

T-cell and antibody response to *Parietaria judaica* allergenic fractions in atopic and nonatopic individuals

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The *in vitro* proliferative response to separated immunologically relevant components of *Parietaria judaica* pollen extract (PjE) was investigated by proliferation assay and limiting dilution analysis, in peripheral blood mononuclear cells from *Parietaria*-allergic subjects and nonallergic controls. In the same subjects, the profile of the antibody response to the PjE fractions was also studied by immunoblotting to evaluate the functional significance of allergen-induced T-cell activation in the two groups. The estimated frequency of PjE-reactive T cells in peripheral-blood mononuclear cells was low in both groups. No difference was found between the *Parietaria*-allergic subjects and nonallergic controls. To assess the overall contribution to the cellular response of PjE components of different molecular weights, we separated the extract by the SDS–PAGE technique, and the fractions were blotted onto nitrocellulose and solubilized. Almost all the 14 fractions tested induced T-cell proliferation, at different degrees of magnitude. Responses were similar in the allergic subjects and nonallergic controls. Immunoblotting demonstrated specific IgG antibodies to the 14 PjE fractions not only in the allergic subjects, but also in the healthy controls, whereas IgE antibodies were found, as expected, only in the sera from atopic subjects. These findings indicate that PjE fractions elicit similar T-cell activation and IgG production in allergic and normal subjects.

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Introduction

The pollen of *Parietaria* spp. is one of the most clinically relevant sources of allergens in the Mediterranean area and is responsible for almost 30% of atopic respiratory tract diseases (4).

In a previous paper (5) we investigated the lymphocyte proliferative response to an allergenic extract of *Parietaria judaica* that had been biochemically and immunochemically characterized (7). The results showed that a T-cell proliferative response to this pollen extract occurred not only in subjects with clinical evidence of hypersensitivity and specific IgE to *Parietaria*, but also in normal individuals. Agreeing with the data obtained from other allergen preparations, such as those of *Dermatophagoides* spp., ragweed, and cat (3, 6, 8, 11, 16, 18), this finding could be due to the widespread occurrence of this allergen and to its long pollination period. An anamnestic *in vitro* proliferative response to it might thus be expressed by lymphocytes from most individuals. Some qualitative or quantitative differences between

atopic and normal subjects might occur, however, in the subpopulations of proliferating T cells.

The lack of difference that we found between *Parietaria*-allergic subjects and healthy controls could also depend on the antigen preparation used in our experiments. This explanation is suggested by reports (20, 21) finding, in contrast to ours, that purified allergen preparations from ragweed or mite extracts activated T cells only in atopic individuals.

The aim of this study was to examine the differences between allergic subjects and controls from different points of view. First we evaluated the frequency of allergen-reactive T cells in *Parietaria*-allergic and healthy subjects, to exclude quantitative differences in the allergen-specific T-cell repertoire. Then we investigated T-cell reactivity in both groups, in response to isolated, immunologically relevant fractions of *P. judaica* pollen extracts.

In a complex protein mixture – such as pollen extract – attempts to identify antigenic structures recognized by specific T cells are hampered by the difficulty of obtaining large amounts of purified, char-

acterized components by conventional biochemical and immunochemical techniques. To overcome this problem, we used a method in which the whole *P. judaica* pollen extract is separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the fractions are electroblotted onto nitrocellulose (NC). The blotted antigens are then screened in T-cell proliferation assays (13, 28).

To evaluate the functional significance of allergen-induced T-cell activation in both allergic and non-allergic subjects, we also studied antibody production in the two groups, by probing the respective sera in immunoblotting. This technique also enabled us to compare T-cell activation patterns and serum IgE and IgG antibody profiles, thus addressing the question of whether proliferating T cells and antibody-producing B cells each recognize different constituents of the *P. judaica* extract.

Material and methods

Subjects

The study group comprised 13 *Parietaria*-allergic patients with a clinical history of rhinitis and bronchial asthma, and positive skin prick tests (SPT) and radioallergosorbent tests (RAST) (Pharmacia, Uppsala, Sweden) for *P. judaica*. None of the patients had received specific immunotherapy. Six nonatopic normal volunteers, with no clinical evidence of allergy and negative SPT and RAST for most relevant allergens, were studied as healthy controls. A serum sample from each subject was collected for use in the immunoblotting detection of specific IgG and IgE.

Pollen extract preparation

P. judaica pollen was kindly provided by Pharmacia (Sweden). The extract (PjE) was obtained as reported for *Parietaria officinalis* (7). The protein content, measured in the lyophilized preparation according to the method of Bradford (2), was approximately 20%. After being reconstituted in culture medium (see below) and filtered through a Millex-GV 0.22- μ m Filter Unit (Millipore S.A., Malsheim, France), the extract was added to the culture at the final optimal concentration of 5 μ g protein/ml, as determined in preliminary experiments.

SDS-PAGE and Western blotting

SDS-PAGE was performed according to the method of Laemmli (12) in a vertical slab gel by a 13% acrylamide gel under reducing conditions. A blank comb having a single reference well for standard protein markers (BIO-RAD, Richmond, CA, USA) was used to apply 2 mg protein of PjE to the gel.

Proteins were electroblotted from gel to NC paper (Schleicher and Schuell, Dassel, Germany), as described by Towbin et al. (24), with a BIO-RAD apparatus.

Blotted foils were used either to prepare the various fractions needed for the proliferation assay or to detect specific IgE and IgG in the sera of the subjects studied.

Preparation of *P. judaica* pollen extract fractions

Fractions for the proliferation assay were prepared from the blotted foil, essentially according to the method of Young & Lamb (28), as modified by Abou-Zeid et al. (1). Briefly, the left and right margins of the NC paper were cut out and stained with amido black (0.05% in isopropanol:methanol:water 1:1:8) to allow accurate localization of the different components. According to the pattern obtained, the paper was cut into 14 sections. Figure 1 reports the SDS-PAGE pattern of PjE and the scheme of the 14 fractions together with their molecular weight ranges. To solubilize the blotted sections of NC, dimethylsulfoxide (1 ml) was added to each section and left for 1 h at room temperature. The dissolved NC was reprecipitated by drop-wise addition, under continuous mixing, of an equal volume of bicarbonate/carbonate buffer, pH 9.6. The preparations were then centrifuged in a Sorvall RC-5 centrifuge (Sorvall Instruments, Du Pont, Wilmington, DE, USA) at 10 000 g, 4°C, washed four times with

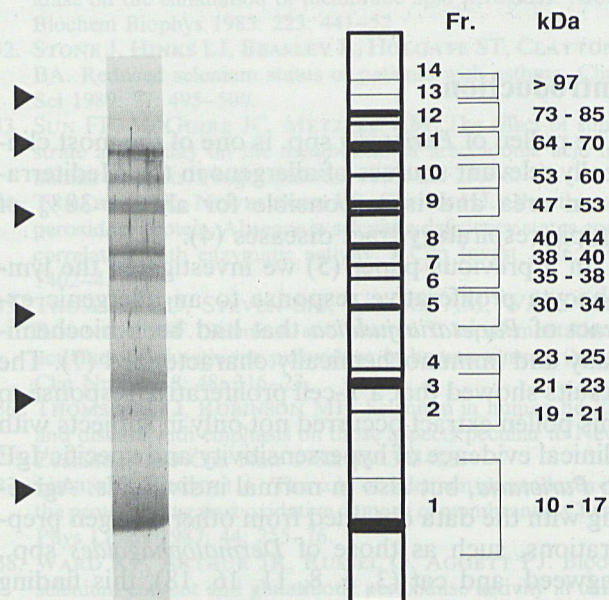


Fig. 1. SDS-PAGE pattern of PjE after electroblotting onto nitrocellulose sheet and staining with amido black (left), and scheme of the 14 fractions with the respective molecular weight ranges (right). Black arrows indicate the molecular mass markers (from top to bottom: 97.4, 66.2, 42.7, 31, 21.5, 14.4 kDa).

sterile RPMI-1640 medium supplemented with antibiotics (see below), and resuspended in a final volume of 5 ml with the same medium. Each sample was divided into aliquots and kept at -20°C until use; these preparations were used throughout the experiments. The optimal amount of NC fractions to be used in proliferation assays was determined in preliminary experiments and found to equal 20 μl /well of the preparation. Equal areas of NC spotted with 250 μg proteins of PjE (positive control) and NC alone (negative control), treated as above, were used as controls.

Immunoblotting

The presence of specific IgG and IgE antibodies in the sera of the subjects studied was investigated by SDS-PAGE and immunoblotting. Blotted strips were incubated overnight with 1.5 ml of individual sera diluted 1:10 for IgG and 1:3 for IgE in washing buffer (50 mmol/l sodium phosphate, 0.05% (v/v) Tween 20, pH 7.2) at room temperature.

For IgG antibody detection, the strips were then incubated for 2 h at room temperature with a peroxidase-labeled, affinity-purified goat antibody to human IgG (KPL, Gaithersburg, MD, USA), diluted 1:500 in washing buffer. The colorimetric reaction was developed with 4-chloro-1-naphthol according to the BIO-RAD instructions.

IgE antibodies bound to NC strips were detected by overnight incubation with ^{125}I -labeled, rabbit anti-human IgE (RAST I, Pharmacia, Uppsala, Sweden; 300 000 counts per minute (cpm)/strip); the reactive bands were developed by exposing the strips to X-ray film at -80°C for 3 days.

Preparation of lymphocytes

A 20-ml blood sample from each subject was collected into heparinized vacuum tubes (Vacutainer, Becton & Dickinson, Meyland, France) and separated by the standard technique with Lymphoprep (Nycomed, Oslo, Norway). Peripheral-blood mononuclear cells (PBMCs) were harvested, washed twice in HBSS (Whittaker, Walkersville, MD, USA), and resuspended in RPMI-1640 (Whittaker), supplemented with 10% pooled, heat-inactivated human AB serum, 2 mmol/l L-glutamine, 5000 u penicillin, 5000 μg streptomycin/100 ml (Flow Laboratories, Rockville, MD, USA) (complete medium).

Proliferation assay

Microcultures were performed in triplicate in 96-well, flat-bottomed culture plates (Costar, Cambridge, MA, USA). PBMCs were cultured in complete medium at a concentration of 500 000 cells/ml,

in a volume of 200 μl /well, with soluble PjE or with the 14 NC fractions. Cultures were incubated for 8 days at 37°C in a humidified atmosphere of 5% CO_2 . DNA synthesis was measured by adding 18.5 kBq ^3H -thymidine per well (specific activity 74 GBq/mmol, Amersham, UK) 16–18 h before harvesting. Cultures were harvested with a semiautomatic, multiple harvester (Skatron, Lierbyen, Norway) onto fiberglass filter paper, and the thymidine incorporation was assessed by liquid scintillation counting in a Beckman LS5000TD counter (Beckman, Fullerton, CA, USA).

Limiting dilution assay

Different numbers of PBMCs (ranging from 3000 to 100 000 cells/well) were cultured with the optimal antigen concentration (soluble PjE, 5 $\mu\text{g}/\text{ml}$) and a constant number of autologous irradiated (6000 rad) PBMCs as feeder cells (10 000 cells/well). For each concentration of PBMCs tested, 24 replicate wells were established with antigen and 12 wells without antigen. Antigen-stimulated cultures in which the ^3H -thymidine incorporation exceeded the mean counts per minute (cpm) plus 3 standard deviations (SD) of the 12 corresponding antigen-free cultures were scored as positive.

The distribution of PjE-reactive T cells was analyzed by plotting the negative logarithm of the fraction of the nonresponding cultures on the y-axis and the cell input on the x-axis. If the PjE-reactive T cells

Table 1. T-cell proliferative response to soluble *Parietaria judaica* extract and nitrocellulose-bound *P. judaica* extract constituents in an allergic and a healthy subject

	B.S. (allergic subject)		F.L. (healthy control)	
	mean cpm $\pm$ SE	% sum	mean cpm $\pm$ SE	% sum
Medium	146 $\pm$ 19	—	371 $\pm$ 71	—
Soluble PjE [#]	14 275 $\pm$ 716	—	8098 $\pm$ 1516	—
NC [@]	901 $\pm$ 146	—	1406 $\pm$ 480	—
Dot-PjE [@]	10 276 $\pm$ 1050	—	4712 $\pm$ 764	—
Fr. 1 [@]	2985 $\pm$ 271	3.9	2791 $\pm$ 943	4.6
Fr. 2	995 $\pm$ 22	1.3	3180 $\pm$ 480	5.2
Fr. 3	1347 $\pm$ 210	1.8	3661 $\pm$ 128	6.0
Fr. 4	1082 $\pm$ 112	1.4	4379 $\pm$ 872	7.1
Fr. 5	1046 $\pm$ 52	1.4	5589 $\pm$ 185	9.1
Fr. 6	4673 $\pm$ 1902	6.1	4564 $\pm$ 187	7.4
Fr. 7	1077 $\pm$ 100	1.4	7149 $\pm$ 1397	11.7
Fr. 8	4867 $\pm$ 1718	6.4	3668 $\pm$ 394	6.0
Fr. 9	3652 $\pm$ 1581	4.8	5290 $\pm$ 732	8.6
Fr. 10	18 325 $\pm$ 540	23.9	3560 $\pm$ 34	5.8
Fr. 11	2558 $\pm$ 148	3.3	3277 $\pm$ 136	5.3
Fr. 12	3198 $\pm$ 1460	4.2	3517 $\pm$ 109	5.7
Fr. 13	30 227 $\pm$ 8416	39.5	5809 $\pm$ 1484	9.5
Fr. 14	3454 $\pm$ 1004	4.5	4752 $\pm$ 974	7.8

[#] Soluble PjE was used at the final concentration of 5 $\mu\text{g}/\text{ml}$ in culture.

[@] NC alone, dot-PjE, and fractions from 1 to 14 suspensions were used at the final dilution of 1/100 in culture.

are assumed to have a random, Poisson distribution, this semilog plot is used to estimate the frequency of precursor cells for straight lines passing through the origin ("single-hit" kinetics). In this case, the frequency of PjE-reactive T cells is the inverse of the number of PBMCs giving rise to 37% negative wells. However, in limiting dilution cultures exhibiting non-"single-hit" kinetics, as in our experiments, the frequency of PjE-reactive T cells can be roughly calculated as described by Lefkovitz & Waldmann (14). Briefly, $F_0 = 0.37$ was plotted from line 0.1 of the logarithmic scale on the y-axis and ΔN cells read directly on the titration curve (frequency = $1/\Delta N$).

Data presentation

The results were expressed as follows:

- Mean counts per minute (cpm) $\pm$ standard error (SE) of the mean for triplicate cultures (Table 1).
- % sum of the 14 fractions (Table 1) for each subject:

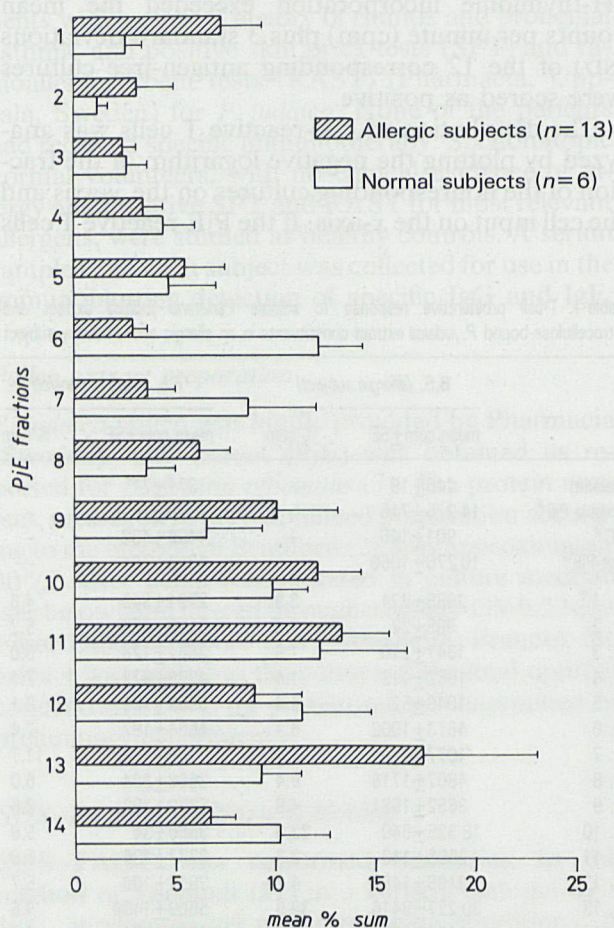


Fig. 2. Contribution of each PjE-fraction in inducing the overall T-cell proliferative response, calculated as % sum (see Table 1). The mean % sums for the 13 *Parietaria*-allergic subjects (▨) and the 6 nonallergic controls (□) are reported.

$$\% \text{ sum} = \frac{\text{cpm value of each fraction}}{\text{sum of cpm values of 14 fractions}}$$

Figure 2 gives the mean % sum for the 13 allergic and the 6 normal subjects.

- Frequency histogram: for each fraction, the percentage of subjects exhibiting a proliferative response exceeding 2 SD of the corresponding NC-induced response (Fig. 3), or the percentage of sera reactive in IgG blotting (Fig. 4), or the percentage of sera reactive in IgE blotting (Fig. 5).

Statistical analysis

The nonparametric Wilcoxon rank sum test for comparing two groups was used.

Results

Frequency of PjE-reactive T cells

The results of the limiting dilution assay used to estimate the frequency of PjE-reactive T cells in PBMCs from four *Parietaria*-allergic patients and four healthy controls showed no differences in the

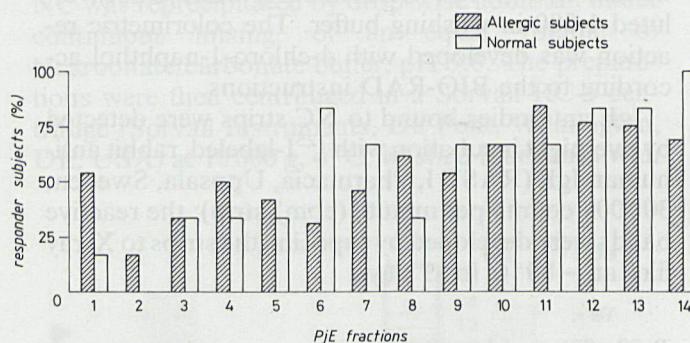


Fig. 3. Frequency histogram, showing for each fraction the percentage of *Parietaria*-allergic subjects (▨) and nonallergic controls (□) exhibiting a T-cell proliferative response exceeding 2 SD of the corresponding NC-induced response.

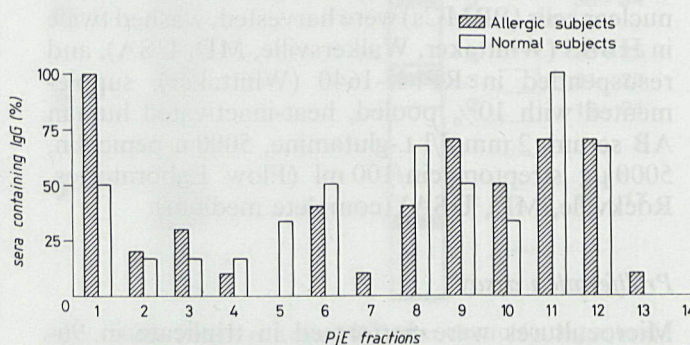


Fig. 4. Frequency histogram, showing for each fraction the percentage of sera from *Parietaria*-allergic subjects (▨) and nonallergic controls (□) containing specific IgG.

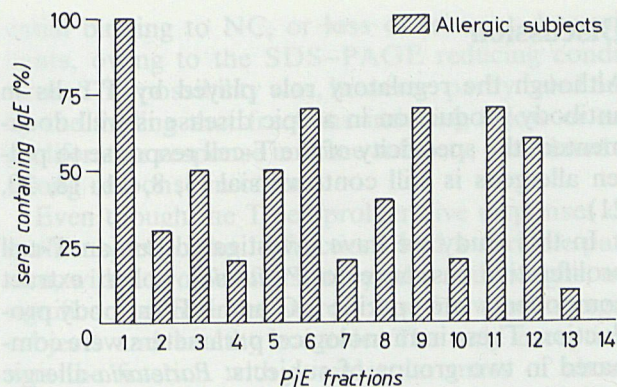


Fig. 5. Frequency histogram, showing for each fraction the percentage of sera from *Parietaria*-allergic subjects containing specific IgE.

frequencies of PjE-reactive T cells between *Parietaria*-allergic and nonallergic subjects (Wilcoxon rank sum test $P > 0.5$). Figure 6 reports the results of a typical assay of PBMCs from an allergic

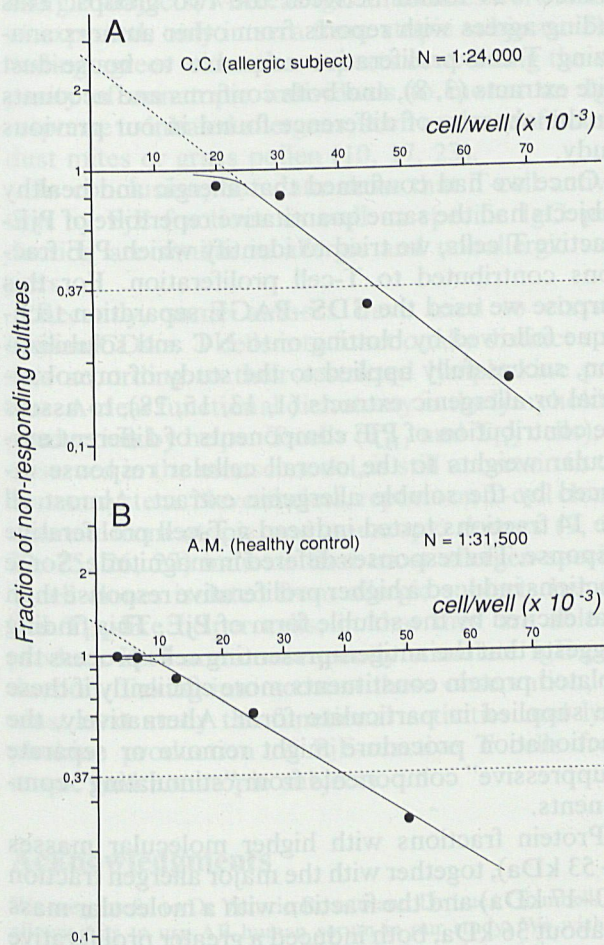


Fig. 6. Limiting dilution assay. Results are shown from two representative experiments performed with PBMCs from one *Parietaria*-allergic subject (A) and one nonallergic control (B).

subject (A) and a control (B), plotted on a semilogarithmic graph. On the linear part of the curve, the frequency of PjE-reactive T cells is roughly estimated as the inverse of the number of T cells at which 37% of the cultures are nonresponding (in this experiment, 1:24 000 for the allergic subject and 1:31 500 for the control).

Table 2 shows the calculated frequencies of PjE-reactive T cells for the eight subjects tested. The regression analysis of the linear portion of the curves gave correlation coefficients ranging from -0.918 to -0.996 .

Reactivity pattern of T-cell proliferative response to SDS-PAGE-fractionated NC-bound PjE

PjE bound to NC after fractionation on SDS-PAGE induced proliferation in T cells from both *Parietaria*-allergic subjects and nonallergic controls. Table 1 shows a representative experiment with lymphocytes from a *Parietaria*-allergic subject and a healthy control. The proliferative responses are expressed as $\text{cpm} \pm \text{SE}$ of triplicate cultures, elicited by the soluble PjE, by a dot preparation of the whole PjE and by the 14 NC-bound fractions. The responses obtained with medium alone and with NC alone were taken as negative controls. The dot preparation and the soluble extract induced similar proliferative responses; NC alone was not mitogenic. Almost all the 14 fractions induced, at different magnitudes, T-cell proliferation. Some fractions induced a higher response than that elicited by the dot preparation, or the soluble form of PjE, or both (see Table 1; fractions 10 and 13 in the allergic subject, and fractions 5, 7, 9 and 13 in the healthy control). To evaluate the contribution of each fraction in inducing the overall response, we also calculated the % sum corresponding to each fraction (Table 1).

Table 2. Frequencies of PjE-reactive T cells in *Parietaria*-allergic patients and healthy controls

Subjects	T-cell frequencies
<i>Parietaria</i> -allergics	
1	1:24 000
2	1:16 000
3	1:25 000
4	1:39 500
Geometric mean	1:24 815
Healthy controls	
1	1:31 500
2	1:9 500
3	1:36 500
4	1:25 000
Geometric mean	1:22 859

Figure 2 reports the mean % sum for the 13 allergic subjects and the 6 nonallergic individuals. An intragroup statistical comparison of the contribution of each fraction to the total response showed that the higher molecular mass fractions (> 53 kDa) elicited greater proliferative responses in both groups (P values ranging from 0.01 to 0.0001). Fraction 1 (molecular mass 10–17 kDa, corresponding to the major allergen) in the allergic group, and fraction 6 (molecular mass about 36 kDa) in the control group induced a proliferative response comparable ($P > 0.05$ in both cases) with that obtained with the higher molecular mass fractions. In the comparison between the two groups of subjects, however, only fraction 6 gave % sum values that were significantly higher in the normal group ($P < 0.01$).

Allergen-induced lymphocyte activation was also considered in terms of the percentage of allergic or normal subjects exhibiting a positive proliferative response to each fraction (i.e. a proliferative response exceeding 2 SD of the corresponding NC-induced response). Results are reported in Figure 3. Fractions with higher molecular masses could induce activation in a higher percentage of subjects, both in the allergic patients and in the healthy controls. Fractions 1 and 6 also proved highly reactive for the T cells of allergic and normal subjects; 54% and 83%, respectively.

Reactivity pattern of specific IgG and IgE response to PjE immunoblots

To investigate B-cell recognition of PjE components, we examined sera from 10 of the 13 *Parietaria*-allergic patients and from 6 healthy controls for IgG and IgE binding to PjE blotted onto NC. Figure 4 shows the frequency histogram of sera from patients and controls containing specific IgG antibodies to each of the 14 SDS-PAGE-separated components of PjE. Sera from normal subjects contained IgG antibodies specific for almost all the 14 fractions, excluding 7, 13, and 14, which were not recognized by IgG from any of the normal subjects. Fraction 14 and fraction 5 were not recognized even by IgG from allergic subjects. Fraction 1, the major allergen, bound IgG from all the patients' sera and from 50% of the control sera.

Figure 5 gives the frequency histogram of specific IgE binding from the allergic sera. As expected, fraction 1 was the PjE component that showed the highest frequency of IgE binding, being recognized by 100% of the tested sera. With the exception of fraction 14, all fractions bound IgE; the frequencies ranged from 10% to 70%. As expected, sera from healthy controls showed no IgE binding (data not shown).

Discussion

Although the regulatory role played by T cells in antibody production in atopic disease is well documented, the specificity of the T-cell response to pollen allergens is still controversial (3, 8, 11, 18, 20, 21).

In this study we have investigated human T-cell proliferative response to *P. judaica* pollen extract components and specific IgG and IgE antibody production. These immunological parameters were compared in two groups of subjects: *Parietaria*-allergic individuals and healthy subjects.

In a previous study (5) we found that PBMCs from both *Parietaria*-allergic subjects and nonallergic controls proliferated in response to soluble PjE. Our aim here was therefore to estimate the frequency of PjE-reactive T cells, by means of limiting dilution technique, to find out whether quantitative differences existed between allergen-reactive T cells from allergic and nonallergic subjects. The estimated frequency of PjE-reactive T cells was low, and no difference was found between the two groups. This finding agrees with reports from other authors analyzing T-cell proliferative response to house-dust mite extracts (3, 8), and both confirms and accounts for the absence of difference found in our previous study.

Once we had confirmed that allergic and healthy subjects had the same quantitative repertoire of PjE-reactive T cells, we tried to identify which PjE fractions contributed to T-cell proliferation. For this purpose we used the SDS-PAGE separation technique followed by blotting onto NC and solubilization, successfully applied to the study of mycobacterial or allergenic extracts (1, 13, 15, 28), to assess the contribution of PjE components of different molecular weights to the overall cellular response induced by the soluble allergenic extract. Almost all the 14 fractions tested induced a T-cell proliferative response. The responses differed in magnitude. Some fractions induced a higher proliferative response than that elicited by the soluble form of PjE. This finding suggests that the antigen-presenting cells process the isolated protein constituents more efficiently if these are supplied in particulate form. Alternatively, the fractionation procedure might remove or separate "suppressive" components from "stimulating" components.

Protein fractions with higher molecular masses (> 53 kDa), together with the major allergen fraction (10–17 kDa) and the fraction with a molecular mass of about 36 kDa, both induced a greater proliferative response and activated PBMCs from a larger number of subjects. Our T-cell proliferation assay might, in theory, have been affected by unequal transfer of the different proteins during electroblotting, differ-

ential binding to NC, or loss of relevant determinants, owing to the SDS-PAGE reducing conditions. This possibility was, however, partly ruled out by the finding that fractions inducing a low or no proliferative response in some subjects, stimulated a strong response in others.

Even though the T-cell proliferative responses of each subject to the 14 fractions had a variable pattern, with a profile of distinct peaks and troughs, no significant differences were found between allergic subjects and healthy controls. The only exception was the fraction with a molecular mass of about 36 kDa, which gave significantly higher proliferation values in the nonallergic group.

Because T and B cells may recognize different components of PjE, we studied the corresponding IgG and IgE antibody specificity detected by immunoblotting in the sera of the same subjects. Because of the smallness of the study group, the result may not be conclusive. Yet, we did identify specific IgG antibodies to the 14 PjE fractions in the sera of the 6 normal subjects, as well as in 10 of the 13 *Parietaria*-allergic subjects, whereas IgE antibodies were found, as expected, only in sera from atopic subjects. This finding agrees with other reports concerning the capacity of nonatopic individuals to mount an IgG response to inhaled allergens, such as those of house-dust mites or grass pollen (10, 17, 23).

In conclusion, our data show that T-cell activation by PjE fractions as well as specific IgG production are similar in allergic and nonallergic subjects.

Recently, some authors have tried to classify human CD4⁺ T cells into functionally distinct subsets according to their restricted lymphokine profiles. A clear functional dichotomy in the lymphokine production by helper T cells (T_{H1} and T_{H2} cells), as observed in the mouse model, is still uncertain in the human system. Nevertheless, reports on T-cell clones isolated from atopic and nonatopic donors (9, 19, 22, 25, 26, 27) indicate that most allergen-specific T-cell clones isolated from allergic subjects behave like T_{H2}-like helper cells, in that they are able to produce IL-4 and consequently stimulate IgE production. Taking into account these observations, we must next study the functional activities and lymphokine production of PjE-reactive T cells from atopic and nonatopic subjects.

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