

Immunological studies *in vitro* and *in vivo* of children with pollenosis given immunotherapy with an aqueous and a glutaraldehyde-treated tyrosine-adsorbed grass pollen extract

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Summary

Ten patients treated with an aqueous Timothy grass pollen extract and given monthly maintenance doses were compared to ten patients given a glutaraldehyde-treated tyrosine-adsorbed grass pollen extract as five weekly injections pre-seasonally. There were four non-treated controls. Both treated groups showed equivalent increases of total IgE, Timothy-specific IgE and IgG, but the concentrations of Timothy-specific IgG antibodies were sustained only in the patients receiving maintenance therapy. An increase of IgE and RAST titres during the pollen season were obtained in all three groups. No consistent changes were seen in the results of leucocyte histamine release tests and naso-conjunctival provocation tests from spring to autumn.

A Sepharose-protein A technique was used for the estimation of Timothy-specific IgG antibodies. The results obtained with this technique correlated excellently with those of the Farr technique and a double-antibody technique.

Introduction

Immunotherapy of patients with pollen allergy has been used for more than 60 years but the mechanism behind the clinical improvement which is usually obtained is still obscure. Hereditary factors and the amounts of allergen given are regarded to be of crucial importance for the immunological response (Connell & Sherman, 1969; Norman, 1969). One factor thought to contribute to the beneficial effect is the achievement of a high concentration of 'blocking antibodies' mainly belonging to the IgG class of immunoglobulins (Lichtenstein, Holtzman & Burnett, 1968). Such antibodies may perhaps explain why no exacerbation of symptoms is seen, although, during the early phase of immunotherapy, the concentration of pollen-specific IgE antibodies increases substantially (Berg & Johansson, 1971).

Ideally, a pollen extract used for immunotherapy should stimulate the production of pollen-specific IgG 'blocking antibodies' but not the production of pollen-specific IgE antibodies. Neither should it give rise to local or systemic reactions. When allergens are administered by injection the production of IgG antibodies is favoured. To obtain

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a more selective stimulation of the production of 'blocking' antibodies and to reduce the risk of complications by the injection therapy attempts have been made to modify the allergens chemically (Marsh, Lichtenstein & Campbell 1970, Berrens, Hénocq & Radermecker 1973, Johansson *et al.*, 1974b) and to develop slowly releasing depot formulae (Fuchs & Strauss, 1959; Johansson *et al.*, 1974b).

In the present study the clinical and immunological effects of a glutaraldehyde-treated tyrosine-adsorbed grass pollen extract (Johansson *et al.*, 1974b) have been compared with those of an aqueous extract of Timothy grass pollen.

A radioimmunoassay for determination of IgG antibody, possibly 'blocking antibody' has been developed and the results obtained are discussed in relation to the clinical effects of treatment.

Material and methods

Grass-pollen sensitive patients, with rhino-conjunctival symptoms attending the out-patient allergy clinic at the Department of Paediatrics, University Hospital, Uppsala, Sweden, were selected for the study. The two groups of ten patients each for immunotherapy (groups A and B) were chosen to be comparable regarding the severeness of their allergic symptoms during the grass pollen season. The four control children (group C) had somewhat less severe symptoms.

Allergens

An aqueous extract of Timothy grass pollen (Vitrum Laboratories, Stockholm, Sweden) was used for all naso-conjunctival provocation tests and for all *in vitro* studies. The stock extract (10^{-1} w/v) was kept at 4°C and for the *in vivo* studies fresh dilutions were made for the tests in the spring and autumn. As the extract contained 0.5% phenol as a preservative the extract was dialysed for 24 hr against 2×1000 ml distilled water and frozen in aliquots at -20°C until used in the leucocyte histamine release studies.

Ten patients (group A) were treated with an aqueous Timothy grass pollen extract (Vitrum Laboratories, Stockholm, Sweden) and another ten patients (group B) with an experimental glutaraldehyde-treated tyrosine-adsorbed mixed grass pollen formulation (GTA) (Beecham Research Laboratories, Sussex, England) (Johansson *et al.*, 1974b).

Immunotherapy

The aqueous extract was used in rush type desensitization (Berg & Johansson, 1971) and a maintenance dose was given every fourth week. The GTA allergen preparation was given pre-seasonally only as five weekly injections (50, 125, 300, 800 and 2000 Noon units).

Time schedule for blood samplings and provocation tests

Venous blood was drawn from all patients in March (before treatment), at the end of May (after initial treatment, before the grass pollen season) and in October (after season). Furthermore, venous blood was obtained from all but two of the patients receiving immunotherapy with the aqueous extract at the end of July (at the end of the season). The serum samples were kept frozen at -20°C until analysed.

Naso-conjunctival challenge tests (Berg, Bennich & Johansson 1971) were per-

formed with serial ten-fold dilutions of the Timothy allergen extract at the end of May and in October.

Leucocyte histamine release

The method used for the preparation of leucocytes and the challenge of the cells with allergen was that described by May *et al.*, 1970. The cells were challenged with allergen concentrations varying from 10^{-4} to 10^{-8} w/v. The histamine released was assayed on an atropinized (10^{-7} g/l) guinea-pig ileum.

Assay of total IgE and allergen specific antibodies

The total IgE concentrations were determined by PRIST (Ceska & Lundkvist, 1972), a direct sandwich-type of radioimmunoassay, as described in detail (Johansson, Berglund & Kjellman, 1976).

IgE antibody to Timothy allergen was determined by RAST (Wide, Bennich & Johansson, 1967) as described previously (Johansson, Bennich & Berg, 1971). A 0.3 ml aliquot of Timothy allergen extract (K569, batch 36, 6%; Bencard, London) was coupled to 100 mg CNBr-activated cellulose particles (Microcrystalline, E. Merck AG, Darmstadt) to make an allergen-polymer complex (APC). To each test tube was added 0.5 mg APC. Each serum sample was tested in duplicate at two dilutions in order to ensure parallelism with the reaginic reference serum. The results were expressed in parts per thousand (‰) of the reference.

IgG antibody to an allergen was measured by a technique based on the adsorption of IgG to a Sepharose-protein A complex followed by the addition of ^{125}I -labelled allergen (Johansson *et al.*, 1974a). Isolated staphylococcus protein A was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala). One millilitre of the Sepharose-protein A complex (Seph-pA), kindly supplied by Professor J. Sjökvist, Uppsala, had the capacity to bind 20 mg IgG of the subclasses 1, 2 and 4. A 0.5 ml aliquot of Seph-pA diluted 1/10 in test buffer (0.1 M phosphate buffer pH 7.4, containing 0.2% bovine serum albumin, 0.1% 'Tween 20' and 0.05% NaN_3) was incubated with 0.1 ml of the serum sample diluted 1/10 in test buffer under vertical rotation for 2–3 hr at room temperature. At higher dilutions the serum sample was diluted in a 10% solution in test buffer of normal serum lacking allergen-specific IgG antibodies. After centrifugation but no washing 0.1 ml of the ^{125}I -labelled allergen preparation corresponding to approx. 100 000 ct/min was added for a second incubation overnight. The Seph-pA complex was then washed five times with saline containing 0.1% 'Tween 20' and the bound radioactivity was measured in a gamma scintillation counter. The results were expressed in parts per thousand (‰) of a reference pool of sera from patients extensively treated by hyposensitization with grass pollen extracts. A similar technique using Sepharose-coupled anti-IgE (Seph-aE) was used in some experiments to investigate the capacity of the ^{125}I -labelled allergen to react with antibody of the IgE type. Seph-aE was prepared by the coupling of 20 mg Na_2SO_4 -precipitated gammaglobulin fraction of specific sheep anti-FcND to 1 g dry CNBr-activated Sepharose 4B. A 0.5 ml aliquot of the Seph-aE preparation diluted 1/25 in test buffer was incubated with 0.1 ml of the serum sample undiluted unless otherwise stated, at room temperature overnight. After five washings the ^{125}I -labelled allergen was added for the second incubation followed by washings and counting as described for the Seph-pA system.

Two further techniques were used to determine IgG antibody. In a double-antibody

procedure 0.1 ml serum sample diluted 1/10 in test buffer, 0.1 ml ^{125}I -labelled allergen representing approx. 80 000 ct/min and 0.1 ml undiluted specific, rabbit anti-IgG were mixed and incubated at 37°C overnight. The precipitate was washed three times with saline containing 0.1% 'Tween 20' by 5 min centrifugation at approx. 3,500 *g*. The activity of the precipitate was measured in a gamma counter and expressed in relation to a standard reference consisting of a highly positive serum sample serially diluted in a diluent which consisted of a 10% solution of a negative serum in test buffer.

Ammonium sulphate precipitation was performed essentially as described by Farr (1958). A 0.5 ml aliquot of the serum sample, diluted in a diluent consisting of 10% solution of bovine serum in 0.1 M borate buffer pH 8.3 and 0.5 ml of the ^{125}I -labelled antigen representing approx. 80 000 ct/min, was incubated at room temperature overnight. For a second incubation of 30 min at 4°C 1.0 ml of a saturated ammonium sulphate solution was added. After centrifugation for 30 min at 4°C at approx. 3 000 *g*, the activity of the unwashed precipitate was measured in a gamma counter. After subtraction of the activity of a buffer control the results were expressed in relation to a highly positive serum sample serially diluted in the diluent.

Iodination of allergen

A Timothy pollen preparation, a pool of Group 3 bands from an acrylamide electrophoresis separation (Mullan, 1974) (kindly supplied by Mr B. G. Overell, Bencard, London) was used. Twenty microlitres of a 5 mg/ml solution was labelled with 2 mCi ^{125}I by the chloramine T method as described (Wide *et al.*, 1967) and separated on a Sephadex G75 column equilibrated by 0.1 M phosphate buffer pH 7.4 containing 0.3% bovine serum albumin. Protein peaks which eluted just after albumin were pooled and used diluted to approx. 100 000 ct/min. Ovomucoid (lot 8, code 96-016, Pentex, Miles Laboratories Ltd, Kankakee, Ill.), 10 μl of a 10 mg/ml solution, and cod fish allergen DS22 (kindly supplied by Dr K. Aas, Oslo), 20 μl of a 1 mg/ml solution, were iodinated with 2 mCi ^{125}I , respectively, as described above.

Results

Methodological experiments

The serum pool from patients intensely treated with grass pollen extracts and tested at a 1/10 dilution under standard conditions bound about 15 000 ct/min of the approx. 100 000 ct/min added (15%) to the Sepharose-protein A (Seph-pA) complex compared to 100–200 ct/min for serum samples from healthy individuals. The binding was influenced by the IgG concentration of the sample to be tested although the Seph-pA complex added to each tube had the capacity to bind twice the amount of IgG in the sample added. Therefore, all samples were analysed at a 1/10 dilution. When a serum had to be diluted more than 1/10 a diluent consisting of a 10% solution of a negative serum in buffer was used.

The antigen specificity of the IgG-antibody-allergen reaction was confirmed by an inhibition experiment. When the Seph-pA and serum complex was preincubated for 2 hr with 10 μl of serial dilutions of an aqueous Timothy allergen extract the maximally bound activity 19,068 ct/min decreased. The 10^{-3} dilution gave 2842 ct/min and undiluted extract 148 ct/min which represented 14.8 and 0.8% respectively of the non-inhibition value.

The amount of isotope bound to allergen was calculated from the capacity of the

Table 1. The capacity of a reaginic serum to bind ^{125}I -labelled Timothy allergen (110,000 ct/min added) to Sepharose anti-IgE

Sample	Volume (ml)	Bound (ct/min)	Percentage of added
Reaginic serum (BC 11092)	0.1	3 510	3.2
	0.2	5 234	4.8
	0.4	7 852	7.1
	0.8	11 566	10.5
	1.6	16 888	15.4
	3.2	20 680	18.8
Normal serum (V.S. 13041)	0.1	565	0.5
	0.2	638	0.6
	0.4	537	0.5
	0.8	573	0.5
	1.6	566	0.5
	3.2	612	0.6
Buffer	0.1	588	0.5

^{125}I -labelled Timothy allergen preparation to react with IgE antibodies. Sepharose anti-IgE (Seph-aE), was incubated with increasing volumes, 0.1–3.2 ml, of a serum containing a high concentration of IgE antibody to Timothy allergen. Normal serum and buffer were used as controls. The immune reactivity for IgE antibody (Table 1) was in the same order as that obtained for IgG antibody in the Seph-pA system.

A comparison was performed between the Seph-pA complex technique and the double antibody method for determination of IgG antibody using ^{125}I -labelled cod fish allergen DS22 (Fig. 1). An excellent correlation ($r = 0.97$) was obtained and the

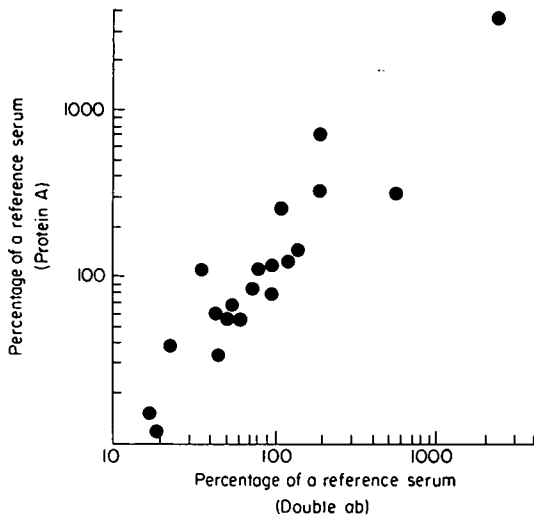


Fig. 1. The correlation ($r = 0.97$) between the concentration of IgG antibody to cod fish allergen DS 22 as measured by the Seph-pA technique (Protein A in the figure) and a double-antibody procedure (Double ab in the figure) in sera from fish allergic patients.

two tests seemed to be equally sensitive. The correlation between the Seph-pA complex test and the ammonium phosphate precipitation technique was also found to be very good ($r = 0.94$) and in this case ^{125}I -labelled ovomucoid was used.

Clinical investigations

At the time of the last blood sampling in October the patients in group A had each received a mean of twenty injections and the patients in group B five injections. One patient in group B developed a local reaction during the first 24 hr after injection no. 3 (300 Noon units) with heat, swelling and redness of the upper arm. The symptoms subsided within a few days without therapy. The next dose was divided into two and given with 1 week's interval. As he had no reaction this time the scheduled injection programme could proceed without further complications.

Symptoms. The patients' symptoms were recorded daily during the grass pollen season but, as no symptom scores were available from the summer before immunotherapy, no comparison was possible. However, in October the patients were asked to give their opinion of the severity and frequency of their allergic symptoms during

Table 2. The reported change of allergic symptoms during the grass pollen season in comparison with the preceding year

Reported change	Group		
	A	B	C
Much improved	1	2	0
Improved	4	2	1
Possibly improved/no change	4	4	3
Worse	1	2	0

the grass pollen season in comparison with those of the previous summer. As seen from Table 2 the reports were rather similar in all three groups.

Provocation tests. Three of the patients in group A, none in group B and two patients in group C had at least a ten times higher sensitivity threshold for Timothy allergen in October than in March as judged from the provocation test. All the other patients reacted to the same allergen concentration. However, two children in group B tolerated ten times more allergen after treatment (end of May) than before treatment and after season. These two patients did not have an improved pollen season. Of the three patients in group A with a decreased sensitivity in October, one was reported to be much improved, another moderately and the third possibly improved by the treatment.

Leucocyte histamine release tests. The maximum percentage of histamine release and the histamine content of the leucocytes did not show any systematic changes during the observation period. In three patients in group A less than 20% histamine was released on challenge with allergen on all three test occasions. These children had RAST values of 133, 533 and 725% respectively in the pretreatment samples. In all other children the maximum percentage of histamine release varied between 24 and 100%. The leucocyte sensitivity, defined as the allergen concentration giving the

Table 3. The mean IgE concentrations and their changes during the observation period. Ranges are given in brackets. NT, not tested

Time of blood sampling	Group A			Group B			Group C		
	units/ml	%	%	units/ml	%	%	units/ml	%	%
Pre-treatment (March)	269 (26-2500)	100		161 (57-1800)	100		243 (45-1300)	100	
Post-treatment but pre-season (May)		126 (19-781)	100		122 (29-932)	100		85 (15-617)	100
Season (July)		305* (43-1338)	243*	NT	—	—	NT	—	—
Post-season (October)		254† (41-1338)	208†		170‡ (37-1180)	139‡		153 (12-700)	180

Significant increase from pre-season: * $P < 0.001$; † $P < 0.005$; ‡ $P < 0.05$.

maximum degree of histamine release from the leucocytes, was generally the same after the season as before treatment in all groups. However, there was a tendency to an increased sensitivity of the leucocytes after treatment but before season. This was seen in one patient in group A, and in five patients in group B. In the patient in group A and in three in group B this difference was at least 100-fold.

IgE. The geometric mean concentration before treatment was highest in group A (Table 3). This high figure was very much influenced by two patients in this group with very high IgE concentrations. The IgE concentrations increased in all but one of the patients from the pretreatment to the postseason sample. The increase of the mean IgE level during pollen season was significant in both group A ($P < 0.005$) and group B ($P < 0.05$). One child in group B showed gradually decreasing IgE levels as well as RAST values and she had a much better season than in the previous year. After treatment the IgE levels had increased slightly in both treated groups and decreased slightly in the control group. The postseasonal values from October probably do not represent the highest seasonal levels since the IgE concentration in the late season samples of group A were approx. 50% higher than the postseasonal level of the same group (Table 2).

Allergen-specific IgE. The pretreatment geometric mean Timothy-RAST value of group A was two or three times higher than that of group B and C (Table 4). The high mean for group A depended to a great deal on the two patients with very high total IgE concentrations who had RAST values of 8000 and 5200% respectively.

The changes in the concentration of Timothy pollen-specific IgE antibodies showed a pattern very similar to that of the total IgE. A slight increase occurred in both treated groups during treatment; in group A the peak level was found at the end of the pollen season and in October a slight decrease had already occurred. The two children in group A with very high RAST values showed less increase than the other children in the group. Late in the season (July) their concentrations were 134 and 250% respectively of the pretreatment values compared to a mean of 336% for the whole group. The increase during the season (May to October) was highest, 136%, in the non-treated group and lowest in group B, 61%. These differences were not statistically significant.

Table 4. The mean Timothy-specific IgE concentrations and their changes during the observation period. Ranges are given in brackets. NT, not tested

Time of blood sampling	Group A			Group B			Group C		
	RAST %	%	%	RAST %	%	%	RAST %	%	%
Pre-treatment (March)	800 (83-8000)	100		342 (65-2750)	100		266 (7-1450)	100	
Post-treatment but pre-season (May)		143 (32-868)	100		129 (19-724)	100		77 (2-573)	100
Season (July)		336* (50-1623)	235	NT	—	—	NT	—	—
Post-season (October)		297* (42-1436)	208*		207† (60-687)	161†		182 (3-902)	236

Significant increase from preseason: * $P < 0.001$; † $P < 0.005$.

Allergen-specific IgG. In the pretreatment samples low concentrations of IgG antibody to Timothy were found in all groups (Table 5). The geometric mean value of group A was higher than that of group B and C depending on high values in two children. One of them had high IgE and RAST values but the other had concentrations below the mean for his group. The mean concentration of IgG antibodies for the remaining eight patients in group A was 28%.

After 5 weeks of immunotherapy the concentration of Timothy-specific IgG antibodies had increased about six times in both groups compared to a 20% decrease in the control group during the same period. Because of the small series and the great individual variations within each group the differences were not statistically significant. After season the patients receiving monthly injections with aqueous extracts (group A) still had high concentrations of IgG antibodies while the patients with pre-seasonal

Table 5. The mean Timothy-specific IgG concentrations and their changes during the observation period. Ranges are given in brackets. NT, not tested

Time of blood sampling	Group A			Group B			Group C		
	%	%	%	%	%	%	%	%	%
Pre-treatment (March)	36.9 (10-255)	100		22.8 (4-83)	100		19.9 (14-32)	100	
Post-treatment but pre-season (May)		642* (46-2385)	100		654* (11-10088)	100		77 (13-211)	100
Season (July)		778† (130-3523)	121	NT	—	—	NT	—	—
Post-season (October)		672 (76-2710)	104		293‡ (16-1447)	45‡		132 (21-518)	171

Significant increase from pretreatment: * $P < 0.005$; significant increase from preseason: † $P < 0.1$
significant decrease from preseason: ‡ $P < 0.05$.

Table 6. The antibody ratio IgG/IgE $\times 10^3$ and its changes during the observation period. Ranges are given in brackets. NT, not tested

Time of blood sampling	Group A			Group B			Group C		
	Ratio	%	%	Ratio	%	%	Ratio	%	%
Pre-treatment (March)	46.1 (4-346)	100		66.7 (9-268)	100		74.7 (11-2759)	100	
Post-treatment but pre-season (May)	449* (56-4033)	100		509§ (58-10222)	100		100 (22-728)	100	
Season (July)	232† (36-3816)	52		NT	—	—	NT	—	—
Post-season (October)	226‡ (26-4813)	50‡		141‡ (25-856)	28‡		73 (17-639)	73	

Significant increase from pretreatment: * $P < 0.001$; § $P < 0.01$; significant decrease from pre-season: † $P < 0.02$; ‡ $P < 0.005$.

treatment only (group B) had markedly reduced levels but not as low as before treatment or as the control group after season.

The ratio of Timothy-specific IgG/IgE. A significant increase of the ratio was found after treatment but before the season in both treated groups (Table 6). Post-seasonally the ratio in group B had decreased to approx. 30% of the preseasonal value compared to 50% in group A. The ratio in group C changed little.

There was no correlation between the IgG/IgE ratio and the degree of clinical improvement as reported by the patients. Attempts to find a mathematical relationship between the concentration of Timothy-specific IgG antibody combined with the IgG/IgE antibody ratio and clinical improvement also failed.

Discussion

The severity of the allergic symptoms during the pollen season depends on the degree of hypersensitivity of the patient and the degree of exposure to the offending allergen. Both these parameters may vary from one season to another and, therefore, it is very difficult to know whether, in a particular patient, an improvement depends on such spontaneous variations or on the therapy given. It is therefore an open question whether the improvement reported by nine of the treated patients in this study was an effect of the therapy. The fact that in most of these patients no decreased sensitivity was found by the provocation test or in the leucocyte challenge test does not support a beneficial effect of the therapy. However, since in the tests the allergen dose in the titration procedure was increased by a factor of ten each time smaller changes of the sensitivity threshold could have been overlooked.

After the 5 preseasonal weeks of immunotherapy the concentration of IgE and Timothy-specific IgE antibody had increased similarly in both treated groups. The values of the control group had decreased further as a result of the absence of Timothy allergen stimulation during that period. The concentration of Timothy IgE antibodies increased during the pollen season in all three groups. Thus, we were not able to suppress by immunotherapy the seasonal rise of IgE as has been claimed by others

(Sadan *et al.*, 1969; Levy *et al.*, 1971). This can be due to the lower dose of allergen given preseasonally in our study but, on the other hand, even a high dose treatment does not consistently prevent the seasonal rise of IgE antibodies (Yunginger & Gleich, 1973). Although no blood samples from late season (July) were available from the patients in group B and C it seems reasonable to believe that the mean concentrations of IgE and Timothy-specific IgE were high at this time and that the results postseasonally (October) represent decreasing values.

The occurrence of Timothy-specific IgG antibodies showed a similar pattern to that of the IgE-antibodies but the initial increase during immunotherapy was thirteen to nineteen times greater. This increase of the concentration of 'blocking' antibodies is in parity with that reported by Sadan *et al.* (1969) using a biological test system. Although the exposure to pollen during the season seemed to stimulate the production of Timothy-specific IgG antibodies this effect was much less pronounced than that caused by the therapy given. In comparison with the natural exposure to pollens the immunotherapy favoured the production of IgG over IgE antibodies; therapy gave approx. a five times increase of the IgG/IgE antibody ratio compared to a 25% decrease during season in the control group. In this respect the aqueous and the depot extract appeared to be equally potent.

In commercially available allergen extracts the allergen usually represents only a minor part of the protein content of the extract (Baer *et al.*, 1970). Even preparations of 'purified' allergens probably contain non-allergenic protein to a great extent, a fact that is of great importance for the *in vitro* determination of 'blocking antibody'. Thus, the appearance during a course of immunotherapy of non-reaginic antibody activity to the allergen preparation used in the assay does not necessarily reflect a production of 'blocking antibody'. The injection of non-allergenic antigens will stimulate an antibody synthesis that will be detected in the test system but is of no obvious benefit to the patient. When isotope-labelled allergen preparations are used an additional hazard is introduced; non-allergenic material might be selectively labelled. Our ¹²⁵I-labelled Timothy allergen preparation had equivalent immunoreactivity with regard to its binding to IgE and IgG antibodies indicating a high percentage of allergenic material labelled. However, the immunoreactivity was rather low, approx. 20%, and the presence of non-allergenic protein labelled which might have influenced the results giving falsely high values of IgG-antibody after treatment cannot be excluded.

Preseasonal treatment only is regarded to be less effective than perennial therapy. However, the former kind of treatment has the obvious advantage of fewer injections and visits to the doctor. Because of the short observation period and the lack of objective criteria for the evaluation of clinical symptoms, our results do not allow any definite conclusions regarding the clinical effectiveness of the two kinds of immunotherapy given. However, the immunological short-term effect regarding changes of the concentration of IgE and IgG antibodies was the same.

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