

Lymphocyte transformation by grass pollen allergens: a study of atopic patients receiving immunotherapy

Part II. Patients during maintenance treatment

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We have previously reported on peripheral blood lymphocyte (PBL) transformation by allergen, PPD as a control antigen and PHA as a mitogen during and after a preseasonal immunotherapy period. The present report describes similar parameters during and after the ensuing maintenance treatment period. Ten patients with grass pollen rhinitis were treated with Allpyral extract and 10 with Conjuvac two-grass mixture. Lymphocyte transformation responses to grass antigen continued to be low for PBL from patients during the maintenance treatment. Postseasonal values were higher during treatment. In late autumn 1980, when treatment had been stopped, there was a spontaneous fall in lymphocyte stimulation values. Occasional high values were noticed in some patients, two of whom had treatment side effects (urticaria). Clinical data during the whole treatment period (skin prick test, provocation tests, serological parameters, total IgE, grass-specific IgE, grass-specific IgG, pollen counts, symptom scores, clinical effect and adverse reactions) have been published separately.

Key words: alginate conjugation; grass pollen; hay fever; immunotherapy; lymphocyte transformation; side effects.

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In a previous paper (3) changes in lymphocyte transformation were described during the initial intensive period of specific immunotherapy. The aim of this investigation was to study the lymphocyte transformation changes during and after the maintenance treatment.

There is a need for a method that monitors the effect of treatment and, if possible, could help select patients suitable (good relief and no side reactions) or unsuitable for further treatment (1, 4).

Most specific index values were low but cells from a few patients showed occasional high values, the reason for which will be discussed. After the first season, treatment was repeated annually for 2½ years. Cellular studies have been done before, during, and after this treatment period. Provocation tests, as well as determinations of specific IgG, total IgE, and specific IgE anti-

bodies were performed. Clinical evaluation, symptom scoring and pollen counts were monitored throughout the study (9).

This paper describes the cellular results from the last 1½ years maintenance treatment and the period after cessation of treatment.

MATERIAL

Twenty patients (12 females and 8 males) aged 19-41 years (mean 27) with classical hay fever entered the study. Three had seasonal asthma in addition to their rhinoconjunctivitis. Their allergy was proven by a typical case history and positive skin prick testing with extracts of both cocksfoot (*Dactylis glomerata*) and timothy (*Phleum pratense*). Each patient also had positive nasal and conjunctival response to challenge with a freshly prepared solution of a dialysed aqueous

extract of the two grasses. Four patients were also clinically birch-sensitive and 14 clinically mugwort-sensitive. Only one patient had a weak positive skin test for moulds. The patients were randomly allocated in blocks of two to the two treatment groups (10 patients on each treatment). All patients had been referred to the clinic and gave their informed consent before admission to the trial.

Allergen extracts

The preparations used were produced by Dome/Hollister-Stier, Division of Miles Laboratories, Stoke Court, Slough, England from grass pollen obtained from Allergon AB, Ängelholm, Sweden. The same batches of pollen were used in the preparation of both extracts.

Conjuvac. An equal mixture by weight of pollen from cocksfoot and timothy was defatted with ether and then extracted with modified Coca's solution followed by hollow fibre dialysis using a nominal 5 kD cut-off. The term "alpha fraction" was used to describe the resultant extract. Analytical isoelectric focussing was used to ensure that dialysis had not removed major proteins.

The mixed anhydride method was used in the conjugation of grass pollen extract to sodium alginate (9). The product was precipitated, washed free of unconjugated protein, redissolved and lyophilised. The conjuvac so produced was approximately four parts by weight sodium alginate to one part allergenic protein.

Allpyral. The same mixture of equal parts cocksfoot and timothy pollen was extracted in pyridine and precipitated with alum. The precipitate was filtered and washed to remove excess pyridine before suspension in Coca's solution with 0.4% phenol.

PNU determination. The Dome standard procedure was used to determine the protein nitrogen units (PNU) contained in the pollen extract. The proteins were precipitated with phosphotungstic acid and the nitrogen in the

precipitate was assayed by the micro-Kjeldahl method (9). The units of protein are related as follows: 100 PNU = 1 µg protein nitrogen which represents, on average, 6.25 µg protein.

Treatment

After the first year's preseasonal therapy period, maintenance therapy was reinstituted in late autumn of 1978. Conjuvac injections were started at 5 PNU, since experience has shown that this would be tolerated by the patients. All hyposensitization injections were given subcutaneously. (For dosage schedule see ref. 9). A lower maintenance dose (5,000 PNU), however, was adopted as serum grass-specific IgG levels during the first year suggested this would be adequate for equivalence with those obtained by the Allpyral-treated patients. The maintenance dose was halved during seasonal treatment in 1979 and was then increased to 10,000 PNU after the season. Treatment was administered annually until May 1980.

The Allpyral dosage schedule was that currently employed in Sweden, i.e. starting with 10 PNU, approximately doubling the dose on subsequent weekly injections to a maintenance dose of either 10,000 PNU or the highest tolerated dose, given once monthly with a similar seasonal dosage reduction as indicated above.

The grass pollen season in Sweden starts at the end of May and continues until the end of July.

Lymphocyte transformation

Samples for lymphocyte cultures were taken on several occasions during and after the maintenance treatment. Samples were taken at an interval of at least one week after the last injection.

Five patients served as atopic controls. They received treatment with antihistamine tablets, eye drops and if this treatment was insufficient, nasal spray (9). Blood donors served as negative controls.

METHODS

Peripheral blood lymphocytes (PBL) were separated from heparinized venous blood by flota-

tion on a Ficoll-Isopaque gradient (2). Blood diluted with 0.9% NaCl was layered over the Isopaque Ficoll by holding the pipette against the tube wall above the fluid meniscus. After centrifugation the blood cells separate into two fractions, a white layer consisting of mononuclear cells (and platelets) at the interphase region, and a bottom fraction containing the erythrocytes and granulocytes. The molecular cells can be easily removed with a pipette.

Cells were washed twice, counted in a hemocytometer and suspended in medium. Cell viability was determined by trypan blue staining. The allergens and antigens were added in appropriate concentrations. Cell concentration in the studies was $1.5 \times 10^6/\text{ml}$.

Culture procedure and assay for DNA synthesis

Cells were suspended in Hepes buffered Eagle's minimal essential medium containing 10% heat-inactivated human AB serum and nutrients. Cells were cultured in triplicate in microtiter plates (3040, Falcon, USA) with 1.5×10^5 cells/well per 0.2 ml. Cultures were incubated for 3 (PHA response) or 6 days in a humidified atmosphere of 5% CO_2 in air 37°C . Twenty-four hours before harvest, $1.0 \mu\text{Ci } ^3\text{H}$ -labelled thymidine with a specific activity of 20 Ci/mmol (Radiochemical Centre, Amersham, UK) was added to each culture. Harvesting was performed with a Skatron harvesting machine (Lierbyen, Norway) and radioactivity was measured by scintillation spectrophotometry (inter-tec, Nanoteknik, Sweden).

The response of cultured cells was expressed as a stimulation index (SI) by dividing the radioactivity (cpm) of stimulated cell cultures by that of non-stimulated cell cultures. A kinetic study with cell culture harvesting from days 3 to 7 with the antigens used in different concentrations was done to titrate antigen concentration and optimal culture time.

Allergens: Lyophilised extract of equal mixtures of cocksfoot and timothy containing 50 PNU/ml (0.1 ml = 5 PNU/culture); solution of birch extract 1/10 (Vitrum) in a final concentration of 1/2000 (per culture).

Antigen: PPD from tuberculin was obtained

from State Serum Institute, Copenhagen, Denmark and used in a concentration of 0.1 $\mu\text{g}/\text{ml}$ final solution in culture. Phytohemagglutinin (PHA) Wellcome Ltd., UK was used in a final dilution of 1:100.

Humoral parameters: Specific IgE was measured by the RAST technique (7) and specific IgG was measured by the Staphylococcus protein A method (6). Samples were analysed by Miles Laboratories and expressed in ng/ml and Trial units/ml (tu/ml).

Statistics: The data were analysed for statistical significance using the Student's test for paired means and the Pearson coefficient of correlation. All calculations were performed on natural logarithmic values.

RESULTS

Blood donors

Cell cultures were stimulated with antigens and mitogens. Index values were low in response to antigens. Most values were below an index of 4. Some blood donors had birch indices above 4, which could indicate latent birch allergy. Responses to mitogens were similar to those obtained with cells from atopic patients.

Pharmacologically treated patients (atopic controls)

Lymphocyte responses to two-grass antigen were low before season and increased after. This pattern was repeated during the 2 following years. High values were obtained in some patients even before season in 1979 and 1980 (Fig. 1a).

Lymphocyte responses to birch antigen were low in all patients (data not shown). The number of pharmacologically treated patients was unfortunately too low to allow statistical analyses of the material.

Patients on immunotherapy

Mean background levels in unstimulated cultures varied throughout the study period (Table 1). Most of the variations are probably methodological. No statistical differences were detected between these values.

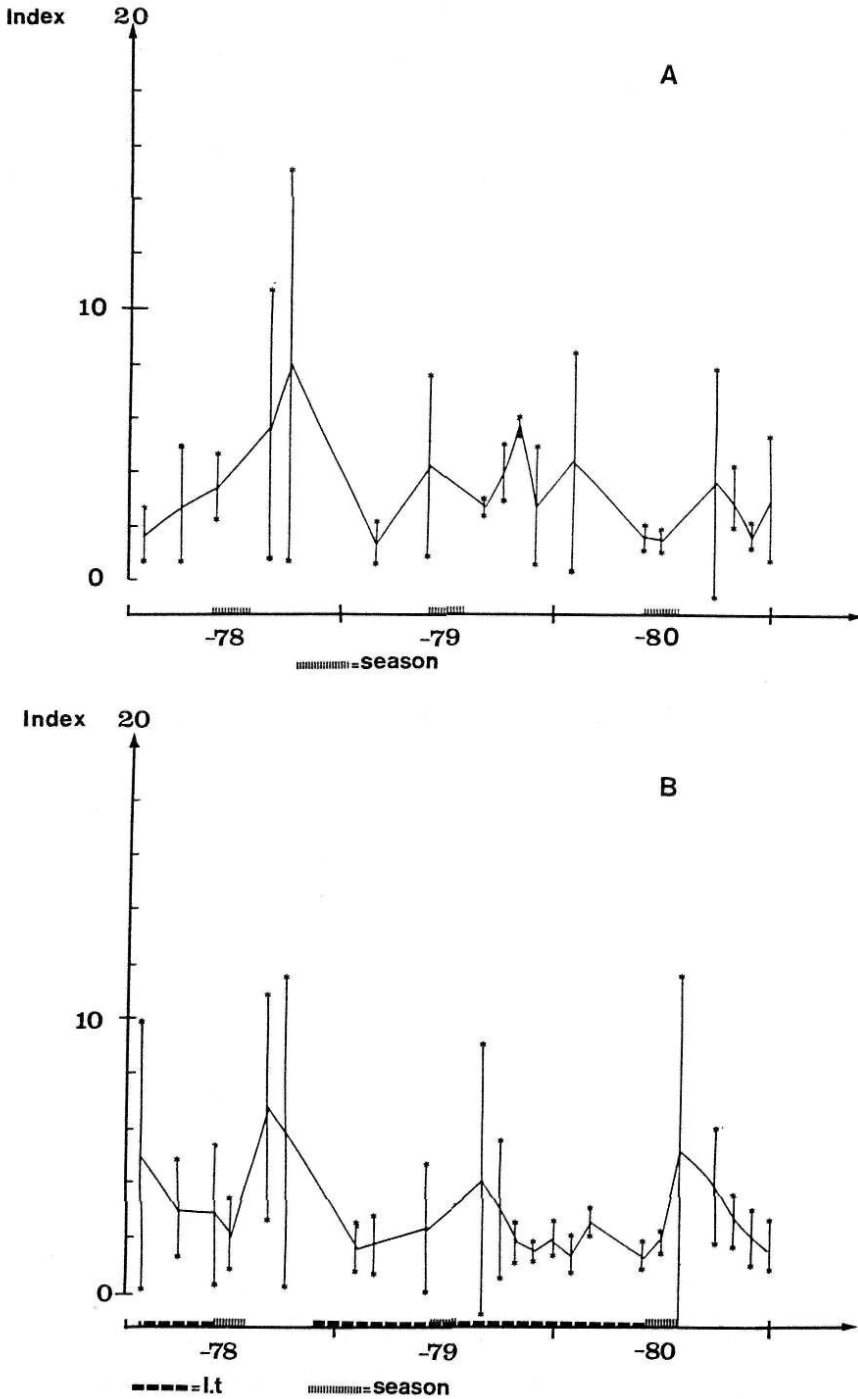


Fig. 1. A) Lymphocyte stimulation (mean ± 2 SD) with 2-grass pollen mixture in pharmacologically treated patients (atopic controls) during the years 1978-81. B) Lymphocyte stimulation (mean ± 2 SD) with 2-grass pollen mixture in Allpyral and Conjuvac treated patients during the years 1987-91.

Lymphocyte response to two-grass antigen

The curves for the whole treatment period are shown in Figs. 1b, 4, and 5. Statistical comparison between lymphocyte stimulation data of the Allpyral and Conjuvac groups was done during year 1 (not included in ref. 3) but no differences were found. Therefore the groups were added together and the values between different occasions compared. Cells from the patients gave low stimulation responses to specific antigen during the maintenance treatment. A significant decrease was seen between August 1978 and January 1979 and between August 1978

and January 1980 (See Tables 2 and 3 and Fig. 1b).

Higher values were again seen after treatment was stopped. This increase was statistically significant (Table 3 on the 5% level).

Lymphocyte response to birch antigen

When Allpyral- and Conjuvac-treated patient groups were analysed together, a significant decrease was seen between August 1978 and January 1980 (in index on the 5% level, not in net increase, see Tables 2 and 3).

Table 1
Background (lymphocytes and culture medium) values in counts

	Aug-78	Jan-79	Jan-80	Aug-80
Natural logarithm of background count values (mean $\pm$ 2 SD)	7.72 $\pm$ 0.57	7.32 $\pm$ 0.57		
	7.71 $\pm$ 0.59		7.54 $\pm$ 0.73	
		7.33 $\pm$ 0.59	7.54 $\pm$ 0.73	
			7.47 $\pm$ 0.78	7.73 $\pm$ 0.77

Natural logarithms of count values are expressed as means $\pm$ SD. Lines connect samples which have been statistically compared. (If no *P* value is expressed on the middle of the line no statistical difference has been detected).

Table 2
Allergen specific stimulation (lymphocyte and culture medium with allergens)

	Aug-78	Jan-79	Jan-80	Aug-80
Allergen specific stimulation index values and statistics. Index values in nat. logarithms (mean $\pm$ 2 SD) 2-grass stim cells	1.21 $\pm$ 0.10	1.07 $\pm$ 0.06		
	1.21 $\pm$ 0.09	<i>P</i> < 0.001	1.07 $\pm$ 0.06	
		1.07 $\pm$ 0.06	1.07 $\pm$ 0.06	
			1.07 $\pm$ 0.06	1.25 $\pm$ 0.45
Birch stim cells index	1.21 $\pm$ 0.11	1.09 $\pm$ 0.08		
	1.12 $\pm$ 0.09	<i>P</i> < 0.05	1.04 $\pm$ 0.05	
		1.10 $\pm$ 0.08	0.98 $\pm$ 0.24	
			1.05 $\pm$ 0.05	1.10 $\pm$ 0.07

Allpyral- and Conjuvac-treated patients are analysed together. Counts are divided by background counts to obtain stimulation index values. Natural logarithms of index values are expressed as means $\pm$ 2 SD. Lines connect samples from different occasions during maintenance treatment which have been statistically compared. (*P* value is expressed on the middle of the line if a statistical difference has been detected.) Mean values change as a few patients did not complete the study.

Lymphocyte response to PPD and PHA

The PPD and PHA lymphocyte stimulation curves (index and net increase) showed close resemblance for pharmacologically and immunotherapy treated patients. All curves are shown graphically (Figs. 2a-3b) and the statistical determinations are therefore not included.

DISCUSSION

The clinical effect of hyposensitization is satisfactory in about 75% of treated patients if patients are treated with pollen and mite extracts (12). There is a need for a method that monitors the effect of treatment and if possible could help select patients suitable (good relief and no side reactions) or unsuitable for further treatment (1, 4).

During immunotherapy complex immunological changes can be observed (10). Other results indicate that a high IgG₄ specific antibody level early in the course of immunotherapy may predict a poor clinical response to treatment (5). Rocklin et al. (11) used a suppressor cell assay to study the possible mechanism of hyposensitization. Since the availability of specific monoclonal antibodies, the character-

ization of different subsets of lymphocytes has now been greatly simplified. The results of Kus et al. (8) indicate that immunotherapy could lead to an increase in the proportion of T₈⁺ cells (or T₈ positive), though the importance of T₈⁺ (T₈ positive), and T₄⁺ (T₄ positive) subpopulation cells in the IgE production *in vivo* has not yet been established.

During the first year (3) the patient material appeared divided into two distinct groups, one giving a lower response (in specific lymphocyte reactivity) after immunotherapy and the other being unchanged or even increasing in reactivity. Three patients were of special interest as the treatment had a beneficial effect but also gave side effects. One of these three patients stopped treatment as he felt "more discomfort from the injections than from the season itself". Two other patients (No. 38 Fig. 4 and No. 36 Fig. 5) acquired urticaria which caused mild symptoms in cold air but exacerbated when the patients came into room temperature. These patients had continued complaints of urticaria during the winter season 1978-79 and 1979-80. Their symptoms disappeared when treatment was terminated.

During maintenance treatment cells from these two patients gave occasional high lympho-

Table 3
Allergen-specific stimulation (lymphocyte and culture medium with allergens)

	Aug-78	Jan-79	Jan-80	Aug-80
Net counts 2 grass stim cells	8.25 ± 2.41	6.37 ± 1.86		
Nat. logarithms (mean ± 2 SD)	8.96 ± 0.86	P < 0.01	6.35 ± 2.94	
		6.47 ± 1.70	6.83 ± 2.40	
			5.72 ± 3.12	P < 0.05 8.19 ± 0.95
Net counts birch stim cells	6.56 ± 3.28	6.74 ± 1.90		
	6.97 ± 2.9		5.62 ± 3.01	
		6.79 ± 1.94	5.62 ± 3.01	
			5.35 ± 3.21	6.81 ± 3.05

Allpyral- and Conjuvac-treated patients are analysed together. Background counts have been subtracted to obtain net counts. Natural logarithms of net counts are expressed as means ± SD. Lines connect samples from different occasions during maintenance treatment which have been statistically compared. (P value is expressed on the middle of the line if a statistical difference has been detected.) Mean values change as a few patients did not complete the study.

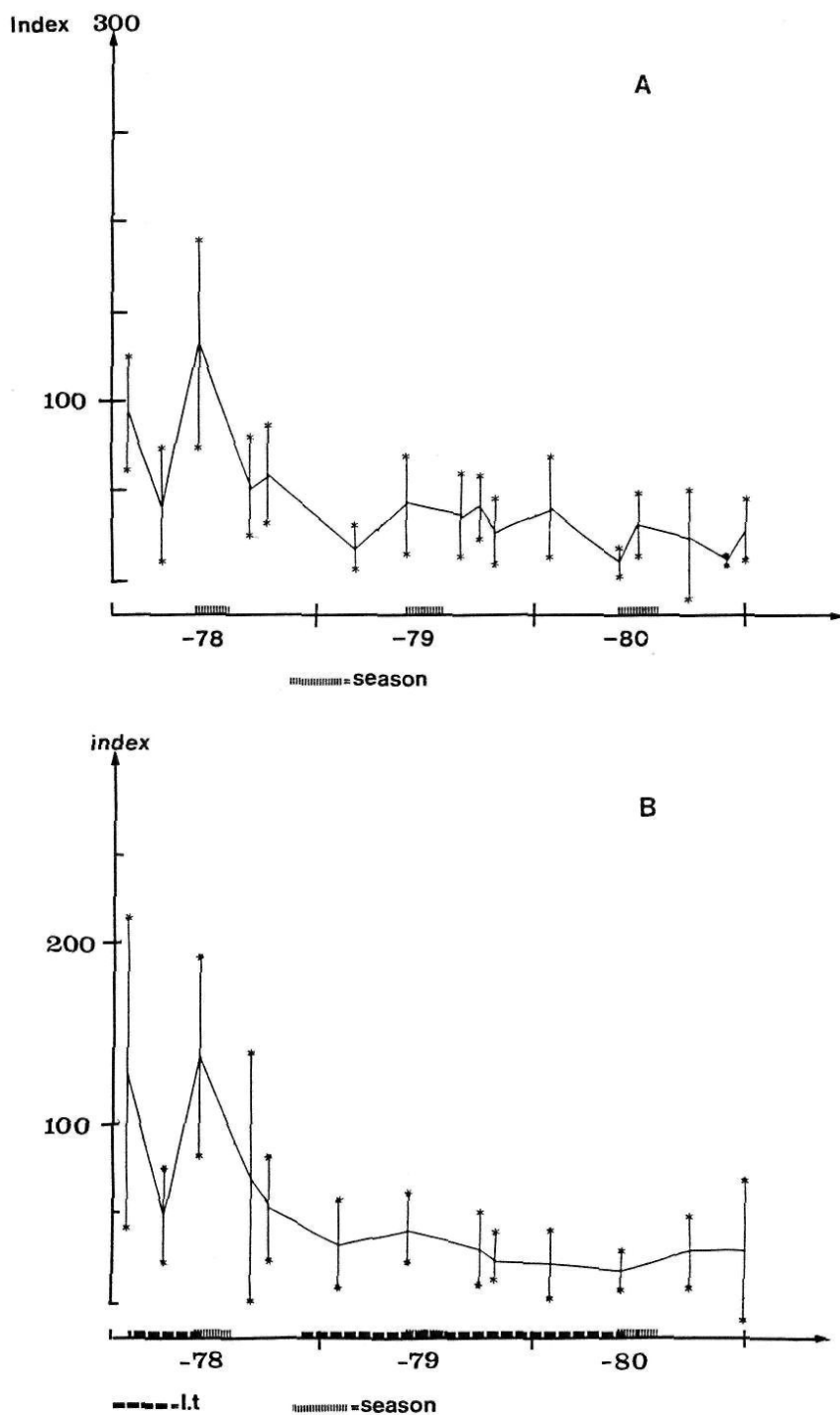


Fig. 2. A) Lymphocyte stimulation (mean $\pm$ 2 SD) with PPD in pharmacologically (atopic controls) treated patients during the years 1978-81. B) Lymphocyte stimulation (mean $\pm$ 2 SD) with PPD antigen in Allpyral- and Conjuvac-treated patients during the years 1978-81.

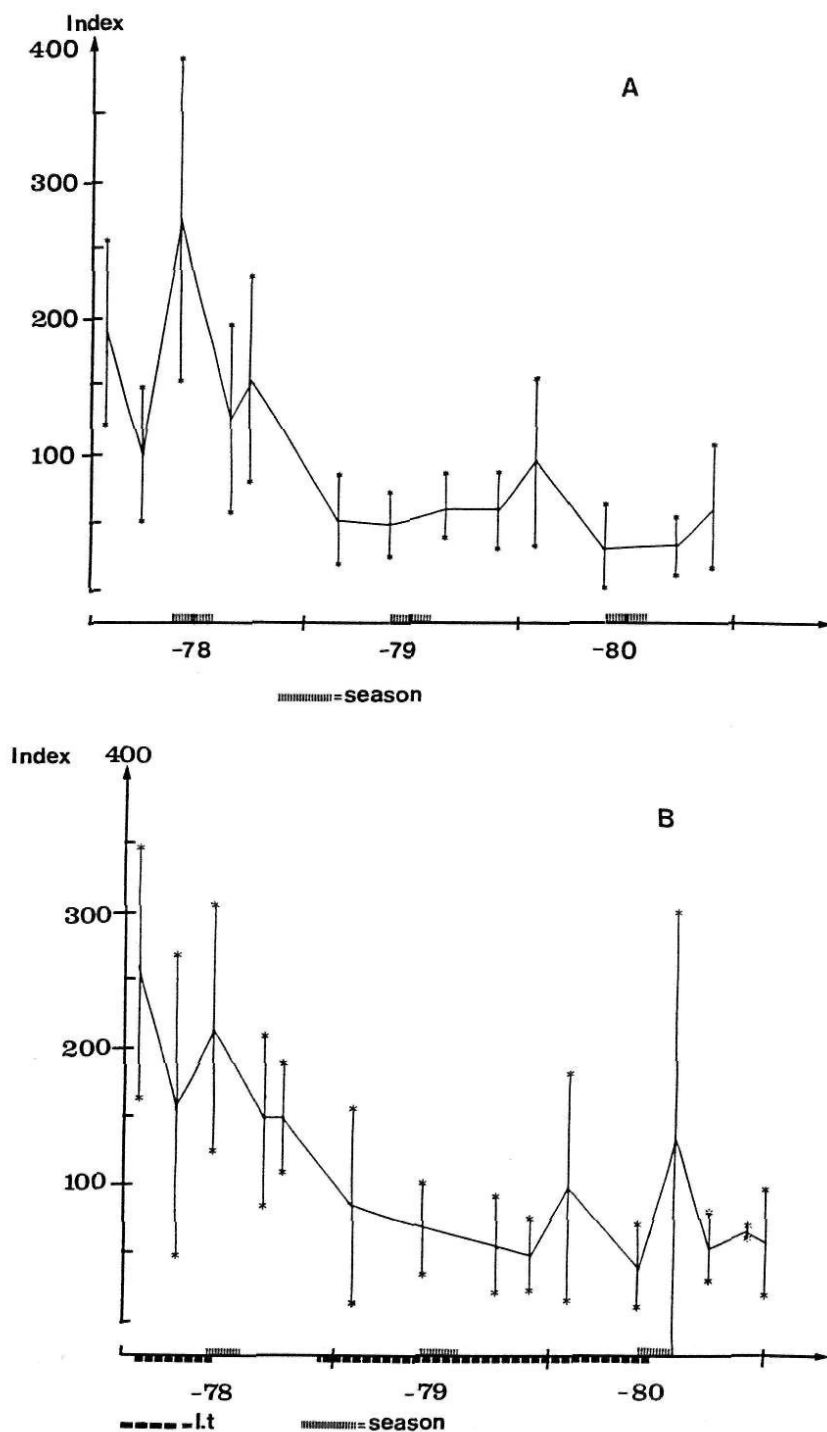


Fig. 3. A) Lymphocyte stimulation (mean $\pm$ 2 SD) with PHA mitogen in pharmacologically (atopic controls) treated patients during the years 1978–81. B) Lymphocyte stimulation (mean $\pm$ 2 SD) with PHA mitogen in Allpyral- and Conjuvac-treated patients during the years 1978–81.

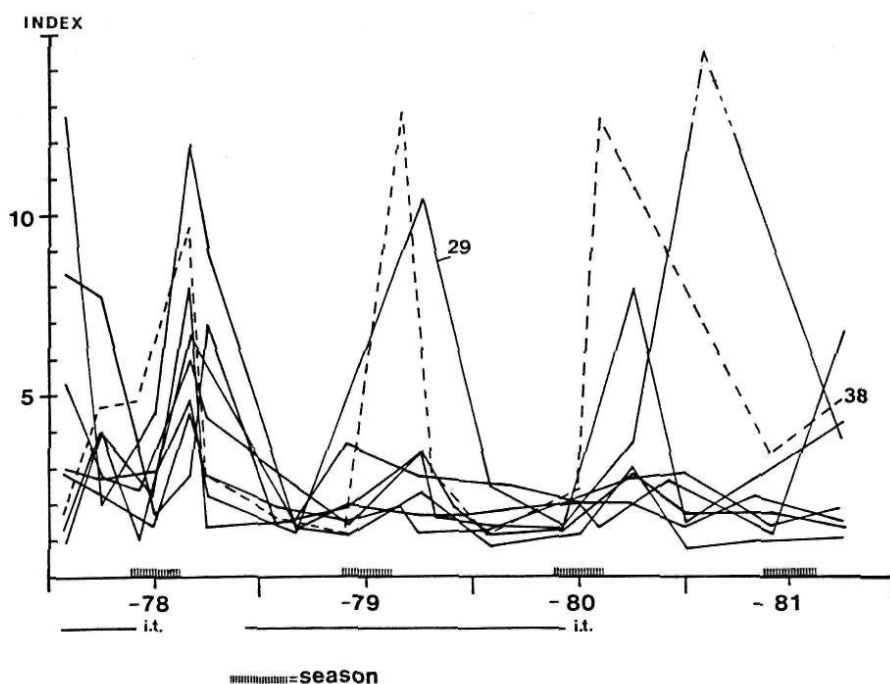


Fig. 4. Lymphocyte stimulation with 2-grass pollen mixture in Conjuvac-treated patients (8/10 patients. 2 patients did not complete the study.)

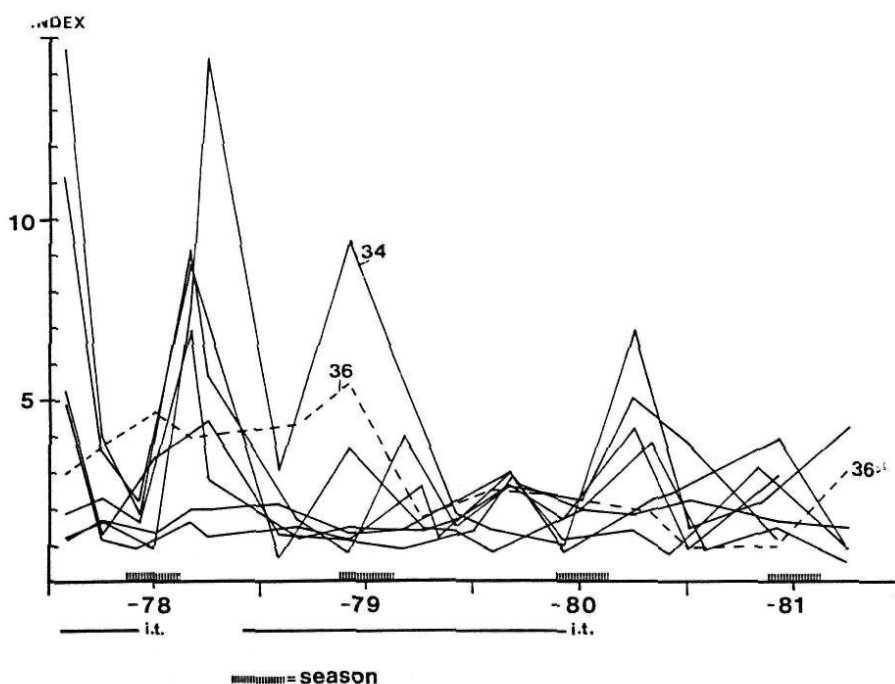


Fig. 5. Lymphocyte stimulation with 2-grass pollen mixture in Allpyral-treated patients (8/10 patients).

cyte stimulation indices (Figs. 4 and 5). (Patient No. 34, who also had an occasional high specific stimulation index value (May 1979), had an adverse reaction (dyspnea and chest irritation) from the injection given 2 weeks previously. Patient No. 29 had a long interval between injection and the sample taken in autumn 1979. Occasional high lymphocyte stimulation values and side effects were thus combined even during the maintenance treatment period. This indicates that the cellular response has predictive value for possible side effects.

In our previous paper (3), describing the intensive immunotherapy period, we observed in most patients a diminution of antigen-specific stimulation in blood lymphocytes and after termination of season an increased response. A post seasonal increase was registered also during maintenance treatment (autumn 1979).

The continued treatment, now given annually, was combined with a rediminution of antigen-specific stimulation. We believe that these changes reflect a recruitment of specifically reactive cells to other lymphoid cell compartments during treatment, whereas after the season cells would reappear in the circulation and be able to give higher stimulation indices due to clonal expansion during treatment and season. During maintenance treatment the dose of antigen administered/time is lower than during the initial therapy phase. This might imply different conditions. The dose/time may now be too low to have any additive effect on the lymphocytes, the changes seen only reflecting the spontaneous alterations, and thus explain the decrease in late autumn 1980.

Moreover, the initial hyposensitization treatment resulted (3) in an obvious difference between the pharmacologically and hyposensitization treated groups (Fig. 1a, 1b). During the following years no marked difference could be visually observed. To ensure that the statistical differences (Tables 2, 3) are due to the maintenance treatment it would have been necessary to compare the results with those of pharmacologically treated patients. Unfortunately, too few pharmacologically treated patients were included in this study, a fact that also compromises other studies.

The main finding in this study is that patients benefiting from specific immunotherapy show decreased cellular reactivity in blood lymphocytes during the whole period of treatment.

The lack of a control patient group for comparison makes the conclusions uncertain. However, there might be a decrease in post seasonal index in subsequent years. The changes could also reflect spontaneous seasonal variation in lymphocyte stimulation values. Two patients with clinical side effects also showed occasional high specific lymphocyte stimulation values (during the maintenance treatment period). These patients were detected within 3 months of the start of immunotherapy (3).

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