Airway function, inflammation and regulatory T cell function in subjects in asthma remission


BACKGROUND: Factors associated with asthma remission need to be determined, particularly when remission occurs in adulthood.

OBJECTIVE: To evaluate airway responsiveness and inflammation in adult patients in asthma remission compared with adults with mild, persistent symptomatic asthma.

METHODS: Adenosine monophosphate and methacholine responsiveness were evaluated in 26 patients in complete remission of asthma, 16 patients in symptomatic remission of asthma, 29 mild asthmatic patients and 15 healthy controls. Blood sampling and induced sputum were also obtained to measure inflammatory parameters.

RESULTS: Perception of breathlessness at 20% fall in forced expiratory volume in 1 s was similar among groups. In subjects with symptomatic remission of asthma, responsiveness to adenosine monophosphate and methacholine was intermediate between mild asthma and complete asthma remission, with the latter group similar to controls. Asthma remission was associated with a shorter duration of disease. Blood eosinophils were significantly increased in the asthma group, and blood immunoglobulin E levels were significantly increased in the complete asthma remission, symptomatic remission and asthma groups compared with controls. The suppressive function of regulatory T cells was lower in asthma and remission groups compared with controls.

CONCLUSION: A continuum of asthma remission was observed, with patients in complete asthma remission presenting features similar to controls, while patients in symptomatic asthma remission appeared to be in an intermediate state between complete asthma remission and symptomatic asthma. Remission was associated with a shorter disease duration. Despite remission of asthma, a decreased suppressor function of regulatory T cells was observed, which may predispose patients to future recurrence of the disease.

Key Words: Airway inflammation; AMP responsiveness; Asthma; Asthma remission; Methacholine responsiveness; Regulatory T cells

Asthma is characterized by variable airway obstruction and hyper-responsiveness (AHR), which is attributed to airway inflammation and structural changes (1,2). We and others have provided evidence that airway inflammation and remodelling begin many years before the onset of asthma symptoms; however, once asthma has been diagnosed, it is considered to persist for life in the majority of patients (3-5).

Although relatively rare in adults, some patients may ‘grow out’ of asthma and, will therefore, be considered to be in asthma remission. There is evidence that even if symptoms have remitted in former mild-to-moderate asthmatic patients, residual airway inflammation on bronchoalveolar lavage or bronchial biopsies (6-8) can be observed in many subjects. Van den Toorn LM et al (7,8) and Warke et al (9) demonstrated persistent subepithelial fibrosis in subjects with symptomatic remission of asthma compared with healthy controls.

The mechanisms involved in symptomatic or complete remission of asthma, particularly in adulthood, are not well understood and need further investigation. The study of this phenomenon could help in determining how to possibly induce the remission of asthma. There is, therefore, a need to characterize these patients in so-called ‘asthma remission’ to determine the factors associated with remission and with recurrence of asthma in adulthood. In the present study, we compared pulmonary function, airway response to methacholine (MIT) and adenosine monophosphate (AMP), perception of respiratory symptoms, immunoglobulin E levels, airway inflammation on induced sputum, and T cell function in patients in symptomatic or complete asthma remission and mild asthmatic patients.

METHODS

Subjects
Patients were recruited from advertisements and through the asthma clinic database. Twenty-six consecutive asthmatic subjects in complete remission (with a provocative concentration of methacholine inducing a 20% fall in forced expiratory volume in 1 s [PC_{20}FEV_{1}] >16 mg/mL, having no asthma symptoms and not having used asthma medication for more than two years) (10), with a history of asthma
confirmed by a physician diagnosis and follow-up, in addition to previous asthma symptoms and asthma medication use, were enrolled (Table 1). Six patients also had a positive methacholine test during their asthma period dating back to before the occurrence of remission. Furthermore, 16 asthmatic subjects in symptomatic remission for more than two years, but still presenting hyper-reactive airways (11,12), 29 subjects with mild asthma not using anti-inflammatory agents and 15 healthy controls with normal airway responsiveness (PC$_{20} >16$ mg/mL) were studied.

All subjects provided informed written consent; the protocol was reviewed by the institutional ethics committee (Clinical Trial registration number: NCT 00526019).

The primary end point of the study was airway inflammation, assessed by airway responsiveness to AMP, and induced sputum eosinophil and neutrophil counts. Secondary end points were the following: airway response to methacholine (in symptomatic remission and current mild asthma compared with normal controls) and baseline FEV$_1$, blood IgE levels and blood eosinophils, diurnal variation in peak expiratory flows (PEF) and the profile of peripheral blood regulatory T cells (Tregs).

Procedures
All patients visited the laboratory at study entry and one week later. They completed a respiratory questionnaire based on the European Community Respiratory Health Survey Questionnaire (13). Patients underwent physical examination, skin prick tests, baseline spirometry and a methacholine challenge according to American Thoracic Society criteria (14) using the method described by Juniper et al (15), with additional measures of modified Borg perception scores for breathlessness and chest tightness. Complete blood count and serum IgE levels were performed on blood samples. Sputum was induced using the method described by Pin et al (16) and modified by Pizzichini et al (17) by inhalation of hypertonic saline.

On the second visit, after baseline spirometry, an AMP inhalation test was performed using the 2 min tidal breathing method described by Cockcroft et al (18) for histamine and applied to AMP by Oosterhoff et al (19).

induced sputum processing
Sputum was processed within 2 h of induction. Briefly, mucus plugs were selected from saliva and treated with four times their volume of dithiothreitol. An equal volume of 1×Dulbecco’s phosphate-buffered saline was then added and the suspension was filtered. Total cell count and viability were determined using the trypan blue exclusion method. Slides were prepared and stained with Diff-Quik (Dade Diagnostics Inc, USA) for differential cell count.

Regulatory T cell isolation
To study regulatory T cell function, eight healthy nonatopic controls, nine subjects with current mild asthma and nine asthmatic subjects in complete remission of asthma underwent peripheral blood sampling (150 mL) out of the allergy period. Peripheral blood mononuclear cells were isolated using lymphocyte separation media (Wisent, St-Bruno, Canada) density gradient centrifugation. Monocytes were separated from total lymphocytes by adhesion for 1 h at 37°C. CD4$^+$CD25$^+$CD127$^-$ cells were isolated using a commercially available CD4$^+$ T cell isolation Kit II and CD4$^+$CD25$^+$CD127dim$^-$ Regulatory T cell Isolation Kit according to the manufacturer’s instructions (Miltenyi Biotec, USA). The purity of the CD4$^+$CD25highFoxp3$^+$CD127$^-$ cells was >90%.

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*Patients were seen regularly by physician during asthma period; †When available. B Breathlessness; Bd Inhaled bronchodilator; CT Chest tightness; C Cough; ICS Inhaled corticosteroids; PC$_{20}$ Provocative concentration of methacholine causing a 20% fall in forced expiratory volume in 1 s; Ph Phlegm production; W Wheezing
Suppression assays
Lyophilized CellTrace (Invitrogen, Canada) carboxyfluorescein diacetate succinimidyl ester (CFSE) was diluted in dimethyl sulfoxide immediately before use (5 mM working solution). Freshly isolated CD4+CD25− cells were resuspended in phosphate-buffered saline/0.1% bovine serum albumin at 1x10^6 cells/mL, and 1 μl/mL of CFSE stock solution was added yielding a final concentration of 5 μM. Labelling was performed according to the manufacturer’s protocol. For the proliferation assay, 96-well round bottom plates (Corning Life Sciences, USA) were coated with 10 μg/mL anti-CD3 antibody (eBiosciences, USA). CFSE-labelled CD4+CD25− T cells were cultured at 2x10^4 cells/well with irradiated autologous monocytes. Unlabelled CD4+CD25high CD127low regulatory T cells at different ratios (1:1 or 1:0.5) were added to CFSE-labelled CD4+CD25− T cells. After five days in culture, cells were harvested, washed and analyzed by flow cytometry for fluorescence signal on a Coulter EPICS XL-MCL flow cytometer using Expo32 software (Beckman Coulter, Canada).

Figure 1) Study flow diagram

Figure 2) Characteristics of asthma and remission. Age at time of diagnosis, duration of asthma, age at time of asthma remission and duration of remission are compared among groups. Closed circles represent atopic patients, open circles represent nonatopic patients.

Statistical analysis
Results were expressed as mean ± SD, geometric means for PC_{20} methacholine and AMP, or percentages according to the distribution of data. PC_{20} values for methacholine and AMP were log transformed for analysis. For statistical comparisons, a PC_{20} >16 mg/mL was considered to be equal to 16 mg/mL. The subjects’ demographic characteristics were analyzed using Fisher’s exact test or one-way ANOVA for categorical and qualitative data, respectively. Posteriori comparisons were performed using Tukey’s multiple comparison test. Relationships between pulmonary function parameters and airway inflammation (sputum eosinophils) were measured using Pearson’s or Spearman’s correlation coefficients. The univariate normality assumption was verified with the Shapiro-Wilk test. The Brown and Forsythe’s variation of Levene’s test statistic were used to verify the homogeneity of variances. P≤0.05 was considered to be statistically significant. All analyses were performed using SAS version 9.2 (SAS Institute Inc, USA).

Results
Of the 92 subjects assessed for eligibility, six did not meet the inclusion criteria. A study flow diagram is presented in Figure 1.

Table 2 summarizes the demographic characteristics of the subjects at study enrollment. Atopy was more prevalent in patients with previous or current asthma diagnosis than in controls. Family history of asthma and/or allergy or their absence did not appear to be a predictor of asthma remission or of symptomatic remission (P=0.31).

The age at time of asthma diagnosis was not significantly different among the groups (P=0.47 [Figure 2]). Patients in the complete remission group were generally younger than those of the symptomatic remission group at time of asthma remission (P=0.03). The duration of asthma was not significantly different between asthma remission and symptomatic remission groups.

When questioned about the circumstances surrounding asthma remission and the possible reasons why they became asymptomatic, patients (complete remission, n/symptomatic remission, n) mentioned the following:

- Environmental improvement by better control of environment and/or reduced exposure to allergens (5/6);
- Increased time allocation for sports and/or training (5/5);
- Less intensive sports or less sport in arenas (2/1);
- Smoking cessation (1/0);
- Puberty (1/0);
- Important weight loss following bariatric surgery (1/0);
Figure 3) Airway responsiveness to methacholine (MIT) and adenosine monophosphate (AMP). Airway response to challenges was not significantly different among groups. Patients in symptomatic asthma remission were significantly less responsive to MIT and ASTHMA than asthma patients, and significantly more responsive to both challenges than patients in complete asthma remission, whose responsiveness was similar to that of controls. Bar represents mean. PC20 Provocative response to methacholine causing a 20% fall in forced expiratory volume in 1 s

Table 3
Expiratory flow and volume data

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<th>Symptomatic remission</th>
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<td>FEV1, % predicted†</td>
<td>103±15</td>
<td>104±13</td>
<td>95±13</td>
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<td>FVC, % predicted†</td>
<td>108±13</td>
<td>112±14</td>
<td>109±13</td>
<td>106±11</td>
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<td>FEV1/FVC ratio</td>
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<td>0.78±0.05</td>
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<td>AM PEF, % predicted†</td>
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<td>94 (85–103)</td>
<td>85 (77–94)</td>
<td>86 (78–94)</td>
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<td>Δ AM PEF, %†</td>
<td>5 (3–9)</td>
<td>7 (5–8)</td>
<td>7 (2–12)</td>
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<td>Δ PM PEF, %†</td>
<td>6 (2–5)</td>
<td>6 (5–8)</td>
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*Data presented as mean ± SD; †Data presented as mean (95% CI). Δ Daily variations in morning (AM) or afternoon/evening (PM) peak expiratory flow (PEF) over a period of one week; FEV1 Forced expiratory volume in 1 s; FVC Forced vital capacity

FEV1 and FVC
Baseline FEV1 and FVC, and FEV1/FVC ratio before methacholine inhalation tests varied slightly but not significantly among the groups (Table 3). Borg scores for perception of respiratory symptoms at 20% fall of FEV1 on methacholine inhalation test were not significantly different in the groups in symptomatic remission of asthma and asthma remission groups whose responsiveness was similar to that of controls.

Diurnal variation in PEF
Daily measurements of morning (AM) and afternoon (PM) PEF over one week are summarized in Table 3. The magnitude of variations of AM and PM PEF was not significantly different between groups (P=0.54 and P=0.48, respectively).

Blood IgE and eosinophils
Blood IgE levels were significantly different between control (median: 19.0 μL/mL) and asthma (166.5 μL/mL) groups (P=0.012 [Figure 4]). Blood IgE levels in the asthma remission (55.0 μL/mL) and symptomatic remission (45.0 μL/mL) groups were not significantly different from control or asthma groups. Besides, nonatopic controls had significantly less blood eosinophils (median 1.0%) than subjects of the remission (median 2.4%; P=0.008), symptomatic remission (median 2.8%; P=0.0026) and asthma (median 3.0%; P=0.0002) groups (Figure 4).

Inflammatory cells in induced sputum
Evaluation of inflammation was performed using induced sputum. Total cell number was not significantly different among the groups (ie, P>0.05, [Table 4]). There were no significant differences in neutrophil or eosinophil percentages among the groups.

Correlation with airway response to AMP
The patients in the symptomatic remission and current mild asthma groups who had negative AMP challenges had lower blood IgE levels and lower blood and sputum eosinophils; PC20 AMP correlated with blood IgE levels (r=−0.347; P<0.0001), blood eosinophils (r=−0.27; P=0.002) and sputum eosinophils (r=−0.58; P<0.0001).

Treg function
There were no significant differences in the percentage of CD4+CD25highFoxp3+CD127- cells among controls, asthma and complete asthma remission groups (9.1±1.8%; 7.8±0.6% and 5.2±0.6%, respectively). The suppressive function of Tregs was analysed by co-culture of these Tregs with CD4+CD25- effector cells. The capacity of Tregs to suppress proliferation of effector T cells was higher in healthy controls (83.1±3.0% of inhibition) than in the asthma group (51.3±4.9%; P=0.0006) or the complete remission of asthma group (34.0±10.9%; P=0.0002). There were no differences in the...
sensitization to house dust mites, airway hyper-responsiveness, female sex, smoking, and early age at onset were factors involved in the persistence of wheezing or relapse. Furthermore, Taylor et al (26) reported data from the latter cohort, showing that one of three asthmatic children was considered to be in remission at 18 years of age, although relapses were not easily predicted. Other studies showed that some children may outgrow their asthma at puberty (27,28), but 30% to 80% redevelop symptomatic asthma in adulthood (28-32). Regarding the factors associated with a recurrence of asthma symptoms, development of chronic airflow limitation in childhood and persistent airway inflammation have been proposed (6-8,33,34). In the present study, airway inflammation evaluated by induced sputum showed no significant difference between asthma and symptomatic or complete asthma remission. This is consistent with the study by Van den Toorn et al (35), who found that residual inflammation exists in asthma remission. There is evidence that even if symptoms have remitted in former mild-to-moderate asthmatic patients, residual airway inflammation often persists. Pumputiene et al (6) reported increased airway T cell and eosinophil activity (assessed by serum interleukin-2 and eosinophil cationic protein levels) in atopic and nonatopic children despite the absence of asthma symptoms. Warke et al (9) showed that eosinophil numbers were raised in the bronchoalveolar lavage fluid of atopic children who had apparently outgrown their asthma compared with healthy controls. However, there was no relationship between duration of remission and degree of airway eosinophilia, but the authors suggested that the latter could be a risk factor for future relapses (9).

Although there were a slightly higher number of inflammatory cells in asthmatic subjects in our study, globally, the number of these cells was not different among the groups. This was also shown in a recent study by Broekema et al (36), in which asthma remission and asthma without an accelerated decline in lung function demonstrated a similar sputum inflammatory profile.

Tregs contribute to the maintenance of immunological tolerance in the airways, evidenced by impaired function of these cells in asthma and allergy (37). Thus, peripheral blood CD4+CD25+ T cells derived from atopic donors were shown to be suppressive in allergen-stimulated cultures. This suppressive activity was similar to healthy controls. Ling et al (38) showed that FOXP3 Treg cells were defective in atopic individuals. This defect was particularly marked during seasonal pollen exposure. Verhagen et al (39) reported an absence of CD4+CD25+FOXP3+ cells in allergic dermatitis, suggesting a dysregulated control of inflammation. We evaluated the function of these cells in a subgroup of subjects in complete asthma remission and compared them with asthmatic patients and controls. Although the number of Tregs was similar in all groups, we found that subjects in complete remission still had decreased suppressive function of Tregs. In asthmatic subjects, this is a feature that may indicate an increased risk of future recurrence of the disease.

To characterize subjects in asthma remission, Vonk et al (40) reported outcomes in a cohort of 119 allergic asthmatic children initially evaluated at five to 14 years of age and followed for a mean of 30 years. In this group, complete remission of asthma was present only

**DISCUSSION**

In the present study, patients in asthma remission presented features similar to controls with normal airway hyper-responsiveness. Remission was associated with shorter disease duration. Patients in symptomatic asthma remission appeared to be in an intermediate state between complete remission of asthma and symptomatic asthma.

Asthma remission may be observed in adolescence, but occurs less frequently in adulthood. There appears to be a continuum of remission, with patients having no symptoms but still evidence of airway hyper-responsiveness or inflammation. In this regard, persistent airway obstruction, increased PEF variability and AHR to methacholine or cold air have been observed during symptomatic asthma remission (20-24).

Sears et al (25) investigated factors predicting persistence or relapse of wheezing in a New Zealand birth cohort, and showed that sensitization to house dust mites, airway hyper-responsiveness, female sex, smoking, and early age at onset were factors involved in the persistence of wheezing or relapse. Furthermore, Taylor et al (26) reported data from the latter cohort, showing that one of three asthmatic children was considered to be in remission at 18 years of age, although...
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