

Clinical and immunological effects of immunotherapy with alum-absorbed grass allergoid in grass-pollen-induced hay fever

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A double-blind, placebo-controlled study of immunotherapy was conducted in 19 patients with grass-pollen hay fever to evaluate the efficacy and safety of a formalinized depot grass allergoid. The patients were assessed before and during IT by clinical (symptom-medication scores during the grass-pollen season, specific nasal and skin reactivity) and immunological (specific IgE, IgG, IgG₁ and IgG₄ antibodies) parameters. High doses of grass allergoid, corresponding to a cumulative pre-seasonal dosage of 46050 PNU, were administered, with only one systemic reaction. The actively treated patients had significantly lower symptom-medication scores than placebo ($p < 0.01$) during the month of May and showed a significant decrease in specific skin ($p < 0.01$) and nasal ($p < 0.05$) reactivity, and a significant early increase in specific IgE ($p < 0.01$), IgG ($p < 0.0005$), IgG₁ ($p < 0.001$) and IgG₄ ($p < 0.05$), with a subsequent decrease of IgE and IgG₁. No differences were detected in any of these parameters in the placebo group. A correlation was found between high IgG₄/IgG₁ ratio and the specific skin reactivity decrease ($r = 0.691$, $p < 0.05$), whereas a high IgG₄/IgG₁ ratio was associated with higher symptom-medication scores ($r = 0.654$, $p < 0.05$). Possible explanations of these apparent discrepancies are proposed.

E. A. Pastorello, V. Pravettoni, C. Incorvaia, M. Mambretti, E. Franck *, R. Wahl *, C. Zanussi

Allergy Center, First Department of Internal Medicine, University of Milan, Italy, and
* Allergopharma Joachim Ganzer KG, Reinbek, Hamburg, Germany

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Dott. Elide A. Pastorello
Clinica Medica I
Padiglione Granelli
Ospedale Policlinico
Via F Sforza 35
20100 Milano
Italy

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Immunotherapy (IT) for inhalant allergens has been shown to be clinically effective in numerous controlled studies employing extracts with high allergenic potency and high doses, but the question of safety remains open. In the 1970s allergoids were proposed as a solution to this problem, since, theoretically, they should have less allergenicity (and consequently a lower rate of adverse reactions) while retaining enough immunogenicity to elicit significant clinical effects and IgG response.

The first studies, by Norman et al. (20), used ragweed formalinized allergoids and reported good clinical effectiveness and a strong IgG response. However, the rate of adverse reactions during treatment with these allergoids was not significantly lower than in patients treated with aqueous conventional extracts. Similar observations were reported by Bousquet et al. (6) in a more recent study with a formalinized grass allergoid.

We evaluated the efficacy and safety of a formaldehyde-modified grass allergoid absorbed by aluminum hydroxide. In this double-blind, placebo-controlled study we assessed subjective and objec-

tive clinical parameters, such as symptom scores during the grass-pollen season, and specific nasal and skin reactivity. We followed immunological parameters such as IgE, IgG, IgG₁ and IgG₄ antibodies specific to a grass-pollen extract to assess their behavior at different times during treatment. Particular attention was paid to the correlation between IgG₄ and IgG₁ antibodies and clinical parameters with the aim of investigating the role of allergen-specific IgG subclasses during IT, which is still a matter of debate.

Material and methods

Patients

Nineteen patients (12 females and 5 males; mean age 27.4 years, range 18–56 years) were selected among outpatients at our Allergy Center on the basis of the following criteria: 1) seasonal rhinoconjunctivitis with or without asthma for at least 3 years; 2) positive skin prick test (SPT) for a mix of grass-pollen extracts (*Dactylis glomerata*, *Festuca elatior*, *Holcus lanatus*, *Phleum pratense*, *Lolium perenne*, *Poa*

pratensis) (Allergopharma Joachim Ganzer KG, Hamburg, Germany), at a concentration of 5000 PNU/ml; SPT had to present a mean area twice that elicited by a SPT with histamine hydrochloride solution at concentration of 1 mg/ml; 3) negative SPT for all the other pollens in our area, such as birch, hazel, alder, mugwort, and wall pellitory; 4) positive RAST for grass pollen (at least class 3, Phadebas RAST, Pharmacia); 5) no previous specific IT with grass-pollen extracts.

Study design

The design was double-blind, placebo-controlled. Patients were randomly assigned to active treatment or to placebo. Active treatment consisted of a grass-allergoid obtained by mild formalinization of a grass pollen extract (six grasses, *Dactylis glomerata*, *Festuca elatior*, *Holcus lanatus*, *Phleum pratense*, *Lolium perenne*, *Poa pratensis*) according to the procedure described by Marsh et al. (19). Residual allergen activity was <1%, as compared with to the unmodified six-grass allergen extract as measured by RAST inhibition assay. The molecular size pattern changed to greater "homogeneity" and higher molecular weights, determined by size exclusion chromatography (HPLC with TSK-250 column). The allergoid was absorbed onto aluminum hydroxide, and the content was expressed in protein nitrogen units (PNU). PNU was determined according to the method of Berthelot (4). The alum hydroxide concentration of allergen and placebo extracts was 1 mg/ml at maximum strength, 0.25 mg/ml at median strength and 0.025 mg/ml at the lowest concentration. Placebo consisted of a caramel NF acid solution (USP) with histamine hydrochloride solution randomly added to approximately 50% of the placebo vials with the following concentrations: maximum strength 0.5 mg/ml, median strength 0.125 mg/ml, lowest strength 0.0125 mg/ml. This was to ensure real double-blind conditions.

IT started in January 1986 with weekly injections of increasing dosages until a top dose of 20 000 PNU, or the maximum tolerated dose, had been reached. Doses were administered weekly until the middle of April, after which a 50% equivalent dose was given every 3 weeks as maintenance.

Clinical efficacy was evaluated by recording seasonal symptoms on diary cards and recording changes in grass-specific nasal and skin reactivity before, during and after IT (Fig. 1).

In May and June patients recorded their daily symptoms and drug consumption in diaries. Each patient had to attribute separate values to nasal, conjunctival and bronchial symptoms with a score from 0 to 3 on the basis of severity. They had at the same time to enter the amount of drugs taken. Pa-

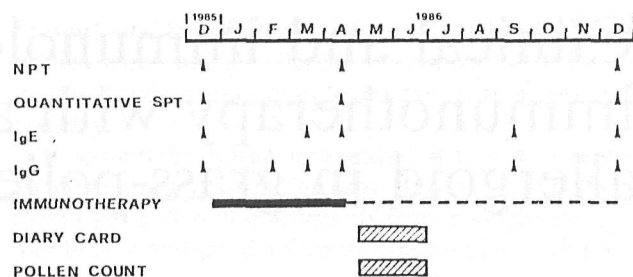


Fig. 1. Study design.

tients were allowed to use only symptomatic drugs, such as xylometazolin as nasal vasoconstrictor, terfenadine as antihistamine, and salbutamol as β -2-agonist. A score was attributed to drug consumption: 1 for each drop of nasal vasoconstrictor, 2 for each antihistaminic tablet, and 1 for each puff of β -2-agonist spray.

To assess specific skin reactivity, a quantitative skin test was done with a grass-pollen extract of the above mentioned six grasses dissolved in glycerinated normal saline and standardized in biological units (BU) (Allergopharma Joachim Ganzer KG). Standardization was performed on the mixed extract with each grass in equal weight proportion according to the method suggested by Nordic Guidelines (13). Four allergenic concentrations (500, 1500, 4500, 13 500 BU/ml) of the grass extract, histamine dihydrochloride solution (1 mg/ml) as positive control, and glycerinated normal saline as negative control were randomly repeated three times on the patient's volar forearm surface. For statistical analysis we considered the mean area elicited by the SPT with the solution at 1500 BU/ml and expressed it as a percentage of the histamine mean wheal. At each subsequent assessment of skin reactivity during IT, we employed the 1500 BU/ml solution as described.

Changes in nasal airway resistances were determined with a passive anterior rhinomanometer (NART, PK Morgan Ltd, Chatham, UK) after allergen challenge. To assess non-specific nasal reactivity, after two baseline determinations, the most patent nostril was challenged with 100 μ l of a human albumin solution nebulized at 4 l O₂/min. If an increase in nasal resistance not greater than 20% of baseline was recorded, the same nostril was challenged with increasing allergenic concentrations (100, 500, 1000, 5000, 10 000 BU/ml) obtained by diluting a starting solution at 100 000 BU/ml. Nasal resistances were recorded five times at four intervals, 2, 5, 10 and 20 min, after administration of each dilution until an increase greater than 500% of the baseline was reached or until the stock solution was administered. For statistical analysis we considered the allergen concentration in BU able to elicit the

desired increase in nasal resistance; in order to have numerical data, a value of 20000 was arbitrarily attributed to a negative test.

For immunological evaluation, changes in grass-specific IgE, IgG, IgG₁ and IgG₄ antibodies were measured before, during and after IT.

Grass-specific serum IgE antibodies were determined by the radioallergosorbent test (RAST) in either undiluted or 1:5 diluted sera. Phadebas RAST (Pharmacia Diagnostic, Uppsala, Sweden) was used, according to the manufacturer's instructions. The reference extract from Pharmacia was used as standard curve. Undiluted sera were used to define RAST classes, while 1:5 diluted sera were used to follow the treatment-related increases. In these cases, results were expressed as a percentage of radioactivity binding. For statistical analysis we took the percentage change from the baseline, considered as 100%.

Grass-specific serum IgG, IgG₁ and IgG₄ antibodies were assayed by an ELISA micromethod in solid phase, using a raw lyophilized extract of *Phleum pratense* (Allergopharma) as antigen. *Phleum pratense* was chosen, as it cross-reacts with all the other grasses, and contains all the relevant allergens according to grass standardization by WHO (15). The in-house *Phleum pratense* extract we used was previously tested against the WHO standard (30). This extract, at a 3 µg/ml concentration, was used to coat alternate rows of microtiter plates (Titertek, Flow Labs), incubated at 37°C for 2 h. The rows not coated were used as control. After aspiration, washing with a 0.1% gelatin and 0.05% Tween 20 solution in PBS, and drying with cold air, all the wells were filled with 0.1% gelatin in PBS for 1 h at 37°C. After aspiration, washing and drying, sera to be tested and a reference serum (a pool of hyperimmune sera with high levels of *Phleum pratense* specific IgG) were diluted in washing solution and each dilution was added to the wells. Sera were incubated overnight at room temperature for total IgG determination, and for 2 h at 37°C for IgG₁ and IgG₄.

After aspiration, washing and drying as above, the technique differed for total IgG determination and subclasses. For total IgG a peroxidase-conjugated rabbit anti-human IgG anti-serum (Miles) diluted in wash buffer was added and incubated for 2 h at 37°C. For IgG subclasses, a monoclonal mouse anti-human IgG₁ (ICN ImmunoBiologicals, Lisle, IL, USA; code 63-080) or IgG₄ (ICN ImmunoBiologicals, Lisle, IL; code 63-063), diluted in wash buffer, was added and incubated overnight at room temperature. After a further wash, a peroxidase-conjugated goat anti-mouse IgG antiserum was added for 1 h at 37°C.

Subsequently, for all determinations, after aspiration, washing and drying, o-phenyldiamine 0.5 mg/

ml and 0.04% H₂O₂ in 0.15 M citrate phosphate buffer pH 5.9 was added for 1 h at room temperature. The reaction was stopped by adding 1 N sulfuric acid and the results were read by a microtiter spectrophotometer (Titertek Multiscan, Flow Labs) at a wavelength of 492 nm. Results were expressed in net optical density (OD), subtracting the value read in the corresponding control well. For statistical analysis, results were expressed as the percentage change from the baseline, taken as 100%. Changes were evaluated by measuring the percentage increase in immunoglobulins over basal values, after normalizing them on a complete dose-response curve of the reference serum obtained from hyperimmunized patients. This was necessary to overcome technical problems with the ELISA method, which does not allow a proper assessment of the increases of IgG antibodies, compared, as baseline. We applied the following formula:

$R_1/R_b * FD_b * FD_1 * 100$ = percentage of increase over baseline of the sample at the assessment in question.

$R_1 = OD_{s1}/OD_{r1}$ where:

OD_{s1} = observed optical density of the sample at the assessment in question;

OD_{r1} = observed optical density of the reference pool where $OD_{r1} - OD_{s1} = \epsilon$ (value as low as possible).

$R_b = OD_{sb}/OD_{rb}$ where:

OD_{sb} = observed optical density of the sample at baseline;

OD_{rb} = considered optical density of the reference pool where $OD_{rb} - OD_{sb} = \epsilon$ (value as low as possible).

$FD_b = 2^n$ = reference pool dilution factor

where n = number of dilutions between OD_{rb} and OD_{r1} ;

$FD_1 = 2^z$ = sample dilution factor

where z = number of dilutions between OD_{sb} and OD_{s1} .

The IgG₄/IgG₁ ratios used in the correlation with clinical parameters and IgE antibodies were calculated by the percentage increases obtained as above, considering baseline as 100%.

The specificity for anti-human IgG₁ and IgG₄ of monoclonal antisera was guaranteed by the manufacturers on the basis of the following observations: positive reactions for normal human IgG₁ and IgG₄ (whole molecule and their Fab); no detectable cross-reactions with other IgG subclasses or IgM determined by ELISA. For coating the concentration of 1 µg/ml of IgG₂, IgG₃ and IgG₄ and 2.5 µg/ml of IgM as regards cross-reactions with IgG₁, and the concentration of 1 µg/ml of IgG₁, IgG₂ and IgG₃

and 5 µg/ml of IgM as regards cross-reactions with IgG₄ were used. We further checked the specificity by measuring the OD values before and after absorption of the reference pool with monoclonal anti-human IgG₄ and, separately, anti-human IgG₁ antiserum. Briefly, this was done by incubating overnight at 4°C the reference pool (consisting of hyperimmunized sera) diluted 1:20 in phosphate buffered saline 0.01 M pH 7.4 with an equal amount of monoclonal anti-human IgG₁ or anti-human IgG₄ antiserum diluted 1:10 in the same buffer. An equal amount of 5% polyethyleneglycol was added to insolubilize the immune complexes. After centrifugation at 3000 rpm for 1 h at 4°C the supernatant phase was tested in duplicate and in parallel against the non-absorbed reference pool at the same dilutions to detect the persistence of its content in specific IgG₁ and IgG₄ according to the ELISA method.

Adverse reactions during IT

Reactions were considered "immediate" when they appeared within 60 min of injection; "late" when they appeared after this time. Reactions were recorded as local or systemic. We considered as a local reaction the appearance of a wheal at least 10 cm in diameter if immediate and 5 cm if late. Systemic reactions were considered mild when only cutaneous reactions occurred; severe when respiratory or vascular symptoms occurred.

Statistical methods

Wilcoxon's signed rank test was used for the evaluation of skin and nasal reactivity or immunoglobulin changes during treatment within each group. Nasal reactivity was further analyzed by a non-parametric Fisher test. The Mann-Whitney U test was used for matching actively treated and placebo groups, and for symptom + medication scores. The non-parametric Spearman rank correlation was used for the coefficients of correlation between clinical and immunological parameters, confirmed by Pearson's test.

Results

IT schedule and adverse reactions

Under grass-pollen allergoid treatment patients received a mean cumulative pre-seasonal dose of 46 050 PNU (range 20 700–54 500 PNU). The mean maximum dose administered in a single injection was 16 250 PNU (range 4500–20 000 PNU). In 7 of the 10 actively treated patients the maximum single dose of 20 000 PNU could be reached without any adverse reaction. In the remaining 3 patients the

maximum dose could not be reached because of repeated late local reactions (4, 2 and 1 reactions) and a mild late systemic reaction (urticaria) in 1 (10% of actively treated patients); all these reactions appeared more than 4 h after the injection.

No patients in the placebo group experienced any adverse reaction.

Clinical efficacy

In May, the primary pollen season, when pollen count is maximum with a mean daily concentration greater than 40 grass-pollen grains/m³, the actively treated patients had significantly ($p < 0.01$) lower symptom and medication scores (Fig. 2). The difference was statistically significant for all the symptom and medication scores considered separately, i.e. nasal ($p < 0.01$), conjunctival ($p < 0.05$) and bronchial ($p < 0.05$). No differences were detected in June, when the pollen concentration in the atmosphere is lower (< 40 grains/m³) and both groups presented low symptom scores.

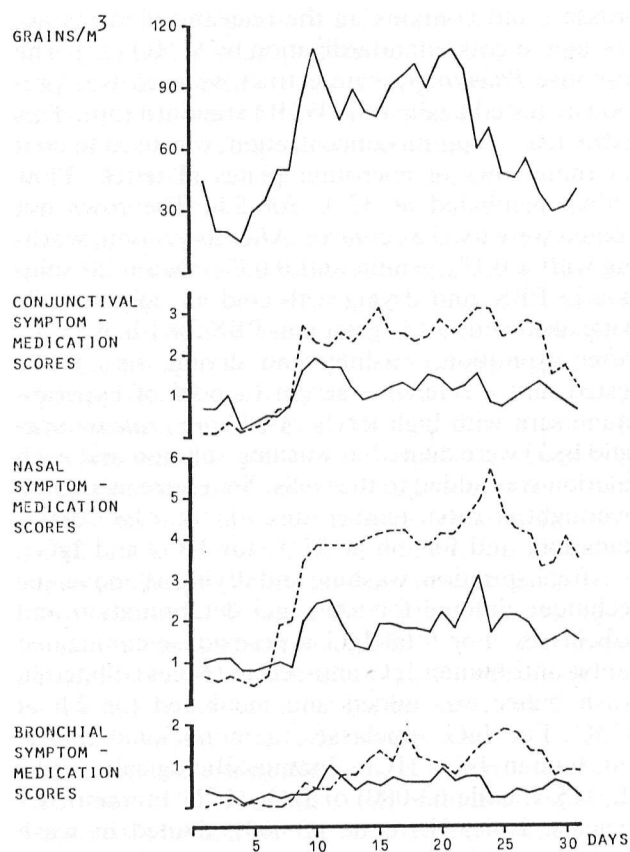


Fig. 2. Grass-pollen count and mean daily conjunctival, nasal and bronchial symptom-medication scores during May 1986 in the actively treated group (solid line) and in the placebo group (dotted line). Conjunctival ($p < 0.05$), nasal ($p < 0.01$) and bronchial ($p < 0.05$) symptom-medication scores were significantly lower in active than in placebo group (Mann-Whitney U test).

Skin reactivity

At baseline no statistical differences were observed in skin reactivity between the actively treated and placebo group (Mann-Whitney U test $p > 0.2$, NS). A significant decrease ($p < 0.02$) in specific skin reactivity to the grass-pollen extract was observed in actively treated patients after 4 and 12 months of IT (Wilcoxon signed rank test) (Figs. 3 and 4); no differences were detected in the placebo group. No change in skin reactivity to histamine was detected in either group.

Nasal reactivity

No differences were detected at baseline between the two groups (Mann-Whitney U test $p > 0.2$, NS). A significant decrease ($p < 0.05$) in specific nasal reactivity was detected in actively treated patients after 12 months of IT (Wilcoxon signed rank test) (Fig. 5). No differences were found between the two groups after 4 months of IT. After 12 months of IT three patients no longer reacted to the top concentration

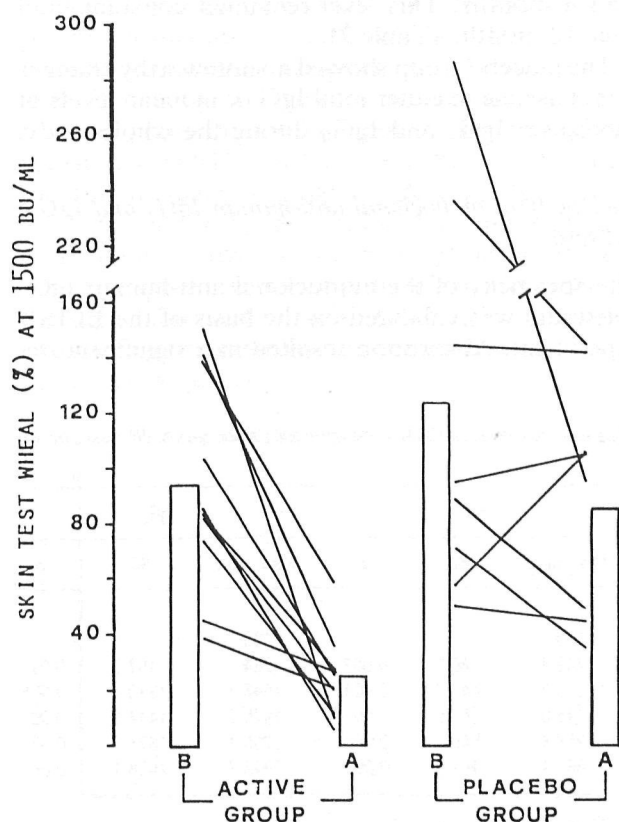


Fig. 3. Changes in the mean wheal area elicited by the prick test done with grass-pollen extract at 1500 BU/ml as a percentage of the histamine wheal before (B) and after (A) 4 months of IT in the active ($p < 0.01$) and placebo (NS) groups (Wilcoxon signed rank test). No differences were observed between the two groups at baseline (Mann-Whitney U test, $p > 0.2$, NS).

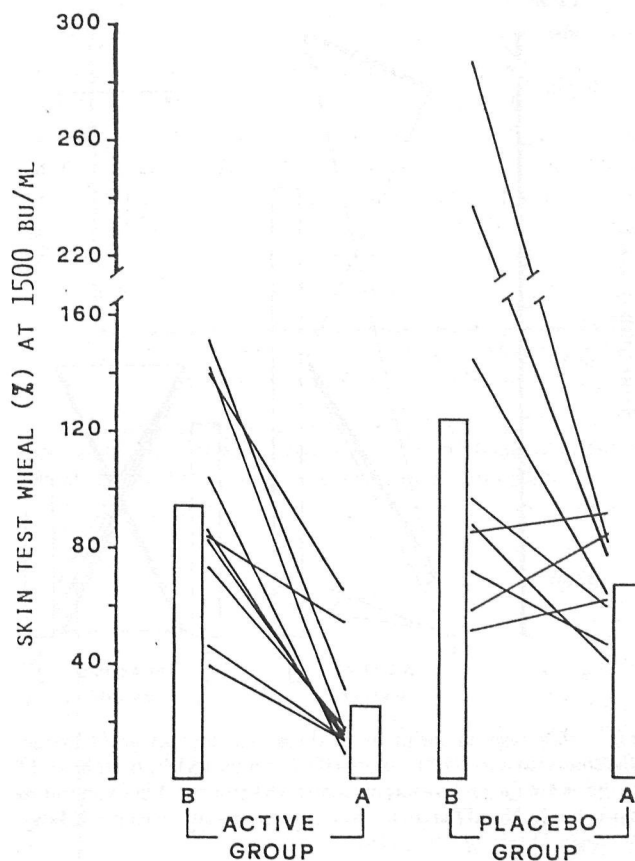


Fig. 4. Changes in the mean wheal area elicited by the prick test with grass-pollen extract at 1500 BU/ml as a percentage of the histamine wheal before (B) and after (A) 12 months of IT in the active ($p < 0.01$) and placebo (NS) groups (Wilcoxon signed rank test). No differences were observed between the two groups at baseline (Mann-Whitney U test, $p > 0.2$, NS).

(10000 BU/ml). No differences were observed in the placebo group.

We also looked at the percentage of patients who presented an improvement in nasal sensitivity, i.e. needing a larger challenge dose to elicit a positive NPT, after 1 year of treatment. This improvement was 80% in the actively treated group and 22% for placebo. This difference was significant (Fisher's non-parametric test; $p < 0.05$) (Table 1).

Table 1. Number of patients (and percentage) who experienced an increase in the threshold dose eliciting a positive NPT. A significant difference was detected between the two groups (non-parametric Fisher test; $p < 0.05$)

	Threshold dose Increased (%)	After therapy Not increased (%)
Depot allergoid	8 (80)	2 (20)
Placebo	2 (22)	7 (78)
Total	10	9

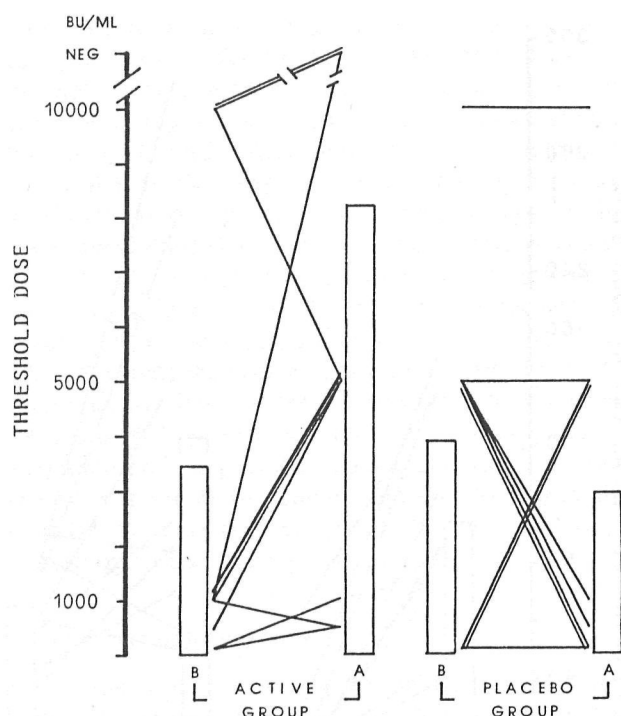


Fig. 5. Changes in mean nasal threshold dose of grass extract eliciting a positive NPT before (B) and after (A) 12 months of IT in the active ($p < 0.05$) and placebo (NS) groups (Wilcoxon signed rank test). No differences were observed at baseline (Mann-Whitney U test, $p > 0.2$, NS).

Grass-specific IgE antibodies

For statistical analysis we considered the results of the 1:5 diluted sera. As compared with baseline, the actively treated group presented a significant increase in specific IgE mean level after 3 ($p < 0.01$) and 4 ($p < 0.01$) months of IT (Wilcoxon signed rank test). After 9 and 12 months of IT this group

showed a decrease in specific IgE mean levels, reaching a value not different from baseline (Table 2).

The placebo group showed no noteworthy changes in specific IgE mean levels throughout the year.

Grass-specific IgG antibodies

The actively treated group showed a statistically significant increase ($p < 0.0005$) in mean IgG level already after 2 months of IT (855 PNU) (Wilcoxon signed rank test). The maximum mean level was reached after 4 months (46 050 PNU). At subsequent determinations, after 9 and 12 months of IT, the level was somewhat below the maximum but the increase was still significant ($p < 0.001$) after 9 months and after 12 months ($p < 0.02$) (Table 2).

Specific IgG₁ antibodies also showed a significant mean increase after only 2 months ($p < 0.001$), reaching the maximum mean level after 4 months of IT and declining slightly at subsequent controls, though still maintaining a significant difference from baseline ($p < 0.001$ after 9 and 12 months) (Table 2).

Specific IgG₄ showed a significant increase ($p < 0.05$) after 2 months of IT, followed by a constant increase, reaching the maximum mean level after 4 months. This level remained constant until after 12 months (Table 2).

The placebo group showed no noteworthy changes from baseline in either total IgG or in mean levels of subclasses IgG₁ and IgG₄ during the whole study.

Specificity of monoclonal anti-human IgG₁ and IgG₄ antisera

The specificity of the monoclonal anti-human IgG₁ antiserum was validated on the basis of the ELISA experiment. Absorption resulted in a significant de-

Table 2. Mean specific IgE, IgG, IgG₁, and IgG₄ levels during the study expressed as percentages of baseline (considered as 100%) in the active and placebo groups. Wilcoxon's signed rank test was used at each assessment to compare results with baseline

	IgE			IgG			IgG ₁			IgG ₄		
	Mean value	SD	<i>p</i>	Mean value	SD	<i>p</i>	Mean value	SD	<i>p</i>	Mean value	SD	<i>p</i>
Active												
Basal	100			100			100			100		
2 mos				349	183	0.0005	249.8	45.5	0.001	234	157.7	0.02
3 mos	150.7	54.8	0.01	808	511	0.0005	819.8	443.7	0.001	1546.7	1310.1	0.005
4 mos	147.7	43.2	0.01	1239	758	0.0005	1145.8	557.5	0.001	1875.7	1414.1	0.001
9 mos	128.9	75.3	NS	987	988	0.01	958.8	545.2	0.001	1769.1	1871.3	0.02
12 mos	108.9	31.1	NS	1001	1196	0.02	881.4	487.6	0.001	1943.7	2428.1	0.05
Placebo												
Basal	100			100			100			100		
2 mos				123	75	NS	123	67	NS	122	106	NS
3 mos	92	11.7	NS	138	63	NS	87	49	NS	127	87	NS
4 mos	95.7	19.1	NS	142	87	NS	91	51	NS	147	149	NS
9 mos	111.2	16.7	NS	133	90	NS	69	64	NS	134	103	NS
12 mos	103.5	21.3	NS	109	71	NS	65	39	NS	143	104	NS

crease in OD values of IgG₁ but not of IgG₄. The OD value for IgG₁ at the dilution considered was 1.42 for the non-absorbed reference pool, and 0.33 after absorption with anti-human IgG₁. IgG₄ OD values remained unchanged after absorption, at 1.34 and 1.29.

Similarly, the specificity of the monoclonal anti-human IgG₄ antiserum was validated by the OD values of the non-absorbed reference pool and of the pool after absorption with monoclonal anti-human IgG₄, being respectively 1.39 and 0.21. No changes were observed in IgG₁ OD values of the pool after absorption, as compared with those of the non-absorbed pool, which were respectively 1.41 and 1.38.

Correlations between clinical and immunological data

We observed some significant correlations between clinical and immunological parameters in actively treated patients. Firstly, after 4 months of IT the higher the grass-specific IgG₄/IgG₁ ratio, the greater the decrease in specific skin reactivity ($r = 0.691$, $p < 0.05$) (Fig. 6) assessed at the same time. Also, the higher the grass-specific IgG₄/IgG₁ ratio in the samples obtained immediately before and after the pollen season (after 4 and 9 months of IT), the more marked were clinical symptoms ($r = 0.654$, $p < 0.05$) (Fig. 7). Finally, we found a significant association between grass-specific IgG₄/IgG₁ ratio and IgE increase ($r = 0.6941$, $p < 0.05$) (Fig. 8); i.e., the larger the IgE increase, the higher the IgG₄/IgG₁ ratio. Two patients were not included in this statistical correlation because of their high IgE levels at baseline. The IgE basal values of these patients exceeded the saturation level of the test (set at 35% radioactive binding), thus preventing any precise calculation of further increases.

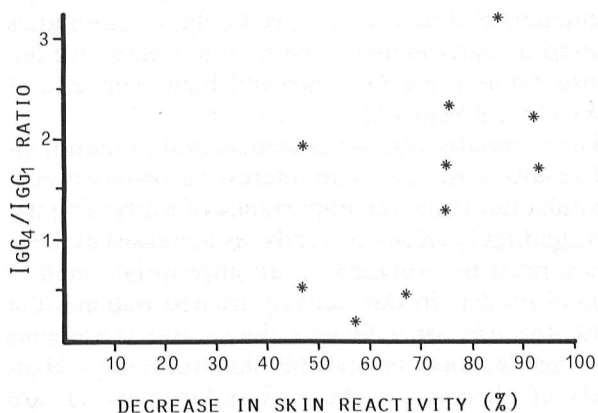


Fig. 6. Correlation between IgG₄/IgG₁ ratio and percentage decrease in specific skin reactivity in relation to baseline after 4 months of IT in each of the 10 actively treated patients ($r = 0.691$, $p < 0.005$; Spearman rank correlation).

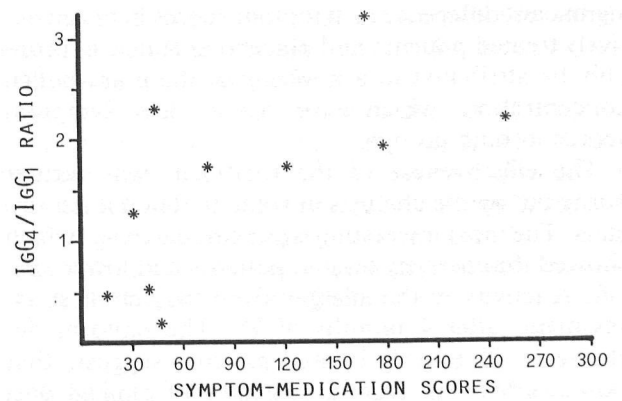


Fig. 7. Correlation between IgG₄/IgG₁ ratio and symptom-medication scores in May 1986 in each of the 10 actively treated patients ($r = 0.654$, $p < 0.05$; Spearman rank correlation).

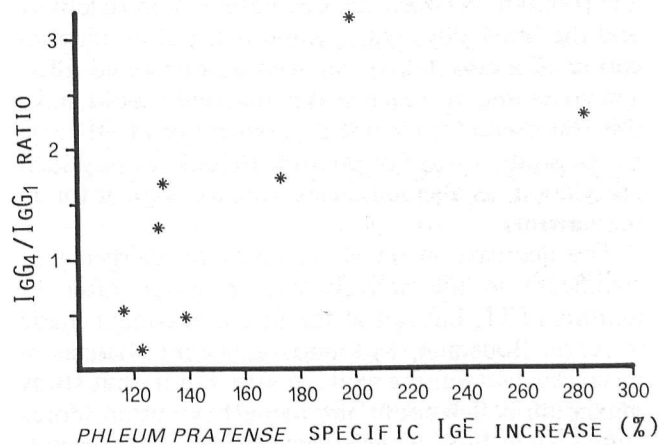


Fig. 8. Correlation between IgG₄/IgG₁ ratio and decrease in specific IgE levels (as percentage of baseline) after 4 months of IT in each of the 8 actively treated patients ($r = 0.6941$, $p < 0.05$; Spearman rank correlation).

Discussion

The efficacy of immunotherapy in grass-pollen-induced hay fever has been demonstrated in several double-blind, placebo-controlled studies (28). Four of these considered modified grass extracts (6, 7, 16, 17) and one employed a formalinized aqueous grass-allergoid (6), which showed good clinical efficacy but still presented the problem of systemic reactions.

The present study shows the clinical efficacy and safety of IT with a formalinized grass allergoid administered in a depot preparation. The treatment was effective during May, when the grass pollen concentration in the atmosphere of Milan (as documented by a pollen count) is at its peak. In May actively treated patients recorded significantly lower symptom scores than the placebo group. This difference was observed for all symptoms considered (nasal, conjunctival and bronchial). The lack of any

significant difference in symptom scores between actively treated patients and placebo in June can probably be attributed to a lowering of the grass-pollen concentration, which gave rise to low symptom scores in both groups.

The effectiveness of the treatment was further borne out by the changes in some functional parameters. The most interesting was skin reactivity, which showed that actively treated patients had lower specific reactivity to the allergen from the very first assessment, after 4 months of IT. The uniform decrease in all actively treated patients suggests that skin reactivity is a good indicator of clinical outcome. A decrease in specific skin reactivity is relatively common during IT. Some authors found it in IT with aqueous (3, 24, 27) and alum-absorbed (5) grass-pollen extracts and others even with formalinized grass allergoid (8). Bousquet et al. (8) reported a correlation between the decrease in skin reactivity and the fewer days with symptoms. Future identification of a correlation between administered allergen doses and decrease in skin reactivity could make this test useful for routine assessment of IT efficacy. In our study it was not possible to look for any such correlation, as allergen doses were too similar for all the patients.

The decrease in nasal reactivity to allergen was significant in the actively treated group after 12 months of IT, but not at the first assessment made in April. Bousquet (8) found significant changes in nasal reactivity in the same month, but in that study maximum pollen count and highest symptom scores were seen in June, while in our study the corresponding period was May. Possibly in our patients the earlier pollen season influenced the response to nasal provocation test (NPT), since a small amount of grass pollen already present in the air in April could have exerted a "priming effect" on the nasal mucosa (10). Bousquet reported a correlation between nasal symptom scores and changes in nasal reactivity to NPT. In our study this could not be proposed because of the different periods in which the two parameters showed the effectiveness of IT, i.e. May for symptom scores and December for nasal reactivity. On the basis of our results NPT appears to be a good way of monitoring IT, but the marked influence of environmental and local factors and the lack of a standardized procedure make it difficult to propose this test for routine follow-up.

In our hands grass allergoid was quite safe. Very high doses, corresponding to a pre-seasonal cumulative dosage of 46050 PNU (with a mean top dose of 16250 PNU) and an annual cumulative dosage of 116045 PNU, were administered with a systemic cutaneous reaction in only one patient and some local reactions in three, requiring dose limitation.

The concept of allergoid is linked to high immu-

nogenicity and low allergenicity. However, the results about safety in studies available on IT with allergoids vary considerably, and though Grammer et al. observed a low rate of adverse reactions with polymerized allergoids (16, 17), other authors had a larger number of both local and systemic reactions with aqueous formalinized allergoids (6, 20, 21). The safety of the different allergoids, including the depot-allergoid we used, cannot be compared because no studies have matched them all. Considering the literature to date, the goal of total loss of allergenicity has not been completely reached by allergoids.

The progress of IgE responses in our trial can be interpreted on this basis. Grass-specific IgE antibodies in actively treated patients showed a significant increase early in IT, with a subsequent return to baseline. This behavior, already described for formalinized allergoids (6, 20, 21), is commonly observed during IT with many kinds of allergen extracts (28).

In line with the report by Normal et al. (20), grass-specific IgG response was very strong, indicating the high immunogenicity of this preparation. In actively treated patients specific IgG antibodies were already significantly increased after the administration of 855 PNU. The close relationship between the increase in dosage and the rise in IgG levels, as reported by Creticos et al. (11), was confirmed.

Grass-specific IgG₁ and IgG₄ antibodies, which had never been evaluated during IT with grass allergoid, rose significantly in the early phase of treatment. IgG₄ antibodies maintained their high levels throughout the study, while the IgG₁ showed a tendency to decline, though remaining significantly higher than baseline. The more evident persistence of IgG₄ response seems to be in accordance with the model of IgG₄ restriction of IgG response in prolonged allergen stimulation suggested by Aalberse et al. (2). Our results confirm our previous findings in house dust mite long-term IT where, despite high maintenance doses, mite-specific IgG₁ antibodies showed an early increase but then a progressive decrease, while the IgG₄ were still high even after 2 years of treatment (22).

The correlation between clinical and immunological results leads to some interesting observations. We must underline the importance of expressing the IgG₁ and IgG₄ values correctly, as increases in these values must be evaluated in an appropriate mathematical model. In our actively treated patients the higher the grass-specific IgG₄/IgG₁ ratio, the greater was the decrease in specific skin reactivity. High levels of allergen-specific IgG induced by IT are known to be related to inhibition of immediate (18) and late (23) responses to the specific skin test. Our results suggest that the effects on immediate skin reactivity are essentially due to the specific IgG₄

subclass. Fling et al. (14) recently reported a strong correlation between high specific IgG₄ levels and suppression of late phase skin reaction during IT with a mountain cedar extract. These findings suggest that the blocking activity of IgG antibodies is essentially due to the IgG₄ subclass and should constitute clinical confirmation of the biological properties of this isotype, which experimental studies suggested was the most reliable blocking antibody (1).

We also found that the higher the grass-specific IgG₄/IgG₁ ratio the higher the seasonal symptom score. As regards this relationship, the few studies published appear to assign this subclass a negative role in clinical outcome of IT. Djurup (12), statistically analyzing the results of some studies on IT, concluded that high levels of allergen-specific IgG₄ were prevalently associated with treatment failure, thus attributing to these antibodies a sensitizing role.

Although we detected a correlation between high IgG₄/IgG₁ ratio and high symptom scores, it is not possible to attribute a bad prognostic meaning to IgG₄. The actively treated group showed a lower mean symptom score than the placebo group, thus suggesting an overall improvement. In the presence of a decrease of specific skin reactivity, a sensitizing role of IgG₄ seems unlikely.

The apparent discrepancy between the two relationships, high IgG₄/IgG₁ ratio and high symptom score and high IgG₄/IgG₁ ratio and low skin reactivity, might be explained on the basis of a further observation. There was a significant correlation between high specific IgG₄/IgG₁ ratio and increase of specific IgE at the maximum dosage of IT. This would suggest a parallelism between specific IgG₄ and IgE after allergen stimulation which could be elicited by the production of IL-4. It is now well known that in mice the activation of B lymphocytes by bacterial lipopolysaccharides induces IL-4 secretion followed by the production of IgE and IgG₁ (may be equivalent to IgG₄ in man) (9, 26, 29). If the structural and functional similarities between mice and human IgG subclasses and the same activation steps could be demonstrated in man in the model of allergenic stimulation, it could reasonably be presumed that parenteral administration of allergen during IT could under particular circumstances induce a parallel production of IgE and IgG₄, as suggested by our own *in vivo* results. Thus, the high symptom score in patients with a high IgG₄/IgG₁ ratio might be due to the rise in IgE induced by IT, more than to IgG₄ antibodies which otherwise showed their blocking activity on the skin.

The role of IL-4 during IT requires thorough investigations and, as underlined by Rocklin in a recent review article (25), "it is hoped that the recent development of *in vitro* models for the study of IgE

biosynthesis by peripheral blood lymphocytes utilizing IL-4 will shed further light on the mechanisms underlying immunotherapy."

In conclusion, our results indicate that IT with an alum-absorbed formalinized grass allergoid is effective and safe. The correlations between changes of specific IgG antibodies elicited by the treatment and clinical parameters suggest that specific IgG₁ and IgG₄ subclasses may play particular roles in inducing the different clinical effects of IT.

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