Symptomatic and asymptomatic airway hyperresponsiveness
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Chapter 6

AIRWAY INFLAMMATION IN SYMPTOMATIC AND ASYMPTOMATIC SUBJECTS WITH AND WITHOUT AIRWAY HYPERRESPONSIVENESS

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ABSTRACT

In a sample from a general adult population, we determined whether differences in airways inflammation were related to the presence of respiratory symptoms (either chronic cough, chronic phlegm, bronchitis episodes, dyspnea, wheeze, or asthma attacks) and/or airway hyperresponsiveness (AHR: \( \text{PC}_{20} \leq 8 \text{ mg/ml histamine} \)). Bronchial biopsies were obtained from 14 symptomatic subjects with AHR, 12 symptomatic subjects without AHR, 12 asymptomatic subjects with AHR, and from 19 asymptomatic subjects without AHR. Immunostainings were performed using antibodies to CD3, CD4, CD8, CD25, CD20/22, EG2, MBP, AA1, NP57, CD68 and ICAM-1, VCAM-1, and E-selectin in combination with anti-CD31. We were unable to show a difference in the inflammatory process between the four groups under study. The presence of chronic respiratory symptoms during the previous three years and not necessarily at the time of the study, and the heterogeneity of characteristics in this sample from a general population might imply that more study subjects are needed to reach sufficient power. A new finding is the lower number of macrophages in symptomatic individuals, especially when atopy was present. Further, more CD8+ T-lymphocytes were observed in current smokers, less mast cells in subjects with airways obstruction, and more eosinophils were observed in the bronchial submucosa of subjects with blood eosinophilia. Our results suggest that asymptomatic hyperresponsive subjects with blood eosinophilia, who are at increased risk to develop respiratory symptoms, may have a more attentive immune response with increased susceptibility upon certain stimuli, capable to activate the eosinophils in the bronchial wall, which results in AHR and respiratory symptoms.

INTRODUCTION

Cross-sectional population studies investigating the relationship between airway hyperresponsiveness (AHR) and respiratory symptoms have shown that AHR is associated with respiratory symptoms, such as wheeze, breathlessness, and chest tightness (1-3). However, a significant proportion of subjects with AHR is asymptomatic although the prevalence in the population is low, varying from 2 to 14% (4).

So far, the clinical meaning of AHR in the absence of respiratory symptoms is unclear. An important finding is that asymptomatic subjects with AHR are more prone to develop respiratory symptoms than asymptomatic subjects without AHR (5-8), especially when peripheral blood eosinophilia is also present (9). In case peripheral blood eosinophilia would be supposed to reflect higher numbers of eosinophils in the airway wall, this suggests that inflammatory changes in the airways may be an underlying mechanism for AHR in asymptomatic subjects. Current studies in asthma suggest that an intricate interaction between different types of inflammatory cells and their respective mediators play a role in the processes that lead to increased airway responsiveness (10-15). Only a few studies have examined the presence of
inflammatory changes in asymptomatic subjects with AHR (16,17). Although the number of eosinophils in peripheral blood was found to be increased in asymptomatic subjects with AHR compared with asymptomatic subjects without AHR (16), AHR was not found to be associated with higher numbers of T lymphocytes, eosinophils, mast cells, and macrophages in the airways (17). In one study it was suggested that more extensive inflammation is present, i.e. detectable interleukin 5 (IL-5) levels in the bronchoalveolar lavage fluid, in symptomatic asthma than in asymptomatic asthma (18).

In this study bronchial biopsies were obtained from a sample of 57 subjects from a general adult population to determine whether differences in inflammation in the airways are related to the presence of respiratory symptoms and/or airway hyperresponsiveness, and to lung function.

METHODS

Subjects
In 1995 and 1996, we examined 57 subjects of whom complete information was available of the final surveys of the longitudinal Vlagtwedde-Vlaardingen Study at Vlagtwedde in 1989 or 1985. The Vlagtwedde-Vlaardingen Study is a longitudinal population study that started in 1965 and was originally designed to determine risk factors for the natural history of chronic obstructive lung diseases (1,19). The initial cohort in Vlagtwedde consisted of a random sample of 450 subjects 40 to 44 years of age and of 1,793 subjects 15 to 39 years of age with baseline measurements in 1965 and 1967, respectively. After the baseline surveys the cohorts participated in follow-up surveys approximately every 3 years. All surveys were carried out during the month of October.

For this study, we invited a subsample of participants of the Vlagtwedde Survey of 1989 or 1985, because asymptomatic individuals with airway hyperresponsiveness are hard to find and in order to include subjects more efficiently. The subjects were selected on their characteristics in the final survey in 1989 or 1985. We invited all participants who experienced respiratory symptoms at the final survey, a random sample of subjects who were asymptomatic without AHR, and a sample of asymptomatic subjects with AHR starting with the more hyperresponsive subjects. In this study, we determined the current symptom and airway responsiveness status of the subjects again and divided them into four groups, i.e. subjects with respiratory symptoms and airway hyperresponsiveness (Sy+AHR+), subjects with respiratory symptoms and without airway hyperresponsiveness (Sy+AHR-), asymptomatic subjects with airway hyperresponsiveness (Sy-AHR+), and asymptomatic subjects without airway hyperresponsiveness (Sy-AHR-). The study protocol was approved by the local medical ethics committee and all participants gave their informed consent at each survey.
Measurements

Information on age, sex, chronic respiratory symptoms, and smoking habits was collected by means of a Dutch version of the British Medical Research Council’s standardized questionnaire (20). Subjects were considered symptomatic if they reported one or more of the following chronic respiratory symptoms: cough or phlegm production on most days or nights for as much as 3 consecutive months each year during winter (chronic cough, chronic phlegm), a period of at least 3 weeks with (increased) cough and phlegm (bronchitis episodes) in the previous 3 years, shortness of breath when walking with other persons of their own age on level ground (dyspnea ≥ grade 3), a wheezing or whistling sound on the chest on most days or nights (persistent wheeze), or attacks of shortness of breath (asthma attacks) in the previous 3 years. Smoking was divided into the categories current smokers, ex-smokers (quitted smoking at least 1 month before the examination), and never-smokers.

Pulmonary function tests included measurements of FEV₁ and IVC using a water-sealed spirometer (Lode Spirograph D53, Lode Instruments, Groningen, The Netherlands). The higher of the values obtained in three technically satisfactory tracings was taken as the baseline measurement. Airway responsiveness was first assessed by 2 minutes inhalation of doubling doses of adenosine 5-monophosphate (AMP) (0.04 to 320 mg/ml). At least 45 minutes later when the FEV₁ value had returned within 95% of the FEV₁ value before the AMP provocation test, the second provocation test was carried out by inhalation of doubling doses of histamine (0.03 to 8 mg/ml). FEV₁ measurements were performed 30 and 90 seconds after inhalation of each concentration of AMP and histamine. When the FEV₁ value did not change or decrease by less than 20%, the doubling dose of AMP or histamine was inhaled three minutes after inhalation of the previous dose. The test was terminated when the FEV₁ value decreased by 20% or more compared with the FEV₁ value before provocation, or when the highest concentration was inhaled. AHR was defined as a PC₂₀ ≤ 8 mg/ml histamine or a PC₂₀ ≤ 80 mg/ml AMP.

Skin test positivity was assessed by intracutaneous skin tests for 12 common inhalation allergens and defined as a mean diameter > 5 mm to one or more allergens. The following allergens were tested: mixed grass pollen (Alopecurus pratensis, Dactylus glomerata, Lolium perenne and Phleum pratensis), mixed tree pollen (a mix of Alnus glutinosa, Betula verrucosa and Corylus avellana, and a mix of Sambucus nigra, Quercus robur, Ulmus glabra and Populus species), mixed weeds (Plantago lanceolata and Artemisia vulgaris), house dust mites (Dermatophagoides pteronyssinus and Dermatophagoides farin), mixed storage mites (Acarus siro, Lepidoglyphus destructor and Tyrophagus putrescentiae), animal danders (dog, cat, horse, mixed feathers of pigeon, parrot, canary, and parakeet, mixed animal danders of rabbit and guinea pig). Before the measurements took place, a blood sample was taken to determine the total white blood cell and differential cell counts.

At least three days later, a bronchoscopy was performed after the assessment of reversibility of airways obstruction by inhalation of 200 µg salbutamol. Bronchoscopy was performed using an Olympus flexible fiberoptic bronchoscope (Olympus Optical,
Tokyo, Japan). Lidocaine (2% and 4%) was used for local anaesthesia. Four to six biopsies were taken from the subcarinae of the left or right lower lobe using a fenestrated forceps (FB-21C, Olympus, Tokyo, Japan).

**Biopsy processing and analysis**

Biopsies were immersed in Tissue Tek® mounting medium, snap-frozen in liquid isopentane and stored at -80°C until use. Serial sections were cut with a thickness of 4 µm. Two sections at an interval of 100 µm were stained for each antigen to be determined. In order to evaluate the bronchial architecture of the sections, we stained each 50th section with Mayer's hematoxylin and eosin (HE). These sections were examined on the following morphological criteria: integrity of the bronchial tissue, thickness of the submucosa beneath the intact basal membrane (>100 µm) on a cumulative length of 1000 µm, presence of at least basal epithelial cells. When none of the HE stained sections satisfied the criteria, the next biopsy was cut till a morphologically adequate serie of 25 sections was available. The sections were stored at -20°C.

Immunostaining was performed using the antibodies to CD3 (polyclonal, DAKO, ITK Diagnostics, The Netherlands) to identify total number of T-lymphocytes, CD4 (Becton-Dickinson, San Jose, USA) and CD8 (own laboratory) for CD4+ and CD8+ lymphocytes, major basic protein (MBP, Monosan, Sanbio, Uden, The Netherlands) for eosinophils, EG2 (Sanbio) for activated eosinophils, AA1 (DAKO, ITK Diagnostics, The Netherlands) for mast cell tryptase, CD25 (Becton-Dickinson) for activated T- or B-lymphocytes, CD68 (DAKO) for macrophages, NP57 (DAKO) for neutrophils (neutrophil elastase), CD20 combined with antibodies to CD20 (CD20: own laboratory, CD22: Becton-Dickinson) for B-lymphocytes. Before immunostaining, the sections were fixed in acetone and endogenous peroxidase activity was blocked by hydrogen peroxide (0.075%) in phosphate buffer. An immunoperoxidase streptavidin-biotin method was used with hematoxylin as counterstain. Further, double immunostaining was performed to visualize all vessels using a mAB against CD31 (Monosan, Sanbio), which is present on all endothelial cells, in combination with mAb against the adhesion molecules ICAM-1 (anti-CD54, Dr. A.W. Boyd, Royal Melbourne Hospital, Australia), VCAM-1 (anti-CD106, Genzyme), and E-selectin (anti-CD62E, Genzyme). Labeling of anti-CD31 was performed by isotype specific biotinylated goat anti-mouse immunoglobulins and subsequently by streptavidin conjugated to alkaline phosphatase, providing a blue reaction product. Labeling of the anti-adhesion antibodies was performed by isotype specific goat anti-mouse antibody conjugated to peroxidase, using 3-amino-4-ethylcarbazole as a reagent, giving a reddish brown reaction product.

Counting was carried out using a light-microscope at a final magnification of 400x and of 250x (for EG2, AA1, ICAM-1, VCAM-1, E-selectin). Positive cells were counted at a depth of 100 µm beneath intact basement membrane (BM) with a cumulative length of at least 1000 µm (21). If the cumulative length of intact basement membrane of one section was not enough, positive cells were also counted in the second section. Cell numbers were presented as the number of
positive cells per millimeter BM, which equals 0.1 mm². The adhesion molecules on bloodvessels were counted differently. All vessels in one section were scored positive or negative for immunopositivity for an adhesion molecule and expressed as a percentage of the total number of CD31 positive vessels (22). When less than 20 vessels were recognized in one section, both sections were quantified.

Data analyses
Differences in cell numbers between the groups were determined using the Kruskal-Wallis test and when statistically significant followed by a Mann-Whitney U test. Logistic regression analyses were performed to determine the independent effects of the inflammatory parameters on the presence of respiratory symptoms and AHR. The risk estimates were adjusted for age, sex, smoking habits, FEV₁ (%predicted), skin test positivity, and AHR or the presence of respiratory symptoms. The percent predicted reference values of FEV₁ were calculated using regression coefficients derived from analysis of our own population data (23). Linear regression analyses were performed to determine whether the inflammatory cell numbers in the airway wall related to the level of FEV₁ or to the retrospective decline in FEV₁ from age 25 to age at the time of this study (mean age of 56 years), independent of age, sex, smoking habits, respiratory symptoms, AHR, and positive skin tests, all determined at the current study. The decline in FEV₁ (ml/yr) was calculated by individual regression on all available FEV₁ measurements from age 25 (SAS version 6.12). All other analyses were performed using SPSS-PC+ (version 5.0.2). Statistics on the cell numbers are expressed as median and range.

RESULTS
The most commonly reported symptoms were bronchitis episodes, chronic cough, and chronic phlegm (Table 1). The majority of subjects with bronchitis episodes (12 out of 17) reported having had such episodes more than once, and 9 of them also experienced at least one of the other respiratory symptoms. Chronic cough and/or phlegm were reported by 13 subjects of whom 2 also reported dyspnea and/or persistent wheeze.
### Table 1. Respiratory symptoms by airway hyperresponsiveness (AHR: PC<sub>20</sub> ≤ 8 mg/ml)

<table>
<thead>
<tr>
<th></th>
<th>AHR-</th>
<th>AHR+</th>
<th>Total group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Chronic cough</td>
<td>5 (41.7)</td>
<td>5 (35.7)</td>
<td>10 (38.5)</td>
</tr>
<tr>
<td>Chronic phlegm</td>
<td>4 (33.3)</td>
<td>6 (42.9)</td>
<td>10 (38.5)</td>
</tr>
<tr>
<td>Bronchitis episodes</td>
<td>5 (41.7)</td>
<td>12 (85.7)</td>
<td>17 (65.4)</td>
</tr>
<tr>
<td>Dyspnea ≥ Grade 3</td>
<td>1 (8.3)</td>
<td>4 (28.6)</td>
<td>5 (19.2)</td>
</tr>
<tr>
<td>Persistent wheeze</td>
<td>0 ---</td>
<td>2 (14.3)</td>
<td>2 (7.7)</td>
</tr>
<tr>
<td>Asthma attacks</td>
<td>2 (16.7)</td>
<td>1 (7.1)</td>
<td>3 (11.5)</td>
</tr>
<tr>
<td>One or more symptoms</td>
<td>12 (100)</td>
<td>14 (100)</td>
<td>26 (100)</td>
</tr>
</tbody>
</table>

The characteristics of the study population are described in Table 2. In short, mean age of this population is 56 years, 25% are current smokers, and 37% had one or more positive skin tests. Symptomatic hyperresponsive subjects (Sy+AHR+) were significantly more frequently current smokers and had a significantly lower lung function (FEV<sub>1</sub> %predicted and FEV<sub>1</sub> %VC) than Sy-AHR- subjects. Airway hyperresponsiveness was more severe in Sy+AHR+ subjects than in Sy-AHR- subjects.

#### Respiratory symptoms and airway hyperresponsiveness

A wide range and a large overlap was observed in the distribution of inflammatory cells (Figures 1a, b, and c) and percentages of vessels positive for adhesion molecules (Figure 2) in the submucosa of bronchial biopsies from symptomatic and asymptomatic subjects with and without AHR. Table 3 describes the median cell numbers (and range) in the airway wall for each group. Significantly higher numbers of CD20/22+ cells were present in the submucosa of Sy-AHR+ subjects than of Sy+AHR- subjects (P=0.03) and in subjects with AHR (3, 0-20) than in subjects without AHR (0.5, 0-16) (P=0.02).

Apart from a tendency to a higher percentage of vessels positive for VCAM-1 (P=0.09) in biopsies from subjects with AHR<sub>(AMP)</sub> (0, 0-29) compared with subjects without AHR<sub>(AMP)</sub> (0, 0-5), no statistical significant differences were observed between subjects with and without AHR to histamine or to AMP.

Symptomatic subjects had significantly (P=0.01) lower numbers of CD68+ cells (49, 13-166) in the bronchial submucosa compared with asymptomatic subjects (72.5, 26-220).
Logistic regression analyses showed that the relation between a higher number of CD20/22+ cells (Odds ratio [OR] = 1.24, 95% confidence interval [CI] = 1.02-1.51) and AHR, and between a lower number of CD68+ cells and the presence of respiratory symptoms (OR = 0.98, 95% CI = 0.96-0.996), were independent of age, sex, smoking habits, lung function, skin test positivity, and respiratory symptoms or AHR, respectively.

Table 2. Characteristics of the Vlagtwedde population of 1995/1996

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Respiratory symptoms and AHR</th>
<th>Total group</th>
</tr>
</thead>
<tbody>
<tr>
<td>n, (% men)</td>
<td>Sy-</td>
<td>Sy-AHR+</td>
</tr>
<tr>
<td>Age, yr</td>
<td>56 ± 7</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>Smoking habits:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non smoker</td>
<td>7 (37)</td>
<td>3 (25)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>10 (53)</td>
<td>7 (58)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>2 (10)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>FEV1, %predicted</td>
<td>124.8 ± 113.4 ± 15.9</td>
<td>118.0 ± 16.4</td>
</tr>
<tr>
<td>FEV1/VC, %</td>
<td>75.8 ± 72.2 ± 6.5</td>
<td>74.2 ± 6.8</td>
</tr>
<tr>
<td>PC20 histamine</td>
<td>4.63 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Reversible airways 1)</td>
<td>0 (0)</td>
<td>5 (42)</td>
</tr>
<tr>
<td>&gt; 1 positive skin test 2)</td>
<td>7 (37)</td>
<td>5 (42)</td>
</tr>
</tbody>
</table>

* p<0.05 compared with Sy-AHR-, § p<0.05 compared with Sy-AHR+, ¶ p<0.05 compared with Sy+ AHR-.
Smoking habits, reversibility and skin test positivity: n (%). Other parameter are expressed as mean ± standard deviation.

1) 9% predicted after 200 µg salbutamol; 2) 12 allergens tested: mixed grass pollen, mixed tree pollen, mixed weeds, houde dust mites, mixed storage mites, animal danders.
Figure 1a. Number of T-lymphocytes in biopsies from subjects with and without respiratory symptoms (Sy) and airway hyperresponsiveness (AHR)

Figure 1b. Number of mast cells, neutrophils, and macrophages in biopsies from subjects with and without respiratory symptoms (Sy) and airway hyperresponsiveness (AHR)
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Figure 1c. Number of eosinophils, B-cells, and IL-2R+ cells in biopsies from subjects with and without respiratory symptoms (Sy) and airway hyperresponsiveness (AHR)

Figure 2. Percentage of vessels positive for ICAM-1, VCAM-1, and E-selectin in biopsies from subjects with and without respiratory symptoms (Sy) and airway hyperresponsiveness (AHR)
Table 3. The number of inflammatory cells in bronchial biopsies from asymptomatic and symptomatic subjects with and without airway hyperresponsiveness

<table>
<thead>
<tr>
<th>Respiratory symptom and airway hyperresponsiveness status</th>
<th>Sy+AHR+</th>
<th>Sy-AHR+</th>
<th>Sy+AHR-</th>
<th>Sy-AHR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median number of cells (range) /mm basal membrane positive for:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>48 (0-300)</td>
<td>59.5 (0-471)</td>
<td>71.5 (2-187)</td>
<td>51 (7-262)</td>
</tr>
<tr>
<td>CD4</td>
<td>23 (0-112)</td>
<td>22.5 (0-437)</td>
<td>26.5 (0-106)</td>
<td>20 (0-305)</td>
</tr>
<tr>
<td>CD8</td>
<td>44 (3-126)</td>
<td>28.0 (9-184)</td>
<td>51.5 (0-98)</td>
<td>34 (1-133)</td>
</tr>
<tr>
<td>MBP</td>
<td>0.5 (0-22)</td>
<td>1.5 (0-30)</td>
<td>1.5 (0-13)</td>
<td>1 (0-28)</td>
</tr>
<tr>
<td>EG2</td>
<td>0 (0-0)</td>
<td>0 (0-3)</td>
<td>0 (0-2)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>AA1</td>
<td>21 (3-44)</td>
<td>19 (2-46)</td>
<td>18 (0-30)</td>
<td>17 (1-39)</td>
</tr>
<tr>
<td>NP57</td>
<td>13.5 (1-29)</td>
<td>13.5 (0-257)</td>
<td>23 (4-137)</td>
<td>17 (3-57)</td>
</tr>
<tr>
<td>CD68</td>
<td>53 (27-142)</td>
<td>108 (26-220)</td>
<td>39 (13-166)</td>
<td>68 (26-134)</td>
</tr>
<tr>
<td>CD20/22</td>
<td>2.5 (0-20)</td>
<td>3.0 (0-11)(*)</td>
<td>0 (0-8)</td>
<td>1 (0-16)</td>
</tr>
<tr>
<td>CD25</td>
<td>0 (0-4)</td>
<td>0 (0-5)</td>
<td>0 (0-1)</td>
<td>0 (0-2)</td>
</tr>
<tr>
<td>% vessels ICAM-1+</td>
<td>3.7 (0-20.7)</td>
<td>1.65 (0-20)</td>
<td>5 (0-11.7)</td>
<td>2.2 (0-25)</td>
</tr>
<tr>
<td>% vessels VCAM-1+</td>
<td>0 (0-29)</td>
<td>0 (0-10)</td>
<td>0 (0-4.8)</td>
<td>0 (0-4)</td>
</tr>
<tr>
<td>% vessels E-selectin+</td>
<td>0 (0-0)</td>
<td>0 (0-33.3)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
</tbody>
</table>

(*) P < 0.10 compared with Sy-AHR- subjects, ¶ P=0.03 compared with Sy+AHR- subjects

Age, sex, smoking habits, pulmonary function, skin test positivity, reversibility of airways obstruction, and peripheral blood eosinophilia

We also studied the differences in inflammatory cell numbers in relation to various other characteristics, i.e. age ≤ 55 years and older, men and females, current- and never smokers, FEV₁ in the lowest quartile (<109.7 % predicted) compared with the other quartiles, FEV₁/VC ≤ 70 % and > 70%, an increase in FEV₁ of ≥ 9% predicted and < 9% predicted after inhalation of 200 µg salbutamol, ≥ 1 positive skin tests compared with no positive skin tests, and the presence or absence of peripheral blood eosinophilia (≥ 275 or <275 eosinophils/µL).

The number of CD3+ cells tended to be lower (P=0.06) in atopic subjects (28, 0-471) than in non-atopic subjects (63, 0-300). The number of CD4+ cells was significantly higher (P=0.047) in women(32, 0-437) than men(19, 0-283). The number of CD8+ cells tended to be higher (P=0.07) in current cigarette smokers (62.5, 13-153)(mean
FEV₁ ± sd = 103 ± 21 % predicted) than in never smokers (47, 1-94) (mean FEV₁ ± sd = 113 ± 9 % predicted). Further, smokers with airways obstruction (FEV₁/VC ≤70%, N=5) had higher numbers of CD8+ cells (98, 30-153) than smokers without airways obstruction (51, 13-126) (N=9, P=0.32).

The number of MBP+ cells was significantly higher (P=0.003) in subjects with peripheral blood eosinophilia (4, 0-30) than in subjects without peripheral blood eosinophilia (1, 0-22).

The number of AA1+ cells was lower (P=0.07) in subjects with airways obstruction (11, 0-44) compared with subjects without airways obstruction (20, 1-46), whereas no relation was found to hyperresponsiveness. Lower numbers of CD68+ cells (P=0.057) were observed in the bronchial submucosa of subjects with one or more positive skin tests (50.5, 20-127) compared with subjects with no positive skin tests (68, 13-220). As described above this difference was also present in symptomatic subjects compared with asymptomatic subjects. The presence of respiratory symptoms and skin test positivity are related to each other and, therefore, we combined these characteristics and compared the numbers of CD68+ cells in the bronchial submucosa in subjects with and without respiratory symptoms and skin test positivity (Figure 3). A significantly lower number of CD68+ cells was found in the symptomatic subjects with a positive skin test (35.5, 20-84) compared with the other three groups (P=0.01).

![Figure 3](image-url)

**Figure 3.** Number of CD68+ cells in the bronchial submucosa of subjects with and without respiratory symptoms (Sy) and skin test positivity (ST).
Inflammatory cell numbers and vascular adhesion molecules in relation to lung function

The relation of the inflammatory cell numbers and adhesion molecules with lung function was assessed using linear regression analyses to adjust for possible confounding variables (Table 4). A higher number of CD25+ cells in the bronchial submucosa was associated with a lower level of FEV$_1$ %predicted and FEV$_1$ %VC, independent of age, sex, and cigarette smoking. A higher percentage of vessels positive for E-selectin was also associated with a lower lung function, but only one biopsy showed vessels positive for E-selectin. The univariate analysis (described above) showed a lower number of AA1+ cells in subjects with a FEV$_1$ %VC $\leq$70% than in subjects with a FEV$_1$ %VC $>$70%. Linear regression showed also the same trend, though not significant, i.e. a lower number of AA1+ cells with a lower FEV$_1$ %VC value (coefficient=0.11, 95% CI=-0.05 - 0.28). Although the absolute number of T-lymphocytes showed no relation with lung function, a higher ratio of CD4+ and CD8+ cells was related to a smaller decline in FEV$_1$ from age 25 onwards. Additional adjustment for respiratory symptoms, AHR, and positive skin tests yielded similar results with regard to FEV$_1$ %predicted, FEV$_1$ %VC, and the decline in FEV$_1$ from age 25 (ml/yr). The other inflammatory cell counts and vascular adhesion molecules (as described in Table 3) showed no relation to the level of lung function.

Table 4. Relations between the lung function parameters FEV$_1$ % predicted, FEV$_1$ % VC, and decline in FEV$_1$ from age 25 onwards and cellular counts in the bronchial submucosa, determined by linear regression analyses

<table>
<thead>
<tr>
<th>change in lung function by 1 unit change in cell counts ($\beta$)</th>
<th>(95% confidence interval)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV$_1$ % predicted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD25+</td>
<td>-4.39$^1$</td>
<td>(-9.40, 0.62)</td>
</tr>
<tr>
<td>FEV$_1$ %VC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD25+</td>
<td>-2.19</td>
<td>(-4.00, -0.37)</td>
</tr>
<tr>
<td>CD68+</td>
<td>-0.04</td>
<td>(-0.09, 0.006)</td>
</tr>
<tr>
<td>Decline in FEV$_1$ (ml/yr) from age 25 onwards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+/CD8+ ratio</td>
<td>-0.92</td>
<td>(-1.73, -0.11)</td>
</tr>
</tbody>
</table>

* Alle estimates are adjusted for age, sex, and smoking habits. Only those cell counts are described with a p-value < 0.10. All cell counts described in Table 2 were tested.

$^1$ The FEV$_1$ value will be 4.39% lower with every extra positive cell for CD25
DISCUSSION

In this study on a sample of a general elderly population, we investigated whether differences in inflammation in the airways are related to the presence of symptoms and/or airway hyperresponsiveness. Although in our population we could not show differences in inflammation in the airways of asymptomatic hyperresponsive individuals compared with symptomatic hyperresponsive individuals or individuals without hyperresponsiveness, we did find differences in inflammatory cell numbers related with sex, age, smoking habits, skin test positivity, airways obstruction, and peripheral blood eosinophilia.

Only a few differences were observed in the inflammatory process in the airway wall in the four groups under study. Several factors may explain the similarity in inflammatory cell numbers between the groups. First, we did not select subjects from a patient population and symptomatic subjects were defined as having chronic respiratory symptoms during the previous three years. Thus, symptoms may actually not have been present at the time of the survey. Therefore, the respiratory symptoms and AHR, and possibly the inflammatory process in the airway wall, may have been less severe than in a patient population. This was also reflected by the normal levels of FEV1 in all groups. Second, the heterogeneity of our study population with respect to atopy, smoking history, and reversibility of airways obstruction, may be responsible for the wide range in inflammatory cell numbers making it difficult to observe unadjusted differences between the groups. Third, the number of 12 to 19 subjects in the groups may have been too small. In general, 8 to 25 subjects are recommended in each group for biopsy studies (24), but these numbers may be too low when confronted with the above described heterogeneity as present in a study population like ours.

A new finding of our study is that we observed lower numbers of macrophages (CD68+ cells) in individuals with respiratory symptoms and skin test positivity. Previous studies have shown higher numbers of macrophages in biopsies from COPD patients compared with biopsies from chronic bronchitis patients and healthy controls (25-27), whereas inconsistent results were found with atopy (28,29). A possible explanation for the lower numbers of macrophages observed in the bronchial submucosa of symptomatic atopic individuals in our study may be that in subjects with a positive skin test an inclination to Th2-like responses exists. In general, cytokines, prevailing in Th2 responses, like interleukin 4 (IL-4), inhibit release of pro-inflammatory cytokines like interleukin 1, TNF-α, and interferon-γ. Indirectly this may reduce the number of local CD68+ macrophages in the bronchial wall. Differences with other studies may very well be related to the severity of inflammation. In more severe inflammation it is conceivable that the epithelium is more extensively involved (as is the case in asthmatic inflammation); thus, the epithelial cells contribute significantly to production of pro-inflammatory cytokines.
The relative effect of "Th2 cytokines" under this circumstance may considerably be reduced.

The total number of T-lymphocytes (CD3) was borderline significantly lower in atopic subjects than in non-atopic subjects, although the range in CD3+ cells in atopic subjects overlaps and exceeds the range in CD3+ cells in non-atopics. The number of Th-lymphocytes (CD4) was significantly higher in women than in males. As there are many external factors related to sex, such as occupational exposure which is not used in this study, it seems likely that an explanation for this difference is to be found by effects related to one of these epiphenomena. The number of CD8+ cells was borderline significantly higher in current cigarette smokers than in never smokers. This confirms findings of other studies which show a higher number of T-lymphocytes in smokers than in non-smokers with a predominance of CD8+ cells (25,30,31). Moreover, smokers with airways obstruction had higher numbers of CD8+ cells compared with smokers without airways obstruction, suggesting a role for CD8+ cells in COPD.

Subjects with at least one positive skin test had higher numbers of MBP+ cells, but this difference was minimal. More important, the number of eosinophils (MBP+) was significantly higher in subjects with peripheral blood eosinophilia than in subjects without peripheral blood eosinophilia. Two possible explanations can be considered for this phenomenon. One explanation is that one or more signals (likely cytokines) induce recruitment of eosinophils into the blood stream concomitantly with recruitment of these cells from the blood into the bronchial wall. Another possibility is that along with blood eosinophilia that is already present, local inflammation in the bronchial wall induces local upregulation of vascular adhesion molecules with (non specific) recruitment of eosinophils together with other white blood cells ("bystander effect").

Previous results showed that individuals with peripheral blood eosinophilia are more likely to have airway hyperresponsiveness than subjects without peripheral blood eosinophilia (OR=2.06, 95% CI=1.28-3.31) (32). In addition, individuals with both peripheral blood eosinophilia and AHR are at increased risk to develop respiratory symptoms compared with individuals with either peripheral blood eosinophilia (OR=4.41, 95% CI=1.91-10.14) or AHR (OR=2.85, 95% CI=1.29-6.30)(9). Altogether, the results suggest that AHR, peripheral blood eosinophilia, and possibly a higher number of eosinophils in the bronchial submucosa, are related to each other and play an important role in the development of respiratory symptoms in asymptomatic adults. These phenomena may result from one underlying signal (such as IL-5 released by T lymphocytes) which is stronger in subjects with both AHR and peripheral blood eosinophilia than in subjects with only AHR or peripheral blood eosinophilia or, more likely, from multiple signals. This may reflect a more attentive immune response ("pre-activation state") with increased susceptibility to certain stimuli that are capable to activate the eosinophils in the bronchial wall and
subsequently cause AHR and respiratory symptoms.

The number of mast cells (AA1) was lower in subjects with airways obstruction compared with subjects without airways obstruction. No relation was found to hyperresponsiveness.

Hyperresponsive subjects had significantly higher numbers of CD20/22+ cells. However, the number of B-lymphocytes is very small, and therefore the biological significance is unclear.

No differences were found in expression of the vascular adhesion molecules ICAM-1, VCAM-1, and E-selectin. This suggests that the differences in cell numbers are not explained by different recruitment (adhesion) of cells via the bloodvessels to the bronchial submucosa.

Taken the information of all subjects together, it was shown that higher numbers of activated T- and B-cells (IL-2R+ cells indicated by anti-CD25) were associated with a lower actual lung function, although only a few CD25+ cells were observed in the biopsies (N=8). Activated T-cells release cytokines like IL-4 and IL-5 for proliferation and differentiation of T and B lymphocytes, and recruitment and activation of leukocytes, including macrophages and granulocytes which will result in oedema, and epithelial damage causing a lower lung function.

In summary, in a sample of an elderly general population we were unable to show a difference in the inflammatory process in the airways of individuals with and without chronic respiratory symptoms and airway hyperresponsiveness. The presence of chronic respiratory symptoms during the previous three years and not at the time of the study per se, and the heterogeneity of characteristics in this sample might imply that the number of study subjects needs to be increased to reach sufficient power for this particular type of study in comparison with studies in asthma and COPD patients. Our study provides new information in that we observed lower numbers of macrophages in the bronchial submucosa of individuals who experienced chronic respiratory symptoms in the previous three years, especially when atopy was present. Further, higher numbers of CD8+ T-lymphocytes were observed in the bronchial submucosa of current smokers, and lower numbers of mast cells in subjects with airways obstruction. More important, higher numbers of eosinophils were observed in the bronchial submucosa of subjects with peripheral blood eosinophilia. Since it is known that asymptomatic hyperresponsive subjects with peripheral blood eosinophilia are at increased risk to develop respiratory symptoms (9), our results suggest that these subjects may have a more attentive immune response with increased susceptibility upon certain stimuli, capable to activate the eosinophils in the bronchial wall, which results in AHR and chronic respiratory symptoms.
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References
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