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Allergen-induced changes of B-cell phenotypes in patients with allergic rhinitis*

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SUMMARY

We investigated sub-populations of B-lymphocytes in nasal mucosa and peripheral blood of 17 patients with seasonal allergic rhinitis (birch pollen) and 10 controls. The study included provocation with allergen during the non-pollen season, during which no participant used medication. Samples were also taken during the pollen season. Subsets of B-cells as expressed by different CD antigens were investigated by immunohistochemistry on frozen sections and by flow cytometry of peripheral blood. Nasal CD23$^+$ B-cells decreased in allergic patients during provocation, indicating that mature virgin CD23$^+$ B-cells switch into a memory B-cell phenotype with loss of CD23 expression. This indicates differentiation towards cells that can represent a local source for IgE synthesis. No decrease was observed during the pollen season when the patients used medication. Serum IgE was significantly higher in allergic patients on all occasions. The observed up-regulation of CD40 expression on peripheral blood B-cells in allergic patients during the pollen season clearly indicate B-cell activation. Furthermore, a relative increase of CD19$^+$ B-cells was observed in peripheral blood during provocation. Up-regulation (by IL-4) of CD40 on B-cells which then may be stimulated by gp39 (CD40 ligand) can constitute an early and important event in the IgE-mediated allergic reaction.

Key words: allergy, allergic rhinitis, B-lymphocytes, cytokines

INTRODUCTION

The pathogenesis, mechanisms and management of allergic rhinitis have been thoroughly investigated, and a vast number of studies and reviews of the subject exist (e.g., Mygind, 1986; Schwartz, 1987; Cadwick et al., 1989; Mackay, 1989; Mygind et al., 1990; Settipane, 1991). A variety of different techniques have been applied in order to scrutinize the intriguing changes in the nasal mucosa in allergic rhinitis. The major sampling techniques used have been nasal mucosa! imprints (Pipkorn and Enerback, 1984), nasal lavage (Naclerio et al., 1983; Bascom et al., 1986), nasal scrapings (Okuda and Otsuka, 1977), nasal brushings (Pipkorn et al., 1988; Bachert et al., 1990), and nasal biopsy (Winther et al., 1987; Fokkens et al., 1989; Stoop et al., 1989; Hameleers et al., 1989; Hellquist et al., 1991, 1992).

The synthesis of IgE is regarded to be intimately associated with differentiation and proliferation of B-lymphocytes, and the existence of T-cell-derived factors (e.g., interleukin-4) that promote B-cell differentiation and production of antibodies have been known since the mid 1970s (Dutton et al., 1971; Schimpl and Wecker, 1972; Kishimoto et al., 1975). This latter system indicates a necessary T/B-cel! contact (Parronchi et al., 1990), or an engagement of the B-cell-specific antigen CD40 (Jabara et al., 1991). The combined effect of CD40 stimulation and IL-4 may mimic a pathway for IgE production as seen *in vivo* in human allergic diseases (Zhang et al., 1991; Gascan et al., 1991).

In this paper we report an investigation on patients with allergic rhinitis. The principal aim was to study sub-populations of B-cells, both in peripheral blood and in the nasal mucosa. CD19 and CD20 are expressed by both unstimulated and activated B-cells, but lost during terminal B-cell differentiation into plasma cells (Darken et al., 1989). CD23 indicates an activation of mature virgin B-cells (Kikutani et al., 1986). CD40 is expressed by B-cells delivering both competence and progression signals, but CD40 (as CD23) is not expressed by terminally-differentiated B-lymphocytes. The purpose was thus to observe any differences or fluctuations of these cells in allergic patients, both during the pollen season and during provocation in the non-pollen season, and to relate the findings to possible stimuli of IgE synthesis.

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MATERIAL AND METHODS

Patients

The records of patients with birch-pollen allergy at the Allergy Clinic at Orebro Medical Center Hospital contain 203 patients. Seventeen (10 females and seven males) fulfilled the criteria with a history of seasonal allergic rhinitis and a positive (3x3 mm or more) skin prick test for birch pollen (Phazet®). The age span was 18 to 63 years, with a mean of 32.3 years. The control group consisted of 10 individuals with no allergic symptoms and a negative skin prick test panel, aged 21 to 36 years with a mean of 26.1 years. Written and informed consent were obtained. The study was approved by the Local Ethics Committee of Orebro Medical Center Hospital.

No patient received medication for his/her nasal allergy at least one month before and during the period of provocation. During the pollen season seven patients used nasal corticosteroids, of which four also used systemic anti-histamines, and another three patients with used anti-histamines only. None of patients in the study had been byposensitized during the last 10 years.

Procedure

The study was performed during winter 1990 and spring 1991, and the patients with seasonal allergic rhinitis were followed from before the pollen season and well into the birch-pollen season. Samples from patients and controls were taken from the nasal mucosa and peripheral blood at three separate occasions, and nasal biopsies from healthy controls on two occasions. All participants, controls included, were challenged with birch-pollen allergen (Aquagen, A.L.K.; 10,000 SQ-E/ml and 1,000 SQ-E/ml), which was administered in the nose by a nasal spray in a standardized way. Both patients and controls were randomly sub-divided into three groups, hence a total of six groups. One sample was taken from every participant before the provocation study started (S_1), and provocations were performed two, three, and four times, respectively (P_1 and P_2). Sampling was performed (also between 9 a.m and 3 p.m.) on two additional occasions, once during the non-pollen season after provocation (S_2), and another sampling was done in the pollen season (S_3), for controls only peripheral blood was collected (Figure 1).

Each participant was provided with a symptom score card when challenged with birch pollen. Symptoms were to be registered on a 0-4 scale, where "0" indicated no symptom at all and "4" severe symptoms. The symptoms registered were nasal blockage, secretion, itching, and sneezing. The symptoms were registered immediately before, 15 min after, 30 min after, and 24 h after the nasal challenge with birch-pollen allergen. The birch-pollen count was determined daily by a pollen trap and expressed as the number of pollen grains per cm^3 of air filtered.

The nasal biopsies were taken with a 2-mm Gerritsma forceps (Fokkens et al., 1988) from an area 1-2 cm behind the frontal edge on the medial-lower border of the inferior turbinate in the challenged nostril. This technique will render high-quality tissue specimens representative for the full thickness of the mucosa (Fokkens et al., 1988). The biopsy specimens were placed on a cork disc, embedded in Tissue-Tek® 4583 OCT compound (Miles Scientific, Elkart, USA) and immediately snap-frozen. The specimens were kept in -70°C for subsequent sectioning and immunohistochemistry. Peripheral blood was examined for IgE by a standardized enzyme-linked immunological technique (Pharmacia Diagnostics), and other tubes of the blood were prepared for flow cytometry.

Immunohistochemistly

Approximately 5-µm thick sections were cut on a cryostat and then transferred to 3-well slides (ph099 black, Cel-line Associates Inc., Newfield, USA). The sections were air-dried at room temperature for 5 min, fixed in acetone for 10 min at room temperature, air-dried again for 5 min, wrapped in aluminium foil, and finally stored at -20°C until stained at a later stage. Immunhistochemical staining was performed according to the APAAP technique (Cordell et al., 1984) without any blocking. The primary incubation was performed at room temperature for 30 min. The antibodies used are listed in Table 1. After washing the second incubation was carried out with rabbit anti-mouse immunoglobulins (Dakopatts Z259; diluted 1:50 in 1:25 diluted normal human serum [Dakopatts 260]) for 30 min. After washing the specimens were incubated with an alkaline phosphatase anti-alkaline phosphatase complex (Dakopatts D651). One repeat step was performed. The reaction was visualized with naphtha! AS-MX phosphate (Fast red; Sigma N4875) for 15 min, resulting in a bright red colour.

Table I. Antibody panel for immunohislochemistry.

epitope	antibody	species	subclass	clone	lot#	source (ref.)
CD19	Dako-CD19	mouse	IgG1,K	HD37	051	Dakopatts (14, 40)
CD20	Dako-L26	**mouse**	IgG2a, κ	L27	091	Dakopatts (33)
CD23	Dako-CD23	mouse	IgG1, κ	MHM6	118	Dakopatts (30)
CD40	CD40	mouse	IgG!, κ	B-B20	0291	Serotec (49)

Dakopatts = Dakopatts A/S, Copenhagen, Denmark; Serotec = Serotec Ltd, Unit 22, Oxford, UK

Thy	-10*		0	1	2	3	4	5	6	7	8	9	10	11	12	13
Ooup 1	S1		P1		P2	S2										
Ooup2	S1		P1		P2			P2			Si					
Ooup3	S1		P1		P2			P2			P2					S2

Figure 1. Design of provocation study(*: 10 days before first provocation; $S_{1,i}$: sampling of nasal biopsy and venous blood; P_1: provocation with 0.1 ml of birch pollen (100,000 SQ-E/ml); P_2: provocation with 0.1 ml of birch pollen [10,000 SQ-E/ml]).

Table 2 Antibody panel for flow cytometry.

epitope	antibody	species	subclass	clone	FITC lot #/PE	source (ref.)
positive						
CD45	anti-HLe-1	mouse	IgG1, K	2D1	FITC P0534	B&D
CD14	anti-Leu-M3	mouse	IgG2b, K	M0	PE	(7, 13)
negative						
	X40	mouse	IgG1, K	SP2/0-Ag 14	FITC P0130	B&D
	X39	mouse	IgG2b, K		PE	
CD3	anti-Leu-4	mouse	IgG1	SK7	FITC N0914	B&D
CD19	anti-Leu-12	mouse	IgG1	4G7	PE	(34)
CD20	anti-Leu-16	mouse	IgG1, K	L27	PE P0856	B&D
CD23	anti-CD23	mouse	IgG1, K	MHM6	FITC* 118	Dakopatts (30, 33)
CD20	anti-Leu-16	mouse	IgG1, K	L27	PE P0856	B&D
CD40	CD40	mouse	IgG1, k	B-B20	FITC B003	Serotec (33, 49)

B&D = Becton & Dickinson, USA; Dakopatts = Dakopatts, Copenhagen, Denmark; Serotec = Serotec Ltd, Unit 22, Oxford, UK
*: Secondary antibody, goat anti-mouse, B&D

Counterstaining was achieved with Mayer's haematoxylin (5-10 s). Evaluation of immunoreactivity was performed by computerized image analysis.

Computerized image analysis

A CCD camera was attached to the microscope and the system (Innovativ Vision, Linkoping, Sweden) operates with 232 grey levels. The contrast was enhanced by an interference filter of 546 nm. The grey-level threshold value between object and background was determined interactively. Both the number and the total area of the positive cells/mm^2 were calculated in each case. Previous studies have shown that measurement of 0.5 mm^2 per section, and three sections per case, gave results representative for the entire biopsy (Karlsson et al., 1994b).

Analytical flow cytometly ofperipheral blood

Fifty µl of blood was incubated in MicroTest tubes (Brand Laboratories, Germany) together with 3 µl of the primary antibody for 10 min. Mild shaking was carried out in the dark at room temperature. One ml of lysing solution (Becton & Dickinson, USA) was added followed by mixing and centrifugation at 400 x *g* for 5 min. After the pellets had been washed twice in PBS and fixed in 1 ml 1% formaldehyde in PBS the samples were transferred to Falcon 2052 test tubes (Becton and Dickinson, USA) before analysis. This procedure results in saturation of epitopes and causes minimal non-specific binding. The antibodies used are listed in Table 2.

Statistics

Flow cytometry data were analysed by Student's t-test for independent samples (allergies versus controls) and for paired samples (within groups). Normality distribution of data was confirmed by Kolmogrov-Smirnov test ($p > 0.05$). The imrnunohistological data were slightly askew and therefore Mann-Whitney U-test and Wilcoxon signed-rank tests were used for differences between groups and within groups, respectively.

RESULTS

All patients and controls were able to participate according to the schedule, and no interruptions occurred. Before provocation none of the allergic patients had a symptom score above "2" in any of the four categories of symptoms. Nasal congestion was the most pronounced subjective symptom, and most prominent in the period 15-30 min after provocation. The symptoms vanished in time and after 24 h the severity of symptoms was significantly reduced, but several patients still had values above pre-provocation status. All healthy control subjects had symptom scores of "0" or "1", and no increase was noticed after provocation (full data available on request).

The allergic patients showed significantly higher serum levels of IgE during the non-pollen season, after provocation, and in the pollen season as compared to controls ($p=0.051$, $p=0.036$, and $p=0.003$, respectively). Allergic patients also had an increased serum IgE level in the pollen season as compared to that after provocation during the non-pollen season ($p=0.008$). However, provocation alone did not induce a significant increase of serum IgE (Table 3).

The pollen count was performed between March 8 and August 29. In this particular area, the main birch-pollen season in 1991 started April 29 and finished May 30. The peak of the year occurred on May 10, and was measured to be 463 birch pollen/m^3/day. The provocation study was performed well before the birch-pollen season started.

Measurements of lymphocytes in the nasal mucosa showed a significant decrease in the amount of CD23$^+$ lymphocytes during provocation ($p < 0.001$) amongst patients with allergic rhinitis compared with before provocation. Concerning lymphocytes staining positively for CD19 and CD20 no significant changes were observed in neither group. In spite of the well-known cross-reactivity with i.a. epithelial cells of the upper respiratory tract mucosa, we tried three different antibodies for CD40$^+$ lymphocytes, but a proper evaluation was not possible (Table 4).

The flow-cytometric lymphocyte gating for the positive control

Table 3. Serum IgE in patients and controls.

	S1	S2	in season		S1	S2
group 1	-	35	30	controls	25	15
		15	30		55	55
		30	50		30	55
		120·	150		15	15
		400	450		—	—
		15	15	mean	31	29
		—	—			
	mean	102	121			
group 2	130·	100	150		1	9
	40	50	50		1	6
	20	15	15		1	7
	35'	30	80		—	—
	160	200	200	mean	1	7
		—	—			
	mean	77	79	99		
group 3		155	250		20	20
		130	190		1	15
		350	300		120	190
		200	300		—	—
		115	140	mean	47	75
		105	140			
		—	—			
	mean	176	220			

Values given in kU/1; normal range 1.6-122 (18.2-263 for persons aged 5-20 years); · = Patient under 20 years of age; S_1 = Sampling before provocation; S_2 = sampling after provocation

Table. 4 Immunohistochemistry of nasal mucosa. Mean and 25% and 75% value within parentheses.

		allergies		controls	
antibody		N/mm^2	area(%)	N/mm^2	area(%)
CD19	non-season	50 (27-73)	2.6 (0.75-4.2)	37 (7.5-62)	1.6 (0.24-3.1)
	provocation	39 (11-55)	1.6 (0.32-2.0)	47 (23-52)	11.9 (0.88-2.2)
	season	70 (40-75)	3.2 (1.5-3.5)		
CD20	non-season	57 (11-101)	2.1 (0.37-2.9)	55 (9.9-73)	1.9 (0.34-1.9)
	provocation	28 (3.1-47)	2.2 (0.11-3.2)	35 (5.7-27)	2.9 (0.18-1.3)
	season	43 (13-65)	1.7 (0.42-2.4)		
CD23	non-season	24 (15-31)	0.74 (0.44-0.90)	17 (2.3-15)	0.67 (0.07-0.76)
	provocation	12 (7.4-14)*****	0.37 (0.21-0.51)****	26 (10-38)	1.4 (0.18-0.99)
	day 3	16 (9.3-14)	0.53 (0.20-0.81)		
	day 8	8.6 (1.7-12)*	0.23 (0.04-0.42)		
	day 13	11 (7.4-13)**	0.34 (0.21-0.52)***		
	season	17 (1.3-37)	0.79 (0.04-0.96)		

* vs. non-season, Wilcoxon signed rank test, p=0.040; ** vs. non-season, Wilcoxon signed rank test, p= 0.023; *** vs. non-season, Wilcoxon signed rank test, p=0.014; **** vs. non-season, Wilcoxon signed rank test, p=0.010; ***** vs. non-season, Wilcoxon signed rank test, p= 0.003

was 97.6%, and for the negative control 99.8%. The flow cytometric analysis of the percentage of $CD19^+$ B-lymphocytes revealed a slight increase in allergic patients at a late stage during the provocation (p=0.014; Table 5). During the pollen season, on the other hand, no change was observed concerning $CD19^+$ and $CD20^+$ B-cells. The mean intensity of CD40 fluorescence as determined by channel number was 28.80±0.68 in the controls, and 32.19±0.94 in allergic patients during the pollen season (p=0.01; Table 5). Evaluation of $CD23^+$ B-cells was not possible due to haemolysis.

Table 5. Flow cytometry of peripheral blood lymphocytes. Mean (± SD) of gated lymphocytes.

		aUergics	controls
antibody		lymphocytes (%)	
CD19	non-season	9.5 (4.4)	8.9 (2.4)
	provocation	11.0 (5.1)*	6.8 (3.6)
	day 3	9.5 (3.6)	
	day 8	12.7 (5.9)	
	day 13	11.7 (6.2)**	
	season	9.2 (3.2)	8.0 (2.8)
CD20	season	9.5 (3.9)	9.8 (3.5)
CD20/40	season	8.7 (2.6)	8.3 (2.6)
		Mean fluorescence intensity (channel number)	
CD20/40	season	32.19 (3.1)***	28.80 (2.2)

*vs.non-season, t-test paired samples p=0.023; ** vs. non-season, t-test paired samples p=0.014; *** vs. controls, t-test unpaired samples p=0.010.

DISCUSSION

In order to study both local and systemic events occurring during the allergic reaction, particularly in relation to time, a well-defined group of patients with seasonal allergic rhinitis was chosen. The participants thus had positive skin prick test for birch pollen, but were negative for perennial allergens. Furthermore, the season for birch pollen is well defined with a clearly defined onset in April, and a defined main duration of approximately four weeks. The provocation was performed well before the pollen season, and the samples taken during the season coincided within the definition of the pollen season, and possible influences of other allergens have thus been minimized. The study comprised adults only, an advantage with regard to the flow cytometric analysis as adults have constant levels of lymphocyte sub-populations, contrary to the age-related sub-populations in children (Erkeller-Yuksel et al., 1992).

Earlier studies have reported that there are relatively few B-lymphocytes in the nasal mucosa as compared with the amount of T-lymphocytes (Stoop et al., 1989; Hameleers et al., 1989). However, B-cells are of interest because they constitute a possible source of lgE production, as well as being potential antigen-presenting cells (Pirron et al., 1990). To our knowledge studies of sub-populations of B-lymphocytes have not been performed in patients with allergic rhinitis, neither in the nasal mucosa nor in the peripheral blood. The progress made during the last 4-5 years in immunocytochemistry and flow cytometrical techniques, and particularly in the manufacturing of reliable monoclonal antibodies for the different CD antigens, have greatly facilitated such studies.

CD23 expression in both mouse and human appears to be restricted to the mature virgin ($sIgM^+$, $sIgD^+$) B-cell population (l(jkutani et al., 1986; Kehry and Hudak, 1989), and is irreversibly lost after isotype switching ($sIgG^+$, $sIgA^+$, $sIgE^+$), nor can it be re-induced by IL-4 stimulation of memory B-cells (Kikutani et al., 1986; Defrance and Banchereau, 1990). Due to the unchanged amount ofB-cells after provocation, the observed decrease of$CD23^+$ B-cell sub-population in the nasal mucosa can be explained by two separate or simultaneous mechanisms. The CD23 epitope could be engaged by a complex consisting of allergen and lgE, hence constituting an initial step in the antigen presenting property of B-cells and the T-cell activation (Pirron et al., 1990). Another explanation would be that mature virgin $CD23^+$ B-cells switch into a memory B-cell phenotype with loss of CD23 expression during the exposure to the allergen (provocation). Both of these two mechanisms may be valid during the allergic reaction, however, we believe the latter mechanism to be the main source for the observed decrease of $CD23^+$ B-cells. The decrease of $CD23^+$ mature virgin B-cells occurred later during provocation, and is thus in agreement with the findings that $sIgE^+$ B-lymphocytes are observed in gut-associated lymphoid tissue only 8-10 days after intraperitoneal antigen stimulation (Auci et al., 1992).

No decrease in the nasal mucosa of $CD23^+$ B-cells occurred during pollen season. The patients participating in the study used nasal corticosteroids and/or anti-histamines during the pollen season. Anti-histamines have a well-known blocking effect on histamine action on H_1 receptors, whilst there is little information available concerning the site and mode of action of corticosteroids on B-cells involved in the allergic reaction. Patients with allergic rhinitis and asthma have an increase of activated Th-cells (Karlsson and Hellquist, 1994a; Corrigan et al., 1988; Azzawi et al., 1990; Hellquist et al., 1992). Recent studies have demonstrated that approximately one week after systemic administration of glucocorticoids a decrease of activated Th-cells occurs, and a level not significantly different from that of controls is obtained (Corrigan et al., 1993). A decreased activation of T-lymphocytes can reduce the phenotype switch of 8-cells to lgE positive via a possible local decrease of IL-4, thus explaining the unchanged level of $CD23^+$ B-cells that we observed during season.

The surface CD40 antigen is a 45-50kDa transmembrane glycoprotein expressed on all resting B-lymphocytes (Gordon et al., 1991a; Dancescu et al., 1992). CD40 is a marker for early B-cell activation and is up-regulated by IL-4 (Bjorck et al., 1991). Engagement of the CD40 molecule provides signals that permit IL-4 to induce lg-isotype switching (Splawski et al., 1993). Furthermore, the recently identified ligand for CD40 is restricted in its expression to T-lymphocytes, mainly $CD4^+$ T-cells (Spriggs et al., 1992). In the present study we observed in peripheral blood an up-regulation ofCD40 expression ofB-cells in allergic patients during the pollen season as well as a slight increase of $CD19^+$ B-cells during late provocation. These findings indicate a B-cell activation. *In vivo* studies in mice have shown that JL-4 with proper co-stimuli can induce lgE secretion (Finkelman et al., 1989). Earlier observations have shown that allergens induce activation and phenotype switching of T-lymphocytes capable of IL-4 synthesis (Karlsson and Hellquist, 1994a), and increased expression ofIL-4 mRNA in nasal mucosa (Durham et al., 1992). Furthermore, the recently described T-lymphocyte restricted synthesis of gp39 (Spriggs et al., 1992)

also demonstrates a close liaison between T-cell activation, B-cells and allergen-induced IgE synthesis. Activated T-lymphocytes produce IL-4 which up-regulates CD40 on B-cells, which then may be stimulated by gp39, mechanisms constituting two important stimuli for lgE synthesis. These mechanisms in the allergic patient may constitute an early event in the IgE-mediated allergic reaction.

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