

Complexes of grass pollen allergens and specific antibodies reduce allergic symptoms and inhibit the seasonal increase of IgE antibody

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Summary

Complexes made from antigen and specific antibodies have been used to suppress specific antibody production. This property is of potential therapeutic interest in immediate hypersensitivity states which are characterized by hyperproduction of IgE antibodies. We report here on the use of antigen–antibody complexes in patients with hypersensitivity to grass pollen. Specific anti-allergen antibodies were prepared by immunoadsorption from the serum of hypersensitive individuals and mixed with grass pollen allergens to form complexes in antibody excess. These complexes were used in a strictly autologous manner for inoculating patients prior to and during a pollen season. The study comprised two randomly defined groups of 15 patients who were inoculated intradermally either with a preparation of allergen–antibody complexes or with the carrier buffer, according to a double-blind protocol. Diary cards were used to follow nasal and ocular symptoms, bronchial asthma and medication intake. Specific IgE antibodies were assayed during the trial and 1 year afterwards.

Inoculation of allergen–autologous antibody complexes was well tolerated. It significantly reduced ocular symptoms (Mann–Whitney *U*-test, $P < 0.05$), bronchial asthma during the first part of the season (Mann–Whitney *U*-test, $P < 0.001$) and drug intake (Mann–Whitney *U*-test, $P < 0.001$). This treatment prevented the seasonal increase in specific IgE antibodies, whose production continued to decrease after the pollen season. These effects were obtained within a few weeks of treatment, using a cumulative amount of allergen 100-fold lower than the amount which would have been used for a conventional hyposensitization.

Inoculation of allergen–antibody complexes might prove to be a valuable alternative for the treatment of immediate hypersensitivity.

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Introduction

Animal experiments have shown that antigen–antibody complexes are able specifically to suppress antibody production, provided that they are formed with an excess of antibody [1] of autologous origin [2]. The mechanism of this suppression has not been entirely elucidated, although a variety of possibly relevant effects have been

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described. Antigen–antibody complexes can induce antigen-specific T suppressor cells [3], stimulate the production of anti-idiotypic antibodies [4], impair the function of antigen-specific B cells [5] or suppress antibody production by clonal competition with the immune response to unmasked antigenic determinants [6].

Immediate hypersensitivity is characterized *inter alia* by the increased production of IgE antibodies; a logical approach to the treatment of IgE-mediated diseases would therefore be to reduce the production of such

antibodies. Animal experiments on IgE regulation cannot be extrapolated to the human situation. However, this does not necessarily apply to the use of antigen-antibody complexes, which may present an effective way of achieving such an objective. In fact immediate hypersensitivity may represent an ideal situation for the use of antigen-antibody complexes, since IgE antibody production is exquisitely sensitive to T-cell suppression [7] and to regulation by idiotype-anti-idiotype interactions [8]. Suppression of IgE synthesis may be easier to achieve than that of other isotypes because of its relatively short half-life and low number of B cells pre-committed to IgE production [9]. In addition, even if the administration of antigen-antibody complexes resulted in an increase in allergen immunogenicity and a consequent stimulation of specific IgG antibody synthesis, then it could be argued that this could in any case benefit the patients since an increase in specific IgG antibodies is the usual result of conventional hyposensitization.

In an attempt to evaluate the immunosuppressive potential of antigen-antibody complexes in human immediate hypersensitivity, we have isolated anti-allergen antibodies by immunoadsorption from the plasma of grass-pollen hypersensitive patients. These antibodies were mixed *in vitro* with the corresponding allergen to form complexes which were administered to the patients from whom the antibodies had been prepared.

The present study is a first appraisal of the reaction to inoculation of complexes in grass-pollen hypersensitivity and evaluates the effect of such therapy on *in-vivo* IgE production during and after the grass-pollen season. Thirty patients were randomly separated into two groups. The first group was treated by inoculations of complexes, while the second group was inoculated with the carrier buffer alone. Symptoms were evaluated during the pollen season together with the level of specific IgE antibodies.

Materials and methods

Patients

Thirty patients (16 females and 14 males, average age 30 yr; range 14–57 yr) participated in this study after informed consent had been obtained. They were all suffering from grass-pollen allergic rhinitis and 18 of them had a past history of bronchial asthma related to pollen exposure. Conjunctivitis was regularly associated with rhinitis while cutaneous symptoms were rare. A retrospective evaluation of the severity of symptoms during the pollen season of the previous year was obtained by means of a questionnaire.

Skin-prick tests were positive in all cases to at least three common grass pollens out of six tested: cocksfoot,

fescue, meadow, rye, sweet vernal and timothy. Twenty-one patients also presented positive skin tests to other allergens, mostly house dust mite and cat (standard extracts containing either *Der pI* and *Der pII* or *Fel dI*).

Seventeen patients (56%) had received conventional hyposensitization with aqueous extracts of grass pollens in the previous few years. This treatment, comprising regular subcutaneous inoculations of allergen for a minimum of two consecutive years, had resulted in only a mild and/or short-lasting clinical benefit.

Grass pollen allergens

An extract of grass pollen (B2), containing 12 pollen allergens commonly encountered in Western Europe, was purchased from Bencard Ltd (Epsom, Surrey, UK), dialysed extensively against phosphate-buffered saline, pH 7.4 (PBS), and concentrated to 3 mg/ml using an Amicon filtration cell (Amicon Corp., Danvers, MA) fitted with YM10 ultrafiltration membrane. The allergen solution was divided into aliquots and stored at -80°C until just before use.

Immunosorbent

B2 pollen extract was coupled to carbodiimide-activated CH1-Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions, using 1 mg protein/ml of Sepharose. After overnight incubation at 4°C , excess reactive groups were neutralized by the addition of 1 M glycine for 2 hr at 21°C . The B2-Sepharose was then washed sequentially with two cycles of acid and alkaline buffers, 3 M potassium thiocyanate and PBS. The final PBS solution contained sodium azide (0.5% w/v) and the B2-Sepharose was stored at 4°C .

Preparation of specific anti-allergen antibodies

Plasma was prepared from 100 ml blood samples from each patient and a 7S-enriched fraction was prepared by precipitation with 180 g/l Na_2SO_4 and gel filtration chromatography over a 5×90 -cm column of Fractogel TSK HW-55F (Merck, Darmstadt, FRG). Anti-grass pollen allergen antibodies were then isolated by immunoadsorption on B2-Sepharose and after extensive washing were eluted with 50 mM citric acid buffer, pH 2.7 [10]. The pH was adjusted to neutrality by dropwise addition of Tris (hydroxymethyl)-aminomethane and the antibody solution then dialysed extensively against PBS.

Preparation of allergen-antibody complexes

The ratio of allergen to antibody in the complexes was calculated from the concentration of specific antibodies

Table 1. Allergen-antibody complex injection schedule

Day	Volume (μ l)	Antibody (μ g)	Antigen (ng)
1	20	0.08	8
7	40	0.16	16
14	80	0.32	32
21	120	0.48	48
28	160	0.64	64
35	20	0.8	80
42	25	1.0	100
49	30	1.2	120
56	40	1.6	160
63	50	2.0	200
70	60	2.4	240
77	70	2.8	280
84	80	3.2	320
Total		16.68	1668

able to neutralize the weal-and-flare reaction resulting from intradermal inoculation of a standard concentration (4 μ g/ml of total protein) of B2 pollen extract. These skin tests were performed on the patients selected for the study 6 months prior to its beginning. It was found that on average 40 μ g/ml specific antibodies complexed with allergen was sufficient to prevent the skin reaction. A ratio of 1/10 (w/w) of antigen to antibody was therefore chosen for all subsequent investigations in this study. However, as not all proteins in the B2 allergen extract are allergens, the actual ratio of antigen over antibody is smaller than 1/10.

Specific antibodies of single patients were diluted to a final concentration of 100 μ g/ml in a solution containing 9 g/l NaCl, 0.3% human serum albumin and 0.4% phenol. An equal volume of B2 solution containing 10 μ g/ml in the same buffer was added and the mixture was incubated for 60 min at 21°C and then overnight at 4°C. The stock solution of complexes prepared at 1/10 ratio of allergen to antibody was then diluted in the same buffer to two working solutions containing respectively 4 μ g/ml antibody with 0.4 μ g/ml B2 pollen extract and 40 μ g/ml antibody with 4 μ g/ml B2 extract. The two solutions were divided into aliquots and stored at -80°C until administration. All injections were made intradermally on the external site of the arm, according to the schedule given in Table 1.

Clinical follow-up

The patients were asked to fill in a daily diary card listing symptoms and medication intake using a discontinuous

intensity scale. Nasal (obstruction, sneezing and rhinorrhea), ocular (redness, itching and watering), bronchial (cough, wheezing, nocturnal and diurnal asthma) and skin symptoms (urticaria and itching) were scored from 0 (no symptom) to 4 (very severe symptoms). Medication intake was graded 0: no drug, 1: local vasoconstrictor, 2: oral antihistamines and 3: oral corticosteroids.

Specific anti-allergen antibodies

Specific anti-grass pollen IgE antibodies in the patients' serum were assayed in a single batch by an ELISA performed as follows. Ninety-six well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 100 μ l grass-pollen allergens diluted to 10 μ g/ml in 50 mM glycine buffer, pH 9.2. After an incubation of 48 hr at 4°C, the plates were washed three times with PBS, and further incubated overnight at 4°C with PBS containing 5 mg/ml bovine serum albumin (PBS-BSA). After three washes with PBS and two with distilled water, 100 μ l of 1/50 dilutions of serum samples in PBS-BSA were added and incubated 24 hr at 4°C. The plates were then washed three times with PBS and 50 μ l of biotin-conjugated anti-human Fc ϵ rabbit IgG [11] added and left for 24 hr at 4°C. After three further PBS washes, 50 μ l of 1 μ g/ml peroxidase-conjugated avidin (Sigma Chemical Co., St Louis, MO) were added and left for 30 min at 21°C, after which the plates were washed again before addition of 50 μ l of a solution containing ortho-phenylene-diamine and 0.2% H₂O₂. Colour development was stopped after 10–15 min by addition of 3 M H₂SO₄ and the plates were read in a Kontron SLT 210 spectrophotometer (SLT-LAB Instruments, Salzburg, Austria).

Specific anti-allergen IgE antibodies were assayed using allergen-coated latex particles (LAST; 12) for the analysis of the serum samples collected 1 yr after the beginning of the trial. Specific IgE results obtained in the ELISA test correlated well with those obtained in the LAST system ($r \geq 0.95$).

Pollen counts

The number of pollen grains per cubic metre was measured daily during the pollen season using a Burkard volumetric trap (courtesy of Dr N. Noland, Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium).

Study design

The patients were randomly allocated into two groups, with the adequacy of randomization being checked by ensuring that no statistically significant differences existed between the two groups with respect to symptoms

described in the previous year and to the number of individuals who had been desensitized by conventional means. Group 1 patients were inoculated with allergen-antibody complexes made from autologous specific antibodies and grass pollen allergens at a ratio of 10/1 (w/w).

Injections were given every week, according to the schedule given in Table 1, for a total of 13 injections, starting 1 month prior to the expected pollen season and carried on over the next three months. Group 2 patients were inoculated with the carrier buffer alone, i.e. which contained albumin and phenol. The same physician (JM), who was unaware of the treatment group to which the patients belonged, performed all the injections.

Statistics

For each group, indices of symptom intensity were calculated as follows. The daily mean of symptom scores was determined from the diary card data by summing the individual scores and dividing by the number of patients in the group. This mean was then multiplied by the percentage of patients within the group who reported non-zero scores, to yield an index of group symptom intensity. It was considered that this index represented the collective symptoms of the group better than the simple means. Non-parametric statistical analyses (Mann-Whitney *U*-test or Wilcoxon matched-paired tests) were used to compare the symptom indices of the two groups and also for the comparison of the levels of specific antibodies in the two groups.

Results

Preparation of specific anti-allergen antibodies

Specific antibodies were prepared as described above, i.e. using fractionation procedures prior to immunoadsorption which would exclude any specific antibodies of IgM isotype. From 100 ml of serum a mean of 0.6 ± 0.05 (SEM) mg antibodies was isolated with an isotypic distribution showing more than 85% IgG, 0.1% IgE with the remainder being IgA. These antibodies retained their functional activity; Fig. 1 shows that the addition of specific anti-B2 antibodies to B2 allergens at a ratio of 10/1 (w/w) results in a nearly complete disappearance of the weal reaction obtained by intradermal inoculation of allergens alone. A mean area of 80 mm² was observed after inoculation of 20 μ l of a solution containing 40 μ g B2/ml complexed with 400 μ g antibodies/ml, while 0.6 μ g/ml of uncomplexed allergen produced a skin reaction of the same size.

Pollen counts

Figure 2 shows the air concentration of grass pollen grains. This shows that the onset of raised pollen counts is

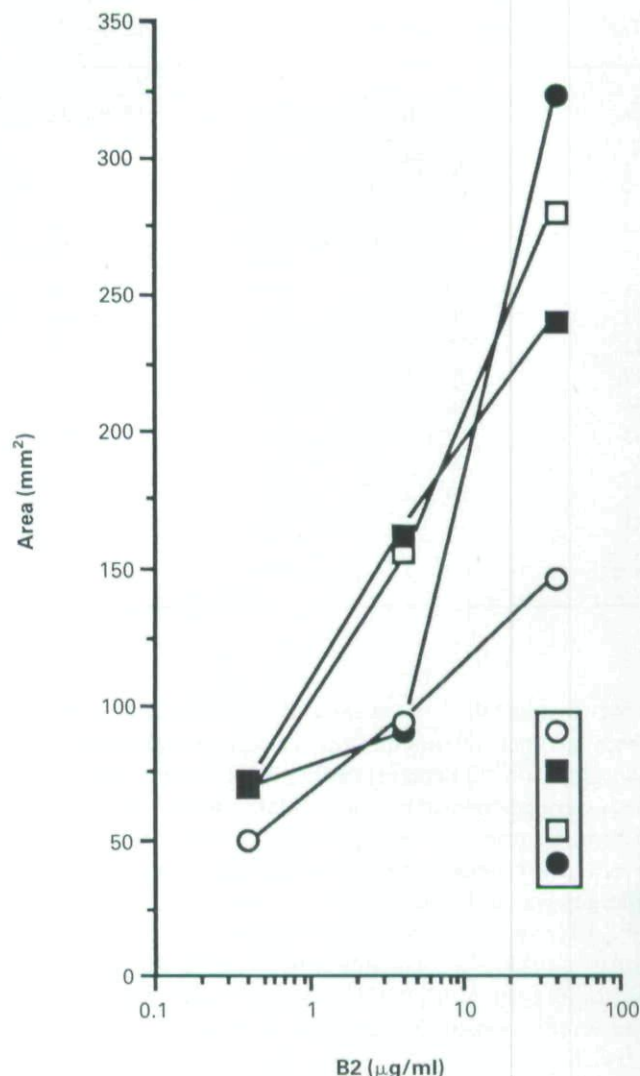


Fig. 1. Weal area resulting from intradermal injection of different concentrations of grass pollen allergens in the back skin of four hypersensitive patients. Framed symbols show the neutralization of 40 μ g/ml B2 by specific antibodies at a 1/10 ratio (w/w).

2 weeks later than usual and the duration of the pollen season shorter than usual (50 days instead of a usual 75). In addition, the period of peak pollination lasted only 4 weeks instead of the usual 7.

Clinical evaluation

Compliance and toxicity. All the patients completed the study, but records of two patients in each group were not sufficiently filled in to allow for meaningful analysis. Injections were well tolerated: the diameter of the immediate local reaction was minimal and in no case exceeded

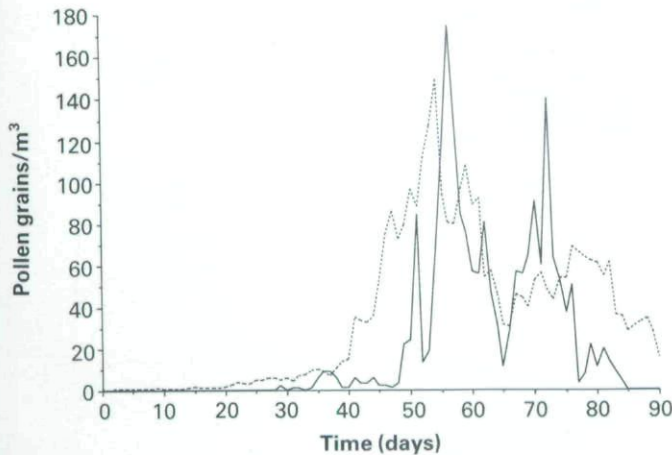


Fig. 2. Grass pollen air concentration measured daily using a Burkard volumetric trap (—), compared to the mean pollen count over the last 15 years (.....).

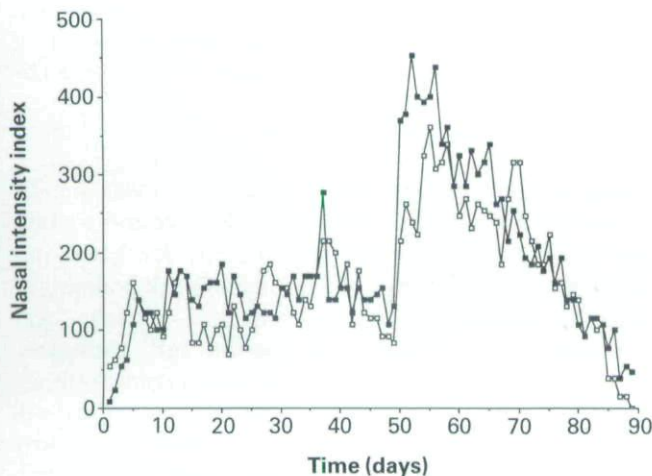


Fig. 3. Evolution of nasal symptoms during the pollen season. Each point represents the daily mean of a score combining sneezing, rhinorrhea and obstruction, multiplied by the incidence of symptoms in the group considered. (□) Treated group; (■) placebo group.

2 cm. No signs of systemic reaction were observed. No effect of previous desensitization could be identified when comparing the clinical evolution of the patients during the trial; however, there was a trend toward an increased drug intake in previously treated patients.

Symptom scores. Nasal symptoms. Nasal scores including obstruction, rhinorrhea and sneezing were calculated by multiplying mean values by the incidence of symptoms.

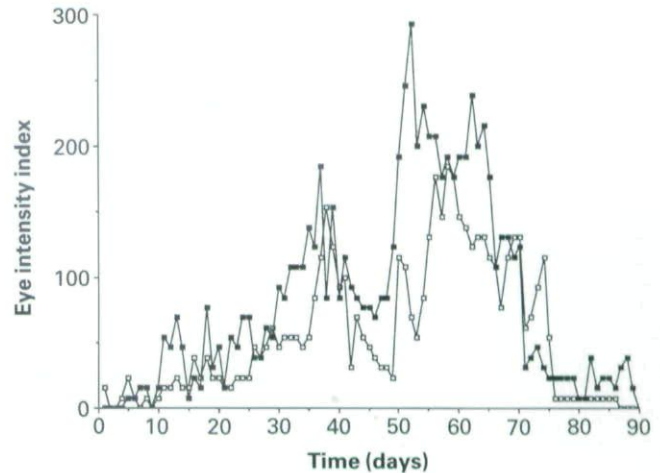


Fig. 4. Evolution of ocular symptoms during the pollen season. Each point represents the daily mean of a score combining itching, erythema and watering, multiplied by the incidence of symptoms in each group. (□) Treated group; (■) placebo group.

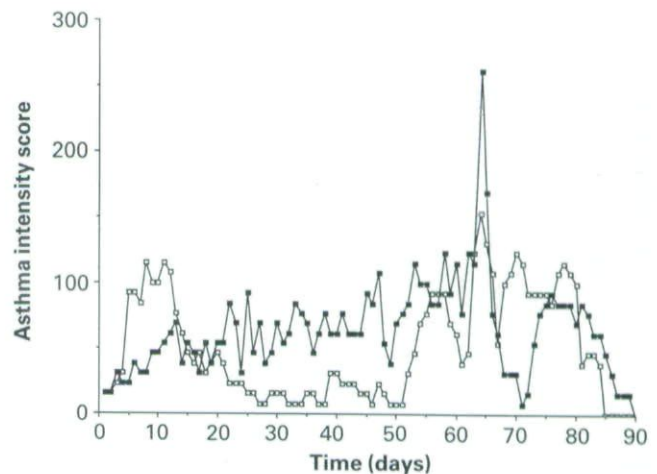


Fig. 5. Evolution of the daily mean bronchial asthma score, multiplied by the incidence of symptoms in each group. (□) Treated group; (■) placebo group.

Figure 3 shows that the increase in nasal symptoms related to pollen exposure was mild in both groups, due no doubt to the low pollen count. In each group only 5/13 patients had a score ≥ 4 , while the maximum theoretical score was 12 (score 4 for obstruction, sneezing and rhinorrhea, respectively). No significant difference was observed between group A and B, but during the period of highest pollen air concentration (days 51–52), treated patients had lower scores than placebos (Mann-Whitney *U*-test, $P < 0.06$). **Ocular symptoms.** Acute conjunctivitis

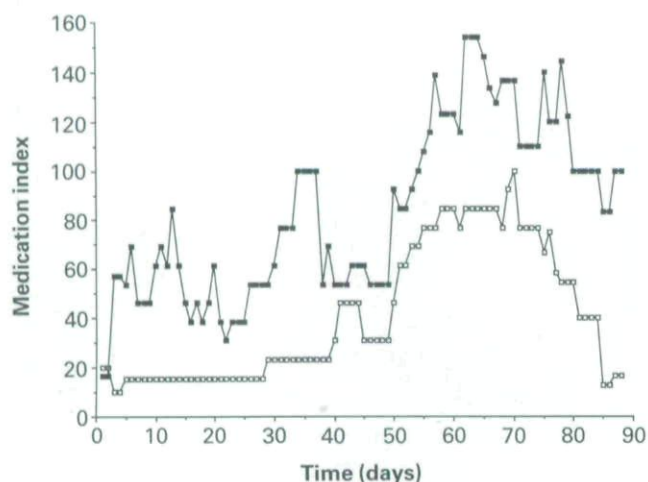


Fig. 6. Mean intake in each group multiplied by the percentage of patients using this medication. (□) Treated group; (■) placebo group.

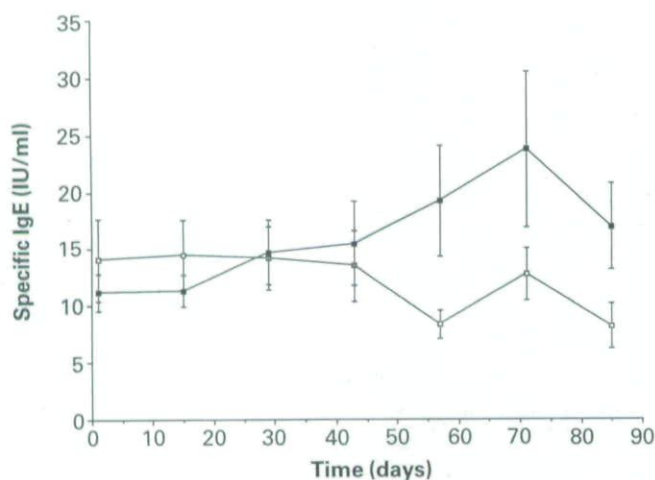


Fig. 7. Evolution of serum specific anti-B2 IgE antibodies assayed by ELISA.

was evaluated by scoring eye itching, watering and erythema. Results given in Fig. 4 illustrate that, although symptoms remained mild in both groups, significant differences between the two groups were observed during the early pollen season (days 20–49) and the first pollen peak (Mann–Whitney *U*-test, $P < 0.01$ and $P < 0.05$, respectively). **Bronchial asthma.** Only 38% of placebo patients suffered from bronchial asthma, while the expected frequency based on report for the previous year was 60%. Figure 5 indicates, however, that treated patients had significantly lower asthma scores during early pollen season (days 20–49, Mann–Whitney *U*-test, $P < 0.001$), but that this difference did not persist during the peak of the pollen season.

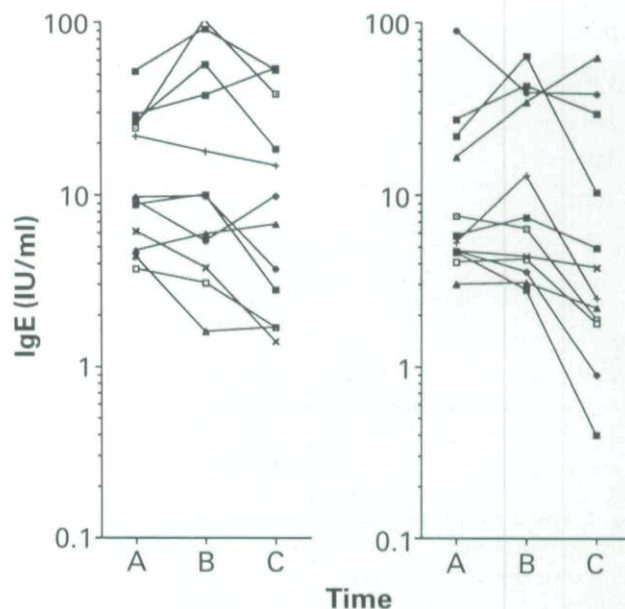


Fig. 8. Specific anti-Rye grass pollen IgE antibodies evaluated before (A) and after (B) the pollen season, and prior to the subsequent pollen season (C). The left panel represents individual data in the placebo group and the right panel patients of the treated group. IgE antibodies were measured in the serum by latex allergosorbent test (LAST).

Medication scores. The use of medications was significantly lower for the treated patients over the entire study period (Fig. 6; Mann–Whitney *U*-test, $P < 0.001$ and $P < 0.001$ for both early pollination and peak pollination periods, respectively). No patient had to take oral corticosteroids. **Evolution of specific IgE antibodies.** Figure 7 shows that inoculation of allergen–antibody complexes effectively prevented the seasonal increase in specific anti-grass pollen antibodies which was clearly observed in placebo patients. Differences between placebo and treated patients were significant for the last three samples (Mann–Whitney *U*-test, $P < 0.05$). In contrast, no significant change was observed in the evolution of total IgE antibody concentrations (data not shown). Figure 8 shows that the specific anti-rye grass IgE concentration decreased after the end of the pollen season, although no further injections were administered to the patients. A significant difference (Wilcoxon matched-paired test, $P < 0.05$) was observed between the concentration of specific IgE in treated patients prior to this study and exactly 1 year afterwards; this was not the case in the placebo group.

Discussion

This study describes the treatment of grass-pollen hypersensitive patients using allergen–antibody complexes

administered prior to and during a pollen season. It was designed to examine whether inoculation of complexes would suppress the production of specific anti-allergen antibodies, whether the treatment would be well tolerated and whether a clinical benefit could be observed.

Allergen-antibody complex inoculations efficiently prevent the seasonal increase in specific anti-allergen antibodies. Although the same observation has been made with conventional hyposensitization therapy [13], several differences can be observed. In the present study, prevention of IgE antibody increase was achieved with a lower amount of allergen, obtained faster and appeared to last even after the pollen season. The allergen preparation used here is the same as that used in conventional immunotherapy, and it can be calculated that the cumulative amount of free allergen administered to patients is on average 350-fold greater than the total amount of allergen used in the present study in the form of complexes with autologous specific antibodies. With conventional immunotherapy, it is unusual to observe a significant decrease in the IgE immune response to pollen exposure before the second year of the therapy [14], whereas this effect was achieved within a few weeks in this study. Finally, our data indicate that, at least for some patients, specific IgE concentrations decreased after the end of the pollen season and therefore after the course of inoculations had ceased. Prior to the subsequent pollen season, the level of specific IgE antibodies in treated patients was significantly lower than before the first season. In the placebo group, IgE concentrations returned to initial values over the next months. We therefore conclude that allergen-antibody complex treatment results in an active reduction in IgE antibody production, rather than a mere prevention of the IgE immune response. This was subsequently confirmed in a study on *Dermatophagoides pteronyssinus* hypersensitivity [15], where a parallel decrease in specific IgE and IgG antibodies was observed.

The inoculations were well tolerated with reaction being limited to an immediate local reaction of moderate size with no delayed-type reaction, at least as reported by the patients themselves; no systemic reaction was observed. Although the number of injections given during this study is too small (13 injections in 15 patients) to allow a definite conclusion, there are several factors which might explain why the treatment with allergen-antibody complexes is safe. The largest amount of allergen administered in a single injection was 320 ng, while a typical maintenance dose of 34 µg of allergen is used during a conventional treatment with the same material. Secondly, as shown in Fig. 1, the specific antibodies present in the preparation efficiently neutralized the weal reaction resulting from intradermal inoculation of allergen. The residual skin reactivity we observed in a few patients

could result either from complex dissociation or a failure to mask IgE-binding epitopes by specific antibody; the immobilization of allergen on a solid support which was used in the immunoadsorption process may have altered or rendered some epitopes inaccessible to antibody, thereby reducing the relative amount of such antibodies in the complex and consequently retaining some allergenicity of the pollen in the complex. Thirdly, the mere fact that inoculations were made intradermally may limit the diffusion rate of allergen and so reduce immediate reactivity.

Modest clinical benefits were demonstrated by both the reduction in symptoms and medication intake. The pollen season was, however, unusual in its low pollen density and short duration, thus reducing both the incidence and the severity of symptoms. Differences between treated and untreated patients were consequently not spectacular and meaningful statistical analyses of differences limited by the low number of patients in each group. The significant reduction in medication intake with no or only marginal effect on nasal symptoms is interesting in this respect: symptoms were mild enough to be tolerated without medication. Likewise, the proportion of patients suffering from asthma related to pollen exposure was much lower than might have been expected based on the patients listing of reactions in previous pollen seasons.

The allergen extract used in the present study is a mixture of 12 of the most common grass pollens found in Belgium. However, it actually contains a large amount of rye grass and timothy grass pollens (data not shown). This may have had two effects. Firstly, whereas every patient had specific IgE antibodies to rye grass, but at concentrations varying from 3 to 50 IU/ml, some patients had no detectable anti-timothy IgE antibodies. The allergen extract used here thus may not have been adequate for every patient, even though there is a large crossreactivity between grass pollens. Secondly, as the pollen season was 2 weeks later than usual, it might be that the proportion of the different pollen species present in the air differed from usual. An indication of the possible mismatching between the allergen extract used in the complexes and the pollen actually present in the atmosphere is given by the excellent control of bronchial asthma only during the first part of the pollen season. The precise identification of pollen species obtained by volumetric traps is exceedingly difficult and was not attempted in this study. For further clinical trials, it might be more appropriate to use pollen mixture more closely matching the IgE 'profile' of the patients or, better, single pollen species as ragweed.

The rationale of this study was to examine the suppressive properties of allergen-antibody complexes on the production of IgE antibodies. At least the *de-novo*

synthesis of IgE antibodies seems to be reduced as a result of the treatment. However, it is difficult to relate the clinical results directly to suppression of IgE, since very low concentrations of specific IgE are sufficient to trigger symptoms upon exposure to the relevant allergen. In another study (J. J. Machiels *et al.*, manuscript submitted), we have shown that allergen-antibody complexes do not result in an increase in specific IgG concentrations. Other factors may therefore be involved in the mechanism of action. For instance, we have observed an increase in the level of anti-idiotypic antibodies specific to anti-allergen antibodies (J. J. Machiels *et al.*, manuscript submitted).

From this preliminary trial, we can conclude that inoculation of allergen-antibody complexes is a safe procedure which results in an effective prevention of specific IgE antibody increase during pollen exposure, even though the quantity of allergens used was much lower than for conventional desensitization. Based on this preliminary study, we believe that this approach warrants further exploration.

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