

A Double-Blind, Multicenter Immunotherapy Trial in Children, Using a Purified and Standardized *Cladosporium herbarum* Preparation

II. *In Vitro* Results

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This double-blind immunotherapy trial in children, using a purified and standardized *Cladosporium herbarum* allergen preparation, has shown that children with mould asthma and/or rhinoconjunctivitis, responded to immunotherapy with a decrease in specific IgE and a significant increase in specific IgG. There was a marked increase in the ratio specific IgG/specific IgE as a result of active treatment. IgE-CRIE radiostaining patterns showed no pronounced changes after 10 months' active treatment and no "new sensitivities" could be detected in the studied patients. IgG-CRIE radiostaining, primarily directed towards the important allergens, was significantly increased in the active group and particularly towards Ag-12 (partially identical to a previously described major allergen in *Cladosporium herbarum*, Ag-54). Children treated with histamine placebo showed no change in antibody patterns during 10 months of treatment.

Key words: *Cladosporium herbarum*; IgE-CRIE; IgG-CRIE; RAST; IgG-RAST; immunotherapy.

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Mould allergy is complex in nature and generally considered to be an important cause of allergic rhinitis and allergic asthma (1). Immunotherapy has been widely used with established clinical efficacy in the treatment of different allergic disorders, especially pollen allergy (17). However, no controlled studies have been previously reported which show the clinical efficacy of immunotherapy in the treatment of hypersensitivity to moulds (19). *Cladosporium herbarum*, the most prevalent mould species in Scandinavia, has been purified, well characterized by using biochemical and immunochemical methods, and biologically standardized by skin prick testing (2, 5, 8). The

effect of immunotherapy in mould allergic patients with such a preparation has been investigated in a trial in adults and one in children. Preliminary data from both these studies have recently been reported (9, 13, 15). In an accompanying paper detailed clinical results of immunotherapy in children, using a purified and standardized *C. herbarum* allergen preparation, is reported (8). 16 children received active treatment with the *C. herbarum* allergen preparation (Ch group) and 14 children histamine placebo (Hp group) in a randomized and double-blind manner.

The aim of the present investigation was to study these children's immune response as regards

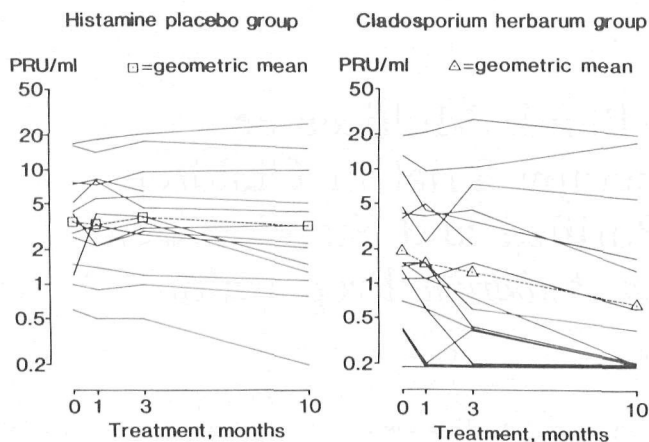


Fig. 1. Specific IgE measured with RAST and expressed as PRU/ml (Phadebas RAST Units) for the patients on active treatment and the placebo group versus time of treatment. Solid lines: individual patients; broken lines: geometric mean of each group.

changes in total specific IgE and IgG antibody levels, and changes in IgE and IgG antibodies directed towards individual antigens of the mould preparation during immunotherapy.

MATERIAL AND METHODS

Antigens

The *Cladosporium herbarum* allergen preparation (Spectralgen®/Pharmalgen®, Pharmacia AB, Uppsala, Sweden) used in this study was a highly purified and standardized material manufactured as previously described (7).

Patients and patient sera

Thirty children, with a mean age of 11 years (range 5–17) participated in the study. All subjects had a clinical history indicating possible mould allergy. Detailed data on the patients included in the study are given in the accompanying paper (8).

Blood samples were drawn four times from each patient immediately before the start of immunotherapy and after 1, 3, and 10 months of treatment. All patient sera were stored at -20°C until analysed.

For identification of previously described important *Cladosporium* allergens in the CIE/CRIE pattern, experiments with two individual patient sera, TdM and TN, containing high

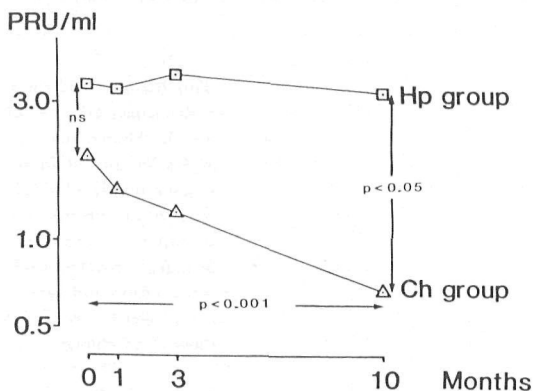


Fig. 2. Geometric means of total specific IgE for the actively treated group (Ch group) and the placebo group (Hp group) versus time of treatment.

amounts of specific IgE antibodies towards Ag-32 and Ag-54 were performed (3).

Rabbit antisera

Antisera to *Cladosporium herbarum* were raised in rabbits by biweekly subcutaneous injections of 300 μg of the crude mould extract. The immunization procedure was performed essentially as described by Harboe & Ingild (12).

Specific IgE and IgG

Specific IgE antibodies towards *Cladosporium herbarum* allergens were measured with

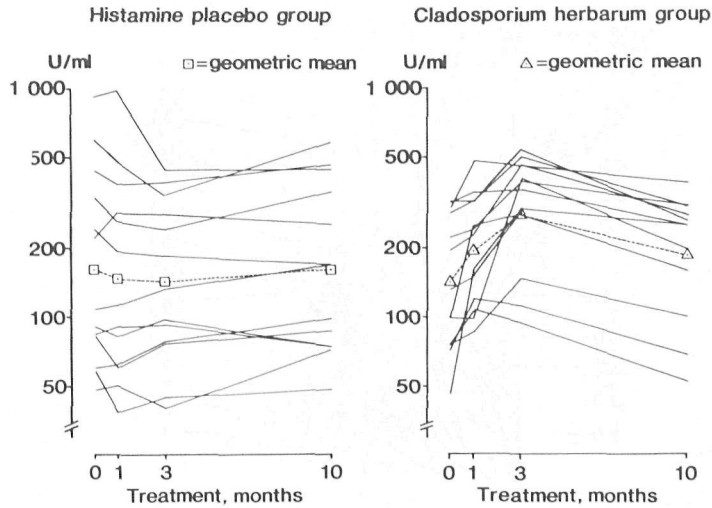


Fig. 3. Specific IgG measured with IgG-RAST and expressed as U/ml (Relative Units) for the patients on active treatment and the placebo group versus time of treatment. Solid lines: individual patient; broken lines: geometric mean of each group.

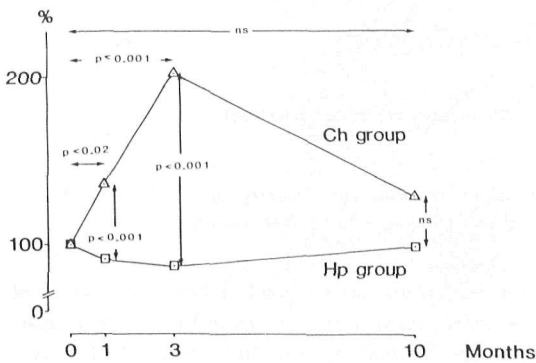


Fig. 4. Geometric means of specific IgG for the Ch group and for the Hp group expressed as percent of the specific IgG before treatment versus time of treatment.

Phadebas RAST® (Pharmacia AB, Uppsala, Sweden) reagents according to the manufacturer's instructions. All tests were performed in duplicate, and if necessary, diluted 5- and 25-fold to improve sensitivity. The results were evaluated between 0.7 and 3.5 Phadebas RAST Units (PRU)/ml (the B and C points on the standard curve).

Specific IgG was determined with a prototype of the Phadebas IgG RAST®. All tests were performed in duplicate and with patient sera diluted 1/200. RAST and IgG-RAST were carried out on patient sera collected before treatment and after 1, 3, and 10 months of immuno-

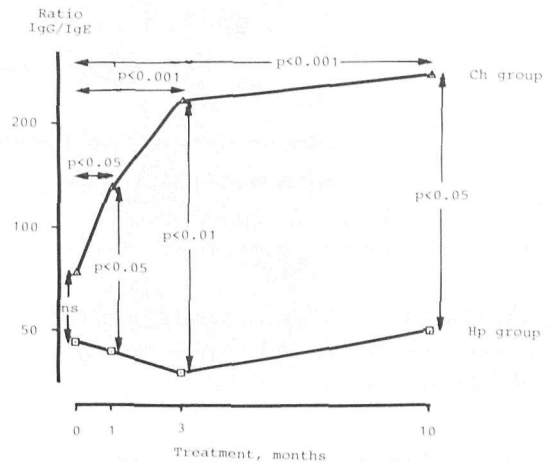


Fig. 5. Ratio between geometric means of total specific IgG/IgE measured with IgG-RAST and RAST versus time of active treatment.

therapy. IgG-RAST data were expressed as arbitrary units (U/ml).

Crossed immunoelectrophoresis (CIE)

CIE was carried out basically as previously described (11, 18) using 0.025 M Tris/Tricine buffer, pH 8.6, in 1% agarose (Agarose A, Pharmacia Fine Chemicals). 5 μ l of *Cladosporium herbarum* extract (60 g/l) was run in the first dimension electrophoresis at 10 V/cm for 25 min. Second dimension electrophoresis was

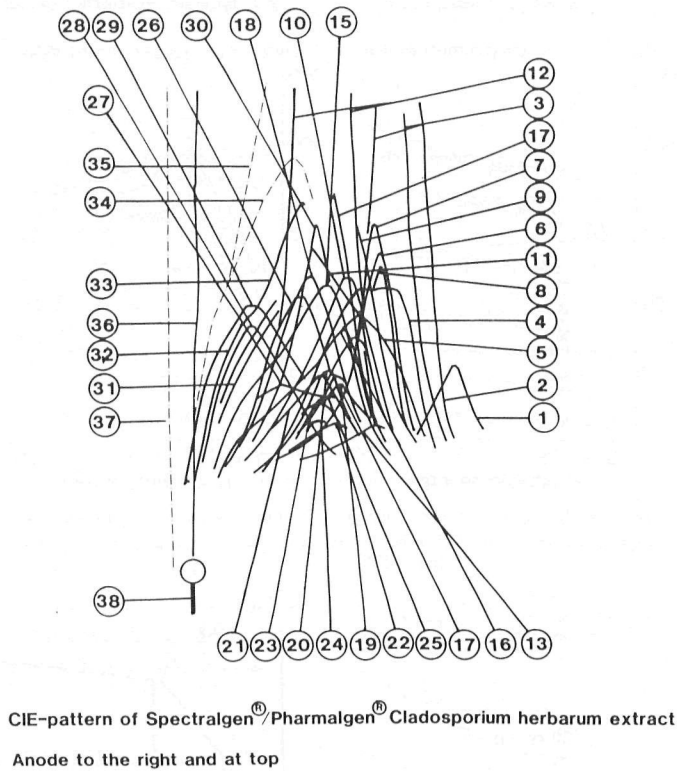


Fig. 6. CIE/CRIE reference pattern of *C. herbarum* extract, a schematic drawing with arbitrary numbers assigned to the individual precipitates. Precipitates marked with dotted lines could only be detected by CRIE radiostaining.

performed at 2–3 V/cm overnight, using 10 µl antiserum anodically and 12 µl antiserum cathodically/cm² gel.

Crossed radioimmunolectrophoresis (CRIE)

IgE and IgG-CRIE were performed as previously described (16, 20). The washed CIE-plates were incubated for 18 h with patient sera, diluted in buffer 1/17 for IgE-CRIE and 1/200 for IgG-CRIE. After several washings the plates were further incubated for 18 h with isotopes (¹²⁵I-anti-IgE or ¹²⁵I-anti-IgG, concentration 72,000 cpm/ml). The plates were then washed, dried and placed on X-ray film. Autoradiography was performed at –70°C using Du Pont Cronex-4 X-ray films in Kodak cassettes equipped with Du Pont Lighting Plus intensifying screens. CRIE-classification was carried out by sequential exposures (5).

To minimize methodological errors, incuba-

tion of patient sera collected before the start of treatment and after 10 months of treatment, were performed at the same time. CIE-plates incubated with saline or with a negative patient serum were used as negative controls.

Statistics

For the statistical evaluation of differences in means the Student’s t-test for paired and independent samples was employed. Correlation analyses were done by using Wilcoxon rank sum test.

RESULTS

Specific IgE

Fig. 1 demonstrates the changes in specific IgE antibodies of the individual patients during treatment with *Cladosporium herbarum*. Some of

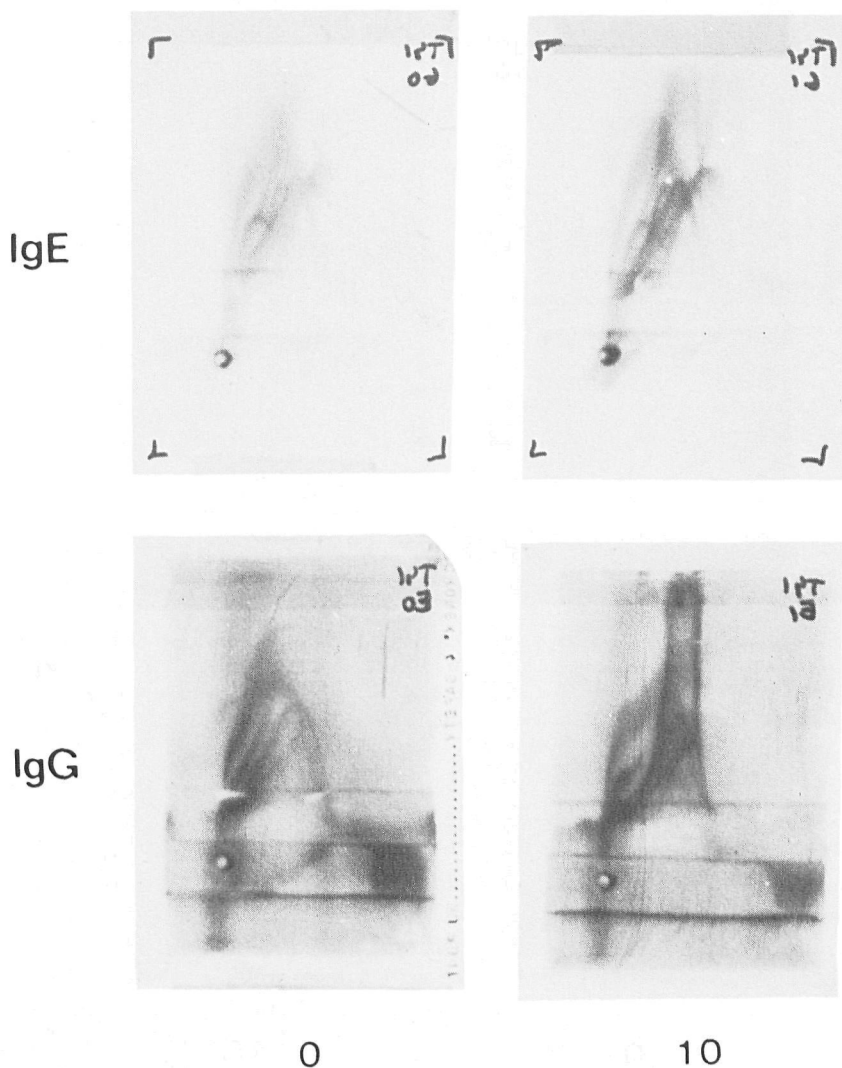


Fig. 7. X-ray films from IgE-CRIE and IgG-CRIE analyses of a patient before and after 10 months of active treatment. (IgE and IgG radiostaining corresponding to CRIE class G and E, respectively).

the patients on active treatment with *Cladosporium herbarum* showed a slight increase in specific IgE antibody level after 1 and 3 months of treatment, but most of the patients exhibited decreased specific IgE levels after 10 months. From the geometric mean it is obvious that there was a significant decrease ($P < 0.001$) during the period of treatment (Fig. 1). For patients receiving histamine placebo (Hp group), the individual IgE levels showed some variations, but as a group, no significant changes were noted.

Initially, the levels of *Cladosporium herbarum* specific IgE antibodies did not differ between the histamine (Hp) group and the *Cladosporium herbarum* (Ch) group. After 10 months of treatment, however, the patients on active treatment showed significantly ($P < 0.05$) lower levels than the control group receiving histamine placebo (Fig. 2).

Specific IgG

Although the levels of specific IgG varied somewhat in the individual Hp-treated patients (Fig.

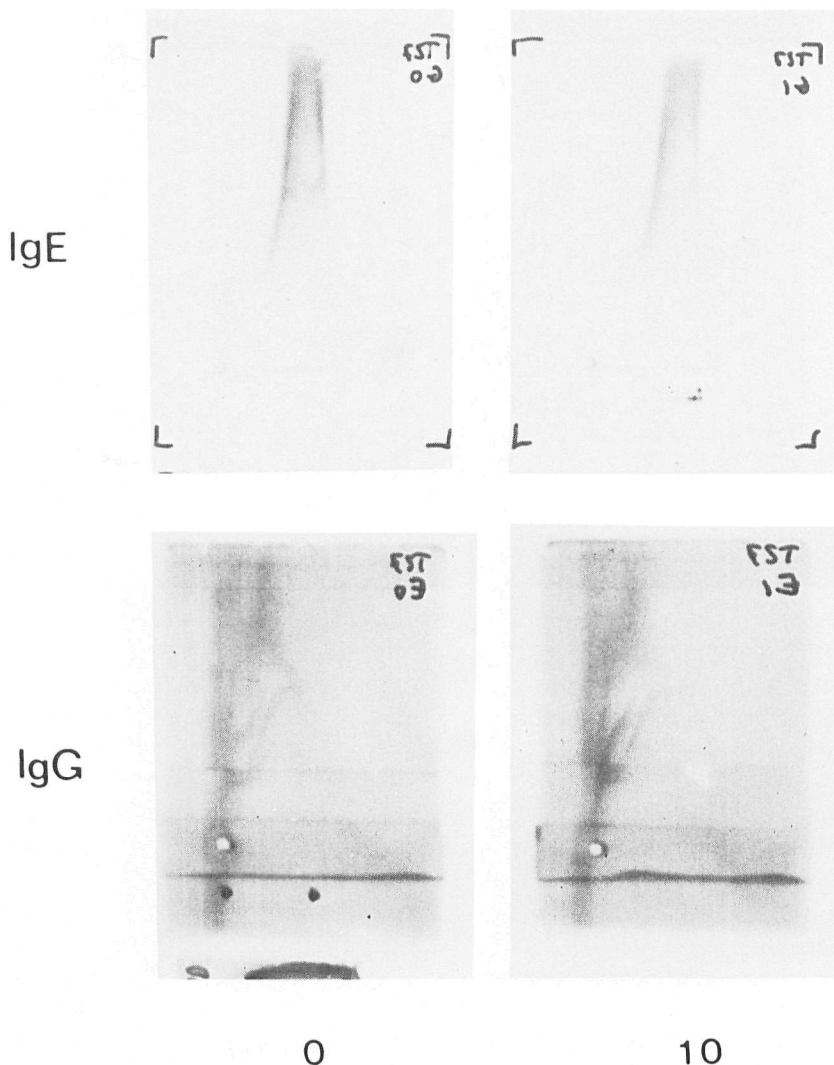


Fig. 8. X-ray films from IgE CRIE and IgG CRIE analyses of a typical patient in the histamine placebo group. (IgE and IgG CRIE radiostaining corresponding to class G and E, respectively).

3) there was no definite trend in the changes observed, the geometric mean remained fairly constant during the observation period (Figs. 3, 4). Patients treated with the *Cladosporium herbarum* allergen preparation showed a distinct increase in the level of specific IgG during the first month of treatment, and the geometric mean increased significantly ($P < 0.02$) (Fig. 4). The highest IgG levels were found after 3 months of active treatment ($P < 0.001$). After 10 months of treatment the IgG levels had decreased, but they were still slightly, but

not significantly, higher than before treatment.

There was no significant difference in IgG levels between the Hp group and the Ch group before treatment. The geometric means were 161.9 U/ml and 142.8 U/ml, respectively. After 1 month of treatment, specific IgG was significantly higher in the Ch group compared with the Hp group ($P < 0.001$). The difference was even more pronounced after 3 months ($P < 0.001$), but after 10 months of treatment there was no longer any significant difference between the two groups.

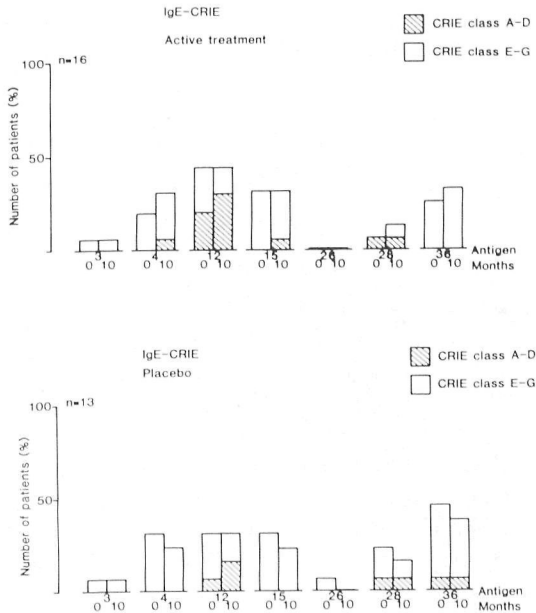


Fig. 9. Allergogram showing average visible IgE-CRIE radiostaining of clinically important allergens before and after 10 months' treatment for the Ch group and Hp group. Hatched bars: strong radiostaining (CRIE-class A-D); open bars: weak radiostaining (CRIE-class E-G).

Ratio specific IgG/specific IgE

Fig. 5 demonstrates the ratio between specific IgG and specific IgE for both groups. In the Ch group the antibody ratio increased already after 1 month of treatment and reached a plateau after 3 months. The IgG/IgE level remained substantially constant between 3 and 10 months of active treatment. The ratio for the Hp group decreased somewhat during the first months but then increased during the peak of the *Cladosporium* season to the initial ratio after 10 months of treatment.

CIE/CRIE analysis

The CIE pattern of the *C. herbarum* preparation used exhibited 38 immunoprecipitates (Fig. 6). Several of the precipitates were weakly protein stained with Coomassie brilliant blue while others (Ag-34, Ag-35, Ag-37) were only detected after radiostaining in CRIE.

Some patients responded to many antigens while others responded to only a few, and

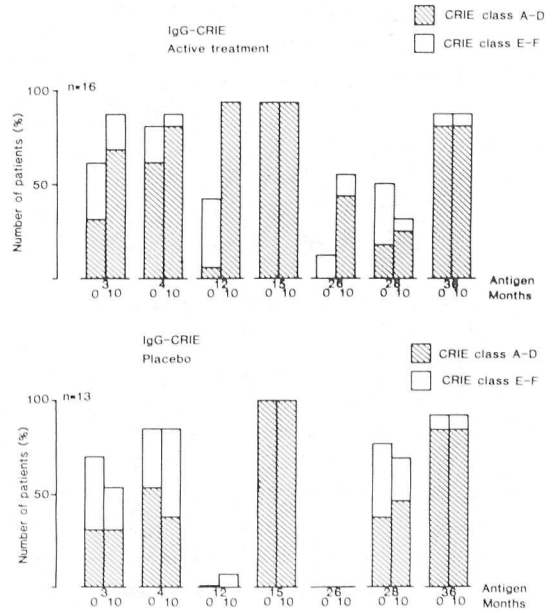


Fig. 10. Allergogram demonstrating average IgG-CRIE radiostaining of clinically important allergens before and after 10 months' treatment for the Ch group and the Hp group. Hatched bars: strong CRIE radiostaining (class A-D); open bars: weak radiostaining (class E-F).

approximately one third of the patients gave no visible radiostaining in IgE-CRIE. Thirteen antigens gave visible radiostaining and, based upon the frequency and intensity of radiostaining in the patient panel studied, seven antigens (Ag-4, Ag-10, Ag-12, Ag-15, Ag-18, Ag-26 and Ag-36) were classified as important allergens. Of these Ag-12 showed the highest radiostaining frequency (38%).

In a previous study on *Cladosporium* two major allergens, Ag-32 and Ag-54, were identified (4). By using specific reagents (patient sera TdM and TN) in the present CRIE study it was possible to show that Ag-12 was partially identical to the previously reported Ag-54. However, it was not possible to identify Ag-32 in our CIE/CRIE system – most likely due to lack of response to this antigen in the rabbit antiserum used.

Figs. 7 and 8 demonstrate two sets of IgE and IgG-CRIE pattern. In Fig. 7 serum from a patient on active treatment has been analysed. Several precipitates are radiostained in IgE-CRIE performed on a serum sample taken

Cladosporium herbarum
Immunotherapy

Patient No.	Top dose BU
5	60 000
9	100 000
21	8 000
22	800
26	4 000
31	3 000
41	10 000
2	100 000
3	60 000
7	100 000
13	60 000
25	45 000
29	4 000
42	50 000
46	100 000
47	50 000

- Patient with IgE towards antigen 12
- Patients with no detectable IgE towards antigen 12

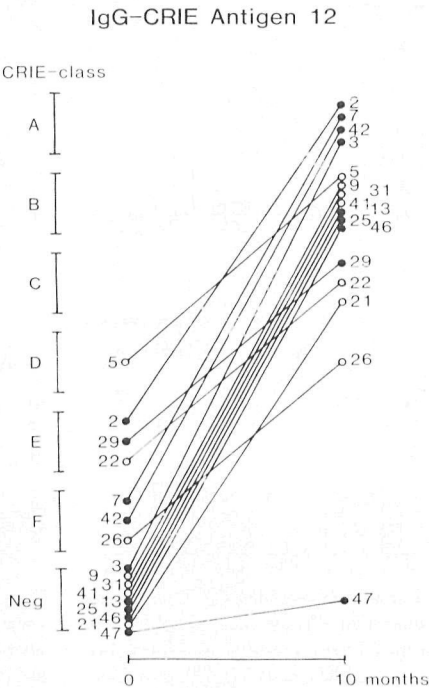


Fig. 11. IgG-CRIE response against a major allergen (Ag-12 partially identical to Ag-54) vs. time of treatment for the patients on active immunotherapy. Each subject is represented with a number in the diagram and the table gives the maximum dose for each individual patient.

before treatment. In this case the radiostaining is also slightly increased after 10 months of treatment. The radiostaining obtained in IgG-CRIE is more intense prior to treatment. After 10 months of treatment the IgG antibody level has further increased, particularly to Ag-12. The CRIE-patterns demonstrated in Fig. 8 are from a patient in the placebo group. This set of patterns differs from that demonstrated in Fig. 7, since the IgG-pattern is unchanged during the observation period.

Both groups showed only slight changes in the visible radiostaining corresponding to IgE CRIE-classes A-G after 10 months of treatment (Fig. 9). In the Ch group three patients actually showed increased levels of IgE-binding to some of the antigens, but none of the negative patients became positive. One patient showed a decreased level of IgE to Ag-12. In the Hp group there were no definite changes in the IgE binding to individual allergens during the observation period.

In general, patients in both the Ch group and in the Hp group had IgG-antibodies directed to many different antigens, and the radiostaining was much more intense than in IgE-CRIE. Fig. 10 demonstrates IgG levels towards some of the antigens before and after 10 months of treatment. Patients in the Ch group responded to immunotherapy with an increase of IgG to several antigens, particularly antigens 3, 12 and 26. However, no changes in IgG levels in the Hp group were detected during the observation period (Fig. 10).

Fig. 11 gives a detailed description of the changes in IgG-CRIE radiostaining towards the major allergen Ag-12 of the sera of patients on active treatment.

All patients except one responded with increased IgG response to Ag-12, independent of the IgG-reactivity towards Ag-12 prior to treatment and of the maximum dose reached after 10 months' immunotherapy. One CRIE-class increase in radiostaining corresponds roughly to

a two-fold increase in the specific IgG-reactivity.

DISCUSSION

This is the first published clinical trial showing the efficacy of immunotherapy with mould extracts (8, 14). In this study a standardized and freeze-dried *C. herbarum* allergen preparation was used for immunotherapy in children with mould-induced asthma and/or rhinoconjunctivitis. The potency of this preparation was at least 100 times higher than the *C. herbarum* allergen extracts previously available.

RAST data showed a significant decrease in specific IgE antibody levels in patients receiving *C. herbarum*, but CRIE experiments revealed no trend towards lower IgE levels (reduced radiostaining). This may be because the low initial RAST scores in many of the patients did not give rise to visible radiostaining in IgE-CRIE. RAST thus appeared somewhat more sensitive than IgE-CRIE. The difference may also indicate that the rabbits had not responded with a production of IgG antibodies to all of the allergens.

The decrease of specific IgE in the mould-treated patients seems to be induced at an early stage compared with previous reports on immunotherapy with pollen preparations, where a decrease of IgE was first observed after several months of immunotherapy (6, 10). This may be due to differences in the immune response to different types of allergens, or to differences in the amount of injected allergens.

On the other hand, the finding of an early increase of specific IgG is in agreement with studies on pollen-allergic patients. In the present study there was a rapid increase in total specific IgG antibodies in the active treatment period. Later, however, IgG dropped, and after 10 months of treatment there was no significant difference in the specific IgG antibody level between the Ch group and the Hp group. Such an early decline in total specific IgG antibodies has not been previously described. As our data are based on results obtained by running all patient sera from the same subject at the same time, we have minimized between-assay varia-

tions and other technical differences. We therefore consider the presented data as relevant.

After 10 months of treatment IgG-CRIE demonstrated a drastic increase of specific IgG to some of the antigens, particularly Ag-12, partially identical to Ag-54, one of the major *Cladosporium* allergens identified by Aukrust (3). Sera drawn after 1 and 3 months have not been investigated by CRIE.

Before treatment there was no significant difference in the IgG/IgE ratio between the Ch group and the Hp group. The antibody ratio increased and remained constant in the actively treated group. It is possible that the IgG/IgE ratio is a better indicator of clinical improvement than IgE and IgG measured separately. After 10 months of immunotherapy, changes in IgG, as shown by radiostaining in IgG-CRIE, primarily involved an increase of IgG antibodies towards Ag-12 (Fig. 11) independently of the top dose reached. Bronchial and conjunctival provocation data and skin sensitivity demonstrated a definite improvement in a majority of the patients after 10 months of treatment with the *Cladosporium herbarum* allergen preparation (8). As 15 of the 16 patients in the active group had responded to immunotherapy with induction of IgG antibodies towards Ag-12, this antigen appeared to be an important allergen in our CIE/CRIE reference system. When we compared the patients T5-T41, where Ag-12 was an important allergen, with the remaining patients T2-T47 where Ag-12 appeared not to be an important allergen, both groups of patients exhibited similar improvement after 10 months' treatment. Consequently, the increase in blocking antibodies to Ag-12 in patients of the former group did not seem to be of critical importance for clinical improvement. However, patients with detectable IgE antibodies towards Ag-12 prior to treatment, in general, received lower top doses (median value 8,000 BU) compared to patients with no detectable IgE reactivity towards Ag-12 (median value 45,000 BU). In the latter group the levels of total RAST-specific IgE decreased during immunotherapy while this was not the case in the former group. There is no direct linkage between laboratory data on

the change in IgE and IgG antibody patterns during immunotherapy and clinical improvement, but as the active treatment period studied here was only 10 months an analysis of the immune response after a longer period may show a more clear correlation.

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