Rhinoviruses (RVs) are the major cause of acute exacerbations of asthma. Human experimental RV infection in volunteers with mild asthma is associated with augmented physiologic and inflammatory responses to allergen challenge, reductions in peak expiratory flow (PEF) and FEV₁, and increases in bronchial reactivity. In agreement, our own previously reported study showed that experimental RV16 infection in asthma induced significant increases in bronchial reactivity, lower respiratory tract symptoms, and lung function impairment and that significant changes in these outcomes did not occur in normal subjects.

Regarding RV-induced airway inflammation, two previous studies of experimental RV infection reported increases in submucosal CD3⁺ lymphocytes and eosinophils in asthmatic and normal groups combined and in subjects with asthma alone. The increased...
number of mucosal CD3+ cells was accompanied by an increase in airway responsiveness and correlated positively with cold symptoms. However, it is unclear whether RV-induced bronchial mucosal inflammatory responses differ between subjects with and without asthma.

In this article, we extend our previous work to compare the nature of the bronchial mucosa inflammatory response to experimental RV infection in subjects with asthma and in normal healthy subjects and explore its physiologic and clinical significance in asthma. We hypothesized that the bronchial mucosal cellular inflammatory response to RV infection will be exaggerated and of a distinctive predominant inflammatory cell phenotype in subjects with asthma compared with normal healthy subjects and that the inflammatory cells recruited to the bronchial mucosa will be associated with virus load and increased clinical symptoms, airways responsiveness, and airflow obstruction associated with an exacerbation.

**Materials and Methods**

**Subjects**

We extended our previously reported investigation of the same 10 subjects with atopic asthma and 15 subjects without atopic asthma (Table 1). These subjects were human RV16 neutralizing antibody seronegative. This study was conducted in accordance with the amended Declaration of Helsinki. All subjects gave written informed consent, and the study protocol was approved by the St. Mary’s NHS Trust Research Ethics Committee (99/BA/345). See e-Appendix 1 for other details.

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**Results**

Inflammatory cells were present in both the bronchial epithelial and the bronchial subepithelial compartments. Representative photographs are shown in Figures 1A-E. Elastase-positive neutrophils (Fig 1A) and CD68+ monocytes/macrophages (Fig 1B) appeared to be more frequent in the bronchial mucosa of subjects with asthma during acute infection compared with baseline (Figs 1C, 1D). Application of irrelevant antibodies for the inflammatory cell markers was negative (Fig 1E).

**Absolute Counts of Inflammatory Cells**

**Total Leukocytes:** The number of subepithelial CD45+ cells in subjects with asthma was significantly greater at both baseline (P = .014) and day 4 infection (P = .025) (Fig 2A) than in normal subjects at the same time points. Compared with their respective
significantly increased at day 4. At this time point, there was a significantly greater number of epithelial neutrophils in the subjects with asthma ($P < .01$) (Fig 2B). EG2$^+$ eosinophil counts were not significantly increased from baseline in either group after infection (Table 2). Compared with normal subjects, subjects with asthma had a significantly higher number of epithelial eosinophils at baseline ($P < .01$), day 4 ($P < .046$), and week 6 ($P < .01$) (Table 2) and showed trends toward a greater number of subepithelial eosinophils at baseline ($P < .07$) and day 4 ($P < .09$), baseline and day 4 values, the number of subepithelial CD45$^+$ cells was significantly less at week 6 postinfection in both normal subjects ($P = .038$ and $P = .006$) and subjects with asthma ($P = .003$ and $P = .0006$) (Fig 2A). There were no significant differences in counts of epithelial CD45$^+$ cells within and between groups.

**Granulocytes:** Compared with baseline, the number of neutrophils in both epithelium ($P = .005$) (Fig 2B, Table 2) and subepithelium ($P = .017$) (Fig 2C) of the subjects with asthma but not of normal subjects were significantly increased at day 4. At this time point, there was a significantly greater number of epithelial neutrophils in the subjects with asthma ($P = .01$) (Fig 2B). EG2$^+$ eosinophil counts were not significantly increased from baseline in either group after infection (Table 2). Compared with normal subjects, subjects with asthma had a significantly higher number of epithelial eosinophils at baseline ($P = .011$), day 4 ($P = .046$), and week 6 ($P = .01$) (Table 2) and showed trends toward a greater number of subepithelial eosinophils at baseline ($P = .07$) and day 4 ($P = .09$).

**Table 1—Baseline Demographic Data**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Male (Female)</th>
<th>Sex</th>
<th>Age, y</th>
<th>Serum IgE, U/mL</th>
<th>FEV$_1$ % Predicted</th>
<th>Positive Skin Pick Test</th>
<th>Histamine PC$_{20}$, mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 15)</td>
<td>8 (7)</td>
<td></td>
<td>27 ± 2.3</td>
<td>27 ± 8.0</td>
<td>104 ± 3.3</td>
<td>0</td>
<td>20 ± 2.5</td>
</tr>
<tr>
<td>With asthma (n = 10)</td>
<td>2 (8)</td>
<td></td>
<td>23 ± 1.4</td>
<td>241 ± 49.8$^*$</td>
<td>106 ± 4.4</td>
<td>9</td>
<td>3 ± 0.7$^*$</td>
</tr>
</tbody>
</table>

Data are presented as counts or mean ± SEM. PC$_{20}$ = 20% fall in FEV$_1$.

$^*$P < .0001 vs normal (Student t test).

**Figure 1.** Immunohistochemistry-stained cells are seen as red fuchsin positivity. A and B, Subject with asthma at day 4 rhinovirus 16 infection demonstrating more elastase-positive neutrophils (A) and CD68$^+$ monocytes/macrophages (B) in bronchial epithelium and subepithelium. C and D, Subject with asthma at baseline showing fewer neutrophils (C) and CD68$^+$ cells (D). E, Negative control sample shows an absence of signal. Scale bar = 20 µm for all images.
The number of subepithelial mast cells of normal subjects at baseline was unexpectedly higher than at day 4 ($P = 0.036$) and week 6 ($P = 0.006$) (Table 2). The number of epithelial tryptase-positive mast cells was significantly greater in subjects with asthma than in normal subjects at day 4 ($P = 0.049$) and week 6 ($P = 0.009$) (Table 2).

Lymphocytes: In subjects with asthma only, the number of epithelial CD3$^+$ cells increased at week 6 from baseline ($P = 0.021$) (Table 2). Compared with normal subjects, subjects with asthma had a significantly greater number of subepithelial mast cells of normal subjects at baseline was unexpectedly higher than at day 4 ($P = 0.036$) and week 6 ($P = 0.006$) (Table 2). The number of subepithelial mast cells of normal subjects at baseline was unexpectedly higher than at day 4 ($P = 0.036$) and week 6 ($P = 0.006$) (Table 2). The number of epithelial tryptase-positive mast cells was significantly greater in subjects with asthma than in normal subjects at day 4 ($P = 0.049$) and week 6 ($P = 0.01$) (Table 2).

Macrophages: Compared with baseline, the number of subepithelial CD68$^+$ cells in both normal subjects ($P = 0.018$) and subjects with asthma ($P = 0.009$) were significantly increased at day 4 of acute infection (Fig 2D). There was a significantly greater number of subepithelial CD68$^+$ cells present in subjects with asthma than in normal subjects at day 4 ($P = 0.021$). No significant differences in counts of epithelial CD68$^+$ cells were found within and between groups (Table 2).

Mast Cells: There were no significant differences within the subjects with asthma in epithelial and subepithelial mast cell counts from baseline to day 4 or week 6 (Table 2). The number of subepithelial mast cells of normal subjects at baseline was unexpectedly higher than at day 4 ($P = 0.036$) and week 6 ($P = 0.006$) (Table 2). The number of epithelial tryptase-positive mast cells was significantly greater in subjects with asthma than in normal subjects at day 4 ($P = 0.049$) and week 6 ($P = 0.01$) (Table 2).
Clinical Symptoms and Airway Hyperresponsiveness: At day 4, epithelial CD4+ counts in subjects with asthma correlated with total chest symptom scores recorded during the 2-week postinfection period \((r = 0.69, P = 0.029)\) (Fig 5A). Those with high subepithelial neutrophil counts had a significantly larger fall in the 10% fall in FEV1 \((PC_{10})\) from baseline to day 6 postinfection \((r = 0.89, P = 0.029)\) (Fig 5B). The PC_{10} at day 6 correlated inversely with subepithelial CD3+ \((r = -0.81, P = 0.016)\) (Fig 5C), CD4+ \((r = -0.67, P = 0.023)\) (Fig 5D), and CD8+ \((r = -0.65, P = 0.03)\) (Fig 5E) cells, and the 20% fall in FEV1 at day 6 was inversely associated with subepithelial CD20+ cells \((r = -0.65, P = 0.03)\) (Fig 5F).

Lung Function: At day 4, higher epithelial CD5+ cell counts in subjects with asthma were significantly associated with a greater maximum fall in FEV1 (percent fall from baseline) \((r = -0.72, P = 0.03)\) (Fig 6A). Of interest, higher subepithelial mast cell counts were significantly associated with a lower maximum percent fall in PEF \((r = 0.8, P = 0.024)\) (Fig 6B). In all subjects at day 4, higher epithelial \((r = 0.64, P = 0.03)\) and subepithelial \((r = -0.53, P = 0.01)\) CD4+ cell counts were significantly associated with a greater maximum fall in FEV1.

Serum IgE: The levels of serum IgE were significantly correlated with subepithelial EG2+ eosinophils in normal subjects at day 4 \((r = 0.64, P = 0.017)\) and in subjects with asthma at both baseline \((r = 0.94, P = 0.005)\) and day 4 \((r = 0.83, P = 0.013)\) (Figs 7A, 7B).
Increased numbers of neutrophils have been detected in the bronchial mucosa of subjects with severe asthma experiencing naturally occurring exacerbations. To our knowledge, however, no attempt has been made to determine whether RV infection alone can initiate bronchial mucosal neutrophilia, and its physiologic and clinical outcomes are unknown.

We reported in a previous publication on the clinical study from which the present biopsy specimens were collected increased numbers of BAL eosinophils during acute infection in the asthmatic group relative to the normal group, with similar, but nonsignificant trends for neutrophils. However, significant increases in BAL or induced sputum inflammatory cells from baseline to acute RV infection within the asthma group were not detected. Consistent with the BAL results, we found increased epithelial eosinophil and neutrophil counts in subjects with asthma vs normal subjects during acute RV infection. In addition, we demonstrated significant increases in the number of both epithelial and subepithelial neutrophils from baseline to acute infection in the asthma group, which were significantly higher than that in the normal group. We did not find increased numbers of neutrophils at week 6, which suggests that virus-induced airway neutrophilic inflammation in patients with mild asthma occurs only during the virus-induced acute asthma exacerbation and not during convalescence. Thus, in contrast to normal subjects, acute experimental RV16 infection selectively increases bronchial mucosal neutrophils in atopic asthma, and the higher numbers of neutrophils are related to the severity of infection and airway hyperresponsiveness. These data suggest that virus infection is one of the major etiologies inducing neutrophilic asthma. Because neutrophils play an important role in innate immune responses to clear virus, they can cause destruction of lung tissues through release of their proteases and other mediators. Therefore, the balance between the protective and pathologic effects of neutrophils in the course of virus-induced asthma exacerbations needs to be clarified when drug treatment to control neutrophilic inflammation is studied in the future.

Whereas we consider that asthma severity and treatment affect the magnitude of the inflammatory response to RV infection, the nature of the inflammation is more influenced by the time at which the biopsy specimens are taken in relation to the onset of RV infection. With respect to the latter, we believe that the results should not have been affected by the subjects with mild asthma studied who had not taken inhaled or oral corticosteroids.

The effect of RV infection on sputum and BAL CD68+ cells in subjects with and without asthma was not investigated in our previously reported study.

There were no significant associations between serum IgE level and other phenotypes of inflammatory cells.

**Discussion**

We demonstrated that compared with normal subjects, subjects with asthma and RV infection had an exaggerated bronchial mucosal inflammatory response particularly characterized by increased numbers of neutrophils and CD68+ monocytes/macrophages. Significant correlations were found between CD45+ cells, neutrophils, and CD68+ cells and virus load and between neutrophils and T and B lymphocytes and airway hyperresponsiveness, chest symptoms, and reduced lung function, whereas mast cells were related to better lung function.

Previous experimental studies have shown that RV16 infection is associated with a significant increase in peripheral blood and BAL neutrophil counts in allergic asthma. In sputum, virus infection in asthma exacerbations has been associated with increased numbers of neutrophils and elevation of both neutrophil and eosinophil degranulation products, and cell lysis has been related to clinical severity. Increased numbers of neutrophils have been detected in the bronchial mucosa of subjects with severe asthma experiencing naturally occurring exacerbations. To our knowledge, however, no attempt has been made to determine whether RV infection alone can initiate bronchial mucosal neutrophilia, and its physiologic and clinical outcomes are unknown.

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Whereas we consider that asthma severity and treatment affect the magnitude of the inflammatory response to RV infection, the nature of the inflammation is more influenced by the time at which the biopsy specimens are taken in relation to the onset of RV infection. With respect to the latter, we believe that the results should not have been affected by the subjects with mild asthma studied who had not taken inhaled or oral corticosteroids.

The effect of RV infection on sputum and BAL CD68+ cells in subjects with and without asthma was not investigated in our previously reported study.
Seymour et al. reported an increase in the number of macrophages seen in bronchial biopsy specimens of normal subjects in response to experimental RV16 infection, but subjects with asthma were not investigated. In contrast, the present study shows that acute RV infection increases the number of subepithelial CD68+ cells of subjects with and without asthma. Notably, the changes in levels observed in the asthma group from baseline to acute infection were significantly greater than those in the normal group. Other work has suggested that impaired macrophage production of T-helper 1-related cytokines is strongly implicated in asthma exacerbation pathogenesis. Therefore, further investigation is needed to discern their role in asthma exacerbations induced by RV infection.

Airway mast cells are key in the immediate allergic response and are long associated with the pathogenesis of stable asthma. Using chloroacetate esterase to identify lysosomal granules in mast cells, Lozewicz et al. found increased mast cell numbers in endobronchial biopsy specimens from subjects with atopic asthma compared with normal subjects. However, the numbers of tryptase-positive mast cells, tryptase-positive and chymase-negative mast cells, and tryptase- and chymase-positive mast cells in bronchial mucosa of subjects with symptomatic atopic asthma was not greater than that in normal subjects. We did not find a difference in the number of tryptase-positive mast cells in the bronchial mucosa between subject groups at baseline, which is consistent with these previous reports. Although there were no changes in numbers of epithelial mast cells within either group during RV infection, we detected a greater number of epithelial mast cells in asthma day 4 and week 6 compared with normal day 4 and week 6, implying that asthma susceptibility to immediate allergic response could be greater after RV infection. The numbers of subepithelial mast cells declined between baseline and acute infection and further declined between acute infection and convalescence in normal subjects. This finding appears to be inconsistent with an earlier study in which the number of tryptase-positive mast cells in bronchial submucosa declined between the infection and convalescent periods, but no initial increase was seen between baseline and infection. Of note, we detected in the present study that in subjects with asthma, those with the highest subepithelial mast cell counts had the lowest maximum percent falls in PEF during acute infection. In this regard, the findings support the results described previously in which the numbers of mast cells in severe asthma correlated with better rather than worse lung function, suggesting that recruitment of mast cells, particularly to the distal lung, may be protective for lung function in severe asthma.
The biologic mechanism for a protective role remains unclear. Rauter et al.\(^2\) notably demonstrated that \(\beta\)-tryptase, a major protease released during mast cell activation, cleaves IgE. They suggested that IgE cleavage by effector cell proteases is a natural mechanism for controlling allergic inflammation. The role of mast cells in the pathogenesis of virus-induced asthma exacerbations clearly merits further study.

We demonstrate for the first time, to our knowledge, in subjects with asthma that epithelial CD4\(^+\) cell counts at day 4 of infection correlates with total chest symptom scores, and the number of bronchial mucosal CD3\(^+\), CD4\(^+\), and CD8\(^+\) T and CD20\(^+\) B lymphocytes are associated with increased airway hyperresponsiveness. Additionally, the subjects with asthma with high epithelial CD8\(^+\) counts had significantly greater maximum falls in FEV\(_1\). The findings suggest the involvement of both T and B lymphocytes in the severity of asthma exacerbations induced by virus infection. The absence of increased counts of CD3\(^+\) and CD8\(^+\) T lymphocytes on day 4 of acute RV infection in subjects with asthma alone is not in keeping with increased subepithelial CD3\(^+\), CD4\(^+\), and CD8\(^+\) cells at day 4, and between PC\(_{10}\) at day 6 and counts of subepithelial CD20\(^+\) cells at day 4 (P) (Spearman rank correlation, n = 10 for all). PC\(_{10}\) = 10% fall in FEV\(_1\); PC\(_{20}\) = 20% fall in FEV\(_1\).

**Figure 5.** A-F. In subjects with asthma alone, correlations between total chest symptom score d 0 to 14 after the rhinovirus 16 infection period and counts of epithelial CD4\(^+\) cells (A), between fall in PC\(_{10}\) (log) and counts of subepithelial neutrophils at d 4 (B), between PC\(_{10}\) at d 6 and counts of subepithelial CD3\(^+\) (C), CD4\(^+\) (D), and CD8\(^+\) (E) cells at d 4; and between PC\(_{20}\) at d 6 and counts of subepithelial CD20\(^+\) cells at d 4 (F) (Spearman rank correlation, n = 10 for all). PC\(_{10}\) = 10% fall in FEV\(_1\); PC\(_{20}\) = 20% fall in FEV\(_1\).
Another study showed no difference in changes of bronchial mucosa inflammatory cell counts between normal subjects and subjects with atopic rhinitis experiencing naturally occurring common cold virus infection. However, to answer this question directly, bronchial mucosa inflammatory responses to experimental RV16 infection in subjects with atopy but not asthma requires further investigation.

We also acknowledge that we have investigated relatively small numbers of subjects, albeit in some detail, and that a large number of statistical tests were carried out; thus, the study may have failed to detect some genuine observations due to limitations of statistical power. However, we consider its strengths to include the comprehensive and meticulous immunochemical approach and rigorous design of the human challenge model, permitting accurate sampling before and during infection of a uniform nature and known time of onset. Despite these caveats, novel data and interesting observations have emerged.

In conclusion, RV16 infection induced a more severe neutrophil and monocyte/macrophage-rich inflammatory response in the bronchial mucosa of subjects high levels of IgE had significantly greater lower respiratory tract symptom scores during the initial 4 days of infection than those with low IgE levels. These patients also had higher total blood eosinophil counts at baseline. Building on these findings, we demonstrate that subjects with asthma with high levels of serum IgE at both baseline and day 4 have significantly greater numbers of submucosal EG2⁺ eosinophils. These findings imply that patients with atopic asthma with high levels of serum IgE could have more EG2⁺ eosinophils in their airway and experience more severe illness during acute RV infection. Anti-IgE therapy, therefore, may be useful for preventing exacerbations of asthma triggered by RV infection.

We acknowledge that an atopic nonasthma group was not included in the present study. Thus, the differences observed between the normal and asthma groups may have been partly due to changes attributable to atopy vs asthma. Previous work reported that compared with normal subjects without atopy, nasal biopsy specimens of subjects with atopy during experimental RV16 infection did not show increases in infiltration of inflammatory cells (including neutrophils).

Another study showed no difference in changes of bronchial mucosa inflammatory cell counts between normal subjects and subjects with atopic rhinitis experiencing naturally occurring common cold virus infection. However, to answer this question directly, bronchial mucosa inflammatory responses to experimental RV16 infection in subjects with atopy but not asthma requires further investigation.

We also acknowledge that we have investigated relatively small numbers of subjects, albeit in some detail, and that a large number of statistical tests were carried out; thus, the study may have failed to detect some genuine observations due to limitations of statistical power. However, we consider its strengths to include the comprehensive and meticulous immunochemical approach and rigorous design of the human challenge model, permitting accurate sampling before and during infection of a uniform nature and known time of onset. Despite these caveats, novel data and interesting observations have emerged.

In conclusion, RV16 infection induced a more severe neutrophil and monocyte/macrophage-rich inflammatory response in the bronchial mucosa of subjects
with asthma compared with normal subjects similarly infected. Numbers of mucosa neutrophils, monocytes/macrophages, EG2+ eosinophils, and CD4+, CD8+, and CD20+ cells in subjects with asthma during acute infection were related to virus load, increasing airway reactivity, and worsening of asthma symptoms and lung function, whereas tryptase-positive mast cells were related to better lung function. These data add to our understanding of the pattern of inflammatory response occurring during RV-induced experimental exacerbations of asthma and, as such, could have an impact on the design of future treatment modalities.

**ACKNOWLEDGMENTS**

**Author contributions:** Drs Zhu and Message had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Dr Zhu: contributed to the counting, data analyses, drafting of the manuscript, and review of the manuscript.

Dr Message: contributed to the subject recruitment, bronchial biopsy specimen and relevant clinical data collection, and writing and review of the manuscript.

Dr Qiu: contributed to the immunohistochemical staining and writing and review of the manuscript.

Dr Mascelli: contributed to the counting, data analyses, drafting of the manuscript, and review of the manuscript.

Dr Contoli: contributed to the subject recruitment, bronchial biopsy specimen and relevant clinical data collection, and writing and review of the manuscript.

Dr Condon: contributed to the counting, data analyses, drafting of the manuscript, and review of the manuscript.

Dr Barnathan: contributed to the subject recruitment, bronchial biopsy specimen and relevant clinical data collection, and writing and review of the manuscript.

Dr Kon: contributed to the study design and writing and review of the manuscript.

Dr Mascelli: contributed to the study design and writing and review of the manuscript.

Dr Papi: contributed to the study design and writing and review of the manuscript.

Dr Stanciu: contributed to the study design and writing and review of the manuscript.

Prof Jeffery: contributed as the co-principal investigator, to the direction of all histologic aspects of the study, and to the writing and review of the manuscript.

Prof Johnston: contributed as the principal investigator, to the direction of all aspects of the study, and to the writing and review of the manuscript.

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**Additional information:** The e-Appendix can be found in the “Supplemental Materials” area of the online article.

**REFERENCES**


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Original Research


