Association between airway hyperreactivity and bronchial macrophage dysfunction in individuals with mild asthma

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Received 24 May 2000; accepted in final form 29 August 2000

Alexis, Neil E., Joleen Soukup, Stefan Nierkens, and Susanne Becker. Association between airway hyperreactivity and bronchial macrophage dysfunction in individuals with mild asthma. Am J Physiol Lung Cell Mol Physiol 280: L369–L375, 2001.—Little is known about the functional capabilities of bronchial macrophages (BMs) and their relationship to airway disease such as asthma. We hypothesize that BMs from asthmatics may be modulated in their function compared with similar cells from healthy individuals. BMs obtained by induced sputum from mild asthmatics (n = 20) and healthy individuals (n = 20) were analyzed using flow cytometry for CD16, CD64, CD11b, CD14, and human leukocyte antigen-DR expression, phagocytosis of IgG opsonized yeast, and oxidant production. Asthma status was assessed by lung function [percent predicted forced vital capacity and forced expiratory volume in 1 s (FEV1)], percent sputum eosinophils, and nonspecific airway responsiveness [provocative concentration that produces a 20% fall in FEV1 (PC20,FEV1)]. Asthmatics with >5% eosinophils (AEo+) had decreased expression of CD64 while CD11b was increased in asthmatics (3, 33–35). As a result, induced sputum is well suited to examine bronchial airway cells from asthmatic subjects. Sputum eosinophilia, described cytologically as >2–5% eosinophils (18, 22), can serve as a reliable inflammatory marker of asthma severity in asthmatics with airway eosinophilia (32, 46, 49). However, many asthmatics do not have airway eosinophilia even following acute exacerbations (18, 23, 47). Asthmatics with eosinophilic sputum have been reported to have increased symptoms of coughing and wheezing, greater airway obstruction, and more severe airway hyperresponsiveness compared with asthmatics without sputum eosinophilia (18). Few studies have attempted to investigate the characteristics and functions of cells obtained by the induced sputum technique (19). In this study, we wanted to determine whether, in mild atopic asthmatics, lung function and sputum eosinophilia, which have been shown to be associated, also correlate with modulated macrophage phenotype and function. Flow cytometry was used to assess the expression of

The immunopathology of allergic asthma is characterized by peribronchial infiltration of macrophages, eosinophils, and T cells in stable and symptomatic periods of disease (2, 5, 37, 39). These cells may all be functionally involved in asthma symptomatology. Among them, macrophages are critical in removing inhaled particulates and microbes that have the potential to exacerbate asthma (6, 11, 43). Previous studies have suggested a link between decreased phagocytic function and asthma (26, 27) as well as enhanced superoxide generation (10, 20) and mediator release [54x332]with PC20,FEV1 (R correlation was found between CD64 expression and BM phagocytosis compared with asthmatics with concentration that produces a 20% fall in FEV1 (PC20,FEV1)]. Asthmatics, and nonspecific airway responsiveness [provocative concentration that produces a 20% fall in FEV1 (PC20,FEV1)]. Asthmatics, and nonspecific airway responsiveness [provocative concentration that produces a 20% fall in FEV1 (PC20,FEV1)]. Asthmatics, and nonspecific airway responsiveness [provocative concentration that produces a 20% fall in FEV1 (PC20,FEV1)]. Asthmatics, and nonspecific airway responsiveness [provocative concentration that produces a 20% fall in FEV1 (PC20,FEV1)].
cell surface receptors known to be modulated by environmental factors as well as by phagocytosis in 20 mild asthmatic subjects and 20 controls.

The data obtained suggest an intriguing correlation between eosinophilic inflammation, lung function, and decreased BM function, associated with decreased CD64 expression, in subjects with mild asthma.

METHODS

Subjects. Subject characteristics are shown in Table 1. Nonsmoking volunteers between 18 and 40 yr of age were recruited for the study. A medical screening exam that included a medical history, psychological questionnaire, physical exam, blood tests, and allergy scratch tests was performed on all subjects on a separate day before the study. All asthmatics and 10 healthy subjects received a methacholine challenge test to assess nonspecific airway responsiveness. The maximum concentration of methacholine used was 40 mg/ml. Seven of ten healthy subjects were able to achieve a nonspecific airway responsiveness (provocative concentration that produces a 20% fall (PC_{20},FEV_{1}) in forced expiratory volume in 1 s (FEV_{1};PC_{20},FEV_{1}) by the maximum concentration. Pulmonary function tests were performed between 8:00 and 9:00 AM on all subjects, and with the exception of one subject, none of the asthmatics had taken any bronchodilators on an as needed basis, and no asthmatic subjects used inhaled steroids within the previous 6 mo. This study was approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina, Chapel Hill.

Induced sputum. The induction procedure of Pin et al. (32) was followed with some modifications. Three 7-min inhalation periods of 3, 4, and 5% hypertonic saline were administered following baseline spirometry. All asthmatics were premedicated with inhaled β_{2}-agonist before saline inhalation, and FEV_{1} was checked following each saline dose. At the end of each 7-min inhalation period, subjects performed a three-step cleansing procedure before a cough attempt as follows: 1) the mouth was rinsed and gurgled with water, 2) the back of the throat was cleared (without coughing), and 3) the nose was blown. The subject was then instructed to perform a "chesty-type" cough without clearing the back of the throat. The sample was expectorated into a sterile specimen cup that was placed on ice throughout the procedure.

Sample processing began immediately according to the method of Pizzichini et al. (34). In brief, mucus plugs were manually selected and weighed, incubated (15 min at room temperature) in four times the plug weight (in mg) of 0.1% dithiothreitol (Calbiochem, San Diego, CA), washed with four times the plug weight (in µl) in Dulbecco's PBS, and gravity filtered through a 48-µm-pore mesh filter (BBSH Thompson, Scarborough, Ontario). Total cell counts were performed with the use of a Neubauer hemocytometer. Visualy identifiable squamous epithelial cells were not counted or included in the total cell count. Cell viability was determined using trypan blue exclusion stain.

Table 1. Subject characteristics and baseline lung function

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Atopy</th>
<th>β₂-Agonist Use</th>
<th>Inhaled Steroid Use in Past 6 Months</th>
<th>%Predicted FEV₁</th>
<th>%Predicted FVC</th>
<th>PC_{20,FEV₁}, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27 ± 2.0</td>
<td>M = 13</td>
<td>F = 7</td>
<td>0</td>
<td>None</td>
<td>112 ± 2.0</td>
<td>116 ± 2.0</td>
<td>11.9 (5–24)</td>
</tr>
<tr>
<td>Asthmatic</td>
<td>27.2</td>
<td>M = 14</td>
<td>F = 6</td>
<td>20</td>
<td>None</td>
<td>104 ± 2.0</td>
<td>109 ± 1.6</td>
<td>1.48 (0.02–20)</td>
</tr>
<tr>
<td>AEo+</td>
<td>28 ± 1.0</td>
<td>M = 7</td>
<td>F = 2</td>
<td>9</td>
<td>None</td>
<td>101 ± 2.0</td>
<td>106 ± 1.0</td>
<td>0.4 (0.02–3.9)</td>
</tr>
<tr>
<td>AEo−</td>
<td>27 ± 2.0</td>
<td>M = 7</td>
<td>F = 4</td>
<td>11</td>
<td>None</td>
<td>108 ± 2.0</td>
<td>113 ± 2.0</td>
<td>2.3 (0.2–20)</td>
</tr>
</tbody>
</table>

Values are means ± SE for age, %predicted forced expiratory volume in 1 s (FEV₁), and %predicted forced vital capacity (FVC) and are geometric mean (range) for nonspecific airway responsiveness (provocative concentration that produces a 20% fall in FEV₁ (PC_{20,FEV₁})), M, male; F, female; AEo+, eosinophilic; AEo−, noneosinophilic. *P < 0.05, significantly different from control. †P < 0.001, significantly different from control. **P < 0.003, significantly different from AEo−. 1P < 0.002, significantly different from AEo−. *P < 0.001, significantly different from control and AEo−.
ing. Differential leukocyte analysis of nonsquamous cells (Diff-Quik stained) was performed on a minimum of 400 cells, provided that squamous cells were <40% of the total cells. Differential cell counts (lymphocytes, neutrophils, eosinophils, monocytes, and macrophages) are expressed as a percentage of total nonsquamous nucleated cells.

Analysis of fluid-phase cytokines and eosinophil cationic protein. Supernatants from the soluble fraction of the spun sputum sample were collected and analyzed for IL-4, IL-5, IL-8, IFN-γ, and eosinophil cationic protein (ECP). Cytokines were measured by specific and sensitive human immunoassays (ELISA, Quantikine; R&D Systems, Minneapolis MN). The lower limits of detection for IL-4, IL-5, IL-8, and IFN-γ were as follows: 35, 3, 10, and 3 pg/ml, respectively. ECP levels in sputum supernatant were measured by means of specific radioimmunoassay (Pharmacia RIA, Uppsala, Sweden). This method has been proven highly specific and sensitive (lower detection limit <2 μg/l), with high intra- and interassay reproducibility.

Flow cytometry. Flow cytometry was performed with a FACSORT (Becton Dickinson) using an argon-ion laser (wavelength = 488 nm). Gain and amplitude settings were set so as to analyze both blood and sputum samples from the same subject to establish reference gates for sputum leukocyte identification. Settings were kept the same throughout the study for each subject. The FACSORT was calibrated with Calibrite (Becton Dickinson) beads (no color, green, and red) before each use. Ten thousand events were counted for all sample runs. Gating of white cells in sputum was based on a combination of light-scatter characteristics and positive/negative expression for relevant surface markers, i.e., lymphocytes CD3+/CD14+, monocytes CD14+/CD3+, neutrophils CD16+/HLA-DR+, macrophages HLA-DR+/CD14+, and the use of reference gates based on isolated (Percoll-separated) leukocytes from whole blood preparation. Side light scatter reflects cell density/granularity and forward light scatter reflects cell size (41). Using these techniques, discrete populations of lymphocytes, monocytes, neutrophils, macrophages, and eosinophils were observed. Fluorescein (FITC-) and phycoerythrin-conjugated nonspecific antibodies of the same isotype as the receptor antibodies were used as controls to establish background fluorescence and nonspecific antibody binding. The mean fluorescence intensity (MFI) of the cells stained with control antibody was subtracted from the mean fluorescence of the cells stained with receptor antibodies to provide a measure of receptor-specific fluorescence.

Immunofluorescence staining. Aliquots of 100 μl (100,000 cells/tube) or 200 μl (200,000 cells/tube) of sputum cell suspension (1 × 10⁶ cells/ml) were stained with 10 or 20 μl, respectively, of saturating concentrations of monoclonal antibodies (Immunotech, Coulter, France) for 60 min in the dark until analyzed by flow cytometry (FACSORT) within 24 h of particle exposure. Particle uptake was identified on histogram analysis as a uniform rightward shift into the M2 region (MFI) from the control (non-particle-exposed) M1 region. Phagocytosis was determined by measuring the MFI of the cells in the particle-exposed region (M2) less the MFI of the cells in the control region (M1). A uniform shift in all sputum phagocyte populations was routinely observed when cells were exposed to particles such that at least 93% of the cells shifted (with particle exposure) into M2 compared with M1 (no particle exposure) on all occasions.

Statistical analyses. Statistically significant differences between subject groups were assessed by one-way ANOVA. Parametric statistics were applied on log-transformed PC20 data for tests of differences between means and for correlations. Simple linear regression analyses were performed to analyze associations between variables, and Pearson’s correlation coefficients (R) were determined from these analyses. A value of P < 0.05 was considered statistically significant.

RESULTS

Characterization of subjects, their lung function, and sputum differentials. Subject characteristics including baseline lung function and nonspecific airway responsiveness are shown in Table 1. All the asthmatics examined had mild, well-controlled disease, i.e., inhaled bronchodilator was used as needed, no inhaled corticosteroids were taken within the previous 6 mo, and a normal range spirometry was present. Sputum eosinophil counts are shown in Table 2. Asthmatic subjects with 5% or greater sputum eosinophils were classified as eosinophilic (AEo+; n = 9), and asthmatic subjects with <5% sputum eosinophils were classified

Table 2. Total and differential cell counts

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total Cells/mg Sputum</th>
<th>Viability, %</th>
<th>Sputum, %PMN</th>
<th>Sputum, %MAC</th>
<th>Sputum, %EOS</th>
<th>Sputum, %LYM</th>
<th>Blood, %EOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5,128 ± 640.0</td>
<td>77 ± 2.0</td>
<td>37 ± 3.0</td>
<td>56 ± 3.0</td>
<td>1 ± 0.3</td>
<td>2 ± 0.4</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>AEo+</td>
<td>4,780 ± 1,193.0</td>
<td>76 ± 3.0</td>
<td>18 ± 4.0</td>
<td>47 ± 3.0†</td>
<td>32 ± 3.0</td>
<td>2 ± 0.6</td>
<td>4.2 ± 0.7‡</td>
</tr>
<tr>
<td>AEo−</td>
<td>3,081 ± 563.0</td>
<td>79 ± 2.0</td>
<td>91 ± 5.0</td>
<td>62 ± 5.0</td>
<td>2 ± 0.4</td>
<td>3 ± 0.6</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. PMN, polymorphonuclear neutrophils; MAC, macrophages; EOS, eosinophils; LYM, lymphocytes. *P < 0.05, significantly different from control. †P < 0.05, significantly different from AEo+; ‡P < 0.004, significantly different from AEo−.
as noneosinophilic (AEo--; n = 11). The arbitrary 5% cutoff for designating sputum eosinophilia was based on prior reports describing sputum eosinophilia in asthmatics (33, 18, 22, 46, 47). Asthmatics demonstrated lower baseline lung function (%predicted FVC, %predicted FEV\(_1\)) and higher nonspecific airway responsiveness (PC\(_{20,FEV1}\)) than healthy controls, and AEo+ subjects demonstrated lower baseline lung function, higher nonspecific airway responsiveness, and greater peripheral blood eosinophils than AEo− subjects. The AEo− subjects demonstrated significantly greater nonspecific airway responsiveness than healthy controls (PC\(_{20,FEV1}\) = 2.3 vs. 11.9 mg/ml, \(P < 0.05\)), verifying their mild asthma status. Significant inverse relationships were observed (data not shown) between the percentage of sputum eosinophils and the percentage of predicted FEV\(_1\) (\(R = -0.5\), \(P < 0.001\)), FVC (\(R = -0.5\), \(P < 0.001\)) and PC\(_{20,FEV1}\) (\(R = -0.5\), \(P < 0.002\)) for all asthmatics.

Sputum differential and total cell counts (TCC) and cell viability are shown in Table 2. All subjects were able to successfully produce an acceptable sputum sample. The samples contained <40% squamous cells and had >60% cell viability based on trypan blue exclusion staining. No difference in TCC was observed between the AEo+ and AEo− subjects, and there was no significant difference in the weight of the selected sputum portion (in mg) between the AEo+ and AEo− subjects (940 ± 260 vs. 1,069 ± 269 mg, \(P < 0.2\)).

**Flow cytometric analysis of induced sputum samples.** The flow cytometer was used to quantify the expression of CD11b, CD14, CD16, CD64, and HLA-DR on BM. The identification of the BM population was easily achieved by the light-scatter properties (forward scatter and side scatter) of the population with no overlap with neutrophils or monocytes. Figure 1 shows the results of surface marker expression of CD11b, CD64, CD16, CD14, and HLA-DR on BMs in healthy and asthmatic AEo+ and AEo− subjects. When subdivided into AEo+ and AEo− asthmatics, however, FcyRI (CD64, \(P < 0.04\)) and CR3 (CD11b, \(P < 0.05\)) expression (MFI) were significantly decreased in AEo+ subjects compared with the AEo− subjects and controls (CD64, \(P < 0.03\); CD11b, \(P < 0.05\)). Expression of CD16, CD14, and HLA-DR on BMs was not altered in subjects with eosinophilic inflammation.

**Sputum macrophage phagocytosis of IgG-opsonized yeast particles.** No difference was observed in the percentage of BM-phagocytosing particles between asthmatics and controls or between asthmatic subgroups. More than 94% of BMs took up particles in all subjects.
When the number of particles taken up by the BM was measured as MFI of the BM population, AEo+ subjects showed significantly decreased phagocytosis compared with the AEo− subjects (P < 0.001) and controls (P < 0.001) (Fig. 2). BM phagocytosis was significantly related to the expression of FcγRI (CD64; R = 0.7, P < 0.01; Fig. 3).

Association between sputum macrophage phagocytosis and markers of asthma severity. Sputum macrophage phagocytosis appeared to be predictive of disease severity. Significant correlations were observed between sputum macrophage phagocytosis and markers of disease severity, such as percentage of eosinophils (R = −0.6, P < 0.01; Fig. 4A), percentage of predicted FEV1 (R = 0.7, P < 0.002; Fig. 4B), percentage of predicted FVC (R = 0.8, P < 0.0001; Fig. 4C), and PC20,FEV1 (R = 0.6, P < 0.007), when all asthmatics were included.

Fluid-phase components. The subjects with eosinophilia (AEo+) demonstrated significantly higher levels of ECP compared with the AEo− subjects (P < 0.05) and healthy controls (P < 0.001), and ECP levels were significantly related to the percentage of eosinophils in all asthmatic subjects (R = 0.7, P < 0.001; data not shown) (Table 3). Cytokines IL-5 and IL-8 were detectable in airway fluid but did not differ between AEo+ and AEo− subjects. IL-4 was not detectable in the same samples.

**DISCUSSION**

This study has analyzed the phenotype and function of BMs obtained from induced sputum from mild asthmatic and healthy subjects. Based on their composition of airway leukocytes, the asthmatic subjects could be separated into those with airway eosinophilia as defined by the presence of >5% sputum eosinophils (mean = 32%) and those with noneosinophilic sputum (mean = 2%). The assessment of asthma severity by nonspecific bronchial responsiveness, baseline pulmonary function, and ECP levels supported the notion that increased levels of eosinophilic inflammation is associated with increased airway responsiveness and therefore disease severity (22). Accordingly, the AEo+ subject group was hyperreactive compared with the AEo− subject group. Our results showed that BMs from AEo+ subjects have decreased phagocytic function and decreased expression of FcγRI (CD64) and CR3 (CD11b) compared with AEo− subjects; however, as one group, the mild asthmatics did not differ from

**Table 3. Fluid-phase components in sputum**

<table>
<thead>
<tr>
<th>Subject</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
<th>IL-8</th>
<th>IL-10</th>
<th>ECP</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51 ± 4.0</td>
<td>204 ± 42.0</td>
<td>1,915 ± 1,587.0</td>
<td>167 ± 12.0</td>
<td>284 ± 73.0</td>
<td>98 ± 10.0</td>
<td></td>
</tr>
<tr>
<td>AEo+</td>
<td>50 ± 7.0</td>
<td>258 ± 90.0</td>
<td>774 ± 761.0</td>
<td>204 ± 42.0</td>
<td>893 ± 85.0*</td>
<td>97 ± 11.0†</td>
<td></td>
</tr>
<tr>
<td>AEo−</td>
<td>39 ± 5.0</td>
<td>603 ± 293.0</td>
<td>2,171 ± 1,743.0</td>
<td>180 ± 27.0</td>
<td>539 ± 127.0</td>
<td>65 ± 7.0‡</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in pg/ml. IL, interleukin; IFN-γ, interferon-γ; ECP, eosinophil cationic protein. ND, not detectable. *Significantly different from control (P < 0.001) and from AEo− (P < 0.05). †Significantly different from AEo− (P < 0.01). ‡Significantly different from control (P < 0.05).
normal subjects. This is the first study to demonstrate that macrophages obtained from a site proximal to asthma pathology are phenotypically and functionally altered. Earlier observations on peripheral blood phagocytes from subjects with asthma and chronic bronchitis have suggested that phagocytic dysfunction may also be present in the circulation of asthmatic individuals (26–28).

Impaired phagocytosis by BMs was found to be associated with cellular and spirometric markers of asthma severity. Relatively strong positive correlations were observed between sputum macrophage phagocytosis and percent predicted FEV$_1$ ($R = 0.7$), FVC ($R = 0.8$) and PC$_{20}$-FEV$_1$ ($R = 0.8$), and an inverse correlation was observed between sputum macrophage phagocytosis and sputum eosinophils ($R = -0.6$). Although it is unlikely that eosinophils directly inhibit macrophage phagocytosis or that phagocytosis directly impacts lung volumes, these correlations do not likely represent an epiphenomenon, but rather the pathogenesis of asthma severity may be related to the ability of the airways to mount a basic host defense response.

We hypothesized that changes in BM phenotype and function were the result of the cellular environment in asthmatic airways. T cells in allergic asthma preferentially produce Th2-type cytokines including IL-4 and IL-5, and cytokine-producing cells can be identified in the mucosa of asthmatic individuals and in bronchoalveolar lavage fluid following allergen provocation (9, 31, 42, 44). In vitro experiments have shown that small concentrations of IL-4 (10 pg/ml) can significantly downregulate the expression of CD64 and inhibit FcγR-mediated phagocytosis by human mononuclear phagocytes (42). Unfortunately, the attempts to measure IL-4 in the sputum supernatant were unsuccessful because most measurements were below the lower limit of detection of the immunoassay (35 pg/ml). Other investigators have also reported difficulty in recovering IL-4 in sputum supernatant (23). Even spike experiments with exogenous purified IL-4 have yielded poor results, suggesting that the sputum contains components interfering with IL-4 immunoreactivity. On the other hand, both IL-4 and IL-5 mRNAs have been detected in the sputum cells of asthmatic subjects, and the number of cells expressing these cytokines was significantly higher in asthmatics than in controls (31). In the present study, immunoreactive IL-5, IL-8, and IFN-γ could be detected in the sputum samples of most of the subjects, but no difference was found in the levels of cytokines between normal subjects and asthmatics or, with the exception of IFN-γ, between the AEO+ and AEO− groups. While induced sputum is a good technique that gives reproducible and valid results on inflammatory cells and most soluble markers of inflammation (IL-8, ECP) (13, 17, 31a, 34, 50), it may not recover cytokines (IL-4, IL-5) that are known to be synthesized and secreted by cells predominantly found in the airway wall, i.e., lymphocytes (14, 25, 54).

The data showed that, along with decreased FcγRI expression, BMs from AEO+ subjects also demonstrated significantly decreased CR3 (CD11b) expression. Since these two receptors interact in the phagocytic process (4, 48, 52), the decrease in uptake of opsonized yeast may be the result of decreased expression of both these receptors. What then may be the significance of decreased BM phagocytic function in asthmatic individuals? They are not known to have increased fungal or bacterial airway infections. However, many microbes, both opportunistic pathogenic and nonpathogenic organisms, are frequently found in asthmatic airways where their presence may lead to increased asthma severity (7). The presence of protease-producing fungi has been shown to lead to asthma symptoms, and histamine release induced by bacteria exacerbates the disease (21, 29). Aspergillus fumigatus is an opportunistic pathogen to which asthmatic subjects are particularly susceptible (21). Furthermore, decreased microbial clearance due to impaired bronchial macrophage phagocytosis may require the involvement of neutrophil inflammation to resist subsequent infection. This process may, in turn, exacerbate asthma. Interestingly, some studies have implicated bacterial infections as causative in the development of asthma severity (30).

In conclusion, this study has demonstrated that, in a population of mild asthmatics, eosinophil inflammation, airway function, and macrophage function and phenotype are interrelated. How these markers of asthma severity link to and affect each other is still an open question.

This study has been reviewed by the National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, and mention of trade names and commercial products does not constitute endorsement or recommendation for use.

REFERENCES


