

A double-blind study of hyposensitization with an alginate-conjugated extract of *Dermatophagoides pteronyssinus* (Conjuvac®) in patients with perennial rhinitis

II. Immunological aspects

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In a 2-year double-blind placebo controlled study an immunological evaluation was carried out on 33 patients (15 males, 18 females, mean age 29.2 years) with mite-induced perennial rhinitis who were submitted to specific immunotherapy (IT) with an alginate-conjugated extract of *D. pteronyssinus*. The behaviour of IgE, IgG, IgG₁ and IgG₄ antibodies specific to *D. pteronyssinus* and its major allergen Der p₁ was characterized by assessment of their changes in serum, and changes in IgG in nasal secretions during the treatment. The placebo-treated patients did not show any significant variation in the levels of specific antibodies, while in the actively treated patients we found: a statistically significant decrease ($P < 0.005$) of specific IgE, a statistically significant increase of specific IgG ($P < 0.005$), IgG₁ ($P < 0.005$) and IgG₄ ($P < 0.005$) in serum and a statistically significant increase ($P < 0.001$) of specific IgG in nasal secretions. The IgG response showed an early relative predominance of the IgG₁ subclass and a late absolute predominance of IgG₄ subclass, that confirmed the model of IgG₄ restriction in prolonged allergen stimulation. No correlation was found between immunological and clinical data.

Key words: alginates; desensitization; immunologic; mites; IgA; IgE; IgG.

Accepted for publication 19 April 1990

The mechanism of action of specific immunotherapy (IT) still presents some unclear aspects. Many immunological changes have been observed, without a clear connection with clinical data, particularly the allergen-specific IgG response, in which the subclasses IgG₁ and IgG₄ seem to represent the most involved antibodies (5), even though their behaviour and meaning are still partially unknown.

Aalberse (1) showed an IgG₄ restriction to prolonged antigen stimulation in the model of IgG response to honey bee venom in non-

allergic beekeepers. Other authors (7, 11, 19) investigated the IgG response during prolonged IT in perennial rhinitis or asthma in both adults and children allergic to house dust mite, and had contrasting results concerning the predominant IgG subclass antibody.

We performed a double-blind placebo controlled group comparative study of IT with a *Dermatophagoides pteronyssinus* (*D. pt.*) extract in patients with perennial rhinitis, the clinical results of which have been previously reported (4). In this paper we report the immunological

changes observed during the treatment concerning IgE, IgG, IgG₁ and IgG₄ specific to Der p₁ and *D. pt.* in serum and nasal secretions. Also, the lack of the association between clinical and immunological results is discussed.

MATERIAL AND METHODS

Patients

A total of 66 patients with perennial rhinitis due to *D. pt.* were studied in a double-blind placebo controlled study of the clinical efficacy of IT with a biological standardized extract of *D. pt.*. The experimental design has been described previously (4). The study involved three centers, Milan and Como in Italy and London in the United Kingdom; however, this report deals only with serum and nasal samples obtained from patients in Italy. Admission criteria required perennial rhinitis to have been present for at least 2 years, a positive skin prick test (SPT) and nasal provocation test (NPT) with *D. pt.* Sera samples were obtained from 33 patients in Italy, 15 males and 18 females with a mean age of 29.2 years (range 17–55 years).

Allergen extracts

Conjuvac mite (Dome/Hollister-Stier) consists of an extract of *D. pt.* which is dialysed to remove components of less than 5,000 Daltons. The extract, known as Alpha Fraction contains 7×10^6 IU mg⁻¹ of the major allergenic component, Der p₁. Following biological standardization the extract is chemically conjugated to a non-toxic alginate carrier which reduces its allergenicity without reducing its immunogenicity (16).

The study injection material (Conjuvac mite) was in the form of single dose vials of lyophilised material containing, on reconstitution, 5.6, 56 or 560×10^3 IU *D. pt.* ml⁻¹. Placebo treatment contained lyophilised sodium alginate diluent (0.2% after reconstitution) used in the manufacture of the active treatment. To preserve the double-blind nature of the study some

placebo vials also contained 5 µg of histamine dihydrochloride to simulate local skin reactions which are often associated with hyposensitization injections. The order of "alginate only" or "alginate + histamine" vials was randomized within each treatment set.

Alphatest mite for skin prick testing on admission to the study contained 90×10^3 IU *D. pt.* ml⁻¹. Stock extract of *D. pt.* for use in nasal testing was in the form of vials of lyophilised Alphabase containing, on reconstitution in physiological saline, 440×10^3 IU *D. pt.* ml⁻¹. This stock extract was further diluted to provide serial concentrations of 220×10^3 (¹/₂), 44×10^3 (¹/₁₀), 22×10^3 (¹/₂₀₀) and 8.8×10^3 (¹/₅₀) IU *D. pt.* ml⁻¹. Lyophilised Alphabase *D. pt.* was also used as the mite antigen in the serological assays. Alphatest mite extract for skin testing, Alphabase mite for nasal provocation testing and *in vitro* tests and Conjuvac mite for hyposensitization injections were all derived from the same parent extract of *D. pt.* Der p₁ was especially prepared from Alpha Fraction mite.

Patients were treated with 11 weekly subcutaneous injections of increasing doses of Conjuvac mite followed by 15 maintenance injections at monthly intervals. Patients on active treatment received 560 to 448,000 IU of *D. pt.*

Symptomatic assessment

Patients were provided with daily diaries on which they were asked to record (on a scale of 0–3) the severity of nasal blockage, sneezing and rhinorrhoea each morning and evening. At these times they also recorded nasal expiratory flow using a low range mini-Wright peak expiratory flow meter (Clement Clarke International Ltd, UK) and face mask (Medishield, size 2).

The diaries were used for four periods of 1 month each, on admission to the study and after 5, 9, and 15 months of injections. At the same four assessment times, passive anterior rhinomanometry using the P.K.Morgan nasal airways resistance tester (NART, P.K.Morgan Ltd., Chatham, UK) was used to measure nasal reactivity to *D. pt.* by the method described by Britton (3). NPT was performed using progres-

sively increasing concentrations of *D. pt.*, and a subjective assessment of nasal symptoms was recorded by patients on visual analogue scales (VAS).

Immunological assays

Immunological tests were performed on serum samples obtained from each of the Italian patients on admission to the study and after 3, 9, 12, and 23 months of IT. Nasal secretion samples were obtained from nine patients receiving active treatment on admission to the study and after 12 months of IT.

Nasal secretions were collected, as described by Merret et al. (8), using 2 ml of hypertonic saline (NaCl 20%) in each nostril. The secretions were collected in glass test tubes containing Polisorbate 20 (Tween 20: Pharmacia, Upsala, Sweden), and centrifuged at 300 rpm for 30 min before being stored at -20°C .

The patients involved in the immunological evaluation received IT for 5 months longer than the group only evaluated clinically and thus received a total of 23 months of IT.

Serum samples were assayed separately for total specific IgG, IgG₁ and IgG₄ against *D. pt.* Alpha Fraction and Der p₁.

The following parameters were assessed: for serum a) Alpha Fraction specific IgE, b) Alpha Fraction and Der p₁ specific IgG, IgG₁ and IgG₄; in nasal secretions total IgA, total IgG, and Alpha Fraction specific total IgG.

ELISA for *D. pteronyssinus* - specific serum IgG antibodies. IgG antibodies specific to *D. pteronyssinus* were detected by an indirect ELISA micro-method utilizing a polyvinyl chloride "U" shaped 96-well microtiter plate (Titertek Flow Labs).

Measurements were separately made for antigen Der P₁ and Alpha Fraction of *D. pt.* Wells were coated with 100 µl of a 500 µg/ml solution of Der P₁, or a 200 µg/ml solution of Alfa Fraction, both in normal saline solution, and incubated at 37°C for 2 h. For each pair of antigen-coated test wells, a pair of control wells received saline only (white wells).

After aspiration, the wells were washed three times using cold-wash buffer (0.1% gelatine - PBS 0.05% Tween 20), followed by air drying. 200 µl of Filler (0.1% gelatine in PBS) were added per well and incubated at 37°C for 1 h. After aspiration, washing and drying, 100 µl of each test serum diluted at 1:10, 1:20, 1:40 were added to the wells in duplicate. After further incubation, wells were aspirated, washed five times and air-dried. Wells were filled with a rabbit anti-human IgG peroxidase-labelled anti-serum (Kirkegaard Labs) diluted 1:1000 in wash buffer which had been previously heated to 37°C and the plates were then incubated for 2 h at room temperature.

After aspiration, five washes, and air drying, the plates were developed with 100 µl/well of 5 mg of O-phenyldiamine in 10 ml phosphate-citrate buffer (0.15 M; pH 5.9) and 4 µl H₂O₂ at room temperature.

The reaction was stopped with 50 µl of sulphuric acid 1 N and absorbance was immediately read in a microtiter plate spectrophotometer (Tikertek Multiscan Flow Labs) at 492 nm.

The results were expressed as net absorbance which was obtained by subtracting the average absorbance of the paired control wells from their corresponding paired antigen-coated wells.

The optical density (OD) values of specific IgG have been expressed in absolute quantitative values (µg/ml) by comparing the results with those from a reference serum at a known concentration of mite-specific IgG, following the formula:

$$\frac{\text{Sample concentration } \mu\text{g/ml:}}{\text{OD [sample]}} = \frac{\text{OD [positive reference serum]}}{\text{positive reference serum conc.}} \times \text{sample dilution factor}$$

The reference serum was obtained by pooling 20 sera from patients treated with IT for 1 year. The IgG content of the reference serum has been evaluated by comparing its OD value with OD value of a known amount of specific anti-*D. pt.* IgG antibodies purified by affinity chromatography.

To obtain the anti-*D. pt.* specific IgG antibodies from the pool serum, we first made a total IgG purification and subsequently specific IgG purification.

Total IgG antibodies were purified as follows: a) preparation and packing of a diethyl amine cellulosa (DEAE 52 Whatman) column for ion-exchange chromatography; b) equilibrium of the column using a phosphate buffer 0.01 M pH 8 (Buffer A); dialysis of the sample serum in the same buffer; c) incubation of the sample on the column; d) elution in Buffer A; e) collection of the purified IgG peak.

The purity of the eluted fraction was tested by double radial immunodiffusion (Outcherlony) (13) and immunoelectrophoresis.

Specific mite Alfa Fraction and Der p₁ IgG antibodies were purified from the total IgG preparation as follows:

a) activation of sepharose CL-B4 column by means of CnBr and subsequent covalent binding with antigen Der p₁ or Alpha Fraction; b) total IgG fraction incubated for 1 h at room temperature and subsequently, overnight at 4°C; c) elution with buffer 0.1 M pH 8 in order to remove the non-specific IgG antibodies; d) further elution with glycine-HCl buffer 0.1 M, pH 2.8, in order to recover the column bound specific IgG antibodies; e) estimation of the amount of eluted specific IgG by an ELISA micromethod previously described (17) but modified in part and described in the following steps: 1) coating polystyrene microplates with rabbit anti-human IgG antibodies diluted with phosphate buffered saline (PBS) (0.1 M, pH 7.8) and incubation overnight at room temperature; 2) aspiration, washing and drying; 3) adding of filler and incubation for 1 h at 37°C; 4) as step 2; 5) addition of: a) human IgG (Miles Ltd) with dilutions from 10 µg/ml to 0.0039 µg/ml as reference, b) column eluted samples to determine IgG content. Incubation for 2 h at room temperature; 6) as step 2; 7) addition of peroxidase-conjugated anti-human IgG antiserum (Dako) 1:10,000 diluted; incubation for 2 h at room temperature; 8) as step 2; 9) addition of substrate OPD 5 µg/ml in 10 ml phosphate-citrate buffer and 4 µl H₂O₂; incubation for 5 min at room temperature; 10) stop-

ping of the reaction by addition of sulphuric acid 1 N; 11) results were read in a microtiter spectrophotometer at 492 nm and expressed as net absorbance generated by subtracting the average absorbance of paired control wells from the corresponding paired antigen coated wells; 12) the OD values of column eluted samples were compared with an IgG reference curve to determine the IgG content in µg/ml by means of the formula described above.

The purified allergen-specific IgG, at known concentration as calibrated above, was used to determine the amount of allergen-specific IgG in a reference serum pool. A serum pool with a known IgG concentration (purified allergen-specific IgG) was used as reference to determine the amount of allergen-specific IgG in test sera before, during and after IT.

ELISA for D. pt.- specific serum IgG₁ and IgG₄ antibodies. Determination of *D. pt.*-specific serum IgG₁-IgG₄ was performed as described above for total specific IgG antibodies with the following exceptions: 1) polystyrene microplates were used, 2) microplates were coated with Der p₁ antigen and Alpha Fraction at 200 µg/ml, 3) serum samples and reference serum were diluted 1:10, 1:20, 1:40, 1:80 and were incubated for 2 h at 37°C. After aspiration, five washes and air drying, 100 µl of a monoclonal anti-human IgG₁ (ICN ImmunoBiologicals, code No.63-080) or IgG₄ (Miles Scientific Code No.63-063) 1:1000 dilution in wash buffer was added per well for overnight incubation at room temperature. After aspiration, five washes, and air drying, 100 µl of rabbit anti-mouse IgG peroxidase labelled antiserum (Miles Ltd) at 1:2000 diluted in wash buffer was added to each well and incubated for 1 h at 37°C. After aspiration and five washes, substrate was added and plates were developed as described above for IgG-specific antibodies. Results were expressed as OD values.

Standardization of the assay

Specificity of the method for D. pt. The specificity of the antigen in question was investigated by

ELISA inhibition after absorption and precipitation of the *D. pt.* reference serum pool.

The method consisted of: 1) absorption of an aliquot of the reference serum pool at a dilution of 1:20 incubated with serial dilutions of allergen; 2) precipitation with 5% PEG and incubation of the samples at 4°C for 1 h; 3) centrifugation at 3000 rpm for 1 h and collection of the supernatant; 4) comparison in an ELISA test of the OD values from the supernatant with those from the non-adsorbed reference pool.

Specificity of the monoclonal antisera anti IgG₁-IgG₄. The monospecificity for human IgG₁ and IgG₄ of monoclonal antisera was guaranteed by the manufacturers on the basis of no detectable cross-reactions with other IgG subclasses or IgM; however, we further checked the specificity by the following method. The specificity of the antiserum for IgG₁ was based on the changes in OD values obtained after adsorption of the reference pool at a dilution of 1:20 with

increasing concentrations of mouse monoclonal anti-human IgG₁ antiserum.

Similarly for the specificity of the antiserum for IgG₄ the reference pool at a dilution of 1:20 was adsorbed with increasing concentrations of mouse monoclonal anti-human IgG₄ antiserum and OD values obtained. The absorption of the precipitate of both antisera employed overnight incubation at 4°C with 5% PEG followed by centrifugation at 3000 rpm at 4°C for 1 h.

Nasal Secretions

ELISA for D. pt. Alpha Fraction-specific nasal secretion IgG antibodies – determination of specific IgG to *D. pt.* Alpha Fraction in nasal secretion was performed as described above for total specific serum IgG antibodies with the following exceptions: 1) Alpha Fraction was coated at 200 µg/ml concentration, 2) nasal secretions were diluted 1:2, 1:4, 1:8, 1:16, 1:32 and incubated overnight at room temperature.

Measurement of IgA, IgG and albumin in nasal secretions. We determined the concentration of total IgA, IgG and albumin in the nasal secretions both before and after IT to evaluate possible protein changes affecting specific immunoglobulin concentrations. We used radial immunodiffusion in agarose gel (LC and Nor-Partigen IgA, IgG and albumin, Institute Behring).

Statistical methods

Unless otherwise stated, the groups were analysed by the Kruskal-Wallis test, or the Mann-Whitney test when there were only two groups, without any adjustments for multiple testing. These tests were used to highlight possible effects, rather than test *a priori* hypotheses.

RESULTS

Clinical results

The clinical results of this study have been reported previously (4), but a brief resumé is given below.

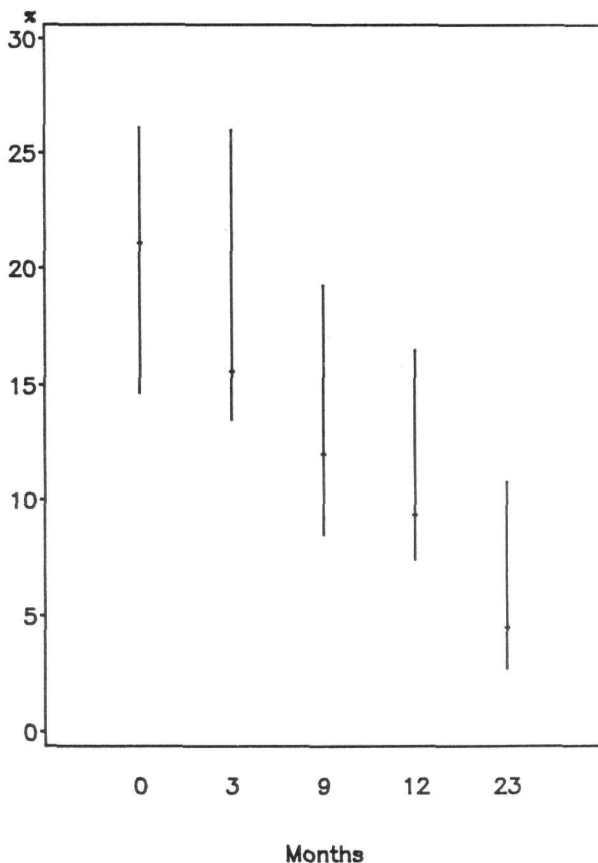


Fig. 1. *D. pt.* Alpha Fraction-specific IgE (percent of radioactive bound), medians and inter-quartile ranges, months after start of IT, actively treated group.

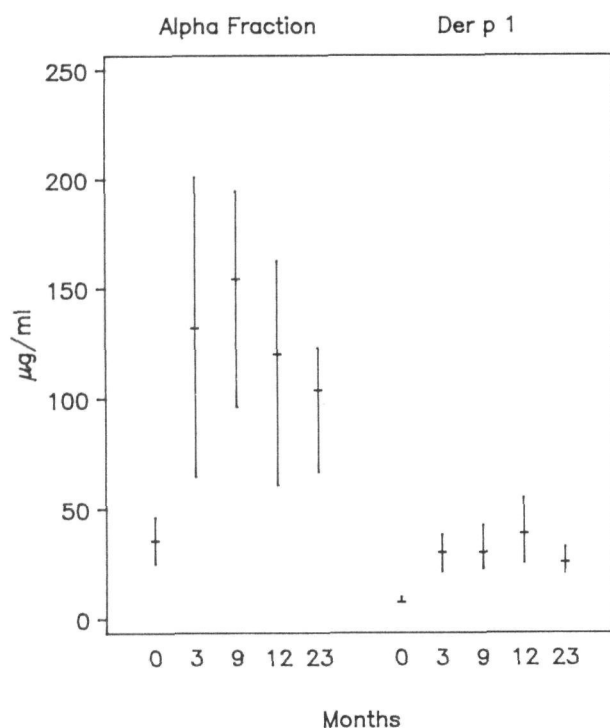


Fig. 2. *D. pt.* Alpha Fraction-specific and Der p_1 -specific total IgG ($\mu\text{g/ml}$), medians and inter-quartile ranges, months after start of IT, actively treated group.

The nasal symptoms recorded in patient daily diaries showed that the actively treated group recorded fewer symptoms than the placebo-treated group. The difference was statistically significant after 15 months of injections ($P = 0.028$). Subjective assessment on visual analogue scales showed a reduction of symptoms during the trial. The actively treated group showed a greater reduction than the placebo group after 15 months of injections ($P < 0.01$). The results of nasal provocation tests showed a significantly greater threshold dose in the actively treated patients compared with those treated with placebo. After 15 months of maintenance injections a large percentage of the actively treated patients tolerated the maximum concentration of *D. pt.* used for the test ($P < 0.001$, chi-square with Yates' correction).

Immunological results

D. pt. Alpha Fraction specific IgE. Serum levels of specific IgE decreased compared with the initial

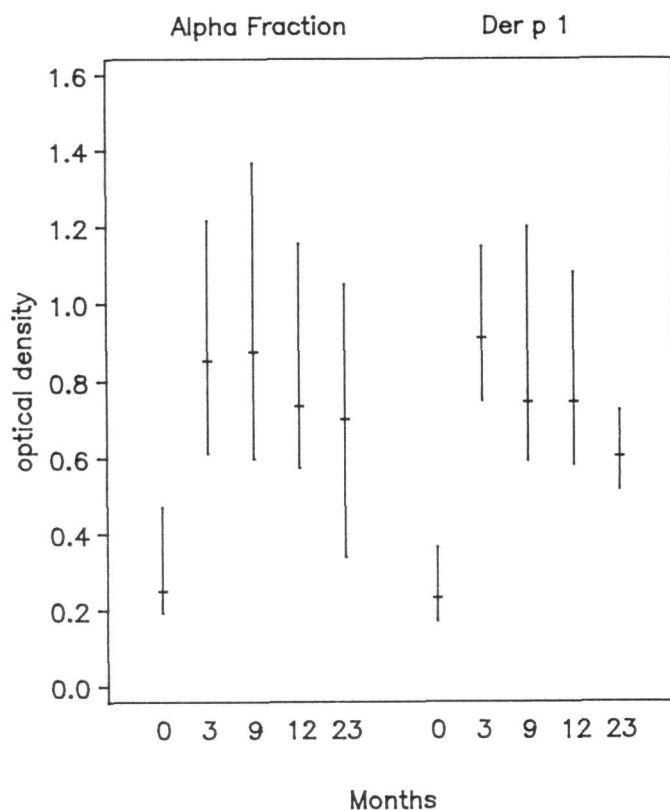


Fig. 3. *D. pt.* Alpha Fraction-specific and Der p_1 -specific IgG₁ (optical density, 1:10 dilution), medians and inter-quartile ranges, months after start of IT, actively treated group.

value in the actively treated group. After 3 months of IT there was a substantial decrease in IgE followed by a continual fall with a halving of the median value after a year of treatment and further reductions in the following samples ($P < 0.005$, assessment during IT compared to entry).

In the placebo-treated group there were no significant changes in specific IgE levels in serum during the entire treatment period ($P < 0.2$).

D. pt. Alpha Fraction specific and Der P_1 specific IgG, IgG₁ and IgG₄. In the actively treated group of patients there was an increase in values of the *D. pt.* Alpha Fraction-specific and Der p_1 -specific total IgG beginning with the first sample after the basal sample ($P < 0.005$, both tests). These values were 132.0 $\mu\text{g/ml}$ for Alpha Fraction and 30.0 $\mu\text{g/ml}$ for Der p_1 and showed percentage increases over the basal values of 372% and 400%, respectively (Fig. 2). However, the maximum value was reached after 9

months of treatment for Alpha Fraction (154.3 $\mu\text{g/ml}$) and after 12 months of treatment for Der p_1 (38.7 $\mu\text{g/ml}$). In the samples taken after 12 and 23 months of IT a slight reduction was seen in levels of total specific IgG with final values of 103.9 $\mu\text{g/ml}$ for Alpha Fraction and 25.7 $\mu\text{g/ml}$ for Der p_1 , which corresponds to an overall increase over the basal value of 293 % for Alpha Fraction, and 342 % for Der p_1 . There were no differences between the assessment after the initial sample for either the measures ($P < 0.1$, both tests).

The specific IgG₁ subclass showed an early increase and reached a maximum value after 3 months with Der p_1 and after 9 months with Alpha Fraction (percent increase compared with the basal value was 353 % for Alpha Fraction and 395 % for Der p_1 , Fig. 3) and the increase was maintained ($P < 0.005$ both tests). In the subsequent samples there appeared to be a gradual but continuing decrease of the median values of this subclass which was not shown statistically ($P > 0.1$, both tests).

The specific IgG₄ subclass showed a slower increase in the median values than occurred

with IgG₁, plateauing after a year of IT (increase at 1 year of 293 % for Der p_1 and 264 % for Alpha Fraction compared with the initial value, Fig. 4). The increases were statistically significant ($P < 0.005$, both tests) in all samples with respect to the initial sample, but with no change thereafter ($P > 0.1$, both tests).

The patients treated with placebo did not show any differences in levels of total IgG, IgG₁ and IgG₄ either with Alpha Fraction or Der p_1 ($P > 0.2$, all tests). The median serum levels we found during the trial were all within the range of the starting values.

Standardization of the assay. The specificity of the method for *D. pt.* allergen was validated on the basis of the ELISA inhibition; the OD value for the non-adsorbed reference pool was 1.820 against 0.250 for the reference pool after adsorption. The specificity of the monoclonal anti-IgG₁ antiserum was validated on the basis of the difference in OD values between the non-adsorbed reference pool and the reference pool

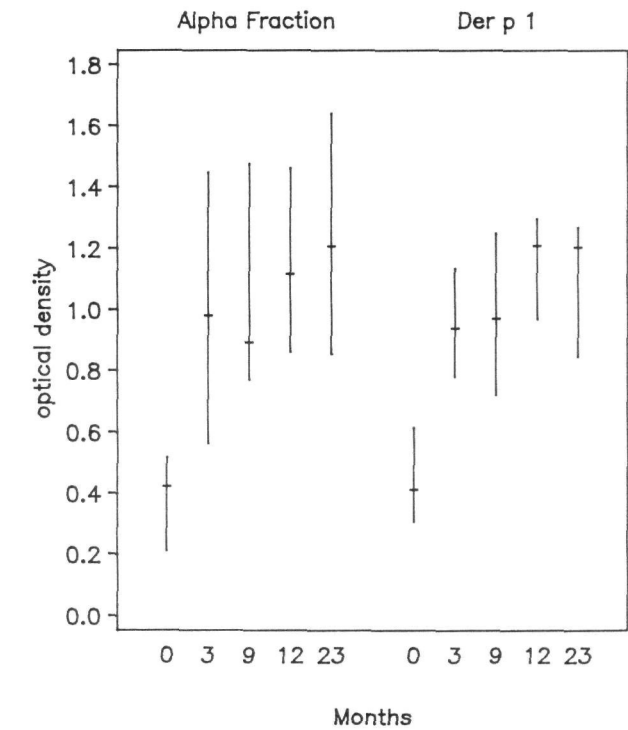


Fig. 4. *D. pt.* Alpha Fraction-specific and Der p_1 -specific IgG₄ (optical density, 1:10 dilution), medians and inter-quartile ranges, months after start of IT, actively treated group.

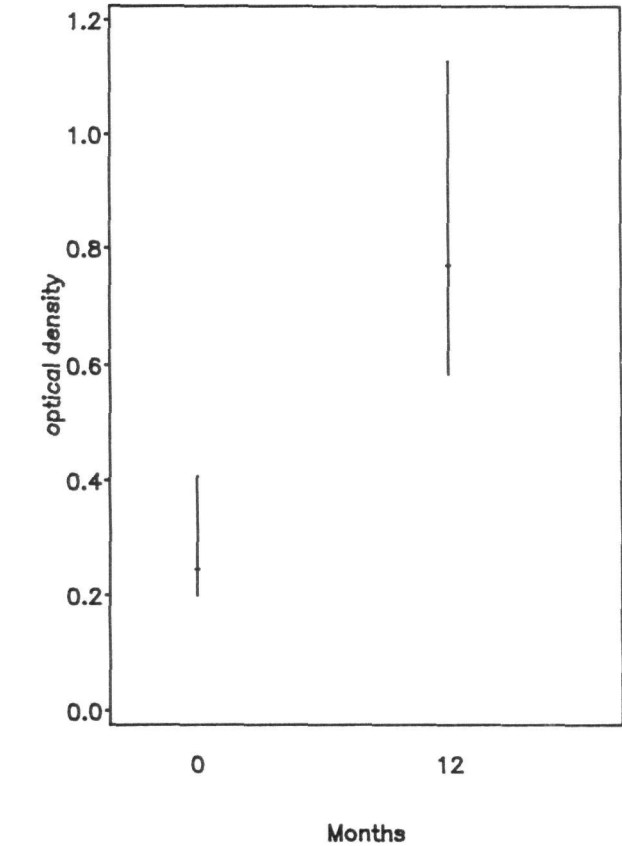


Fig. 5. *D. pt.* Alpha Fraction-specific IgG in nasal secretions (optical density, 1:2 dilution), medians and inter-quartile ranges, months after start of IT, actively treated group.

Table 1

Study schedule showing the intervals when nasal secretions and/or blood samples were collected																		
Months	0	1	2	3	4	5	6	7	8	9	10	11	12		21	22	23	
Blood sample	X			X						X			X				X	
Nasal secretions	X												X					
Immunotherapy	-----																	
	starting therapy									maintenance doses								

after adsorption with anti-human IgG₁, respectively 1.426 and 0.330. The OD values of the reference pool after adsorption with anti-human IgG₄ antiserum were 0.020, and 0.679 for the non-adsorbed reference pool.

Total IgA, total IgG and Alpha Fraction-specific IgG in nasal secretions. In patients receiving active treatment there was, after 12 months of IT, a significant increase ($P < 0.0001$ Mann-Whitney) in Alpha Fraction-specific IgG in nasal secretions compared with the initial value by 314 % (Fig. 5). Total IgG in nasal secretions did not show any significant changes between the two samples ($P > 0.2$). Whereas total IgA in nasal secretions showed a decrease between pre-treatment (median 16.25 mg/dl) and after 12 months (median 9.75 mg/dl) of active treatment ($P = 0.02$ Mann-Whitney).

DISCUSSION

In this study we have demonstrated the progress of the IgG response during IT with *D. pt.*

Firstly, we observed that in actively treated patients there was a significant increase in IgG both for *D. pt.* Alpha Fraction and antigen Der p₁. This is particularly important considering that in this type of treatment the extract dosage should be expressed in terms of the major allergenic component, Der p₁. Some reports of controlled studies with *D. pt.* have been unable to demonstrate a good IgG response (2, 15, 18), but in these studies the doses were expressed in Noon units and no information on Der p₁ content was given. It is likely that it is the allergenic potency and the content of major allergens which influences this type of response.

There have been few previous reports on the behaviour of IgG subclasses during IT with *D.*

pt. The first by Etievant et al. (7), who observed increases in all four IgG subclasses, and this was especially evident for IgG₃. The large increase in that subclass led the authors to suggest that IgG₃ were the antibodies mainly responsible for the clinical improvement seen as a result of IT with *D. pt.* However, it is possible that an ELISA assay using anti-IgG subclass antiserum, which is polyclonal and partially cross-reactive, could be the cause of this positivity. Subsequent studies have shown how the IgG response to the administration of allergens during IT, if evaluated with monoclonal antibodies of high specificity and used at the correct dilutions, is limited exclusively to IgG₁ and IgG₄ (5, 6, 10). Consequently, later studies with *D. pt.* have been conducted solely with these two subclasses.

In a recent study by Nakagawa et al. (11) the changes in IgG₁ and IgG₄ were evaluated in groups of patients treated with *D. pt.* for a period of about 2 years. An increase in both subclasses was noted after 6 months of treatment, after which the IgG₄ continued to increase whilst the IgG₁ levels remained constant.

In another recent study Wahn et al. (19) reported only small increases in specific IgG₁ but large and sustained increases in IgG₄ in patients treated with either whole mite culture or mite bodies of *D. pt.*

In our study we observed a more vigorous response in IgG subclasses in that after 3 months of IT the IgG₁ had increased by about 300 % and the IgG₄ by 200 % as compared with the 50 % and 100 %, respectively, reported by Nakagawa.

In our patients the initial increase was more marked with IgG₁ than IgG₄, peaking after 9 months of IT, or earlier. After that we saw a

progressive but continuous decline in IgG₁ compared with the maintenance of high IgG₄ levels. These findings offer confirmation of the concept expressed by Aalberse (1) concerning the restriction of IgG₄ response to long-term administration of allergen.

Certainly the most striking immunological event in our study was the progressive fall of the *D. pt.*-specific IgE levels. In other studies on the immunological response to house dust mite IT, specific IgE showed no changes or increases from the baseline (2, 11, 15, 18). It is therefore possible that the extract type (an alginate-modified one in our study), might play an important role in conditioning the immunological response. The progressive decrease of specific IgE subsequent to the early predominance of specific IgG₁ antibodies is in accordance with the hypothesis conceived by Djurup (6) who attributed to IgG₁ subclass a favourable role in successful IT for grass pollen, exerted by promoting immunological mechanisms (induced by the binding of IgG₁ with macrophages and lymphocytes) that elicit a decreased production of specific IgE. However, it seems unlikely that the outcome of immunotherapy could be related only to the decline of specific IgE for which a 99% decrease from the baseline is not sufficient to ensure a disappearance of allergic sensitization, as recently pointed out by Norman (12).

In this study we have not been able to establish statistical associations between clinical and immunological parameters. An association between successful treatment and high levels of specific IgG has been found only for venom immunotherapy in the literature (8). However, we were led to seek these correlations in our study by some interesting correlations between clinical outcome and changes in IgG subclasses observed in studies of grasspollen IT by Djurup et al. (6), Moss et al. (10) and ourselves (14). Nevertheless, it should be realised that the clinical severity of symptoms in patients with perennial disease is difficult to evaluate. Whereas in subjects with pollinosis the difference between active and placebo treated patients can be made at the time of maximum pollen concentration, which is therefore of defined but short duration, in patients with perennial symptoms this is not

feasible. The evaluation is done periodically without reference to the level of environmental allergen exposure to which the symptoms can be related.

It is therefore possible that the lack of correlation between clinical and immunological parameters is dependent on the absence of a reliable clinical parameter to use and the relatively small number of patients' data available. However, in general, the maximum clinical improvement (after 9 months of maintenance IT) was obtained when the IgG response was at its maximum.

As to the increase in specific IgG in secretions, we found no significant correlation in individual patients but there was a statistically significant difference between the groups of actively treated and placebo-treated patients.

We can therefore conclude that in this double-blind placebo controlled study conducted over 2 years we have characterized the progress of *D. pt.* specific IgE, IgG and subclasses IgG₁ and IgG₄. Specific IgE antibodies showed a constant tendency to decrease during active treatment. The IgG response was very vigorous from the first months of IT with an early relative predominance of IgG₁ subclass and a late absolute predominance of IgG₄ subclass. The lack of any statistical correlation between clinical and immunological data prevents us from making any assumptions about the role of IgG in IT with *D. pt.*

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