–87.5)
Lymphocyte %	0.9 (0.3–1.8)	0.3† (0–1.3)	0.5 (0.0–0.8)
Columnar epithelial cell %	4.9 (1.7–8.5)	0.8* (0–2)	4.5 (0.3–13.0)

Data is Median (IQR); * $p < 0.001$ vs Stable asthma: bacterial load $< 10^6$ cfu/mL; † $p < 0.05$ vs Stable asthma: bacterial load $< 10^6$ cfu/mL; ‡ $p < 0.05$ vs Healthy controls.

Table IV. Stable asthmatics: inflammatory phenotype in sub-groups with and without a significant load of potentially pathogenic bacteria.

	Bacterial load $< 10^6$ cfu/mL, *n* (%)	Bacterial load $> 10^6$ cfu/mL, *n* (%)	*p*-value
Inflammatory phenotype:			
Neutrophilic	6 (7)	5 (33)	0.008
Eosinophilic	45 (49)	2 (13)	0.011
Mixed	7 (8)	4 (27)	0.048
Paucigranulocytic	33 (36)	4 (27)	0.568
Neutrophilic asthma (including neutrophilic and mixed phenotypes)	13 (14)	9 (60.0)	< 0.001
Eosinophilic asthma	45 (49)	2 (13)	0.011

sputum samples at one time point, did not establish that subjects were colonized over time, we did observe that the presence of a significant load of key potentially pathogenic bacteria at a single time point significantly correlated with enhanced airway inflammation. A significant bacterial load was associated with significantly higher total cell and absolute neutrophil counts, proportion of leukocytes that were neutrophils and sputum levels of IL-8. The role of increased airway levels of neutrophils and pro-inflammatory cytokines, such as IL-8, in asthma is unclear. Indeed, several studies have described increased neutrophilic airway inflammation in a sub-group of asthmatics [26,31] who demonstrate persistent symptoms and AHR in the absence of sputum eosinophils [26,31,32]. The most severe forms of asthma [33] involve elevated neutrophils. Furthermore, neutrophil counts increase with asthma severity [34] and sputum neutrophils correlate with both lung function (%FEV1) and the degree of airflow obstruction in asthma [26]. Therefore neutrophilic inflammation in asthmatic airways appears to be clinically important.

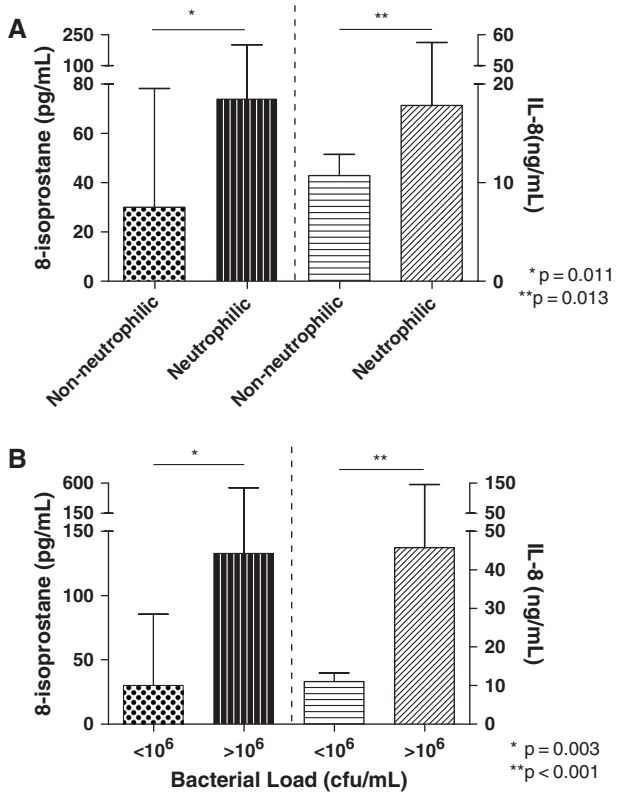

Figure 1. Induced sputum levels of 8-isoprostane and IL-8 in stable asthmatics (A) with and without airway neutrophilia and (B) with and without a significant load of potentially pathogenic respiratory bacteria. In graphs, bars represent median values, extended lines indicate inter-quartile range.

Importantly, a significant load of key potentially pathogenic bacteria was not cultured from the sputum of all subjects with neutrophilic asthma. In our study, 22 subjects with neutrophilic asthma were identified and a significant load of potentially pathogenic bacteria was found in nine (41%) of these subjects. This may indicate that bacteria were present that did not grow under the experimental conditions used in this study. Alternatively, other mechanisms may have led to airway neutrophilia, independent of typical bacteria. Stimuli such as viruses and atypical bacteria lead to increased levels of IL-8, neutrophils and neutrophil degranulation [35]. Similarly, stimuli such as ozone, endotoxin and air pollution can lead to neutrophilic inflammation via mechanisms such as the activation of the Toll-like receptor pathway that may sense non-infectious stimuli [36]. Thus, mechanisms other than the presence of typical bacteria may have led to the development of airway neutrophilia in these subjects.

The link between bacteria, impaired innate immune responses, airway neutrophilia and asthma is supported by examination of children with severe wheeze. In one study, young children with severe wheeze were reported to have a dominance of neutrophils in the airways [37,38]. In another study, risk of recurrent wheeze and asthma in early life was associated with bacterial isolation from the airway [39]. Recent investigations of adults with stable asthma have shown increased expression of toll like receptors and inflammatory cytokines in patients with neutrophilic asthma, suggesting a defect in the innate immune response in the neutrophilic sub-type of asthma [17]. A study in mice showed that effective clearance of *S. pneumoniae* was dependent on toll-like receptor 2 (TLR2) [40]. As genetic variation in TLR2 has been linked to asthma [41], it is

Table V. Multivariate predictors of $\log_{10}$ IL-8 and $\log_{10}$ 8-isoprostane.

	Variable	*R*-squared	Coefficient	*p*-value
$\log_{10}$ 8-isoprostane	Overall model	0.148		0.004
	Neutrophilic asthma		0.2747	0.124
	Bacterial load $> 10^6$ cfu/mL		0.4692	0.023
$\log_{10}$ IL-8	Overall model	0.314		<0.001
	Neutrophilic asthma		0.2739	0.075
	Bacterial load $> 10^6$ cfu/mL		0.7715	<0.001

plausible that modifications in TLR2 contribute to defective bacterial clearance in asthma. Impairment of alveolar macrophage phagocytosis has also been demonstrated in children with poorly controlled asthma, providing evidence of another innate immune defect that may lead to the presence of bacteria in the airways [42].

Our data show that the presence of a significant load of key potentially pathogenic bacteria in the airways in asthma is associated with an increase in airway oxidative stress, measured by induced sputum levels of 8-isoprostane. Subjects with neutrophilic airway inflammation also had an increase in 8-isoprostane levels, independent of the bacterial load. However, regression analysis revealed that the presence of a significant load of bacteria, and not airway neutrophils, was a significant predictor of both 8-isoprostane and IL-8. Increased oxidative stress has important effects on asthma pathophysiology, including increased airway smooth muscle contractility, induction of airway hyper-responsiveness, mucus hypersecretion, epithelial shedding and vascular exudation [12]. Furthermore, reactive oxygen species activate intracellular signalling cascades involving the transcription factors NF-κB, JAK-STAT and Raf-1 [12]. Importantly, it appears that the presence of a significant load of key potentially pathogenic bacteria in the airways leads to oxidative stress in asthma. The importance of bacteria as a driver of oxidative stress in asthma may explain the heterogeneity of data published to date, both describing the level of oxidative stress and the efficacy of antioxidant interventions in asthma. Our data suggest that subjects with a significant load of key potentially pathogenic bacteria present in the airways may be most likely to benefit from antioxidant supplementation.

The prevalence of bacterial isolation varies in different chronic airway diseases. Significant loads of key potentially pathogenic bacteria were not cultured from the sputum of any of the healthy controls in our study, while in asthma, significant loads of bacteria were cultured from 15% of subjects, of which 53% were neutrophilic asthma. Reported bacterial isolation rates in other diseases are: COPD 30–40% [6,8] and bronchiectasis 48% or greater [43]. Not all subjects with these diseases develop bacterial infection and the contributing factors are not well defined. We have found that the presence of a significant load of key potentially pathogenic bacteria in asthma is associated with neutrophilic bronchitis and increased inflammation and oxidative stress, which we have previously linked to local innate immune dysregulation [17]. Thus, innate immune factors may contribute to the presence of bacteria in asthma and should be evaluated in the other obstructive airway diseases associated with bacterial isolation from the lower airways. This study did not identify whether the inflammatory changes occurred as a consequence of, or a precursor to, the presence of these key potentially pathogenic bacteria in the airways and prospective studies are needed to further evaluate these possibilities. It should be noted that the definition of a significant load of bacteria in our study was a level which resulted in growth to $> 10^6$ colony forming units (cfu)/mL for any individual potentially pathogenic respiratory bacteria. This cut-point has also been used previously by others [44]. However, we acknowledge that changing this cut-point may result in different

Table VI. Inflammatory status of asthmatic subjects with a significant load of potentially pathogenic bacteria: smokers vs non-smokers.

	Never-smokers (n = 9)	Ex-smokers (n = 8)	*p*-value
Total cell count ($\times 10^6$/mL)	11.9 (6.4–20.0)	6.4 (4.8–10.3)	0.149
Neutrophils %	73.8 (34.8–97.5)	67.5 (32.5–80.8)	0.643
Neutrophil count ($\times 10^4$/mL)	909 (190–1645)	568 (162–728)	0.165
Eosinophils %	0 (0–17.3)	0.63 (0.3–2.1)	0.813
Eosinophil count ($\times 10^4$/mL)	0 (0–257.4)	3.3 (1.4–33.5)	0.724
Macrophages %	12.3 (2.5–39.0)	25.4 (16.7–60.4)	0.132
Lymphocyte %	0 (0–0.3)	0.8 (0–1.6)	0.297
8-isoprostane (pg/mL)	114 (42–949)	133 (47–189)	1.000
IL-8 (ng/mL)	45.8 (18.6–225.0)	46.5 (20.5–65.1)	0.670

Data is Median (IQR).

estimates for the prevalence of significant bacterial load in the airways in stable asthma. We believe that by using this relatively high cut-point, we are taking a conservative approach, which will ensure that all subjects deemed to have a significant bacterial load have a level of bacteria that is biologically important. This gives us confidence that we are truly addressing our hypothesis, which predicts neutrophilic inflammation will be increased when a significant load of key potentially pathogenic bacteria is present in stable asthma.

There were a number of significant differences in the clinical characteristics of the groups of asthmatic subjects with and without a significant load of key potentially pathogenic bacteria. While both groups had long standing asthma (median duration >25 years), the group with a significant bacterial load were older. The risk of bacterial isolation from the lower airway has been shown to increase with age, due to various factors, including changes in lung structure and function and a decline in immune function [45]. Structural changes may include defects in ciliary function. This may increase susceptibility to bacterial persistence, as mucociliary clearance is impaired and airborne pathogens are not quickly and effectively removed [46]. Altered immune responses with ageing include a gradual deterioration of acquired immunity and a modest increase in innate immune markers [47]. These include an increase in the number of lymphocytes and neutrophils in bronchoalveolar lavage fluid [48], a shift in T-cell sub-sets and activation markers, increased immunoglobulin and IL-6 concentrations and increased alveolar macrophage (AM) ROS production [48]. Neutrophil phagocytosis may also be impaired in the elderly [49]. Ageing is also associated with a decline in macrophage function, such as reduced expression of Toll-like receptors, impaired phagocytosis, decreased generation of nitric oxide and impaired secretion of certain cytokines and chemokines [50]. Each of these changes may reduce host protection against bacteria, which in turn may lead to enhanced neutrophil responses.

The majority of subjects with a significant load of potentially pathogenic bacteria had chronic bronchitis characterized by mucus hypersecretion. Chronic bacterial infection is known to damage airway epithelial cells, inducing an inflammatory response which further damages lung tissue and mucociliary clearance and enhances mucus secretion [14]. Similarly, neutrophilic inflammation leads to the release of elastase, which promotes mucus hypersecretion. Lung function and airflow obstruction were worse in this group, probably resulting from a variety of factors, including older age, as well as the presence of chronic bronchitis. It has previously been reported in COPD that the isolation of bacteria from the lower airway is associated with an increased lung function decline [16]. This raises the possibility that, in asthma, the presence of a significant bacterial load may also be associated with enhanced decline in lung function and this should be examined in longitudinal studies.

None of the asthmatics in this study were current smokers and less than half the subjects were ex-smokers. Smoking has been shown to increase the prevalence of lower airway bacterial isolation [51], which results from the deleterious effects on lower respiratory tract clearance mechanisms [52]. Our data suggest that the presence of a significant load of potentially pathogenic bacteria in the lower airways occurs in asthma, due to mechanisms that are distinct from the smoking-induced mechanisms involved in development of bacterial isolation in COPD. This is further supported by the analysis of subjects with a significant bacterial load, according to smoking status, which demonstrated that never-smokers had a similar inflammatory profile to ex-smokers.

The group with a significant load of key potentially pathogenic bacteria were taking twice the daily maintenance dose of inhaled corticosteroids (ICS) compared to the group without. This may be due to increased disease severity or because in subjects with a significant bacterial load, symptoms are being driven by mechanisms that respond poorly to ICS. The neutrophilic inflammatory pattern in this group is typically relatively resistant to ICS treatment [31,53,54]. Thus, a higher ICS dose may be required to make a clinical difference in this group. Indeed, subjects with airway neutrophilia also tended to have increased ICS use in our study, although this was not statistically significant (data not shown). Glucocorticoids may promote neutrophilic inflammation, due to their ability to increase survival (delay apoptosis) and functional responsiveness of neutrophils, which may lead to the persistence of neutrophils in tissues [55].

Alternatively, it is possible that ICS may facilitate bacterial growth and/or persistence in the airways. Recent data in COPD [54–56] show that ICS use is associated with an increased risk of pneumonia. Indeed, ICS use has been shown to impair local anti-microbial defences with increased mucosal candidiasis [56]. However, the mechanisms behind this effect are unclear. Glucocorticosteroids inhibit the synthesis and release of pro-inflammatory cytokines by alveolar macrophages, including IL-lβ, TNF-α, IL-8 and GM-CSF [57]. However, recent evidence suggests that glucocorticoids alter innate immune responses to bacterial infection. *H. influenzae* activates TLR2, thereby stimulating pro-inflammatory cytokine release from epithelial cells. Dexamethasone accentuates this effect, thereby increasing levels of TNFα, IL-1 and IL-8 [58], which may perpetuate neutrophilic inflammation. The implications of this amplified inflammatory response on bacterial isolation rates requires further investigation. Defence against infections is dependent on phagocytosis of invading pathogens. *In vitro* results indicate that budesonide attenuates AM capacity to phagocytose bacteria, which may be a mechanism by which ICS

may lead to increased susceptibility to bacterial colonization [57]. Interestingly, ICS are not the only factors influencing the presence of bacteria, as high doses were also being used by the group without a significant bacterial load. The relationship between the dose and duration of ICS use and bacterial isolation has not specifically been examined in asthma. However, an increased risk of invasive pneumoccocal disease has recently been identified in asthma [21]. This important issue requires further evaluation.

A limitation of this study is the possibility that induced sputum samples may have some contamination of bacteria from the upper airways. However, all efforts are made to select the opaque mucous portions of the sample leaving the saliva. Furthermore, we used selection procedures that have previously been validated to ensure minimal salivary contamination and maximise isolation of lower respiratory material. The use of induced sputum for detecting the presence of bacteria in lower airway samples is a well accepted and commonly used technique, due to the relative ease and non-invasive nature of sample collection [8,59].

In summary, a significant load of key potentially pathogenic bacteria is present in the airways of a proportion (15%) of subjects with stable asthma and this is associated with increased neutrophilic inflammation and increased oxidative stress in the lower airways. This has significant treatment implications for this subgroup of asthmatics. Subjects with chronic activation of innate immune responses are likely to be resistant to standard ICS treatment. The role of bacteria in potentiating neutrophilic asthma warrants further investigation, since therapies such as antibiotic and antioxidant treatment may be more effective in asthmatics with bacterial persistence in the lower respiratory tract.

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