

The effect of immunotherapy on T-cell subsets in peripheral blood and bronchoalveolar lavage fluid in pollen-allergic patients

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The effect of immunotherapy (IT) on T-cell subsets in peripheral blood and bronchoalveolar lavage fluid (BAL) was examined in 15 patients with rhinoconjunctivitis and asthma caused by sensitivity to birch pollen. They were treated with IT for 3 years. Seven patients were treated with highly standardized birch-pollen extract (Pharmacia, Sweden). Eight untreated patients served as controls. Histamine challenge, blood sampling, and BAL were performed before (January, February), and at the peak of, the birch-pollen season (May). The subpopulations of T cells in peripheral blood and BAL fluid were investigated by immunocytochemistry and flow cytometry. During the birch-pollen season, the percentage of CD3+ and CD4+ cells of blood mononuclear cells in the IT patients increased significantly ($P < 0.03$ and $P < 0.02$, respectively). The percentage of CD8+ cells remained unaltered. In control patients, no changes of T-cell subsets in the peripheral blood were observed. T-cell subsets in BAL did not change during the season in relation to preseasonal values for either IT-treated or non-IT-treated patients.

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The mechanism of action of allergen immunotherapy (IT) is unknown. Induction of the specific blocking IgG antibodies produced in response to injected allergen has been suggested as the mechanism, but subsequent studies revealed poor correlation between specific IgG antibody levels and the clinical symptoms of the allergic disease in IT-treated patients (3, 17). The induction of tolerance in the IgE-producing B cells has also been suggested (3). The effect of IT on the regulatory T cells in blood has been supported by some findings (4, 21), while other studies could not find any change in the T-cell subpopulations (5).

In recent years a number of studies have produced evidence of the important role of eosinophils in the pathology of asthma (9). We have performed studies focusing on the attraction, presence, and activity of eosinophils in the model of seasonal asthma (11, 18, 19). We found that during the natural pollen season, increased eosinophil chemotactic activity and increased eosinophil numbers are present in BAL, and we postulated that secreted eosinophil

cytotoxic proteins contribute to the increase in bronchial hyperresponsiveness. Evidence has shown that IT prevents all these actions (11, 18, 19).

More recently, it has been suggested that cytokines produced by T cells upon antigen activation could cause eosinophil involvement and action as effector cells in asthmatic inflammation (14). Indeed, the study of Frew & Kay on late skin responses (8) and that of Azzawi et al. on bronchial biopsies of asthmatic patients (1) support the view linking T cells to eosinophil activity. Increasing evidence indicates that T cells and their products interact with eosinophils and their actions (14).

The present study examines the changes in T-cell subsets in peripheral blood and BAL caused by natural exposure to pollen and to IT treatment. We have used two different methods, namely a simple immunoperoxidase staining on fixed cells attached to glasses, and flow cytometry, which permits analysis of a large number of cells without prior purification procedures.

Patients and methods

Patient selection

Fifteen patients with rhinoconjunctivitis and asthma participated in the study: nine men and six women of mean age 28 (range 18–46) years. Four were occasional smokers. The diagnosis was based on history of allergic symptoms caused by birch pollen, confirmed by a positive skin test with birch extract and RAST. No patient had perennial allergy. They participated in the successful IT study for 3.5 years, showing significant decreases in symptom scores, use of medication, and specific sensitivity in skin and nose (19). Seven were treated with a well-standardized birch-pollen extract (Pharmacia, Uppsala, Sweden) and remained on the highest maintenance dose of the extract (100 000 BU/ml). The other eight were non-IT controls. Besides IT, the patients were allowed β_2 -agonists for the airways symptoms occasionally; for ocular and nasal complaints, symptomatic local medication was mainly used in season. The clinical data of the participating patients are described elsewhere (19).

Design

The study consisted of two test occasions; one during winter (pollen-free) and the other at peak birch-pollen season as defined by pollen counts. At each occasion, a histamine provocation test was performed, as described by Löwhagen & Lindholm (16), followed (after 1–2 days) by bronchoalveolar lavage (BAL). On the BAL day, 50 ml heparinized blood and 5 ml blood in EDTA-tubes for flow cytometry were drawn and sent to laboratory at room temperature.

All patients gave their consent, and the study was approved by the ethics committee of the University of Uppsala.

BAL procedure

A chest radiograph and an electrocardiogram were taken before each BAL. Premedication consisted of morphine-scopolamine and two puffs of a 0.1-mg dose of salbutamol 15 min before the examination. Bronchoscopy was performed through the mouth with a fiberoptic bronchoscope (Olympus BF1 T) under local anesthesia with 2% xylocaine (Astra, Sweden). The bronchoscope was wedged in the middle lobe bronchus. In 50-ml portions, 200 ml sterile saline at 37°C was instilled, gently aspirated into siliconized plastic tubes, and put on ice. The same investigator performed all bronchoscopies, with the same bronchoscope and at the same time of the day before and during season. The patients were observed at the clinic for 4 h after BAL had been performed.

The cells were pelleted at 400 g for 10 min at 4°C and then resuspended in 8–10 ml RPMI 1640 medium, supplemented with gentamycin 50 µg/ml and 15% fetal calf serum. After transport to the laboratory (on ice), suspensions were centrifuged and resuspended again in Earle's balanced salt solution (BSS) without phenol red, supplemented with gentamycin (50 µg/ml) and 15% fetal calf serum. Total cell counts were performed with a Bürker chamber. Viability of the cells was determined by trypan blue exclusion; it was 84–90% for all cells counted. Cytocentrifuge preparations were made with Cytospin 2 (Shandon, UK). Approximately 5×10^4 cells suspended in 100 µl BSS were cytocentrifuged at 500 g for 3 min, and the slides were stored at -70°C. Cytocentrifuge preparations were stained with May-Grünwald-Giemsa stain for differential cell count; 400 cells were counted. The results were expressed as a percentage of total cell number.

Separation of mononuclear cells

Peripheral heparinized venous blood was diluted 1:1 with PBS at pH 7.4. Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lymphocyte Separation Medium, Flow Lab., Irvine, UK), and washed three times in PBS before resuspension, at a concentration of 10^6 cells/ml, in PBS with 0.1% bovine serum albumin (BSA). Twenty microliters of the suspension was dropped into each of the eight spots of a microscope slide (Cooke glass, Novakemi, Sweden). The slides were then air-dried before storage at -70°C.

Immunoperoxidase staining

Immediately after storage, the slides from both peripheral blood preparations and BAL were fixed in cold acetone (4°C) diluted 1:1 in water for 30 s, followed by final fixation in cold 100% acetone for 5 min. After air-drying, the slides were washed in PBS for 5 min. The peroxidase-antiperoxidase (PAP) staining was performed as previously described (13) with the following mouse monoclonal antibodies, all from Becton Dickinson (Sunnyvale, CA, USA): Leu-4 (IgG1k, working dilution: 1:256; cluster determinants: CD3; main cellular reactivity: pan T cells), Leu-3a (IgG1k, 1:256; CD4; T-"helper/inducer" cells and some macrophages), Leu-2a (IgG1k, 1:32; CD8; T-"cytotoxic/suppressor" cells), and anti-HLA-DR (IgG2ak, 1:128; activated T cells, B cells, macrophages, and monocytes). Rabbit antimouse immunoglobulins and mouse PAP (Dakopatts, Glostrup, Denmark) were used as link and labeled antibodies, respectively. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in PBS for 15 min, and nonspe-

cific staining was prevented by adding normal rabbit serum 1:10 in PBS + 4% BSA for 30 min before applying the monoclonal antibodies. All incubations were performed at room temperature in a humid atmosphere. Omission of the primary antibody was used as a negative control. The cell preparations were exposed to Mayer's hematoxylin for nuclear staining and mounted in Keiser's glycerin-gelatin (Merck, Darmstadt, Germany). The percentage of positive cells was determined by counting at least 500 cells. Only cells with a distinct circular membrane staining pattern were considered to be positive.

Flow cytometry

EDTA blood was collected from the same subjects before the BAL procedure (15 patients). Ten microliters of each monoclonal antibody was added to 100 µl blood incubated for 30 min on ice. The erythrocytes were then specifically lysed by adding 2 ml NH₄CL-EDTA (Lysing Reagent, Ortho Diagnostic Systems, Inc., Raritan, NJ, USA). The leukocyte suspensions were incubated for 2–5 min at 15°C and then centrifuged at 300 g for 5 min at 4°C. The leukocyte pellets were finally resuspended in 1 ml ice-cold PBS-EDTA and kept on ice until analyzed.

Lymphocyte surface antigens were detected by FITC-conjugated monoclonal antibodies against the following: CD3 (5 µg/ml, IgG2a), CD8 (2.5 µg/ml, IgG2a), and CD4 (5 µg/ml, IgG2b) (Ortho Diagnostic Systems, Inc., Raritan, NJ, USA). FITC-conjugated mouse IgG2 (Becton Dickinson) in comparable concentrations served as a background control.

Cells were analyzed in an Ortho Spectrum III flow cytometer (Ortho Diagnostic Systems, Inc.). On the basis of light-scattering properties, each cell is represented by a point in a rectangular coordinate system in which lymphocytes, granulocytes, monocytes, and macrophages are represented by well-separated clusters. In this study, a gate around lymphocytes allowed analysis of cell-surface markers within the lymphocyte population (12). In addition to detection of the light-scattering properties of the cells, fluorescent signals from the fluorochrome could be detected. The instrument gives the absolute number, the percentage, and the actual number of fluorescent cells.

Statistical methods

FEV₁ and PC₂₀ were expressed as medians and actual ranges. Total recovery volumes, total cell numbers and percentage of differential cell count, and other laboratory data were given in means and $\pm$ SD.

For comparison of data within and between the groups, Wilcoxon's rank sum test and the Mann-Whitney U test were used, respectively.

Results

All patients included in the study completed BAL without complications. FEV₁ values and PC₂₀ methacholine before and during season are shown in Table 1. FEV₁ values remained unaltered during the season in relation to preseasonal values. Although PC₂₀ did not change significantly during the season in any group, it increased in 3/7 IT-treated and 6/8 non-IT-treated patients.

Blood

Immunoperoxidase staining of mononuclear cells in peripheral blood. When we compared the percentages of CD3, CD4, CD8, and HLA-DR positive cells before and during the pollen season, no statistically significant differences were observed between IT and non-IT patients (Table 2). In the IT group, however, 5/6 patients showed increase of CD3+ and CD4+ cells and decrease of CD8+ cells during the season. In non-IT patients, a decrease was noted in 6/8 patients for CD3+, 5/8 for CD4+, and 6/8 for CD8+ cells, while an increase in 7/8 patients was found for HLA-DR+ cells in the seasonal values over the preseasonal.

Flow cytometry analysis of peripheral blood lymphocytes. A significant increase in percentages of CD3+ ($P < 0.03$) and CD4+ ($P < 0.02$) cells was observed in IT patients during the pollen season (Table 3). No significant changes were noted in non-IT patients. All values obtained were within normal range.

BAL

Differential cell count of BAL cells. The volumes of recovered BAL fluid were similar for both groups

Table 1. Clinical data: FEV₁ and PC₂₀ histamine median and ranges before and during pollen season

	FEV ₁ l/s		PC ₂₀ mg/ml histamine	
	BS ¹	lnS ²	BS	lnS
IT n = 7	3.85 (2.52–5.48)	4.09 (2.46–5.08)	3.9 (0.69–32)	1.06 (0.03–32)
Non-IT n = 8	3.86 (2.64–4.46)	4.12 (2.8–4.72)	1.5 (0.48–5.19)	0.35 (0.026–16)

¹ BS: before season.

² lnS: in season.

Table 2. Immunoperoxidase staining of mononuclear cells in peripheral blood in percentage of total cell number

	CD3		CD4		CD8		HLA-DR	
	BS ¹	InS ²	BS	InS	BS	InS	BS	InS
IT								
Mean	59.0	66.1	50.8	55.0	23.6	21.2	39.2	33.8
±SD	11.9	2.3	3.0	6.0	5.4	5.4	5.4	12.2
	n = 6	n = 7	n = 6	n = 7	n = 6	n = 7	n = 6	n = 7
Non-IT								
Mean	64.1	58.5	51.4	52.2	26.4	21.0	37.1	43.2
±SD	6.5	10.4	4.4	6.9	9.0	8.2	9.1	7.6
	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8

¹ BS: before season.² InS: in season.

Peripheral blood mononuclear cells were isolated by density-gradient centrifugation. At least 500 cells were counted.

Table 3. Flow cytometry on peripheral blood lymphocytes in percentage of total lymphocyte population

	CD3		CD4		CD8	
	BS ¹	InS ²	BS	InS	BS	InS
IT						
Mean	67.3	72.7*	39.6	45.6**	31.3	31.6
±SD	10.8	11.0	7.7	5.8	6.8	5.8
	n = 7	n = 7	n = 7	n = 7	n = 7	n = 7
Non-IT						
Mean	73.6	74.0	40.1	40.7	30.9	33.3
±SD	5.7	6.9	3.6	5.8	6.1	11.8
	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8
Normal range %	58–63		32–62		17–40	

¹ BS: before season.² InS: in season.* Significant increase in percentage of CD3⁺ cells in blood of IT-treated patients during the pollen season over preseasonal values ($P < 0.03$).** Significant increase in CD4⁺ cells in blood of IT-treated patients during the pollen season over preseasonal values ($P < 0.02$).

before and during the pollen season (about 145 ml). The total cell numbers represent all cells in recovered volume.

In Table 5 the mean of individual total cell numbers and $\pm$ SD are given for each group and BAL. The numbers of recovered cells were similar for each treatment group before and during the pollen season. The differential cell count from lavages before and during the pollen season is shown in percentage of total number of cells. No significant differences were found between the groups.

Immunoperoxidase staining of cells in BAL fluid. There was a clear dominance of CD4⁺ and HLA-DR reactive cells in the BAL; mean values were above 80% (Table 4). Most of these cells were clearly macrophages. No significant changes from preseasonal values were detected in the percentages of CD3⁺, CD4⁺, CD8⁺, and HLA-DR⁺ in any of the patient groups during the pollen season.

Table 4. Immunoperoxidase staining of cells in BAL fluid in percentage of total cell number

	CD3		CD4		CD8		HLA-DR	
	BS ¹	InS ²	BS	InS	BS	InS	BS	InS
IT								
Mean	16.3	16.6	84.1	86.0	6.2	5.6	87.9	87.4
±SD	12.6	11.0	15.7	8.1	4.8	2.8	7.9	10.3
	n = 7	n = 7	n = 7	n = 7	n = 7	n = 7	n = 7	n = 7
Non-IT								
Mean	12.8	11.2	89.1	87.3	5.6	6.1	87.7	88.3
±SD	10.2	5.6	5.9	8.5	5.1	4.2	6.4	6.5
	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	n = 7	n = 8

¹ BS: before season.² InS: in season.

At least 500 cells were counted.

Table 5. Total cell number and differential cell count in recovered BAL fluid of IT ($n = 7$) and non-IT patients ($n = 8$), expressed as percentage of total cell number in mean and $\pm$ SD

IT	BS ¹	lnS ²
Total number cells (10^5)	7.77 (± 4.44)	8.2 (± 6.89)
Macrophages	82 (± 12.2)	86.7 (± 8.4)
Neutrophils	0.39 (± 0.31)	0.26 (± 0.49)
Eosinophils	0.73 (± 0.71)	1.14 (± 1.08)
Lymphocytes	14.65 (± 12.17)	11.08 (± 8.29)
Non-IT		
Total number cells (10^5)	6.58 (± 3.69)	6.22 (± 2.97)
Macrophages	87.05 (± 10.7)	86.95 (± 8.05)
Neutrophils	0.30 (± 0.6)	0.19 (± 0.29)
Eosinophils	0.66 (± 0.4)	0.56 (± 0.31)
Lymphocytes	11.38 (± 10.4)	12.02 (± 7.86)

¹ BS: before season; ² lnS: in season.

A total of 400 cells were counted. The small percentage of other cells not shown in the table which made up 100% were epithelial cells.

Discussion

To our knowledge, this is the first attempt to investigate T-cell subsets derived not only from peripheral blood but also from the BAL of IT-treated, mildly asthmatic patients.

There are substantial differences between the two methods used in this study. The method of immunoperoxidase staining is simple and does not require sophisticated equipment. The slides are easy to store, the staining is permanent, and the structure of the cells can be noted (13).

In the immunoperoxidase method in which the cells are acetone-fixed, the cytoplasm of the cells also stains, while flow cytometry as used here analyzes only the cell surface binding of the antibodies. Flow cytometry allows the examination of selected lymphocyte populations and can thus distinguish between CD4+ T cells and monocytes/macrophages. This methodological difference is reflected in the data obtained where CD4+ monocytes are excluded (Table 3), but not in Table 2. Moreover, the flow cytometry method has the advantage of analyzing a large number of cells; this advantage may account for the significant differences obtained in IT-treated patients for CD3 and CD4+ cells (Table 3). With the immunoperoxidase staining method, a similar tendency was observed, but it was not statistically different (Table 2).

We believe that the increases in CD3+ and CD4+ cell populations were due to IT, and not to exposure to inhaled allergen. Lack of changes in the nontreated group during the season supports this assumption. The birch-pollen season in our study was of low intensity but still evoked changes in bron-

chial hyperresponsiveness in 6/8 untreated patients. Generally, the amount of antigen in the present study caused very mild clinical symptoms, as mirrored by unaltered FEV₁ and nonsignificant changes in PC₂₀ during the season (Table 1). No changes were observed in the percentage of differential cell count of cells derived from BAL of the non-IT patients in this study, as opposed to a pollen season with a high load of pollen during which the eosinophil numbers were increased (18).

There are contrasting data reporting total lymphocyte numbers retrieved from BAL. Thus, some studies have reported an increase of lymphocyte number in BAL from asthmatic patients (15), while in most studies the numbers in mild asthmatic patients are similar to those of normal controls (2). Moreover, after inhalation antigen challenge, the lymphocyte numbers in BAL did not increase (2), while an increased number of lymphocytes, mainly CD4+ cells, was found by Fick et al. after local antigen challenge (7). Approximately 10% of cell population in lavage in our study were lymphocytes, and the percentage remained unaltered during the season (Table 5). We did not find any T-cell subset changes in BAL in our patients during seasonal antigen exposure, nor did IT affect the subpopulation numbers.

In most previous studies, antigen challenge in the laboratory setting has been used as a model of allergic disease (2, 7, 10, 20). Clearly, exposure to natural antigen during the pollen season differs from laboratory antigen challenge in that the amount of the antigen given in one dose is much higher in experimental antigen challenge. The experimental antigen inhalation occurs once, unlike the prolonged low-level antigen exposure during a pollen season. The kinetics of recruitment and activation of different T-cell subsets is unknown. The timing of measurements but also the intensity of antigenic exposure could be of critical importance for the appearance of cellular changes (18).

Atopic asthma is not associated with the base-line abnormalities of T-cell subsets (20). We found normal range of CD3, CD4, and CD8+ cells in mild asthmatic patients, as compared with nonasthmatic controls (Table 3). Gerblach et al. found that after bronchial provocation with antigen, CD4+ cells in the blood were reduced in number and stayed so for 72 h (10).

Earlier studies of the peripheral blood of IT patients (21) suggested restoration of the CD8+ population after successful IT. In agreement with Fennerty et al. (6), we did not find any significant changes in CD8+ cells in the peripheral blood of IT patients. With immunoperoxidase staining, we noted a tendency for CD3, CD4, and CD8+ cell populations to decrease in percentage in most non-IT patients during the season (Table 2).

In conclusion, specific IT of sensitized patients induces increases in the percentages of CD3+ and CD4+ blood mononuclear cells, as measured with flow cytometry. No other changes were observed in peripheral blood or BAL fluid in treated and untreated patients before or during the investigated pollen season with any of the methods used.

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