

Cytokine production in peripheral blood cells during and outside the pollen season in birch-allergic patients and non-allergic controls

K. Wosinska-Becler*, H. Plewako*, L. Håkansson† and S. Rak*

*Asthma and Allergy Research Group, Department of Respiratory Medicine and Allergology, Sahlgrenska University Hospital, Göteborg, Sweden and

†Department of Medical Sciences, Clinical Chemistry and Asthma Research Center, University Hospital, Uppsala, Sweden

Summary

Background The naturally occurring pollen season permits observation of the kinetic changes in the process of allergic inflammation. We examined cytokine production in peripheral blood (PB) T cells and monocytes obtained from birch-allergic patients both during and outside the pollen season.

Methods PB from 16 patients and six healthy controls was obtained during the alder pollen season, at the beginning and the peak of the birch pollen season and outside the pollen season. Mononuclear cells (MNC) were stimulated with allergen and polyclonal activators. For flow cytometric analysis, MNC were stained with monoclonal antibodies (MoAbs) against the cell surface markers CD3, CD8, CD14 and the intracellular cytokines IL-4, IL-5, IL-10, IL-12, IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN- γ .

Results In allergic patients, significant increases in clinical symptoms, use of medication, eosinophil numbers and birch-specific IgE were found during the pollen season. *In vitro* allergen stimulation increased the number of GM-CSF⁺ monocytes ($P < 0.01$) and this increase was dependent on allergen exposure. The IL-4/IFN- γ ratio rose ($P < 0.001$) at the peak of birch pollen season and the ratio correlated with symptom scores during the birch season. In the CD4⁺ cell population, the numbers of GM-CSF⁺ cells were higher throughout the alder and birch seasons compared with outside the pollen season ($P < 0.05$). No such changes were seen in the healthy controls.

Conclusions The main finding of our study was the increased percentage of GM-CSF⁺ monocytes in atopic subjects compared with healthy controls. In allergic patients, natural seasonal pollen exposure resulted in increased numbers of GM-CSF⁺ cells among both monocytes and CD4⁺ T cells. We have also shown that a seasonal change in Th2/Th1 cytokine ratio requires an adequate and prolonged allergen stimulation that is seen late in the pollen season.

Keywords cytokines, kinetics, monocytes, seasonal allergy, T cells

Submitted 14 May 2003; revised 15 August 2003; accepted 10 October 2003

Introduction

The strength of seasonal studies in the investigation of allergic disease is a continuous antigenic pressure during a limited time period, in contrast to experimental antigen challenge where a large dose of antigen is given at a single time-point. The time aspect is of importance because it allows observations of different phases of the allergic inflammation. The birch pollen season in Sweden is preceded by hazel and alder pollination. Most patients sensitized to the birch pollen also react to hazel and alder pollen, consequently, when allergic subjects are exposed to the high amount of birch pollen they have already been primed with low levels of pollen from these other trees. Thus, the allergic patients are exposed to varying and increasing amounts of allergen during a period of 2–2.5 months.

The systemic nature of allergic disease permits the examination of cellular activation and subsequent cytokine production in peripheral blood cells. The relationship between findings in peripheral blood (PB) and local mucosal inflammation is, however, complex and not very clear. The number of studies investigating seasonal variations in cytokine production from PB cells is limited and of those published, most utilized mixed blood mononuclear cells (MNC). Previous investigations have reported both an increased and decreased release of IL-4 and IL-5, increased release of IL-13 and decreased production of IFN- γ from mixed peripheral blood mononuclear cells (PBMNC) as well as a decreased ability of PBMNC to proliferate during the pollen season [1–4]. The differing results might be explained by the choice of methods, including stimulation modes and sampling time-points. To our knowledge, no previous studies have examined the cytokine production in T cells and monocytes separately throughout the pollen season. We have therefore undertaken a study where the cytokine production in a defined population of PBMNC was examined during the alder pollen season, at the beginning and at the peak of the

Correspondence: Sabina Rak, Asthma and Allergy Research Group, Dept. of Respiratory Medicine and Allergology, Sahlgrenska University Hospital, 413 45 Göteborg, Sweden.

E-mail: sabina.rak@medfak.gu.se

birch pollen season and outside the pollen season. The findings in allergic patients were compared with those of healthy controls.

Methods

Patients and study design

Sixteen patients with a history of seasonal rhinoconjunctivitis during the previous two seasons and six healthy subjects participated in the study. The characteristics of the subjects at the time of enrolment (January–February) are presented in Table 1. All patients had a positive skin prick test (SPT) and positive RAST (at least class 2) to birch pollen.

PB samples from the allergic patients were collected during four test periods established according to the daily pollen count (Fig. 1); test period 1 – the alder season (19 March–18 April), test period 2 – beginning of the birch season (20–29 April), test period 3 – peak of the birch season (30 April–15 May) and test period 4 – outside the pollen season (10 October–11 December) (Fig. 1). Blood samples from the healthy subjects were obtained three times; during the alder season (period 1), at the peak of the birch pollen season (period 3) and outside the pollen season (period 4).

The study was accepted by the Ethics Committee of Göteborg University.

Table 1. Characteristics of the subjects participating in the study

	Sex (f/m)	Age (years)	SPT (mm ²)	Specific IgE (kU/L)	Eosinophils ($\times 10^9/L$)
Patients	11/5	36	5.9	7.6	0.19
		(21–49)	(4.5–9.5)	(0.9–100)	(0–0.5)
Controls	5/1	35	0	0	0.17
		(27–49)			(0–0.4)

Total eosinophil count and serum birch-specific IgE concentration are presented as median and range. Other values are presented as mean and range. f, female; m, male; SPT, skin prick test.

Pollen counts

The pollen count was registered by means of a volumetric pollen trap (Burkard Scientific Ltd, Uxbridge, UK). Pollen counts were measured daily and expressed as the mean number of grains per cubic metre of air per day.

Clinical symptoms and medication score

One week before the expected start of the birch pollen season, the allergic patients started to register eye and nose symptoms and the use of medication. The registration was completed one week after the peak of the pollen season. Daily symptom registration was also performed during two weeks outside the pollen season. Symptoms were scored according to the scale: 0 – none; 1 – mild; 2 – moderate; 3 – severe. The daily rhinoconjunctivitis symptom score was calculated for each patient as the mean of eye and nose symptoms experienced each day.

The rescue medication for rhinoconjunctivitis was scored as follows: acrivastine capsule (Semprex[®], Glaxo-Wellcome, Middlesex, UK) 8 mg, 1 point/capsule; Antasten-Privin[®] (Novartis Ophthalmics, Hettlingen, Switzerland) eye drops, 1 point/1 drop and levocabastine (Livostin[®], Janssen Pharmaceutica NV, Beerse, Belgium) nasal spray, 1.5 points/dose. The daily medication score was calculated for each patient as the mean of all drugs used that day.

Allergen-specific IgE assay and differential cell count

The level of birch pollen-specific IgE in serum was measured using the Pharmacia CAP System test (Pharmacia Diagnostics AB, Uppsala, Sweden). Differential blood cell counts were performed using a Cell-dyn 4000 (Abbot Diagnostics, St Clara, CA, USA).

MNC isolation and stimulation

MNC were isolated from heparinized blood (20 mL) by density gradient centrifugation in Percoll (density 1.084 g/mL) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After

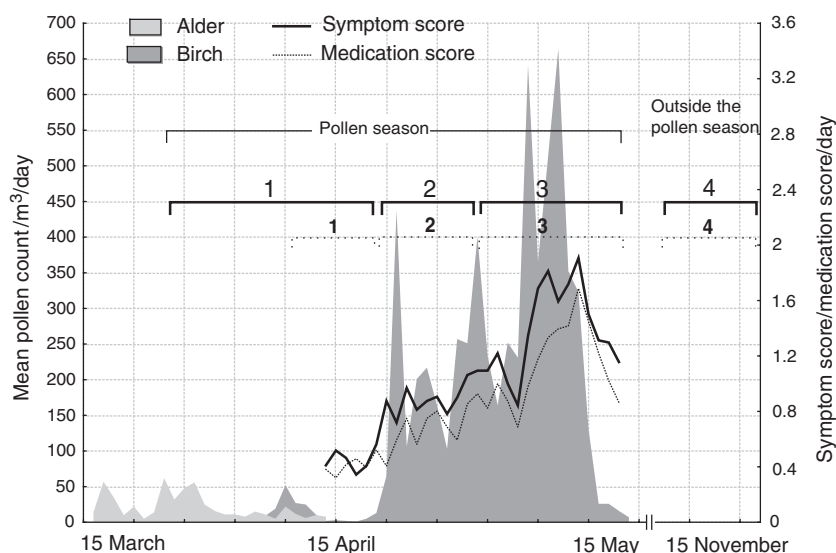


Fig. 1. Clinical data in relation to the pollen season. Symptoms and medication scores were evaluated (dotted lines with numbers) and blood samples taken (continuous lines with numbers) during three periods throughout and once outside the pollen season.

separation, the cells were resuspended in RPMI, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 µg/mL gentamicin (Invitrogen Life Technologies Ltd, Paisley, UK) (complete medium) at a concentration of 2×10^6 cells/mL.

Cytokine production in monocytes was assessed after a two-step stimulation. MNC were first cultured for 7 h with birch pollen extract, 5 µg/mL Bet v 1 (Alk-Abelló, Hørsholm, Denmark), or adequate amount of phosphate-buffered saline. Subsequently, phytohaemagglutinin (leucoagglutinin) (PHA-L) (5 µg/mL) (Sigma-Aldrich, St Louis, MO, USA) and brefeldin A (GolgiPlug, PharMingen Int., San Diego, CA, USA) were added to all cultures, and incubation at 37 °C was continued for 12 h.

When MNCs were stimulated with birch allergen alone for 7 h followed by 14 h of incubation (at 37 °C) in the presence of protein release inhibitor, the frequency of cytokine-producing CD4⁺ lymphocytes was too low to be detected by intracellular staining (results not shown). Therefore, lymphocyte stimulation was performed by the addition of phorbol 12-myristate 13-acetate (PMA) 10 ng/mL, ionomycin 0.5 µg/mL and monensin (GolgiStop, PharMingen Int.) to MNC followed by incubation for 4 h at 37 °C.

Intracellular staining of cytokines and flow cytometry analysis

Following culture, cell surface staining was performed with peridinin chlorophyll protein-conjugated anti-CD3 and fluorescein isothiocyanate-conjugated anti-CD8 antibodies (lymphocytes) or anti-CD14 antibody (monocytes) (PharMingen Int.). Subsequently, the MNC were fixed in phosphate-buffered 2% paraformaldehyde (v/v) and permeabilized with saponin (Perm-Wash solution, PharMingen Int.). Intracellular staining was carried out with mouse monoclonal phycoerythrin (PE)-conjugated anti-IL-4, IL-5, IL-10, IL-12 (p70/p40), IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ antibodies (0.12 µg /10⁶ cells) (PharMingen Int.).

All samples were analysed using a Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA, USA). The control level was set at 0.1% of the cell population stained with PE-conjugated isotype control antibody (PharMingen Int.).

Lymphocyte and monocyte gates were established on the basis of the characteristic side versus forward scatter patterns. As stimulation with PMA/ionomycin is known to decrease CD4 expression on lymphocytes, CD4⁺ lymphocytes were recognized as CD3⁺CD8⁻ cells within the lymphocyte population [5].

Anti-CD14 antibody was used to identify monocytes within the monocyte gate. The relative amount of cytokine-positive monocytes after allergen exposure was calculated by subtracting the relative number of cytokine-positive cells after PHA stimulation (basic level) from the relative numbers of cytokine-positive monocytes after stimulation with allergen and PHA.

Statistical analysis

The Wilcoxon matched pairs test was used to evaluate the changes between different time-points within the groups. Comparison of results from allergic patients and healthy

controls was performed using the Mann–Whitney *U*-test. Correlations between different parameters were evaluated by the Spearman rank-order correlation test. A *P*-value of <0.05 was considered statistically significant. All statistical tests were performed on a personal computer using the Statistica software package (StatSoft Inc., Tulsa, OK, USA).

Results

Clinical data

All the patients were symptom-free outside the pollen season. The median symptom scores increased significantly during the beginning and at the peak of the birch season compared with the alder season ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 1). Furthermore, the symptom scores at the peak of the birch season were significantly ($P < 0.001$) higher than at the beginning of the birch season. The clinical symptoms correlated significantly with the pollen count during the season ($r = 0.61$ and $P < 0.001$). Simultaneously, the patients increased their use of medication at the beginning and peak of the birch season compared with the alder season ($P < 0.01$ and $P = 0.001$, respectively).

Eosinophils

In allergic patients, the blood eosinophil count increased significantly at the peak of the birch season compared with the alder season, the beginning of the birch season and outside the pollen season ($P < 0.01$) (Table 2). An unaltered blood eosinophil count throughout the study was found in the control group. Both the blood eosinophil count and the proportion of peripheral blood eosinophils (%) were significantly higher in allergic patients compared with controls during the peak of the birch season ($P = 0.01$) (Table 2). At the peak of the birch season, a positive correlation between the symptom score for rhinoconjunctivitis and the relative number of blood eosinophils was found in the patient group ($r = 0.78$, $P < 0.001$).

Birch-specific IgE in serum

The serum concentration of birch pollen-specific IgE was significantly greater in allergic subjects compared with control subjects both during (periods 1–3) and outside the pollen season (period 4) ($P < 0.001$) (Table 2). In the patient group, the IgE concentration increased significantly ($P < 0.05$) outside the season as compared with values during the pollen season (Table 2). All controls had specific IgE concentrations below the detection level (0.3 kU/L) at all periods. In allergic patients, the birch pollen-specific IgE concentration correlated positively with the blood eosinophil count ($r = 0.52$, $P < 0.05$) and symptom score at the peak of the birch season ($r = 0.55$, $P < 0.05$).

Cytokine production by monocytes

The relative number of monocytes producing GM-CSF was significantly higher in the patient group at each test period compared with the control group (at least $P < 0.05$) (Table 3a). In the allergic group, the number of GM-CSF-positive monocytes was significantly higher during the peak of the birch pollen season compared with outside the pollen season

Table 2. BEC, RBEC, and serum birch-specific IgE during the four test periods

	Alder season (period 1)		Beginning of the birch season (period 2)	Peak of the birch season (period 3)		Outside the pollen season (period 4)	
	Patients <i>n</i> = 16	Controls <i>n</i> = 6	Patients <i>n</i> = 14	Patients <i>n</i> = 16	Controls <i>n</i> = 6	Patients <i>n</i> = 15	Controls <i>n</i> = 6
BEC ($\times 10^6/L$)	186 (40–540)	166 (50–240)	180 (30–720)	236 ^{***,****,*****} (60–820)	114 (50–200)	169 (50–240)	139 (30–260)
RBEC (%)	2.8 (0.8–7.1)	3.0 (0.7–6)	2.9 (0.6–9)	3.6 ^{***,****,*****} (1.4–12.3)	1.7 (0.8–4.1)	2.6 (0.8–3.6)	1.9 (0.5–5.8)
IgE (kU/L)	7.6 ^{*****} (0.86–100)	<0.3	11.7 (1.1–100)	12.2 ^{*,*****} (1–100)	<0.3	13.1 ^{*****} (1.3–100)	<0.3

Values are presented as median and range. Significant differences in relation to test period 4 (outside the season).

* $P < 0.05$, ** $P < 0.01$; in relation to test period 1 (alder season), *** $P < 0.01$; and between patients and healthy controls in each test period, **** $P = 0.01$, ***** $P < 0.001$ are indicated. BEC, blood eosinophil count; RBEC, relative blood eosinophil count.

Table 3. Proportion of cytokine-producing monocytes (a) and lymphocytes (b) during the four test periods

	Alder season (period 1)		Beginning of the birch season (period 2)	Peak of the birch season (period 3)		Outside the pollen season (period 4)	
	Patients <i>n</i> = 16	Controls <i>n</i> = 6	Patients <i>n</i> = 14	Patients <i>n</i> = 16	Controls <i>n</i> = 6	Patients <i>n</i> = 15	Controls <i>n</i> = 6
(a) Cytokine-producing monocytes [†]							
IL-10	8.4 (0–20.4)	11.1 (3–13.9)	8.4 (0–19.2)	8.3 (0.5–20.7)	9.7 (5.5–15.5)	10.5 (3.3–20.7)	9.4 (3.6–16)
IL-12	0 (0–10.78)	8.3 (0–13.2)	0.6 (0–13.9)	0 (0–11.2)	3.0 (0–16.0)	0 (0–6.4)	4.7 ^{***} (1.7–15.5)
GM-CSF	6.7 ^{***} (0–12.6)	1.7 (0–3.6)	4.2 (0.9–11.1)	7.1 ^{*,***} (0.3–14.2)	0.5 (0–3.8)	3.3 ^{**} (0.7–10.4)	1.0 (0–7.7)
(b) Cytokine-producing lymphocytes [‡]							
IL-5	0.6 (0.2–1.8)	0.5 (0.1–0.8)	0.5 (0.3–1.3)	0.5 (0.4–1.2)	0.4 (0.4–0.6)	0.8 (0.5–1.4)	0.9 (0.6–1.3)
IL-10	2.3 ^{*****} (1.7–7.91)	2.6 (1.9–4.7)	2.6 (1.1–4.3)	2.7 (1.2–4.2)	2.2 (1.2–4.1)	1.6 (0.9–4.4)	2.8 (1.5–4.2)
IL-13	3.3 (1.8–10)	3.5 (2.5–6.4)	3.3 (1.2–5.2)	3.3 ^{****} (2.2–7.3)	3.2 (2.5–3.9)	3.1 (0.8–4.2)	3.4 (2.5–3.6)
GM-CSF	10.2 ^{*****} (3.7–20)	7.1 (3.8–16.4)	9.9 ^{*****} (2.7–23.6)	9.5 ^{*****} (3.3–25.2)	10 (7.2–12.7)	7.2 (1.6–14)	8.5 (3.6–15.2)

Values are presented as median and range of the relative number of positive cells (%).

[†]Significant difference in the cytokine production in relation to test period 4 (outside the season). * $(P = 0.01)$; and between patients and healthy controls in each test period, ** $(P < 0.05)$ and *** $(P < 0.01)$ are indicated. [‡]Differences in cytokine production in relation to test period 4 (outside the season), **** $(P < 0.05)$ are indicated. GM-CSF, granulocyte macrophage-colony stimulating factor.

($P = 0.01$). In the same group, the proportion of IL-12-producing monocytes during the alder and birch seasons tended to be lower than that of the control group ($P = 0.06$). The difference between groups in the percentage of IL-12⁺ cells reached statistical significance outside the pollen season ($P < 0.01$).

The percentage of IL-10⁺ monocytes in both groups remained unchanged between different test periods. There was also no difference between allergic and healthy subjects (Table 3a).

Cytokine production by CD4⁺ lymphocytes

In the patient group, the proportion of IL-4-producing lymphocytes increased significantly, at the peak of the birch season (Fig. 2a), while the proportion of IFN- γ -producing lymphocytes decreased at the same time-point, when compared with the alder season (Fig. 2b). The percentage of

IL-4⁺ lymphocytes at the peak of the birch season was also significantly higher compared with outside the pollen season (Fig. 2a). In the same group, the number of lymphocytes producing IL-10, IL-13 and GM-CSF was unchanged during the alder and birch seasons (periods 1–3), but decreased significantly after the pollen season (Table 3b). During the birch pollen season (periods 2 and 3), a positive correlation was found between IL-4⁺ and IL-13⁺ ($r = 0.85$, $P < 0.001$) lymphocytes. In the control group, no seasonal changes were observed in the proportion of lymphocytes (CD4⁺) producing investigated cytokines.

After stimulation with polyclonal activators, patients and controls showed similar relative numbers of lymphocytes producing the cytokines examined. The relative number of CD4⁺ cells within the T lymphocyte population (CD3⁺) did not change at any investigated time-point and there was no difference between patients and controls (results not shown).

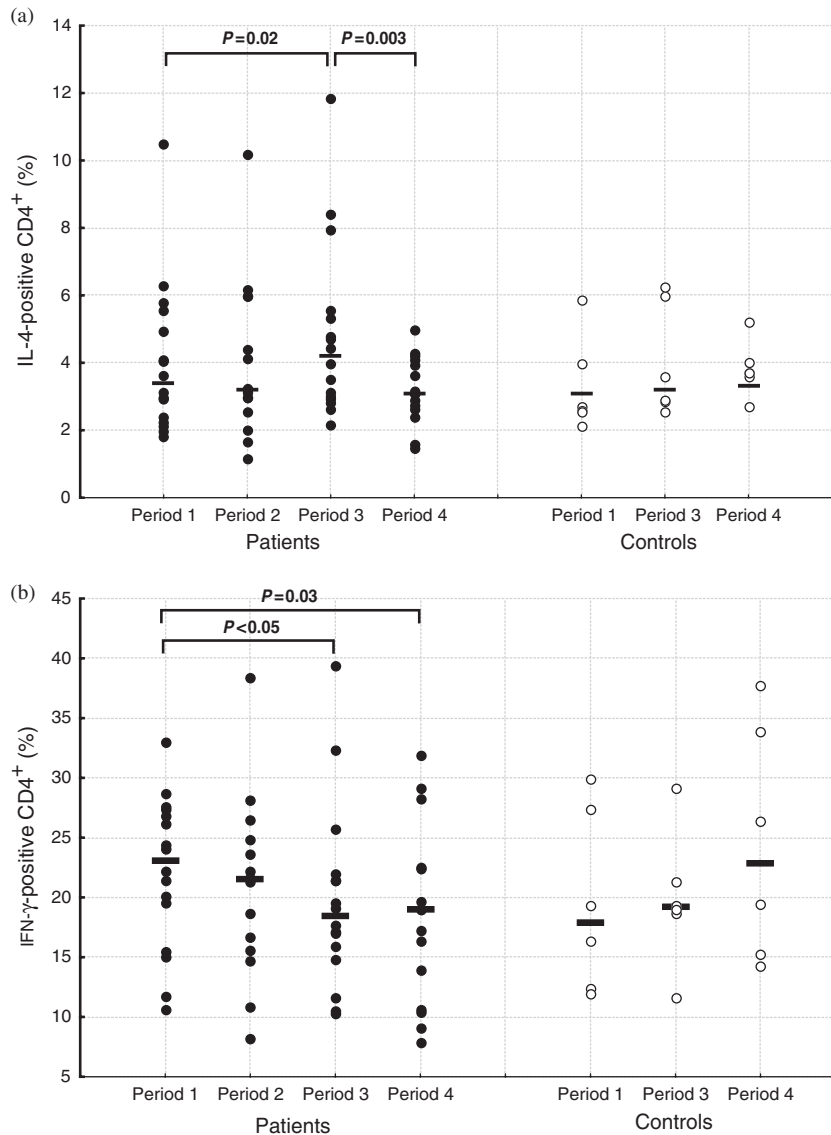


Fig. 2. Production of IL-4 (a) and IFN- γ (b) in CD4⁺ lymphocytes obtained from allergic subjects and healthy controls during three periods throughout and once outside the pollen season. Median values and significant differences between test periods are indicated.

Ratio of IL-4/IFN- γ -positive lymphocytes

The ratio of IL-4/IFN- γ -positive lymphocytes in the patient group significantly increased at the peak of the birch season compared with the alder season ($P<0.001$), the beginning of the birch season ($P=0.002$) and outside the pollen season ($P=0.004$). In the group of allergic patients, the ratio of IL-4/IFN- γ -producing lymphocytes during the birch season (periods 2 and 3) correlated with symptom scores $r=0.49$, $P<0.01$ and with the mean pollen count $r=0.37$, $P<0.05$.

Discussion

The present study demonstrated an increased proportion of GM-CSF⁺ cells and reduced numbers of IL-12-producing monocytes following *in vitro* allergen stimulation in allergic subjects compared with healthy controls. The increase in the

relative number of GM-CSF⁺ monocytes was dependent on allergen exposure. In addition, an increased ratio of IL-4/IFN- γ in stimulated lymphocytes (CD4⁺) from exposed allergic subjects at the peak of the birch pollen season was demonstrated. Moreover, the IL-4/IFN- γ ratio correlated with the clinical symptoms of seasonal allergic rhinitis. The relative numbers of lymphocytes positive for the Th2-type cytokines IL-4 and IL-13 were significantly correlated during the birch pollen season. This study also confirmed that in allergic subjects, an adequate seasonal exposure to pollen results in significant increases in clinical symptoms and use of medication [6]. As expected, higher numbers of PB eosinophils and augmented levels of specific IgE in serum were found in the patient group compared with healthy control subjects [6].

We have found an increased proportion of GM-CSF-positive monocytes upon allergen stimulation in allergic subjects. Previous studies have shown that monocytes from subjects with allergic asthma produced more GM-CSF and

had a reduced ability to produce IL-12 compared with those from healthy subjects [7, 8]. Another study on whole-blood cultures obtained from subjects with rhinitis demonstrated similar levels of GM-CSF, induced by lipopolysaccharide stimulation, during and outside the pollen season [9]. This is in agreement with the results of the present study, where stimulation of MNC with PHA did not alter the number of GM-CSF⁺ monocytes during the pollen season (results not shown). Specific stimulation with allergen was required, in addition to PHA, in order to obtain the aforementioned changes.

The production of GM-CSF together with IL-4 in the microenvironment contributes to enhanced monocyte survival and facilitates antigen presentation [10]. GM-CSF has also been shown to influence the development of T cells towards the Th2-type [11] and to promote differentiation of eosinophil progenitors [12]. Furthermore, GM-CSF is believed to be the main enhancing factor for eosinophil survival [13, 14]. The results of this study suggest that monocytes from allergic patients could be an important source of GM-CSF.

We also report a lower IL-12 production in monocytes from allergic subjects compared with healthy subjects. IL-12 from antigen-presenting cells has been implicated in the differentiation of naïve T cells into the Th1-type because it suppresses the transcription of IL-4 and stimulates IFN- γ production [15]. Thus, the degree of activation and profile of subsequent cytokine production following natural allergen exposure suggests an important role for monocytes in the pathogenesis of seasonal allergic disease.

In the present study, cytokine production in monocytes was assessed after stimulation with allergen followed by mitogen (PHA). The stimulation of MNCs with PHA, compared with the medium alone, increased the numbers of GM-CSF⁺ and IL-12⁺ monocytes. PHA activates lymphocytes via T cell antigen receptor and CD2; the enhancing effect of PHA on cytokine production in monocytes is thus likely to be T cell mediated. Nevertheless, the difference in cytokine production between allergic and healthy subjects was observed only after incubation of MNCs with allergen prior to mitogen stimulation. Thus, it seems unlikely that stimulation with non-specific mitogen (PHA), used in the present study, does more than amplify the effects of allergen itself. Monocytes express the high-affinity IgE receptor (Fc ϵ RI) and ligation of this receptor can induce IL-12 production [16]. This possibility was not investigated in the present study but the direct effect of allergen on monocyte function cannot be ruled out.

IL-4, together with IL-13, provides the necessary signal for the induction of IgE production from B cells [17]. Additionally, IL-4 promotes the differentiation of monocytes into more efficient antigen-presenting cells and favours the differentiation of naïve T cells into the Th2-type. IFN- γ , on the other hand, is required for the development of Th1-type cells. Both *in vitro* allergen stimulation [18] and allergen challenge in allergic subjects result in an increase in the IL4/IFN- γ ratio in PBMNC [19]. Munoz-Bellido et al. [1] reported similar results from polyclonally stimulated PBMNC during natural pollen exposure, while another group observed an increased release of IL-4 but unchanged IFN- γ production [20]. In contrast, one investigation utilizing flow cytometry to measure the numbers of IL-4- and IFN- γ -producing CD4⁺ and CD8⁺ lymphocytes found no seasonal variation [21].

In order to follow the kinetics of cytokine production, we chose to carry out multiple sampling throughout the pollen season. Although birch pollen is the main pollen of deciduous trees, the season starts with pollination of hazel and alder, involving allergens that both cross-react with T cell clones directed against birch pollen [22]. The alder pollen is a very potent allergen and even low pollen concentrations, like that recorded around the first sampling period, give rise to clinical symptoms in allergic subjects. Interestingly, a significant increase in the IL-4/IFN- γ ratio was found only at the peak of the birch pollen season, i.e. well into the pollen season. In agreement with previous *in vitro* studies [23], we suggest that a certain degree of allergen stimulation (pollen count) is necessary in order to evoke an adequate Th2 response that can be measured in PB. The varying conclusions of previous studies investigating seasonal cytokine generation from PBMNC after polyclonal activation are probably due to variations in allergen exposure, timing of blood sampling or other undefined factors. In this study, the ratio of IL-4/IFN- γ -producing lymphocytes and the symptom score over the whole birch pollen season showed a significant relationship. Furthermore, the ratio from the beginning of the birch season had a significant relationship with symptom score during the peak of the birch season ($r = 0.71$ and $P < 0.01$). The latter relationship suggests that cytokine production precedes the development of clinical symptoms.

IL-10 promotes the differentiation of Th0 cells into Th2 type and enhances IgE synthesis in B cells [24]. The present study demonstrated slightly elevated numbers of IL-10⁺ and GM-CSF⁺ lymphocytes before the start of the birch pollen season, i.e. during alder pollination and during the birch pollen season. Previously, increased production of IL-10 after stimulation of PBMNC with birch pollen extract was found in birch-allergic subjects compared with non-allergic controls [25] although the exact cellular source of IL-10 was not established.

Earlier studies investigating GM-CSF production were mainly performed on supernatants from allergen-stimulated PBMNC or isolated allergen-specific CD4⁺ T cell clones [26, 27].

In agreement with one previous report [4], no seasonal increase in IL-5 production was demonstrated in the present study. Contrary to others, we found no relationship between IL-5 and symptoms of rhinoconjunctivitis at any time-point [28, 29].

The absolute frequencies of cytokine-positive cells found in the present study after *in vitro* PMA/ionomycin stimulation are comparable to the frequencies reported by others using similar experimental protocols i.e. short incubation time and relatively low concentration of PMA [21, 30]. Nevertheless, the frequency of IL-5⁺, IL-10⁺ and IL-13⁺ lymphocytes after *in vitro* PMA/ionomycin stimulation was lower than might be expected in response to allergen stimulation. Also, the number of IL-5⁺ lymphocytes after this potent polyclonal stimulus was much lower compared with IL-4⁺ and IFN- γ ⁺ T cells. The reason for this could be that the regulatory pathways for IL-5, IL-10 and IL-13 production are distinct from those of IL-4 and IFN- γ . Stimulation with PMA and calcium ionophore activates protein kinase C and triggers Ca²⁺ mobilization and both factors are required for IL-4 and IFN- γ production [31]. While stimulation with PMA and CD28 leads

to IL-5, IL-10 and IL-13 synthesis [31, 32], a negative effect of calcium signalling on the production of these cytokines has been demonstrated [31, 33, 34]. We suggest that although PMA/ionomycin is a strong and frequently used activator in studies of cytokine production, it should not be assumed that this mode of stimulation faithfully mirrors all aspects of allergen-induced cytokine production.

In conclusion, our findings emphasize the importance of monocytes as a source of cytokines participating in the development of allergic inflammation. We suggest that the seasonal increase in GM-CSF production, which was found in both monocytes and T cells, possibly contributes to the augmentation of eosinophil numbers during the pollen season. We have also shown that the seasonal change in Th2/Th1 cytokine ratio requires an adequate and prolonged allergen stimulation that is seen late in the pollen season. Thus, the time-point of blood sampling should be considered as an important factor in studies examining the effects of seasonal allergen exposure on peripheral blood cells activation and cytokine production.

Acknowledgements

We thank registered nurses Yvonne Backberg and Yvonne Andersson for their skilful assistance. This study was supported by Swedish Asthma and Allergy Association and the Medical Faculty of the University of Göteborg.

References

- Munoz-Bellido FJ, Monteseirin FJ, Escribano MM, Delgado J, Velazquez E, Conde J. Effect of seasonal exposure to pollen on nonspecific interleukin-4, interleukin-5, and interferon-gamma *in vitro* release by peripheral blood mononuclear cells from subjects with pollinosis. *Allergy* 1998; 53:420–5.
- Tsuda M, Ohashi Y, Washio Y et al. Seasonal changes in phytohemagglutinin-induced cytokine synthesis by peripheral blood lymphocytes of patients with seasonal allergic rhinitis due to Japanese cedar pollens. *Acta Otolaryngol Suppl* 1998; 538:156–68.
- Gabrielsson S, Soderlund A, Paulie S, Rak S, van der Pouw Kraan TC, Troye-Blomberg M. Increased frequencies of allergen-induced interleukin-13-producing cells in atopic individuals during the pollen season. *Scand J Immunol* 1998; 48:429–35.
- Jepsen KF, Nielsen L, Olsen OT, Bendtzen K, Poulsen LK. Seasonal variations in T-lymphocyte response to grass pollen allergens from pollen-allergic patients and healthy controls. *Exp Clin Immunogenet* 1998; 15:144–53.
- Petersen CM, Christensen EI, Andresen BS, Moller BK. Internalization, lysosomal degradation and new synthesis of surface membrane CD4 in phorbol ester-activated T-lymphocytes and U-937 cells. *Exp Cell Res* 1992; 201:160–73.
- Winther L, Moseholm L, Reimert CM, Stahl Skov P, Kaergaard Poulsen L. Basophil histamine release, IgE, eosinophil counts, ECP, and EPX are related to the severity of symptoms in seasonal allergic rhinitis. *Allergy* 1999; 54:436–45.
- Hallsworth MP, Soh CP, Lane SJ, Arm JP, Lee TH. Selective enhancement of GM-CSF, TNF-alpha, IL-1 beta and IL-8 production by monocytes and macrophages of asthmatic subjects. *Eur Respir J* 1994; 7:1096–102.
- Tomita K, Lim S, Hanazawa T et al. Attenuated production of intracellular IL-10 and IL-12 in monocytes from patients with severe asthma. *Clin Immunol* 2002; 102:258–66.
- Larsson S, Hansson L, Greiff L et al. Natural allergen exposure does not diminish the sensitivity of cytokine production to glucocorticosteroids in blood cells of seasonal allergic asthma and rhinitis patients. *Respir Med* 2002; 96:927–33.
- Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and down regulated by TNF-alpha. *J Exp Med* 1994; 179:1109–18.
- Pulendran B, Smith JL, Caspary G et al. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc Natl Acad Sci USA* 1999; 96:1036–41.
- Shalit M. Growth and differentiation of eosinophils from human peripheral blood CD 34+ cells. *Allerg Immunol (Paris)* 1997; 29:7–10.
- Okubo Y, Hossain M, Sekiguchi M. Cytokine production in patients with mite-sensitive bronchial asthma. *Int Arch Allergy Immunol* 1994; 104 (Suppl. 1):46–8.
- Becler K, Håkansson L, Rak S. Treatment of asthmatic patients with a cysteinyl leukotriene receptor-1 antagonist montelukast (Singulair), decreases the eosinophil survival enhancing activity produced by peripheral blood mononuclear leukocytes *in vitro*. *Allergy* 2002; 57:1021–8.
- Manetti R, Parronchi P, Giudizi MG et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993; 177:1199–204.
- Katoh N, Kraft S, Wessendorf JH, Bieber T. The high-affinity IgE receptor (FcepsilonRI) blocks apoptosis in normal human monocytes. *J Clin Invest* 2000; 105:183–90.
- Broide DH. Molecular and cellular mechanisms of allergic disease. *J Allergy Clin Immunol* 2001; 108 (Suppl.):S65–71.
- Imada M, Simons FE, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon-gamma production in primary culture: responses of subjects with allergic rhinitis and normal controls. *Immunol* 1995; 85:373–80.
- Gabrielsson S, Paulie S, Roquet A et al. Increased allergen-specific Th2 responses *in vitro* in atopic subjects receiving subclinical allergen challenge. *Allergy* 1997; 52:860–5.
- Lagier B, Pons N, Rivier A et al. Seasonal variations of interleukin-4 and interferon-gamma release by peripheral blood mononuclear cells from atopic subjects stimulated by polyclonal activators. *J Allergy Clin Immunol* 1995; 96 (Part 1):932–40.
- Majori M, Rossi G, Caminati A, Bosoni A, Corradi M, Pesci A. Seasonal variations of T-cell cytokine pattern in peripheral blood from atopic subjects. *J Asthma* 2001; 38:469–76.
- Ebner C, Ferreira F, Hoffmann K et al. T cell clones specific for Bet v I, the major birch pollen allergen, crossreact with the major allergens of hazel, Cor a I, and alder, Aln g I. *Mol Immunol* 1993; 30:1323–9.
- Marshall JD, Wen Y, Abrams JS, Umetsu DT. *In vitro* synthesis of IL-4 by human CD4+ T cells requires repeated antigenic stimulation. *Cell Immunol* 1993; 152:18–34.
- Kobayashi N, Nagumo H, Agematsu K. IL-10 enhances B-cell IgE synthesis by promoting differentiation into plasma cells, a process that is inhibited by CD27/CD70 interaction. *Clin Exp Immunol* 2002; 129:446–52.
- Moverare R, Elfman L, Stalenheim G, Björnsson E. Study of the Th1/Th2 balance, including IL-10 production, in cultures of peripheral blood mononuclear cells from birch-pollen-allergic patients. *Allergy* 2000; 55:171–5.
- Nagata M, Yamamoto H, Tabe K, Sakamoto Y, Matsuo H. Eosinophil-adhesion-inducing activity produced by antigen-stimulated mononuclear cells involves GM-CSF. *Int Arch Allergy Immunol* 2000; 122 (Suppl. 1):15–9.
- Parronchi P, Manetti R, Simonelli C et al. Cytokine production by allergen (Der p I)-specific CD4+ T cell clones derived from a

- patient with severe atopic disease. *Int J Clin Lab Res* 1991; 21:186–9.
- 28 Nakai Y, Ohashi Y et al. Allergen-induced mRNA expression of IL-5, but not of IL-4 and IFN-gamma, in peripheral blood mononuclear cells is a key feature of clinical manifestation of seasonal allergic rhinitis. *Arch Otolaryngol Head Neck Surg* 2000; 126:992–6.
- 29 Till S, Dickason R, Huston D et al. IL-5 secretion by allergen-stimulated CD4⁺ T cells in primary culture: relationship to expression of allergic disease. *J Allergy Clin Immunol* 1997; 99:563–9.
- 30 Madden J, Howarth PP, Godfrey RR, Frew AA. The kinetics and stimulant dependence of cytokine production by blood and bronchoalveolar lavage T cells evaluated at the single cell level. *Cytokine* 1999; 11:510–7.
- 31 Harada Y, Watanabe S, Yssel H, Arai K. Factors affecting the cytokine production of human T cells stimulated by different modes of activation. *J Allergy Clin Immunol* 1996; 98 (Part 2):S161–73.
- 32 Rafiq K, Bullens DM, Kasran A, Lorre K, Ceuppens JL, Van Gool SW. Differences in regulatory pathways identify subgroups of T cell-derived Th2 cytokines. *Clin Exp Immunol* 2000; 121: 86–93.
- 33 Schandene L, Alonso-Vega C, Willems F et al. B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *J Immunol* 1994; 152:4368–74.
- 34 van der Pouw Kraan TC, Boeije LC, Troon JT, Rutschmann SK, Wijdenes J, Aarden LA. Human IL-13 production is negatively influenced by CD3 engagement. Enhancement of IL-13 production by cyclosporin A. *J Immunol* 1996; 156:1818–23.