

T cell responses to a *Parietaria judaica* pollen extract: comparison between *Parietaria*-sensitive patients, other atopics and healthy controls

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We investigated the *in vitro* proliferation of peripheral blood mononuclear cells (PBMC) from *Parietaria*-allergic subjects, atopic patients with other sensitizations and healthy controls to *Parietaria judaica* pollen extracts. PBMC from almost all 44 subjects, divided into five groups (*Parietaria*-, grass-, *Parietaria* and grass-, *Dermatophagoides*-sensitive patients and normal individuals) were able to proliferate in response to the extract without statistically significant differences between groups. Mean values of the stimulation indexes for the five groups were respectively: 10.73, 3.18, 5.50, 10.56, 9.28. The results of separation experiments showed that the responding cells were T lymphocytes. Mitogenic effect of the *Parietaria* pollen extract was excluded by the absence of proliferative PBMC response from cord blood of seven newborns. These results indicate that *Parietaria*-sensitive, other atopics and normal individuals have *Parietaria*-specific T cells able to proliferate *in vitro* to *Parietaria* allergens.

Key words: allergens; *Parietaria judaica*; proliferation; T lymphocytes.

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Our laboratory has previously reported biochemical and immunochemical studies on *Parietaria* sp. pollen allergens (10). *Parietaria* sp. is one of the most clinically relevant allergens in the Mediterranean area and is responsible for almost 30% of atopic respiratory tract diseases (8, 27).

In this paper we investigate the lymphocyte proliferative response to *Parietaria judaica* allergenic extracts previously biochemically and immunochemically characterized.

Cell response to allergenic extracts is controversial. Many authors have described lymphocyte proliferative responses to several environmental allergens from pollens (1, 6, 17, 18, 22, 25, 26, 28, 29), mites (13, 21, 24) and animal dander (23). The aim of most of these studies was to introduce a new tool in the

diagnosis of allergic disorders or a new method for assessing the efficacy of specific immunotherapy. Conflicting results were obtained regarding the specificity of the allergen-induced activation, using both crude extracts and allergens at various grades of purification, such as Amb e I of *Ambrosia*, Der p I of *Dermatophagoides pteronyssinus* and Lol p I of *Lolium* (16). Some authors (18, 22, 24, 26) could demonstrate greater responsiveness from atopic subjects than from normal controls, while others (1, 6, 12, 23) found no differences in lymphocyte proliferation between allergic and normal individuals. This discrepancy was still evident in experiments performed on separated T cells (12, 19, 24). To rule out any possible mitogenic effect of the allergenic preparation, experiments per-

formed with cordblood lymphocytes which have not previously encountered allergens, gave opposite results with different allergens (6, 12).

Several studies have been performed using ragweed, grass, birch and plane pollens. We found it of interest to evaluate the lymphocyte proliferation induced by a pollen extract from a species not previously studied e.g. *Parietaria* sp. This species differs from those previously studied due to its long pollination period.

MATERIAL AND METHODS

Subjects

Forty-four subjects participated in this study: they were divided into five groups on the basis of their clinical history and positivity in skin prick test (SPT) and RAST (Pharmacia, Uppsala, Sweden) to different allergens. Subjects were grouped as follows: 1) 14 subjects with hypersensitivity to *Parietaria* (PAR), seven of whom had received specific immunotherapy (SIT) for at least 1 year; 2) six subjects positive to grass pollen extracts (GRA), none of them treated; 3) four subjects positive to both previous pollens (PAR + GRA), none of them treated; 4) nine *Dermatophagoides pteronyssinus* - hypersensitive subjects (DPT), three of whom had received specific immunotherapy; 5) 11 non-atopic normal volunteers (NOR), with no clinical evidence of allergy and negative SPT and RAST. Lymphocytes from cord blood of seven newborns were used as negative controls. Subjects are characterized in Table 1.

Experiments were performed during the height of the *Parietaria* sp. pollination period (March-July in Rome) (5).

Antigens

Parietaria judaica pollen was kindly supplied by Pharmacia (Uppsala, Sweden). The extract (PjE) was obtained as previously reported for *Parietaria officinalis* (10). The protein content, determined by using the Bradford assay (3) on the lyophilised preparation, was approx. 20 %. Antigen, freshly reconstituted in culture medium and filtered through a Millex-GV 0.22 µm Filter Unit (Millipore S.A., Malsheim, France), was added in culture at the final concentrations of 0.5, 5, 50 µg protein/ml.

Phytohemagglutinin (PHA, Burroughs Wellcome Ltd., Research Triangle Park, NC), concanavalin A (ConA, Flow Laboratories, Rockville, MD) and pokeweed mitogen (PWM, Gibco, Paisley, UK) were used as proliferative response control in each experiment at previously found optimal concentrations (0.2 µg/ml, 5 µg/ml and 1 µg/ml in culture, respectively).

Cell separation and culture conditions

A 20 ml blood sample from each subject was collected into heparinized vacuum tubes (Vacutainer, Becton & Dickinson, Meylan, France), and separated by the standard technique using Lymphoprep (Nyegaard & Co., Oslo, Norway). Mononuclear cells were harvested and

TABLE 1
Features of all PBMC donors

Groups	n	Age (range)	PAR ^a	GRA ^a	DPT ^a	SIT (PAR) ^b
PAR	14	(21-42)	+	-	-	7
GRA	6	(22-53)	-	+	-	/
PAR + GRA	4	(21-56)	+	+	-	/
DPT	9	(13-48)	-	-	+	/
NOR	11	(27-52)	-	-	-	/
Newborns	7	/	/	/	/	/

^aAllergen hypersensitivity was assessed by clinical history and positivity in SPT (wheal > 5 mm) and RAST (Pharmacia class 3 or more).

^bSeven *Parietaria*-sensitive patients underwent SIT with alum-adsorbed *Parietaria* pollen extract for at least 1 year.

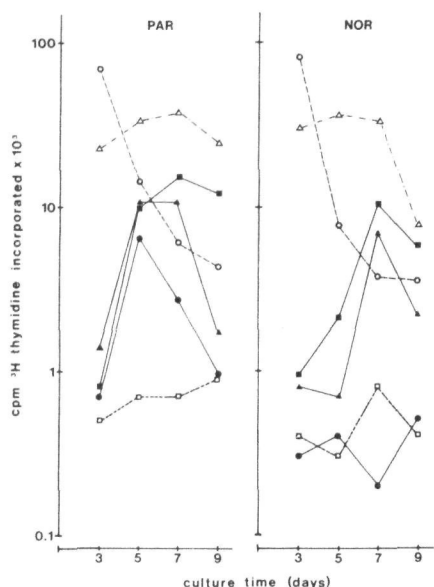


Fig. 1. Time-course of proliferative response to PjE. Results are shown from two subjects, one *Parietaria*-sensitive (PAR) and one healthy control (NOR), cultured with 0.5 (●—●), 5 (▲—▲), 50 (■—■) µg/ml protein final in culture of PjE, PHA (○—○), PWM (△—△), and with medium alone (□—□). Values are expressed as GM of triplicates.

washed twice in RPMI 1640 Dutch Modification (Flow Laboratories, Rockville, MD), and then resuspended in RPMI supplemented with 10 % pooled heat-inactivated human AB serum, 2 mM L-glutamine, 5,000 U penicillin and 5,000 µg streptomycin/100 ml (Flow Laboratories, Rockville, MD). Cells were cultured at a concentration of 500,000 cells/ml, in a volume of 200 µl/well, in 96-well flat-bottomed culture plates (Nunc, Roskilde, Denmark). Triplicate cultures were incubated at 37°C in a humidified atmosphere containing 5 % CO₂ and harvested at days 3, 5, 7, 9. DNA synthesis was measured by adding 0.5 µCi ³H-thymidine per well (specific activity 2 Ci/mM, Amersham, UK) 6 h before harvesting. The cultures were harvested onto glass-fiber filter paper (Skatron, Lier, Norway), and ³H incorporation was measured by liquid scintillation counting (Filter-Count, Packard Instruments, Groningen, The Netherlands). T cells were enriched by rosetting with 2-aminoethylisothiuronium bromide (AET) (Aldrich Chemical Co., Milwaukee, WIS) treated sheep red blood cells (SRBC) and by centrifugation over Lymphoprep (15). After SRBC lysis, the cells in the pellet were referred

to as T cells. The purity of the T cells, assessed by rosetting on the separated T cell population, was > 95 %. The nonrosetted cells were γ-irradiated at 2,000 rads and thereafter referred to as non-T cells. T cells alone or together with irradiated non-T cells in 80/20 and 90/10 ratio, were cultured under the same conditions as PBMC.

Presentation and statistical analysis of data

Proliferation assay results were expressed as the geometric mean (GM) of cpm obtained in triplicate cultures. Since the subjects showed variable levels of proliferative background, stimulation index (SI) was used in order to allow comparison of results from different experiments:

$$SI = \frac{\text{GM cpm in cultures stimulated with antigen}}{\text{GM cpm in unstimulated cultures}}$$

Statistical evaluation was made using Wilcoxon's nonparametric rank sum test to compare two groups.

RESULTS

Proliferative response of PBMC

A proliferative response induced by PjE was detected in PBMC from the majority of subjects in the five groups studied, with variable SI values. Fig. 1 shows a representative time-course PBMC response from a PAR-allergic subject and a normal individual to PHA and to PjE at the three doses, compared with unstimulated culture controls. PHA response showed maximum response at day 3 of culture, reaching 68,650 and 82,700 cpm (GM) respectively in these subjects, whilst PWM induced a broader response with a maximum between days 5 and 7. PjE-induced proliferative response showed a slower increase in culture time than mitogenic response, starting from day 3 and reaching a peak on day 7 with cpm (GM) values of 14,650 (PAR) and 10,290 (NOR), while unstimulated cultures did not reach values higher than 1,000 cpm (GM) until day 9.

The maximum responses of all 44 subjects' PBMC expressed as SI are shown in Fig. 2. Mean SI values of the five categories ranged from 3.18 (GRA) to 10.73 (PAR); mean SI of PAR-allergic subjects was close to that of the DPT group (10.56); mean SI of the NOR group was 9.28. Lowest values were obtained in GRA (3.18) and PAR + GRA (5.50) groups. No statistically significant differences ($P > 0.05$) were seen when the PAR group was compared with each of the other groups, and when SIT treated and untreated subjects in the PAR group were compared (10.46 vs. 10.95, mean SI values from 7 treated and 7 untreated patients).

To assess the potential mitogenic effect of PjE, proliferative response experiments were conducted on PBMC from cord blood samples of seven newborns. Assuming that newborns have not previously encountered the antigen, cord blood was used because of the lack of memory T cells recognizing the antigen in newborns' PBMC. Results (Table 2) show that no proliferative response was obtained in culture stimulated with PjE at the same concentrations used in adult cultures, while PHA- and PWM-stimulated cultures gave positive SI values

ranging from 4.06 to 47.92 (PHA) and from 3.90 to 28.22 (PWM).

Dose response analysis

Dose response analysis of PjE-induced proliferation is shown in Fig. 3. The three doses were chosen on the basis of preliminary experiments in which concentrations ranging from 0.05 to 500 $\mu\text{g/ml}$ were tested; while the 0.05 $\mu\text{g/ml}$ concentration was ineffective for eliciting proliferative response, a toxic effect on cellular viability was detected in culture stimulated with 500 $\mu\text{g/ml}$ of antigen (data not shown). Fig. 3 shows that only seven of 44 subjects had maximum SI with the lower antigen concentration (0.5 $\mu\text{g/ml}$), giving at the same time the lowest mean SI value. The 5 $\mu\text{g/ml}$ concentration elicited the peak response in 21 subjects with an overall mean SI value of 7.67, while the 50 $\mu\text{g/ml}$ dilution caused maximum response in 16 subjects with the highest mean SI value of 12.54. No statistically significant differences were revealed among the mean SI values obtained with 0.5 and 5 $\mu\text{g/ml}$ or with 5 and 50 $\mu\text{g/ml}$ doses ($P > 0.05$), while the mean SI values obtained with 0.5 and 50 $\mu\text{g/ml}$ were significantly different ($P < 0.01$).

Almost half of the subjects had maximum SI on day 7 (20/44 subjects) with a mean value of 7.79. Highest SI mean value was obtained on day 9 (11.29) in 11 of 44 subjects.

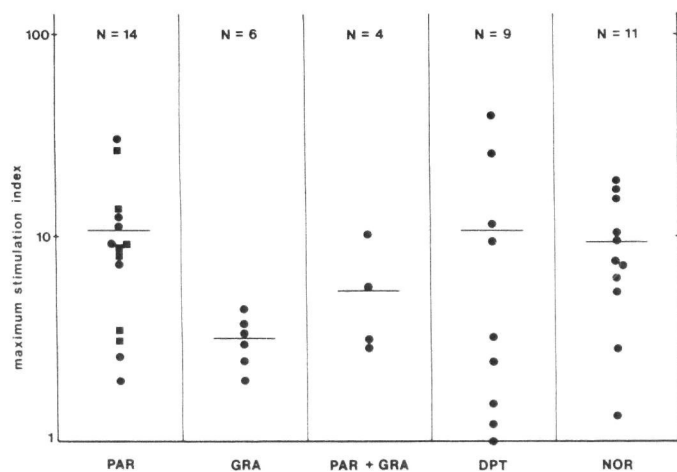


Fig. 2. Maximum SI of the 44 subjects studied, divided in five groups: *Parietaria*-sensitive (PAR), Grass-sensitive (GRA), *Parietaria*- and Grass-sensitive (PAR + GRA), *Dermatophagoides pteronyssinus*-sensitive (DPT) and healthy controls (NOR). Maximum SI values were obtained on different days of culture and with different doses of PjE. Lines (—) are mean values of each group. ■ = *Parietaria*-sensitive subjects receiving SIT. Intergroup comparison by non-parametric Wilcoxon test did not reveal statistically significant differences.

Proliferative response by separated T cells

T cell separation by SRBC-AET-rosetting was performed on PBMC from seven subjects (4 PAR and 3 NOR). Two representative experiments are shown in Table 3. All experiments showed that T cells alone were unable to proliferate in response to antigen during culture (mean $\pm$ SD maximum SI for the 7 subjects: 1.68 ± 0.24 ; range: 1.32–1.96; $P < 0.01$ vs. PBMC and vs. T 80% + non-T 20%). When T cells were recombined with irradiated (2,000 R) non-T cells at different ratios (80% T + 20% non-T, 90% T + 10% non-T), a

TABLE 2
Stimulation of PBMC from newborns with PjE, PHA and PWM

Newborn	Day of culture	PjE ^a (μg/ml) ^b	PHA ^c	PWM ^c
1	3	1.05 (5)	6.57	3.90
	7	0.90 (5)	2.56	1.31
2	3	0.84 (50)	47.92	27.10
	7	0.80 (5)	1.75	0.57
3	3	1.05 (0.5)	40.32	28.22
	7	1.02 (0.5)	1.21	2.55
4	3	1.41 (50)	11.70	13.31
	7	0.52 (5)	ND	ND
5	3	1.11 (5)	24.54	15.32
	7	0.49 (0.5)	1.23	9.23
6	3	0.96 (0.5)	7.73	14.83
	7	0.76 (0.5)	1.04	5.32
7	3	0.91 (0.5)	4.06	13.94
	7	0.90 (0.5)	1.29	3.72

^aResults are expressed as maximum SI.
^bCorresponding PjE concentration in culture, in terms of protein content, are given in parentheses.
^cResults are expressed as SI.

proliferative response (mean±SD maximum SI for the 7 subjects: 5.67±2.45; range: 2.38–8.12, at the ratio 80 % T/20 % non-T) comparable with that of unseparated PBMC was obtained (mean±SD maximum SI for the 7 subjects: 9.42±5.20; range 4.55–18.05; *P* > 0.05), indicating a requirement for antigen-presenting cells.

DISCUSSION

The results obtained in our experiments show that a proliferative response to *P. judaica* pollen extracts of unfractionated lymphocyte populations could be obtained in subjects with clinical evidence of hypersensitivity and specific IgE to *Parietaria*, in those sensitive to other pollens or mites, and in normal individuals. This antigen-induced proliferation can still be obtained in the T lymphocyte-enriched population, as demonstrated by separation experiments followed by recombination with irradiated non-T cells as antigen presenting and/or cytokine producing cells. However, the possibility that non-T cells can also be activated by allergens when total

PBMC are cultured with PjE cannot be excluded on the basis of such experiments.
Our study subjects were chosen in order to assess the influence on cell proliferation of such

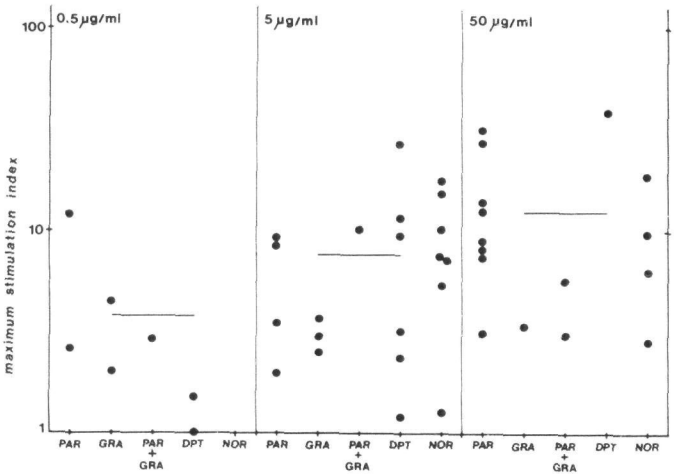


Fig. 3. Maximum SI of the five groups of subjects studied, grouped according to the doses of PjE in culture. Lines (—) are mean values of maximum SI obtained with each dose of PjE regardless of culture day. Seven of 44 subjects had maximum SI with 0.5 μg/ml 21/44 with 5 μg/ml and 16/44 with 50 μg/ml. Comparison by nonparametric Wilcoxon test of responses obtained with each dose revealed that only the values at 0.5 μg/ml and at 50 μg/ml were significantly different.

parameters as monosensitivity to either *Parietaria* or grass, the association of both pollenoses, and sensitization to mite, an example of perennial allergen; healthy individuals were also included as controls.

The subjects grouped according to the above-mentioned parameters did not significantly differ either in the dose of antigen required for optimal stimulation (doses 5 and 50 µg/ml inducing the maximum proliferative response in 84 % 37/44) of the subjects) or in the mean SI of the various groups.

The lack of a significant difference among the groups could be explained by the presence of mitogenic substances in our *P. judaica* pollen extract, as Anfosso et al. (1) showed in *Platanus acerifolia* pollen extract. To rule out this hypothesis, PBMC from cord blood of seven newborns were tested with the same PjE and were found to be unresponsive while showing a normal proliferative response to such classic mitogenic stimuli as PHA and PWM. Moreover, the time-course experiments showing that the peak response elicited by PjE was between days 5 and day 9 of culture while PHA response always peaked on day 3 for all subjects gave further, even if indirect, evidence of antigen- rather than mitogen-induced response.

It is evident from the above that proliferating allergen-specific T cells are present in almost all 44 subjects tested and are involved in the regulation of the immune response. Our finding that almost all the normal subjects had a proliferative response could be due to the idiosyncracies of *Parietaria* sp. as a source of allergen. Its long pollination period, February through

November (5), is responsible for its long-lasting presence in the environment, in contrast to the short pollination period of trees, most grass species and ragweed. This persistence of antigenic stimulus might produce a perennial stimulation of the immune system, a situation similar to that found with *Dermatophagoides* sp., a clear example of a perennial antigen. Using limiting dilution experiments, Halvorsen et al. (12) recently reported that T cells from both *D. farinae*-allergic subjects and normal individuals were able not only to proliferate to the mite extract, but also had a similar frequency of antigen-specific proliferating T cell precursors. Moreover, specific IgG has been reported in the sera of subjects allergic to *Dermatophagoides* sp. and in the sera of healthy controls (7, 9, 20).

The lack of difference between the proliferative response of *Parietaria* allergic subjects and the other subjects can also be explained by the complexity of the extract preparation used throughout the experiments. If we assume that different components in the pollen might prime for either IgE or IgG production, as indicated by Nakada et al. (20) with a mite extract and by Grote et al. (11) with immunocytochemical mapping on birch pollen grains, the use of a purified allergen could result in a prevalent proliferation of the T helper cell involved in the IgE regulation (24). Rawle et al. (24) showed that the major allergen of *D. pteronyssinus* (Der p I) induces a T cell proliferative response only in mite-sensitive patients.

However, this hypothesis is not confirmed by the results of Buckley et al. (6) who found that healthy controls, newborns and agammaglobu-

TABLE 3
PjE-induced proliferative response in separated cell populations

Cell populations	Expt. 1 (PAR) ^a			Expt. 2 (NOR) ^a		
	0.5	5	50	0.5	5	50 (µg/ml) ^b
Unseparated PBMC	1.25	11.05	13.49	1.68	4.55	3.17
T cells alone	1.02	1.43	0.85	1.00	0.91	1.33
T + 20 % irr. non-T	1.03	7.09	2.63	0.81	3.16	2.56
T + 10 % irr. non-T	ND	ND	ND	1.24	2.37	6.68
Irr. non-T cells	1.03	1.13	1.50	ND	ND	ND

^aResults are expressed as SI obtained on day 7 for one subject in each experiment.

^bPjE concentration in culture in terms of protein content.

linemic subjects' PBMC were able to proliferate in response to highly purified mitogen-free Amb e I from ragweed. Similar results were obtained by Phillips et al. (22), who demonstrated that a proliferation in both allergic and normal subjects is induced by purified Amb e I during the first week of culture of PBMC from both allergic and normal donors. Interestingly, non-specific proliferation could be transformed into a specific response by extending the culture time and boosting the cells with fresh antigen. Preliminary data obtained in our laboratory do not indicate any differences in the proliferative response of *Parietaria*-allergic and normal subjects on *in vitro* stimulation with a partially purified fraction of PjE (data not shown). Further work with a panel of highly purified and characterized major and minor allergens together with the availability of *Parietaria* allergen-specific cloned T lymphocyte subpopulations should allow a more precise characterization of the regulatory mechanisms involved in allergen-specific immune response.

Although the PBMC proliferative assay has been widely studied to assess its diagnostic value and to monitor specific immunotherapy (2, 4, 13, 14, 17, 18), our results rather suggest the importance of this test as an experimental model in the study of mechanisms involved in immune response against allergens.

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