

Diagnosis and immunotherapy of mould allergy

VII. IgG subclass response and relation to the clinical efficacy of immunotherapy with *Cladosporium*

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The IgG subclass response was evaluated by a sensitive allergen- and subclass-specific solid phase immunoradiometric assay during a 1-year placebo-controlled, double-blind study of immunotherapy with *Cladosporium herbarum* in 22 adult asthmatics. The IgG response was mainly restricted to subclasses 1 and 4 but a few patients were IgG₂ and IgG₃ responders. An intense and early IgG₁ response was observed during the first clusters of injection followed by a levelling down of the titer. The IgG₄ response had a later onset and showed slowly increasing levels during the 12 months of immunotherapy. The graded clinical efficacy estimated by symptom-medication score was significantly correlated to the preseasonal IgG₁ value, with high values indicating a deleterious response of immunotherapy (deterioration of disease activity). Likewise, the fold increase of IgG₁ and IgG₄ after two clusters of immunotherapy (i.e. after 4 weeks) was significantly related to the clinical outcome. Little or no increase of IgG₁ and IgG₄ was associated with improvement, i.e. decrease in symptom-medication score. The magnitude of the IgG₁ response during the dose-increase phase was directly correlated to the number of systemic side effects. No relation of IgG₁, IgG₄ or IgG₄/IgG₁ ratio to changes in the IgE-mediated parameters (skin prick test, bronchial challenge and circulating specific IgE) was observed. Our data, which are based on few patients and only one allergen system, do not support the hypothesis of IgG acting as blocking antibody being the immunologic mechanism of immunotherapy. The association between high IgG₄ values and a deleterious efficacy of immunotherapy might be caused by IgG₄ acting as sensitizing antibodies. This explanation, however, is opposed by the lack of relation to systemic side effects.

Key words: *Cladosporium* allergy; clinical efficacy; IgE antibodies, IgG subclass antibodies; immunotherapy; side effects.

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CLINICAL ASPECTS

The relation between IgG subclass response and clinical efficacy/side effects was evaluated in a 12-month immunotherapy study with *Cladosporium*. An inverse correlation between the magnitude of the IgG₁ response and a beneficial effect of immunotherapy was observed. Further, a direct correlation was observed between the IgG₁ increase and the number of systemic side effects. These preliminary data point to a deleterious role of high IgG₁ values in immunotherapy. Without implying that IgG antibodies are involved in the immunologic mechanisms of immunotherapy, the early IgG₁ response might be a valuable predictor of the clinical outcome of immunotherapy in patients with allergic asthma. These observations, after being confirmed by more extensive studies including other allergens, might be useful when deciding the early termination of immunotherapy.

The induction of heat-stable "blocking" antibodies in the course of immunotherapy has been known since the mid-30s (3). The "blocking" activity of this antibody was shown to belong to the IgG class of the immunoglobulin (6, 18). A statistical relation between the titer of specific IgG and clinical protection in hymenoptera venom immunotherapy has been published (15), although not confirmed by others (20). Concerning inhalant allergen immunotherapy, the significant rise in the serum level of specific IgG antibodies has been considered the logical immunologic explanation for clinical efficacy (26, 27). However, this statement might be questioned as both the rise in IgG and the clinical efficacy are consequences of the allergen administered but not with causal coherence *per se*. This point of view is supported by a number of studies that were unable to show a relation between the rise in the IgG and the degree of clinical improvement (2, 11, 12, 16, 28, 31). A few studies have demonstrated group-relation between the magnitude of IgG increase and decreased bronchial and conjunctival provocation (34). In immunotherapy antigen-specific IgG may take part in at least three immunologic mechanisms, i.e. neutralization of allergen, inhibition of IgE-synthesis, and sensitization of the allergic type I effector cells (10). Therefore, in recent years special attention has been paid to a differentiated immunologic function of the four IgG subclasses (7). The IgG subclass response in immunotherapy is mainly restricted to subclasses 1 and 4 (5, 8, 9, 10, 14, 20, 33). The relation between IgG subclass and clinical efficacy has only been evaluated in a few studies (5, 8, 13, 16, 20). An association has been reported between failures of immunotherapy with inhalant allergens and high levels of IgG₄ (8, 13), while others present data in favour of a beneficial role of IgG₄ antibodies (16, 24).

The present study describes the IgG subclass response in a 1-year placebo-controlled, double-blind study of immunotherapy with the mould species *Cladosporium herbarum* and the relation to the graded clinical efficacy, side effects, and change in the IgE-mediated parameter, i.e. bronchial provocation test (BPT),

skin prick test (SPT), and circulating specific IgE (RAST).

MATERIAL AND METHODS

Patients

Eleven adult asthmatics in both the *Cladosporium* and the placebo group participated in the double-blind immunotherapy study. The patients were randomized according to SPT and BPT sensitivity and further, showed no significant difference with respect to age, sex, duration of mould allergy, associated allergies, and severity of asthma. A detailed description of patient data has previously been published (22).

Allergen extract

In the IgG subclass assay, the same preparation of a lyophilised, partially purified, and biologically standardized extract of *Cladosporium herbarum* (Pharmalgen®, Pharmacia, Uppsala, Sweden) as used for immunotherapy was employed (22). The extract contained approximately 60 antigens and 10 allergens estimated by crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis, respectively.

Study design

After performing initial tests and randomization, immunotherapy was started in March 1982. Data concerning the clustered dose-increase phase, maintenance treatment, and *in vivo* tests have previously been published (22). Serum was drawn at each cluster prior to the injections, at maintenance dose (preseasonally), after the *Cladosporium* season, and after 1 year of immunotherapy. Serum was stored in small aliquots at -20°C until analysed, and only frozen and thawed once. Results of SPT, BPT and RAST were evaluated according to (21, 23), and the clinical efficacy as described in (22). The following score system was used for calculations: +2 indicated improvement in both symptom and medication score; +1 denoted improvement in either symptom or medication

score, and the other unchanged; 0 is improvement in one score and deterioration in the other, or unchanged symptom and medication score; -1 is the result of deterioration in one score and the other unchanged.

Quantitation of IgG subclass antibodies to Cladosporium

IgG subclass antibodies to *Cladosporium* were quantitated by a modification of the solid-phase immunoradiometric assay described by Djurup et al. (9). Maxisorp test tubes (Nunc, Roskilde, Denmark) were incubated for 2 h at 37°C with 0.5 ml *Cladosporium* extract diluted in PBS (0.01 M phosphate, 0.15 M NaCl, and 1.5 mM NaN₃ at pH 7.5) to 1,600 BU/ml. After aspiration of non-bound antigen, the tubes were washed five times with PBS containing 0.1% Tween 20 (PBS/T). The tubes were filled with PBS/T and stored at 4°C for up to 1 week before use. On the assay day, the PBS/T was aspirated, and the *Cladosporium*-coated tubes were incubated for 2 h at 37°C with 0.5 ml serum diluted 1:80 or more, if the IgG content exceeded the standard curve, in an incubation buffer (IB) consisting of 0.1 M potassium phosphate, 0.15 M NaCl, 1.5 mM NaN₃, 20 mM EDTA, 0.5% (w/v) Tween 20, 0.5% (w/v) bovine gamma globulin (Sigma, St. Louis, Mo, USA), and 5% (w/v) freshly thawed rabbit serum (Gibco, Paisley, Scotland). The tubes were then washed six times with PBS/T and incubated for 2 h at 37°C with mouse monoclonal anti-IgG₁ (BAM 15), anti-IgG₂ (BAM 10), anti-IgG₃ (BAM 08), or anti-IgG₄ (BAM 11) (Unipath, Bedford, UK) diluted in IB to 1:1,000, 1:1,000, 1:32,000, and 1:32,000, respectively. At these dilutions the assay shows equipotency (9). The tubes were washed six times with PBS/T and finally incubated overnight at room temperature with about 25 ng ¹²⁵I-labelled affinity purified rabbit anti-mouse immunoglobulin antibody (RaMIg) in 0.5 ml IB. After washing, the radioactivity was counted for 1 min in a gammacounter.

The assay was calibrated by use of dilutions of one of the patients' sera (No. 124) obtained after 1 year of immunotherapy, containing a

high level of IgG₄ to *Cladosporium*. This serum was arbitrarily ascribed a value of 80 units IgG₄ to *Cladosporium* per ml undiluted serum. Non-specific binding for each subclass was obtained with pooled normal human serum (7). Specific binding was calculated by subtraction of non-specific binding from total binding. A point-to-point standard curve was calculated by computer, using standards containing 0.01, 0.02, 0.05, 0.10, 0.50 and 1.00 U IgG₄ to *Cladosporium* per ml, and the antibody concentrations in the sample in question were estimated by linear interpolation. All determinations were performed in duplicate and successive samples from the same patient were tested in the same set up. Values below 2.0 U/ml undiluted serum were considered insignificant.

To assess antigen-specificity of the assay the reference serum was diluted 1:100 (final dilution) in IB containing 4% (w/v) polyethylene glycol 6,000 and incubated overnight at 4°C with increasing amounts of *Cladosporium* extract or mite extract. The tubes were then centrifuged for 25 min at 2,000 × *g* at 4°C. The supernatants were assayed for IgG₄ to *Cladosporium* as described above.

Statistics

Between-group comparison of quantitative data was performed by Mann-Whitney U-test and chi-square test was used concerning nominal data. Correlations were calculated by Spearman's Rho.

RESULTS

Quantitation of IgG subclass antibodies to Cladosporium

The antigen-specificity of the assay was documented by complete inhibition or specific binding of the reference serum by preincubation with 2,500 BU/ml *Cladosporium* extract. Mite extract (50,000 BU/ml) did not inhibit specific binding. The specificity of the monoclonal antibodies and of the ¹²⁵I-labelled RaMIg used in the assay has previously been documented (9).

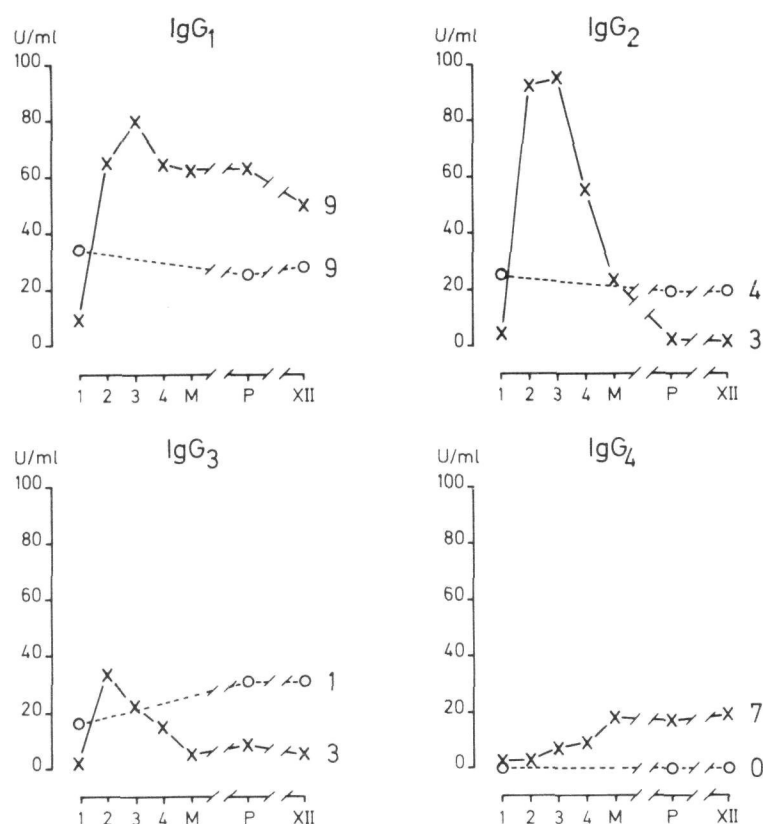


Fig. 2. Median values of the four IgG subclasses in patients with detectable values in the *Cladosporium* group (X) and placebo group (O). The number of IgG responding patients is given to the right. Serum samples were obtained prior to the injection of cluster 1-4, at maintenance dose = pre-season (M), after season (P), and after 12 months of treatment (XII).

majority of patients (8), however, did not show an IgG₂ response to *Cladosporium* antigens. The IgG₃ subclass response was identical to the IgG₂ response, although the magnitude of the IgG₃ increase was minor. Sixty-four per cent (7/11) of the patients were IgG₄ subclass responders and a modest but steady increase was observed during the 12 months of *Cladosporium* immunotherapy. The increase was median 6-fold. In the placebo group, no significant changes were found in patients with detectable pretreatment IgG-values, and no patients without pretreatment IgG became responders.

Relation between IgG subclass response and clinical efficacy of immunotherapy

The pre-seasonal IgG₁ level and the degree of clinical efficacy estimated by symptom-medication score in the subsequent season was significantly inverse correlated ($Rho -0.68$, $P < 0.05$) (Fig. 3). Likewise, the best clinical efficacy was found in patients with low values of IgG₄ just

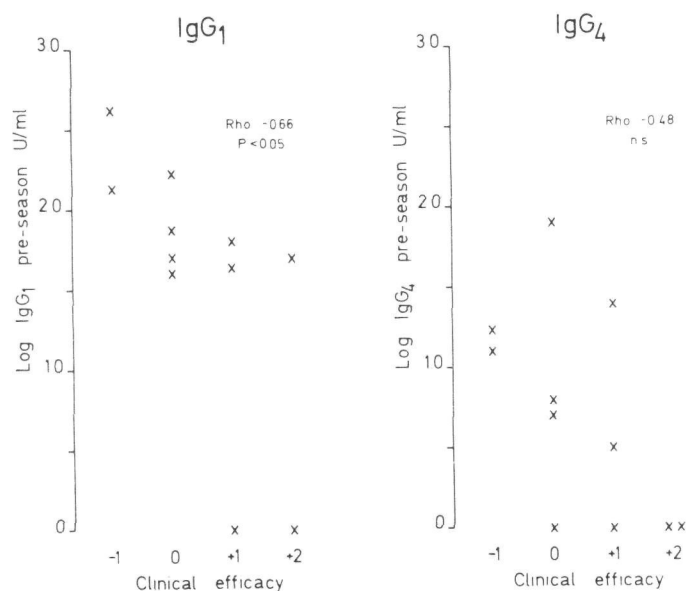


Fig. 3. Relation of absolute titer of IgG₁ and IgG₄ (log U/ml) at pre-season in *Cladosporium*-treated and the graded clinical efficacy estimated by symptom-mediation score in the subsequent season. -1 denotes deterioration of either symptom or medication score, 0 = unchanged clinical status, and +1 and +2 improvement in one or both disease activity scores.

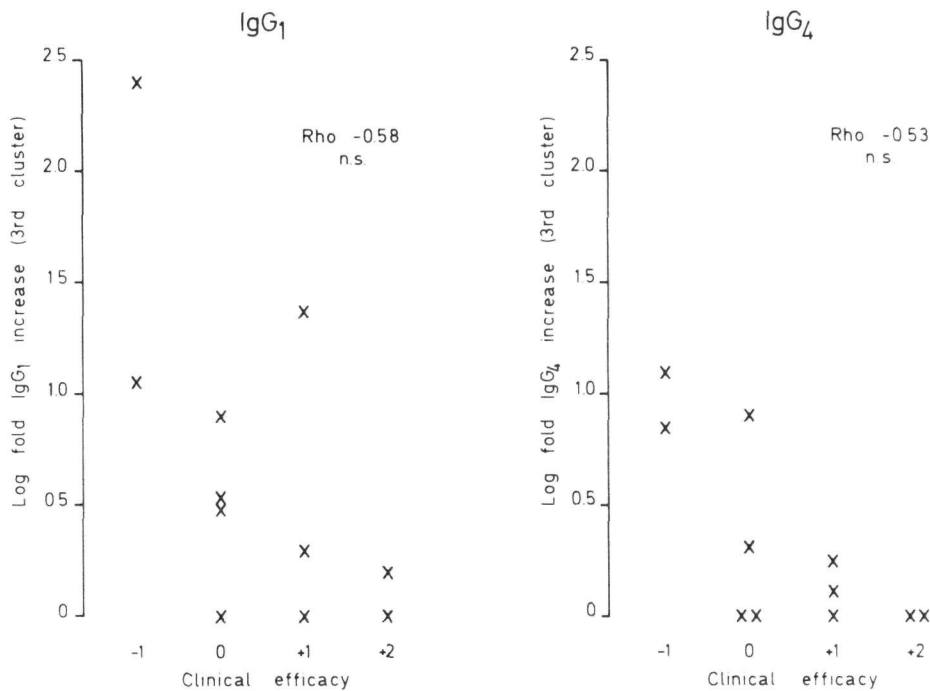


Fig. 4. Relation of increase in IgG₁ and IgG₄ (log fold increase) from pretreatment to the 3rd cluster (after 4 weeks treatment) and clinical efficacy (graded as in Fig. 3) in *Cladosporium*-treated patients.

before the monitoring season. The correlation, however, did not reach a statistically significant level (Fig. 3).

To evaluate the prognostic value of the early IgG₁ and IgG₄ subclass response as a predictor of the clinical efficacy of immunotherapy, the increase of IgG after two clusters of *Cladosporium* injections, i.e. after 4 weeks of treatment, was related to the clinical efficacy. Both concerning IgG₁ and IgG₄, an intense early increase in IgG was associated to a negative response of immunotherapy, and little or no increase to a beneficial effect of the treatment (Fig. 4). The correlation,

however, was not statistically significant although the IgG₁-response approached the 5% level.

A significant association ($\chi^2 = 12.9$, $P = 0.012$) was found (Table 2) when relating the magnitude of the IgG₁ response after two clusters to the clinical response (deteriorated, unchanged, and improved). Increments less than 3-fold were associated with an improved clinical status. Increases between 3–10-fold indicated unchanged clinical condition, and an increase > 10-fold related to deterioration. The magnitude of the IgG₄ response was less than the IgG₁ increase, and the prognostic value of the early IgG₄ response (< 2 times, 2–5 times, and > 5 times) in relation to the clinical outcome of immunotherapy just approached significant levels ($\chi^2 = 9.4$, $P = 0.051$).

Table 2

Relation between IgG₁-response after two clusters of immunotherapy (4 weeks treatment) and clinical efficacy stated as deteriorated (–1), unchanged (0) and improved (+1 and +2); for further explanation see text

		Clinical efficacy		
		Deteriorated	Unchanged	Improved
Fold IgG ₁ increase	> 10	2	0	1
	3–10	0	3	0
	< 3	0	1	4

$\chi^2 = 12.9$, $P = 0.012$

Relation between IgG subclass response and side effects of immunotherapy

An increase from IgG₁ pretreatment values during the dose-increase phase of immunotherapy was significantly correlated to the number of systemic side effects (Rho = 0.65, $P < 0.05$) (Fig. 5). Of lesser interest, and not statistically significant, was the positive rela-

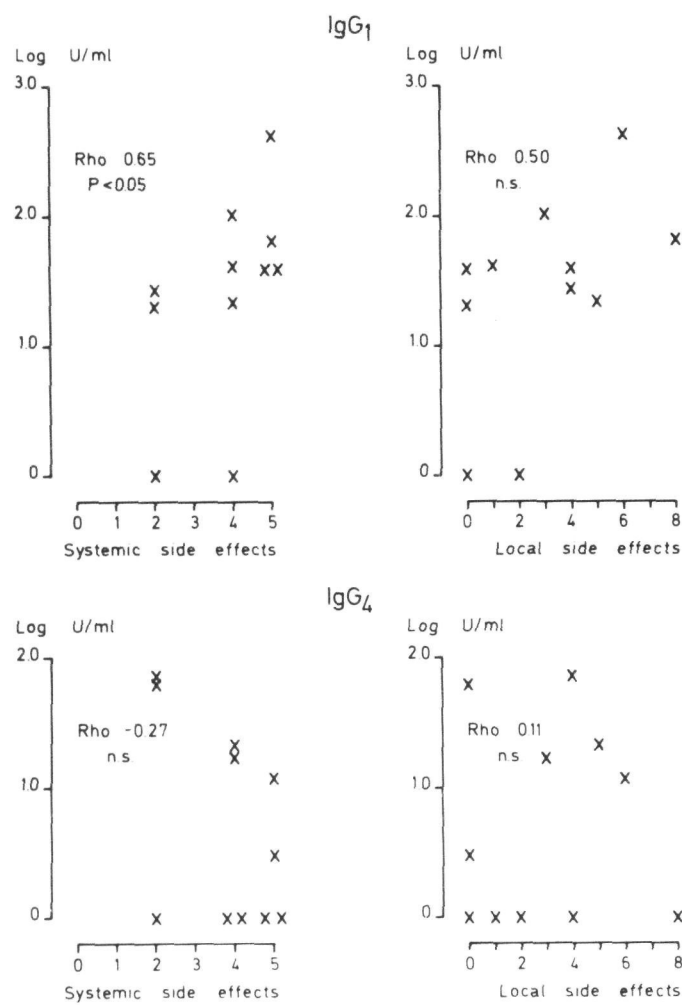


Fig. 5. Relation between increase in IgG₁ and IgG₄ (log U/ml) from pretreatment to pre-season (dose-increase phase) and the number of systemic and local (i.e. > 8 cm) side effects in *Cladosporium*-treated patients.

tion between the changes in IgG₁ during dose-increase and local side effects (defined as swelling > 8 cm). No relation between the IgG₄ subclass response and side effects was observed (Fig. 5).

Relation between IgG subclass response and change in SPT, BPT, and RAST

The changes in IgG subclasses 1 and 4 during the 12 months of immunotherapy were not related to alterations in SPT, BPT, and RAST (Rho -0.04, -0.01, 0.43, and -0.51, -0.11, -0.09, respectively). No tendency towards a down-regulating capacity of an intense, early IgG₁ response on the IgE values could be found.

The median ratio IgG₄/IgG₁ in responders was 0.4 before the start of treatment, declining

to 0.03 after the first cluster. The ratio increased steadily to 0.15 at maintenance dose, 0.50 postseasonally, and 0.56 after 1 year of immunotherapy (data not shown). However, compared to the individual subclasses, the IgG₄/IgG₁ ratio had less prognostic value as an indicator of clinical efficacy and side effects. Furthermore, the ratio was not related to changes in SPT, BPT, and RAST.

DISCUSSION

In the present study we have investigated the IgG response not only to non-allergenic antigens but to all antigens in the *Cladosporium* extract. This overall estimate of the IgG response in relation to clinical efficacy and side effects was preferred, as the exact role of IgG antibodies in immunotherapy is controversial (10), and as IgG directed towards non-allergenic antigens may be involved in the beneficial immunological mechanism. Estimation of IgG to a few major allergens is insufficient as this represents only a fragment of the complex immunological events.

IgG subclass response during immunotherapy

Contrary to what is seen in immunotherapy with grass pollen (5, 8, 14) and hymenoptera venoms (9, 20, 33), the response to *Cladosporium* does not seem predominantly restricted to subclasses 1 and 4. This phenomenon was also found with house dust mite (13). A few patients showed a pronounced increase in IgG₂ which

Table 3
Relation between IgG₄-response after two clusters of immunotherapy (4 weeks treatment) and clinical efficacy according to Table 2

		Clinical efficacy		
		Deteriorated	Unchanged	Improved
Fold IgG ₄ increase	> 5	2	1	0
	2-5	0	1	0
	< 2	0	2	5

$\chi^2 = 9.4, P = 0.051$

might be caused by one of the important antigens in *Cladosporium* extract being a glycoprotein (32). As no patients were responders in all four subclasses, the subclass response seems in some way individually restricted. The selectivity might be caused by a genetic restriction (10, 13), probably a consequence of subclass-specific regulatory T-cells recognizing separate antigenic determinants on *Cladosporium* allergens (19).

The evolution of the IgG₁ and IgG₄ subclass response during immunotherapy is in accordance with previously published studies (5, 8, 14, 33). Using a clustered dose-increase regimen with a fortnight's interval and an aqueous extract of Yellow Jacket, Malling et al. (20) observed a pronounced and early IgG₁ response and declining values after extended immunotherapy. The IgG₄ response had a later onset and continued to rise during the first year of immunotherapy. Compared to single, weekly injections of alum-absorbed extract (8, 9), the clustered regimen seems to favour the IgG₁ response (16, 20). Thus, the time sequence of the IgG subclass response seems to be a consequence of the immunizing regimen rather than a variation in the response of patients with an atopic predisposition (mould-allergic patients) or non-atopics (insect-allergic patients) (10).

The present study does not confirm the observation of Nordvall et al. (25) of a covariation of IgE₁ and IgG₄ with IgE in the grass pollen season. Regarding IgG₁, the nine placebo-treated patients with detectable pretreatment values did not show any significant change from pretreatment to the sample taken after the *Cladosporium* season. No patients in the placebo group had raised IgG₄ antibodies during immunotherapy.

IgG subclass response and clinical efficacy

The immunotherapy-induced rise in the serum level of specific IgG antibodies has been claimed to be the logical immunologic explanation for clinical efficacy, and consequently that "demonstration of a significant rise in the level of IgG-antibody should be considered a demonstration of efficacy" (27). This statement is based solely on the fact that a rise in IgG is the only constant immunologic phenomenon in a number of studies with demon-

strated clinical efficacy. A definite proof of a causal coherence between IgG response and clinical efficacy is missing, and both phenomena might simply be a non-related consequence of successful high-dose immunotherapy.

Using grass pollen, neither Dorward et al. (11) nor Stevens et al. (31) found a relationship between high specific IgG and clinical efficacy. Like the former, Reid et al. (28) found no significant correlation between symptom-medication score and either IgG or IgE/IgG ratio. In a mite allergen study (2), no correlation was observed between the symptom score and the level of IgG, and the same is reported in the only published study of *Cladosporium* immunotherapy in children (17). The study of Hedlin et al. (16), using cat and dog, showed higher mean values of IgG and IgG₄ in patients with subjective improvement compared with subjects without improvement. However, no direct relationship was found. Valovirta et al. (34) described a more pronounced increase in IgG in patients with decreased conjunctival or bronchial sensitivity compared with patients with unchanged/increased sensitivity after 4 months of therapy. The clinical efficacy, however, was not evaluated. Among studies dealing with the IgG subclass response, a non-significant relation between a reduced mean IgG₄ titer and a good clinical response was described by Etievant et al. (13). Djurup & Østerballe (8) reported a significant correlation between the IgG₄ antibody level after 12 weeks of immunotherapy and symptom score during the season 3 years later (after termination of immunotherapy). In contrast, higher mean levels of IgG₄ were observed by Devey et al. (5) in a subpopulation of patients with good clinical response in grass pollen immunotherapy. The same has been reported in a preliminary publication by Nakagawa (24).

The present *Cladosporium* immunotherapy study in adult asthmatics has revealed an inverse relationship between the IgG subclass 1 and 4 response and a graded clinical response. The relation was significant regarding preseasonal IgG₁ and change in symptom-medication score in the following season. Likewise, the magnitude of the early IgG₁ response (after 4 weeks of treatment) was significantly related to the clinical

response. The role of IgG₁ as a predictor of a deleterious clinical response to immunotherapy in patients with extrinsic asthma has never been published before, and as the results are based on one single allergen system (*Cladosporium*) and few patients, this observation has to be confirmed by other studies before general acceptance. From an immunologic point of view, the present study does not elucidate a possibly regulatory role of IgG₁ nor any direct relation to the mechanisms involved in the clinical efficacy of immunotherapy. In theory, the observed deleterious role of IgG₁ could be explained by a localized type III reaction in the bronchial mucosa (no patients showed systemic type III reaction) induced by immune complexes (IgG₁ + allergen) activating complement resulting in formation of anaphylatoxins (C3a and C5a) and thereby histamine release from mast cell. The inverse relation of the magnitude of IgG₄ and a poor result of immunotherapy is in accordance with other studies (8, 13). These findings might support the hypothesis of a capacity of IgG₄ to act as sensitizing antibodies in some patients, perhaps, as suggested by Stanworth (29), not because of IgG₄ being essentially cytophilic but rather through an "opsonic effect" promoting the IgE-mediated allergic reactions. Furthermore, it could be speculated whether a subgroup of allergics either have high avidity IgG₄ receptors on lung mast cells or a high density of IgG₄ receptors with low avidity, resulting in a sensitizing capacity of IgG₄. However, the lack of correlation between the magnitude of the IgG₄ response and the occurrence of systemic pulmonary side effects does not support this hypothesis. The apparently paradoxical role of IgG₄ antibodies acting either anaphylactically or protectively, might reflect a molecular heterogeneity within this isotype. This might be caused by a similar phenomenon, as the IgE-binding factor, depending on the state of glycosylation, might enhance or suppress the IgG-antibody formation (30). Galactosylation of the IgG₄ heavy chain might switch IgG₄-blocking antibody response to an anaphylactic (30).

IgG subclass response and side effects

Very little attention has been paid to the rela-

tion between IgG response and side effects in immunotherapy. In the study of Stevens et al. (31), no correlation was found between grass pollen-specific IgG and side effects. In our previous study with Yellow Jacket venom (20), side effects were not related to either IgG₁ or IgG₄. The present study showed an interesting, significant relation between the number of systemic side effects and the increase in IgG₁. Of less interest is the positive relation between IgG₁ and local side effects. Regarding IgG₄, no relation at all was found to side effects. This finding does not support the hypothesis of IgG acting as blocking antibody by its capacity to neutralize the allergen before reaction with cell-bound IgE (10). Time course analysis of the antigen-binding capacity of IgG, even in an excess of IgG, indicates that the interaction of IgE on the mast cell could not be blocked rapidly enough to prevent histamine release (2).

In a study of combined active and passive immunization (1), patients receiving allergen and ragweed hyperimmune specific IgG experienced systemic reactions 10 times more often than patients only actively immunized. On the other hand, a direct sensitizing capacity of IgG₁ does not seem reasonable as this isotype does not bind to mast cell (10).

IgG subclass response and IgE reactivity

Our data do not elucidate any significant regulatory role of the IgG subclasses on IgE-mediated parameters (SPT, BPT, or RAST). Only in the case of IgG₄, was a negative relation approaching statistically significant levels found between the magnitude of IgG₄ and change in skin reactivity. This might be caused by a sensitizing capacity of IgG₄ as a result of the binding of allergen-specific IgG₄ antibodies to Fc(gamma4) receptor located near the FC(epsilon) receptor on mast cells amplifying the IgE antibody-mediated allergic reaction in the skin (30). As discussed earlier, the data on IgG₄ and side effects do not support this theory.

Our data on *Cladosporium* immunotherapy do not confirm the down-regulatory role of IgG-antibodies on IgE-mediated parameters as published with grass pollen (8) and partially with

Yellow Jacket (9). The results are in accordance with other studies (16, 34). Kinetic analyses of the IgE and IgG response do not favour a down-regulatory role of IgG, since the time-response curve is shifted to the right, i.e. the maximum IgG response is later and requires a higher allergen dose than that for IgE. According to Creticos et al. (4), the IgE antibody response is more sensitive to both stimulation and suppression by allergen administration than is the IgG response.

Conclusion

Based on a few patients and a single mould allergen extract, we found the IgG response in *Cladosporium* immunotherapy to be primarily that of subclasses 1 and 4, but a few patients mounted an IgG₂ and IgG₃ response. A poor clinical response to immunotherapy seems to be predicted by an intense and early IgG₁ response, and partly by an IgG₄ response. However, this does not imply any direct involvement of IgG subclasses in the clinical efficacy, but might be of value in deciding an early termination of immunotherapy. The predictive value has to be confirmed in more extensive studies and for other allergen systems. A deleterious role of IgG₁ is supported by the finding of a direct relation between the increase in IgG₁ and the occurrence of systemic side effects. No regulatory role of IgG₁, IgG₄, and IgG₄/IgG₁ ratio on IgE parameters was found. The mechanisms involved in the clinical efficacy of immunotherapy do not seem directly related to the IgG response, but might be caused by the individual and, with time, varying complex interactions of IgG, IgE and target cell sensitivity in terms of affinity, and serum and shock organ levels of antibodies.

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