

Activation of B-lymphocytes during pollen season. Effect of immunotherapy

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Summary

Background B-lymphocytes play an important part in the allergic reaction as producers of IgE antibodies.

Objective To investigate the cell surface expression of the activation antigens CD23, CD40 and HLA-DR on B-lymphocytes in birch pollen allergic patients before and during birch pollen season and to study the effect of immunotherapy.

Methods The study included 24 birch pollen allergic patients half of whom were treated with immunotherapy against birch pollen before the start of the season. Eleven of the 24 patients had asthma. Blood samples were taken and lung function was registered before the season began and before the immunotherapy treatment in January to February and during the season in May. The relative number of B-lymphocytes (CD19⁺) of the lymphocyte population and the cell surface expression of CD23, CD40 and HLA-DR on B-lymphocytes was measured by the use of flow cytometry.

Results In the control group of patients the relative number and concentration of B-lymphocytes, the cell surface expression of CD23, CD40 and HLA-DR on B cells, and the serum concentration of IgE increased during season compared with before season. In contrast, in the immunotherapy treated patients no changes in the number of B cells or cell surface expression of CD23, CD40 and HLA-DR were demonstrated.

Conclusion The elevated expression of CD23, CD40 and HLA-DR on B cells, combined with increased levels of IgE in allergic patients during season could be explained by the effect of cytokines produced by activated TH₂ cells. A shift from TH₂ to TH₁ cells might be the mechanism after the absence of signs of B-cell activation in immunotherapy treated patients. The prevention of increased cell surface expression on B cells by immunotherapy may constitute a significant mechanism behind the beneficial effects of immunotherapy in the treatment of pollen atopy.

Keywords: B-lymphocyte, pollen season, immunotherapy, CD23, CD40, HLA-DR

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Introduction

Exposure to allergen induces an allergic inflammatory response in sensitized individuals, which activates several of the white blood cells in the body, such as mast cells, T

cells B cells, monocytes and eosinophils. The B-lymphocytes produce IgE antibodies to the allergen, which are important for the triggering of the response. B-lymphocytes express the low affinity Fc_ε-receptor II on their cell surface [1–3]. The role of this receptor for B cells is uncertain, but recent research suggests that CD23 is involved in cellular adhesion and antigen uptake and presentation [4–6]. In this context it has been demonstrated that CD23 mediates antigen specific IgE-mediated enhancement of antibody

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responses. The HLA-DR antigen which is one of the human MHC class II molecules is involved in the initial recognition of an antigen on the cell surface of an antigen presenting B cell by the T-cell receptor on a T-helper cell [3]. The HLA-DR-antigen is also known to be spatially associated with CD23 on the cell surface of B-lymphocytes [7]. This association is also functionally important since anti-CD23 inhibited allogeneic mixed lymphocyte reactions [8]. The cell surface antigen CD40 is one of the accessory molecules which stabilizes the interaction between the T-helper cell and the B cell and co-ordinates the responses of the cells. Engagement of the CD40 antigen provides signals that are absolutely essential for the immunoglobulin isotype switch [9]. CD40 on B cells binds to the CD40-ligand (CD40 L) on T helper cells [10,11].

Previous studies of B-lymphocyte cell surface expression have shown an increased expression of CD23, the Fc ϵ R2, on lymphocytes from allergic children and adults compared with non-allergic controls [12,13]. In the study of the allergic children the percentage of CD23 B cells was demonstrated to decrease after hyposensitization [12]. Another study on grass pollen induced proliferative responses of lymphocytes showed that hyposensitization prevented the increased proliferative responses and enhanced CD23 expression on B cells in sensitized allergic individuals [14].

Studies on the effect of hyposensitization on different aspects of the allergic inflammatory response have demonstrated an increase of allergen-specific IgG antibodies, a moderate impairment of the synthesis of allergen specific IgE, reduced basophil reactivity to allergens, reduced lymphocyte proliferation and cytokine production, generation of allergen-specific suppressor T cells, and reduced number and activity of eosinophils [15,16]. Lately, since the discovery of TH₁ and TH₂ cells it has been proposed that hyposensitization induces a shift from TH₂ to TH₁ cells. Such a shift would result in a reduced production of IL-4, IL-5 and IgE and increased production of IL-2, IFN γ and IgG. A recent publication has also demonstrated that coaggregation of Fc ϵ RI and Fc γ RIIB by immune complexes inhibits activation of mast cells via Fc ϵ RI [17]. Which mechanism or mechanisms that are responsible for the beneficial clinical effects of hyposensitization is, however, far from clear.

The aim of the present study was to investigate whether activation of B-lymphocytes during natural pollen exposition induced changes of the cell surface expression of CD23, CD40 and HLA-DR on circulating B-lymphocytes, and whether the cell surface expression was influenced by immunotherapy. Furthermore, the relationship between B-lymphocyte cell surface expression and blood eosinophil number and activity measured as serum concentration of ECP was investigated. The investigation included 24 birch

pollen allergic patients, who were studied before and during the pollen season. Half of the patients were treated with immunotherapy against birch pollen.

Materials and methods

Patients

Twenty-four birch pollen allergic patients participated in the study. They were 11 men and 13 women, mean age 31 years (range 21–42 years). They all had a history of rhino-conjunctivitis, and 11/24 of asthma, during the birch pollen season. The diagnosis of allergy was based on a positive skin-prick test of >3 mm with birch pollen extract (*Betula verrucosa*, ALK, Hørsholm, Denmark) and RAST. Some patients were also sensitized to grasses and animal dander but none was exposed to pets. Patients sensitive to mites and moulds were not allowed to participate in the study. Patients with asthma presented bronchial hyperresponsiveness of PC₂₀-methacholine <16 mg/mL outside the season. Their disease required inhaled β_2 -agonists occasionally and inhaled steroids were used by some only during previous seasons. None of the patients were treated with immunotherapy earlier in life.

The study was approved by the local Ethics Committee and the patients gave their informed consent.

Treatment design

The study was a double-blind controlled group comparison, where active treatment was subcutaneous immunotherapy (IT) with birch pollen extract Alutard (ALK, Hørsholm, Denmark) and the control treatment was topical nasal steroid (Budesonide, Astra-Draco, Lund, Sweden) 400 μ g once daily over 2 weeks preseasonally and the whole birch pollen season. The IT treatment was initiated after pre-seasonal examination with skin-prick test, RAST, methacholine challenge and blood sampling. All patients reached maintenance dose of 1 mL of 100 000 SQ/mL before start of the birch pollen season. The last maintenance dose before the season was given to the patients at approximately the same time and repeated after 6 weeks (at the end of the season).

Methacholine challenge and blood sampling were performed again at the peak of the birch pollen season.

Throughout the season patients recorded symptoms of rhinoconjunctivitis, PEF measurements using a Wright peak flow meter (in triplicate), and use of medication morning and evening. Per oral antihistamines (acrivastine Wellcome, London, UK) and eye drops of levocabastin (Janssen Pharmaceutica, Beerse, Belgium) and antazolin (Ciba Vision AB, Askim, Sweden) were allowed for treatment of rhinoconjunctivitis and inhaled β -agonists for treatment of asthmatic symptoms.

Methacholine challenge

Methacholine challenge was performed as described elsewhere [18]. Briefly, methacholine chloride was diluted in saline and delivered in nebulized form by Pari Inhalerboy nebulizer (output 0.75 mL/min) (Pari Ritzau Pari Werken, Starnberg, Germany) via a mouthpiece using tidal breathing. The test started with lung function measurements (Vitalograph, UK), followed by saline inhaled for 2 min and methacholine solutions starting from 0.03 mg/mL and increasing at 5 min intervals. FEV₁ was measured 2 and 4 min after each dilution until a fall of 20% was obtained.

Methods

Isolation of mononuclear leucocytes

Twenty millilitre citrated (0.129 mol/L) blood was mixed with an equal volume of PBS with citrate (0.129 mol/L) in a 50 mL tube and 15 mL of a Percoll solution (67% [v/v]) was applied in the bottom of the tube. After centrifugation for 30 min at $1000 \times g$ at 20°C the mononuclear cells above the Percoll gradient were carefully removed and transferred to a new tube. The cells were washed twice with PBS with citrate and human serum albumin (1 g/L) and counted. Finally the cells were centrifuged at $600 \times g$, the supernatant was removed and the cells were resuspended in freezing medium containing 20% FCS, 10% DMSO, and 70% RPMI and immediately transferred to -70°C. After 2 h at -70°C the tubes were stored in liquid nitrogen until analysis.

Flow cytometry

The tubes with frozen cells were rapidly thawed and diluted 10 times with PBS containing 1% (v/v) newborn calf serum (NBS). After two washes with PBS in 1% NBS the cells were diluted to a concentration of $3-5 \times 10^6$ /mL. Fifty microlitre of the cell suspension containing mononuclear leucocytes were incubated with 10 µL of optimally titrated monoclonal antibodies to CD19 (FITC-conjugated) combined with CD23 (RPE-conjugated), HLA-DR (RPE-conjugated), and a RPE-conjugated negative control, respectively, for 30 min at 4°C. After two washes with PBS the cells were analysed in an EPICS Profile II flow cytometer (Coulter Company Inc., Hialeah, FL, USA). Furthermore, 50 µL of the cell suspension containing mononuclear leucocytes was incubated with 10 µL of optimally titrated monoclonal antibodies to CD40 for 30 min at 4°C. After two washes with PBS the cells were incubated with RPE-conjugated goat antimouse Ig for 30 min at 4°C, washed twice with PBS, and incubated with FITC-conjugated anti-CD19 for 30 min at 4°C. After two final washes the cells were analysed in an EPICS Profile II flow cytometer (Coulter Company Inc., Hialeah, FL, USA). Mononuclear leucocytes from each patient isolated before and

during season were analysed on the same day. Based on the pattern of forward and side scatter a lymphocyte gate was set. B-lymphocytes were identified as CD19⁺ lymphocytes. The relative amount of CD19⁺ lymphocytes, and of CD19⁺ cells that were positive for CD23, CD40 and HLA-DR, respectively, were determined. Furthermore, the mean and median fluorescence intensity of CD23, CD40, and HLA-DR on B-lymphocytes were determined.

Determination of birch specific IgE

The serum concentration of specific IgE antibody for birch pollen was determined using the Pharmacia CAP System according to the protocol of the manufacturer (Pharmacia AB, Diagnostics, Uppsala, Sweden).

Serum concentration of ECP

Serum samples obtained for the measurement of ECP were handled according to the instructions of the manufacturer. The serum concentrations of ECP were assayed by means of double antibody RIAs (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). The intra- and inter assay coefficients of variation of the ECP assay are less than 11%, and the detection limit is less than 2 µg/L.

Pollen count

The number of air borne pollen was counted by the Palynological Laboratory, Swedish Museum of Natural History, Stockholm between 1 March and 30 June 1993. The recording of pollen was done by the use of a Burkhard 7-day recording volumetric spore trap [19,20]. The trap was placed on the roof of the hospital of Eskilstuna. The pollen count is expressed as the mean number of pollen per m³, per 2 h interval over the day (Fig. 1).

Statistics

Results are expressed as median and lower and upper quartiles, or median and range, respectively. Statistical evaluations were made using Mann-Whitney *U*-test, and Wilcoxon's matched pair test, and Spearman rank order correlation test was used to analyse relationships between different variables. All calculations were done by the use of CSS Statistica (StatSoft Inc., Tulsa, OK, USA).

Results

Blood leukocyte counts and lung function

The birch pollen counts of the pollen season in spring 1993 were remarkably high and were preceded by high alder pollen counts (Fig. 1). These pollen loads induced considerable responses in sensitized individuals. The non-asthmatic patients demonstrated a significant increase of

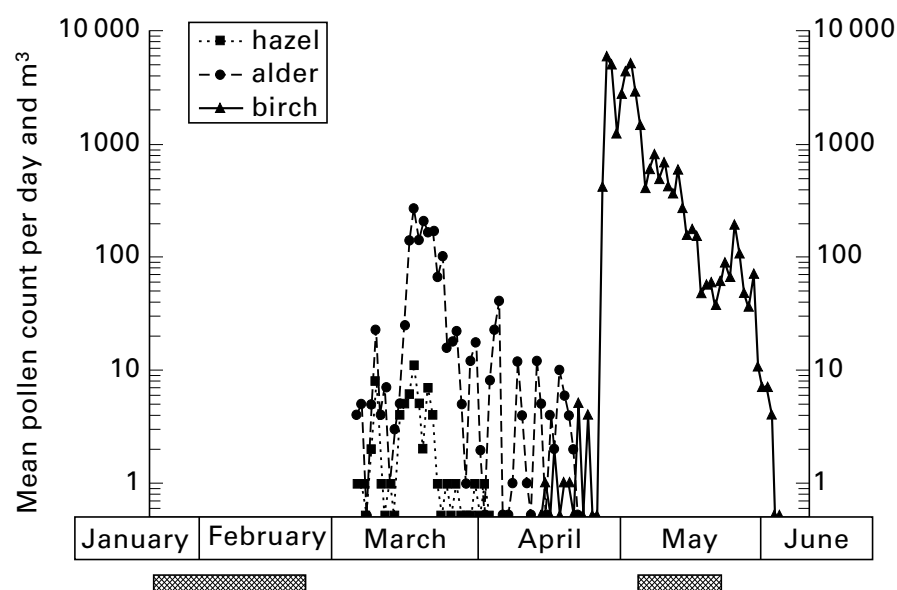


Fig. 1. Mean count of hazel, alder and birch pollen during the period of the study in 1993. The two test periods, before season during January and February and during season in May are indicated by filled boxes.

the non-specific bronchial reactivity during the season, irrespective of treatment (Fig 2). There was a significant difference between the PC_{20} -methacholine levels of non-asthmatic and asthmatic patients both before and during

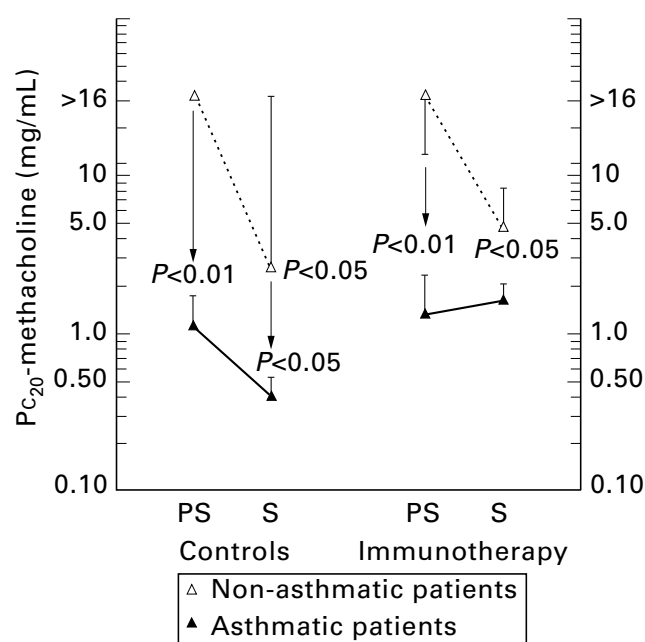


Fig. 2. Non-specific bronchial reactivity of the patients measured as PC_{20} -methacholine. The cut-off point for bronchial hyperresponsiveness is 8 mg/mL. Results shown are medians and upper or lower quartiles of the respective groups. Significant differences between the values of asthmatic and nonasthmatic patients are indicated by arrows and significant changes during season compared with before season are also shown.

the season in the control group of patients. In the IT-treated group of patients there was a significant difference between the PC_{20} -methacholine levels of non-asthmatic and asthmatic patients before the season, but in contrast, not during the season. The concentration of lymphocytes in peripheral blood demonstrated a significant decrease during season compared with before season in both the control and IT-treated group of patients (Table 1). The blood eosinophil counts increased significantly in both patient groups during season, while the blood neutrophil counts were unchanged during season as compared with before birch pollen season. When evaluated in detail it was

Table 1. Blood leukocyte counts

Group	Lymphocytes ($\times 10^9/L$)	Neutrophils ($\times 10^9/L$)	Eosinophils ($\times 10^6/L$)
Controls Before season	1.82 1.05–2.68	2.9 1.2–5.5	145 90–210
Controls During season	1.66 ¹ 0.94–2.37	2.5 1.8–4.9	425 ³ 130–1090
IT-treated Before season	1.97 1.27–3.29	3.2 2.2–4.8	135 100–560
IT-treated During season	1.76 ² 1.01–2.61	2.8 1.9–4.3	280 ⁴ 90–770

The presented results are the medians and ranges of the respective groups. Significant changes during season compared with before season are indicated by ¹($P < 0.05$), ²($P < 0.05$), ³($P < 0.01$), and ⁴($P < 0.01$).

Table 2. B-lymphocyte counts

Group	Relative amount B cells CD19 ⁺ lymphocytes (%)	Concentration on B cells ($\times 10^6/L$)
Controls	9.1	173
Before season	3.1–16.7	63–288
Controls	13.7 ¹	219 ²
During season	4.9–30.2	81–716
IT-treated	11.0	202
Before season	6.1–27.6	85–604
IT-treated	10.2	140
During season	7.7–19.2	102–338

The relative amount of B-lymphocytes (CD19⁺) of the lymphocyte population was measured by flow cytometry. The concentration of B-lymphocytes in blood was calculated from the blood lymphocyte concentration and the relative amount of B-lymphocytes in the lymphocyte population. The presented results are the medians and ranges of the respective groups. Significant changes during season compared with before season are indicated by ¹($P < 0.02$) and ²($P < 0.03$).

demonstrated that only the non-asthmatic IT-treated patients demonstrated a significant ($P < 0.05$) increase of the blood eosinophil count during season, 315 (140–470) $\times 10^6/L$ (median and range) vs 145 (100–330) $\times 10^6/L$.

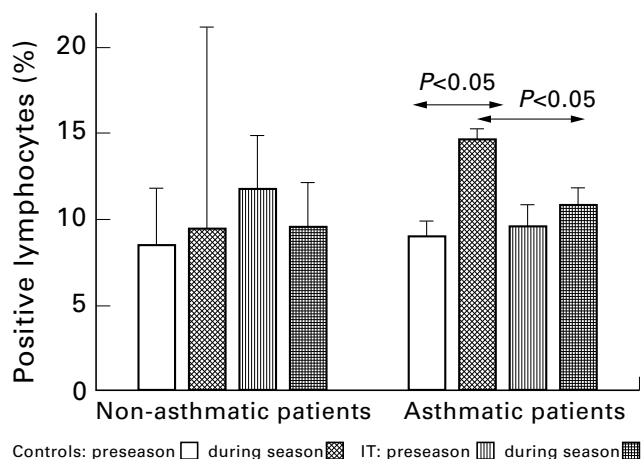


Fig. 3. The relative amount of B-lymphocytes in the lymphocyte population measured as CD19 expressing lymphocytes. Results shown are medians and upper quartiles of the respective groups. Significant differences between values before and during season and between controls and IT-treated patients are indicated.

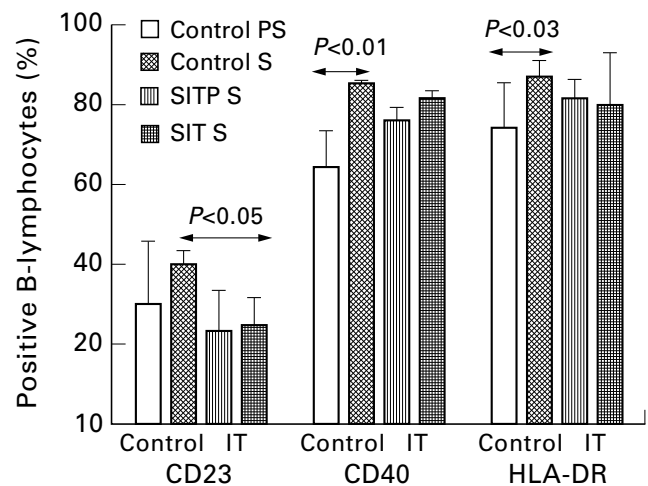


Fig. 4. The relative amount of CD23, CD40 and HLA-DR expressing B-lymphocytes. B-lymphocytes were identified as CD19⁺ lymphocytes. Results shown are medians and upper quartiles of the respective groups. Significant differences between values before and during season and between controls and IT-treated patients are indicated.

Cell surface markers on B-lymphocytes

The relative number of CD19⁺ lymphocytes, i.e. B-lymphocytes, and the concentration of B cells in blood increased significantly in the control group during the season compared with before the season, but was unchanged in the group of IT-treated patients (Table 2). A closer analysis demonstrated that the relative amount of B-lymphocytes was increased during the season compared with before the season only in the asthmatic patients of the control group (Fig. 3). The relative number of CD19⁺ lymphocytes was also significantly higher in the control group than in the IT-treated group of asthmatic patients during the season. The concentration of B-lymphocytes in blood increased significantly ($P < 0.05$) during the season compared with before the season, 226 (186–314) vs 173 (77–288) (median and range), in the asthmatic patients of the control group, while it was unchanged in all other groups (results not shown).

The relative amount of CD23⁺ B cells was significantly higher in the control group than in the IT-treated patients during season (Fig. 4). The relative amount of CD40⁺ B cells increased significantly during season in the control group but not in the group of IT-treated patients. A detailed analysis of B cells from non-asthmatic and asthmatic patients in the two treatment groups showed a significant increase of CD40⁺ B cells during the season in the non-asthmatic control patients and that the asthmatic control patients had a significantly higher number of CD40⁺ B cells than the IT-treated patients during the season (Fig. 5). Examination of the HLA-DR expression demonstrated a

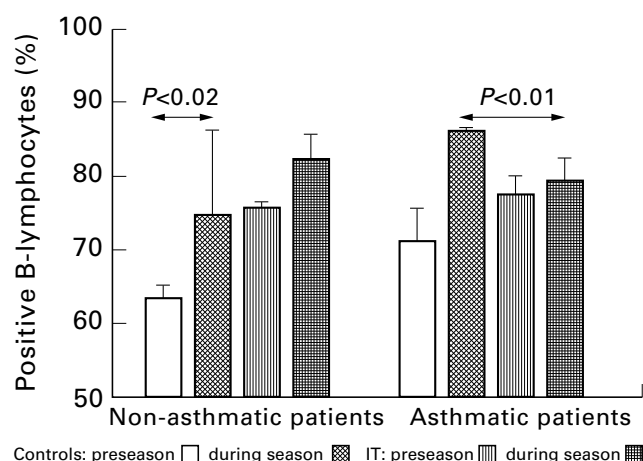


Fig. 5. The relative amount of CD40 expressing B-lymphocytes. B-lymphocytes were identified as CD19-positive lymphocytes. Results shown are medians and upper quartiles of the respective groups. Significant differences between values before and during season and between controls and IT-treated patients are indicated.

slight but significant increase of HLA-DR⁺ B cells in the control group during the season but no significant changes in the other groups (Fig. 4).

Birch specific IgE

The serum concentration of birch specific IgE increased significantly during the season in both non-asthmatic and asthmatic patients in the control group and the non-asthmatic IT-treated patients (Fig. 6). The IT-treated asthmatic patients did not show any increase of birch specific IgE in serum during the season and their level of birch specific IgE was significantly lower than that of the asthmatic patients in the control group.

Relationship between B-lymphocyte cell surface markers and eosinophils

The cell surface expression of CD40 on B cells, measured as median fluorescence, demonstrated a significant relationship to the blood eosinophil count ($r = 0.711$, $P < 0.02$), and to the serum concentration of ECP ($r = 0.728$, $P < 0.03$), respectively, during season in the control group of patients.

Discussion

The present study showed a significant rise of the number of B-lymphocytes, and increased expression of CD23, CD40 and HLA-DR on B cells, during pollen season in the control group of birch pollen allergic patients which did not receive IT. This paralleled with an increased concentration of birch

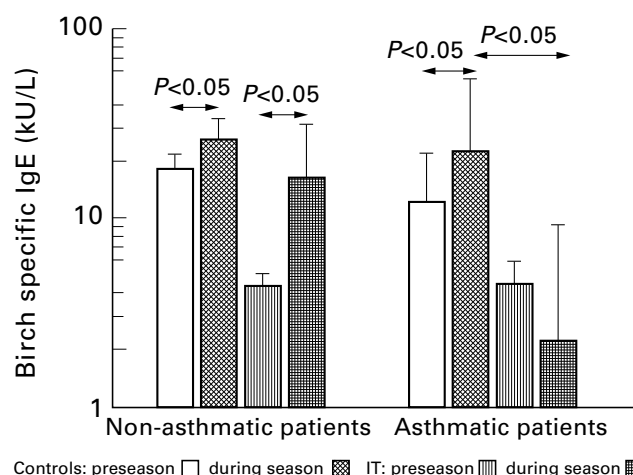


Fig. 6. Concentration of birch specific IgE in serum of the patients. Results shown are medians and upper quartiles of the respective groups. Significant differences between values before and during season and between controls and IT-treated patients are indicated.

specific IgE. The increase of B cells was demonstrated both as a relative increase of CD19⁺ cells in the lymphocyte population and in absolute concentration, in spite of the fact that the total concentration of lymphocytes decreased during season. Further analysis demonstrated that the asthmatic patients showed the most pronounced changes in case of the relative number and concentration of B cells and cell surface expression of CD40. In the IT-treated group of patients there was no change in the number of B cells, neither the relative nor the absolute concentration, and the cell surface expression of CD23, CD40 and HLA-DR on B cells was unaffected. In case of the cell surface expression of CD40 the pattern was complicated by the fact that the IT-treated group showed a seemingly higher, but statistically insignificant, expression already before the season. This pattern might be explained either by an inherent higher expression or by the influence of other immunological stimuli than allergens.

The fact that an increased number of B cells and, in addition, an increased cell surface expression of CD23, CD40 and HLA-DR on the B cells was detected in the blood from the control group of patients, indicates that although the actual allergic inflammatory response is not taking place in the circulation, signs of B-cell activation can be detected in blood. Increased expression of CD23 during allergen exposition has also been demonstrated in previous studies on children and adults allergic to grass pollen and house dust mite [12,13,21]. The most significant mechanism for the B-cell activation seen in blood in the present study is probably the activation of TH₂-cells, recently described in allergic individuals, and the subsequent production of cytokines such as IL-4, IL-5 and IL-13. IL-5 is known to enhance B-cell proliferation and IL-4 and IL-13 to induce

IgE-production and upregulation of CD23 of the cell surface of B cells [2,13,22–24]. IgE alone and in combination with IL-4 or IL-13 is also known to upregulate CD23 [2]. The upregulation of CD23 is mainly due to protection against degradation, but can under certain conditions be due to increased synthesis. Furthermore, recent investigations *in vitro* and *in vivo*, in humans and mice, demonstrate that IgE complexed with antigens amplify the production of IgE in a process which is dependent on CD23 [5,6,25,26]. This process termed facilitated antigen presentation may constitute a vicious circle involving IgE and CD23. IL-4 and IL-13 have also been shown to upregulate the MHC II expression on B cells. It has also been demonstrated that IL-4 in combination with activation of CD40 on B cell is able to induce IgE secretion [9].

It is well known that immunotherapy inhibits the production of IgE which is induced by pollen exposition in sensitised individuals [15,27]. A reduction of CD23 expressing B cells has also been demonstrated in previous investigations [12,14]. In the present investigation it is also shown that other signs of B-cell activation such as increased number of B cells and increased expression of CD40 and HLA-DR are inhibited by immunotherapy. The lack of increased B-lymphocyte cell surface expression of CD23 in the IT-treated patients during season might just be secondary to the decreased IgE production. The parallel decrease of CD40 and HLA-DR, however, corresponds better to the idea that immunotherapy induces a shift from TH₂ to TH₁ cells, which has been suggested by other investigators [16,28,29]. A shift to TH₁ cells would decrease the secretion of IL-4, IL-5 and IL-13 and result in the secretion of IFN γ , which is known to inhibit the expression of CD23 on B cells [13]. Furthermore, the prevention of an elevated cell surface expression of CD23, HLA-DR, and CD40 combined with a decrease of the IgE production would by itself break the vicious circle of IgE-mediated enhancement of antibody responses through CD23.

Immunotherapy prevented the expected increase of specific IgE in serum during season, but only in the asthmatic group of patients. The non-asthmatic group of IT-treated patients actually showed a significant increase of specific IgE during season. A similar kind of pattern was indicated in case of relative B cell number and expression of CD40. Furthermore, the investigation of eosinophil adhesion performed on the same patients [30] demonstrated that priming of eosinophil adhesion during season was prevented by immunotherapy in the asthmatic allergic patients, in contrast to the patients with allergic rhinitis. These results might appear to disagree with the general idea that patients with allergic rhinitis respond better to immunotherapy than patients with allergic asthma [31]. The symptoms of the patients is, however, influenced by several other mechanisms apart from B cell and eosinophil functions.

The present investigation also included assays of different aspects of eosinophil activation, which are described in detail in other publications [31,32]. Evaluation of possible relationships between eosinophil and B cell activation during season demonstrated relationships between the intensity of the cell surface expression of CD40 and number of blood eosinophils, and serum concentration of ECP, respectively. The relationship between serum ECP and expression of CD40 might be explained by the relationship between blood eosinophil count and CD40 expression, since the serum concentration of ECP is partly related to the number of eosinophils, but also to eosinophil activation [33]. The relationship could, however, partly be explained by parallel effects of cytokines produced by TH₂ cells.

In conclusion, the present investigation has demonstrated increased numbers of B cells and increased expression of receptors related to antigen presentation and IgE synthesis on B cells in the blood from birch pollen allergic individuals during season. Pretreatment of allergic patients with immunotherapy prevented the increase of B cells as well as the increased cell surface expression of the antigens CD23, HLA-DR and CD40, especially in the asthmatic patients.

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