

## Immunotherapy to deciduous tree pollens: specific IgE and IgG antibody patterns

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### Summary

Thirty-nine children with birch pollinosis were given immunotherapy (IT) for 3 years with a purified, characterized and biologically standardized pollen preparation made from either birch or a mixture of birch, alder and hazel. Levels of IgE antibodies against birch, alder and oak increased early during IT ( $P < 0.01$ ) and then decreased below the initial level ( $P < 0.01$ ). Birch-specific IgG antibodies also increased ( $P < 0.01$ ) but not as rapidly as birch-specific IgE antibodies and the levels remained increased throughout IT. There were only weak correlations between immunochemical findings before IT or during early IT on one hand and the improvement during IT on the other hand. The ratio birch-specific IgG:IgE after 1.5 and 2.5 years of IT correlated with symptom scores the following season ( $P < 0.01$ ). In 60% of the children below 13 years of age, new IgE specificities developed during IT as demonstrated with crossed radio-immunoelectrophoresis. No such new IgE specificities were found in older children. These 'new sensitivities' did not appear to have any clinical implication.

### Introduction

The well-known cross-reactivity among allergens from pollens of different deciduous trees has been documented *in vivo* [1-4] as well as *in vitro* [2, 5, 6]. In a previous paper we showed that for immunotherapy (IT) of children with rhinoconjunctivitis due to birch pollinosis, a mixed pollen allergen preparation of birch, alder and hazel (M) was more effective than a preparation of birch only (B) measured by symptom scores ( $P < 0.001$ ). There was a similar reduction in skin and conjunctival sensitivity in both groups [7].

The aim of the present work was to study the changes in the amount of specific IgE and IgG antibodies as measured by RAST, as well as the IgE and IgG patterns by crossed radio-immunoelectrophoresis (CRIE) during IT, and to correlate these changes to the clinical outcome.

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## Subjects and methods

### *Patients*

Forty children with hay fever due to birch pollinosis were included [7]. In addition, some children had minor sensitivities to other allergenic substances, mainly grass pollens or animal danders.

The children were allocated to one of us (S.D.) into pairs based on symptom score, conjunctival provocation test (CPT), skin-prick test (SPT), age and sex. Within each pair one of the children was randomly assigned to treatment with B and the other with M. As one of the patients dropped out before IT there were nineteen complete pairs left.

### *Allergen preparations and rabbit antiserum*

Partially purified, characterized, biologically standardized and freeze-dried birch and alder pollen allergen preparations (Pharmalgen®, Pharmacia AB, Uppsala, Sweden), and a crude but biologically standardized and freeze-dried oak pollen allergen preparation, with potencies in biological units per ml (BU/ml) were utilized for SPT and CPT. For IT either the same birch preparation (B group) or a similar preparation made from a mixture of birch, alder and hazel (M group) was used.

For crossed immunoelectrophoresis (CIE) crude pollen extracts of the same origin were used with protein concentrations of 2.7 mg/ml for birch and of 1.1 mg/ml for oak, as determined by a modified standard Lowry protein assay [8]. Rabbit antiserum against birch and oak was raised essentially as described by Harboe and Ingild [9].

### *Total IgE, RAST and CIE/CRIE*

Total IgE was determined by Phadebas PRIST® (Pharmacia AB, Uppsala, Sweden) and expressed in units per ml (U/ml), and specific IgE against birch (B-IgE), alder (A-IgE) and oak (O-IgE) was determined by Phadebas RAST® (Pharmacia AB) according to the manufacturer's instructions and expressed in Phadebas RAST units per ml (PRU/ml). Specific IgG towards birch allergens (B-IgG) was determined with a prototype of Phadebas IgG RAST® (Pharmacia AB) with specially coupled birch allergen discs and <sup>125</sup>I-anti-IgG antibodies. All tests were performed in duplicate and with patient sera diluted 1/200. The radioactive uptake was compared with a standard curve obtained by dilution of a reference serum. The IgG antibody concentration was expressed in arbitrary units/ml (U/ml).

Sera used for CIE/CRIE analysis were collected before and after 3 years of IT. Ten pairs of children were selected based on their changes in symptoms scores from 1979 to 1982 and 1983. In two of the pairs both children had improved moderately, in two of the pairs both children had improved greatly, in three of the pairs the children treated with B had improved much more than those treated with M, and in the remaining three pairs the children treated with M had improved much more than those treated with B.

Crossed immunoelectrophoresis was carried out on a 5 × 7 cm gel bond film (Pharmacia Fine Chemicals, Uppsala, Sweden) using 0.023 M TRIS/Tricine buffer (Bio Rad), and 0.3 mM Calciumlactate, pH 8.6, in 1% agarose (Agarose A, Pharmacia Fine Chemicals) essentially as previously described [10,11]. Samples of 11 µg protein from birch or white oak pollen extracts were run in the first dimension electrophoresis at 10 V/cm, 15°C for 30 min (birch) and for 25 min (oak). The second dimension electrophoresis was performed using 20 µl and 14 µl rabbit anti-birch serum/cm<sup>2</sup> and

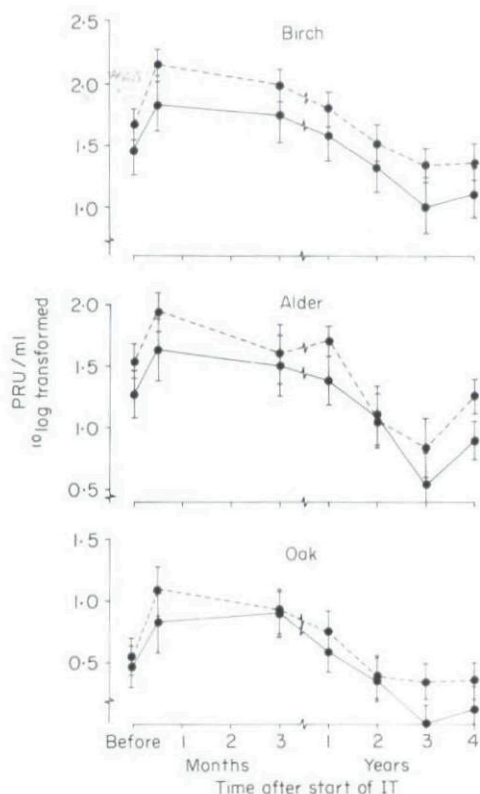


Fig. 1. Tree-specific IgE. (—) B group, (---) M group. The median and s.d. are shown.

35  $\mu$ l and 18  $\mu$ l rabbit anti-oak serum/cm<sup>2</sup> in the anodic and cathodic antibody-containing compartments, respectively. The second dimension electrophoresis was run at 2.5 V/cm for 16 hr at 15°C in both cases.

Determination of IgE- and IgG-CRIE as well as CRIE classification were performed essentially as previously described [11]. For calculations, CRIE class G was assigned one point up to CRIE class A, which was assigned seven points.

#### Statistical methods

Due to skewness, some of the values obtained were transformed for calculations, i.e. symptom scores were square-root transformed and antibody levels and potencies of allergen preparations were 10-logarithm transformed. The differences between the two treatment groups were analysed with Student's *t*-tests except for CPT results, which were tested with chi-square tests (with Yates' correction). Changes within groups were analysed with Student's paired *t*-tests except for CPT results when the sign test was used. Pearson's product-moment correlation coefficient was used for correlations. Multiple regression performed with the computer program 'Statistical Package for Social Sciences, version 9.0' was used for analysis of differences in changes between groups. All tests were two-tailed.

#### Results

After 2 weeks of IT, B-IgE, A-IgE and O-IgE increased ( $P < 0.01$  for all trees in both

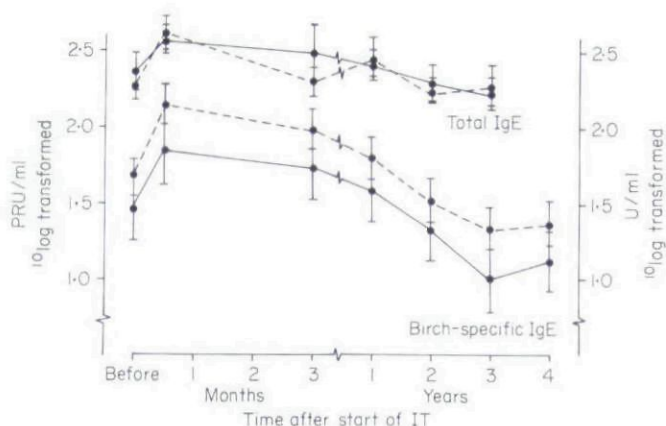


Fig. 2. Total IgE compared with birch-specific IgE. (—) B group, (---) M group.

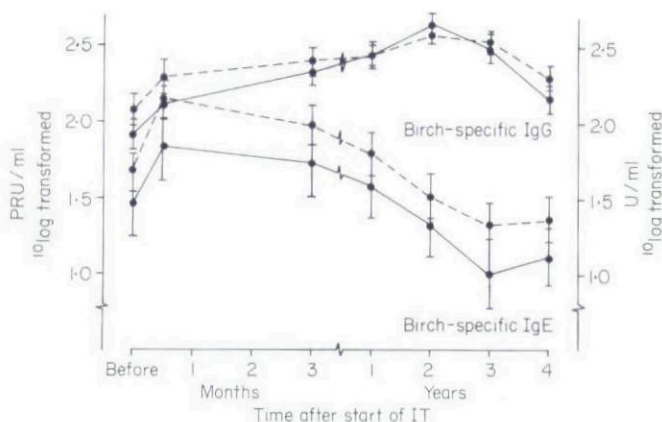


Fig. 3. Birch-specific IgG in comparison with birch-specific IgE. (—) B group, (---) M group.

treatment groups). The antibody levels remained increased during the first year of IT and then decreased to levels below the initial ones ( $P < 0.01$  for all trees in both groups; Fig. 1). A similar pattern, although less pronounced, was seen for total IgE (Fig. 2). In the beginning of IT, B-IgG also increased ( $P < 0.01$ ) but maximal levels were not reached until after 2 years of treatment and the levels remained above the initial values throughout IT (Fig. 3). After the cessation of IT there was a tendency for IgE to increase ( $P = 0.06$  for birch,  $P < 0.01$  for alder,  $P = 0.07$  for oak) and for B-IgG to decrease ( $P < 0.01$ ; Figs 1 and 3). Before IT the M group had higher antibody levels compared with the B group but the changes were similar for the groups during the treatment.

None of the antibody levels in the samples before IT correlated with the improvement in symptom scores from 1979 to 1982. As can be seen in Table 1, some of the antibody levels in the B group after 2 weeks and 3 months of IT correlated with the improvement in contrast to the M group. The ratio between birch-specific IgG and birch-specific IgE, in the samples taken approximately 4 months before the pollen

**Table 1.** Correlations in the B group between antibody levels and improvement

|                                                            |           | Significance for correlation<br>with improvement to 1982 | Significance for difference<br>from M group |
|------------------------------------------------------------|-----------|----------------------------------------------------------|---------------------------------------------|
| 2 weeks after<br>start of IT                               | Total Ig  | n.s.*                                                    | n.s.                                        |
|                                                            | B-IgE     | 0.02                                                     | 0.06                                        |
|                                                            | A-IgE     | 0.02                                                     | 0.1                                         |
|                                                            | O-IgE     | 0.04                                                     | 0.08                                        |
|                                                            | B-IgG     | 0.02                                                     | 0.1                                         |
| 3 months after<br>start of IT                              | Total IgE | 0.02                                                     | 0.07                                        |
|                                                            | B-IgE     | 0.02                                                     | 0.03                                        |
|                                                            | A-IgE     | 0.01                                                     | 0.01                                        |
|                                                            | O-IgE     | 0.01                                                     | 0.03                                        |
|                                                            | B-IgG     | 0.05                                                     | n.s.                                        |
| Maximum                                                    | Total IgE | 0.1                                                      | n.s.                                        |
|                                                            | B-IgE     | 0.06                                                     | n.s.                                        |
|                                                            | A-IgE     | 0.02                                                     | 0.08                                        |
|                                                            | O-IgE     | 0.06                                                     | 0.1                                         |
|                                                            | B-IgG     | 0.1                                                      | n.s.                                        |
| Increase from<br>start of IT<br>to after 2<br>weeks of IT  | Total IgE | n.s.                                                     | n.s.                                        |
|                                                            | B-IgE     | 0.1                                                      | n.s.                                        |
|                                                            | A-IgE     | 0.02                                                     | 0.04                                        |
|                                                            | O-IgE     | 0.1                                                      | n.s.                                        |
| Increase from<br>start of IT<br>to after 3<br>months of IT | B-IgG     | 0.1                                                      | n.s.                                        |
|                                                            | Total IgE | 0.1                                                      | n.s.                                        |
|                                                            | B-IgE     | 0.1                                                      | n.s.                                        |
|                                                            | A-IgE     | 0.01                                                     | <0.01                                       |
|                                                            | O-IgE     | 0.03                                                     | 0.07                                        |
|                                                            | B-IgG     | n.s.                                                     | n.s.                                        |

\* n.s. denotes a  $P$  value  $>0.1$ .

season, correlated with the improvement in symptom scores from 1979 to 1982 and to 1983 ( $P < 0.01$ ) but there were no significant differences between the groups.

The highest dose reached during rush IT correlated with B-IgE, A-IgE and O-IgE before IT and after 2 weeks of treatment in the M group ( $P < 0.01$ – $P = 0.4$ ). In contrast, the correlations were inverse in the B group, i.e. low levels of antibodies correlated with a high dose ( $P < 0.01$ – $P = 0.1$ ). The difference between the groups was also statistically significant in most cases for the three trees on the two occasions ( $P = 0.01$ – $P = 0.07$ ). In both groups the dose correlated with B-IgG after 3 months of IT ( $P < 0.05$ ).

After 3 months and after 1 year of IT the conjunctival sensitivity against birch, as measured with CPT, had decreased ( $P < 0.01$ ) with a negative correlation to the change in birch-specific IgE (non-significant).

Figure 4(a) demonstrates the CIE pattern of the birch pollen extract stained with Coomassie brilliant blue and Fig. 4(b) is a schematic drawing of the CIE reference pattern of birch. Twenty-five antigens were identified in the extract. They were arbitrarily numbered from the anode to the cathode. The demonstrated reference pattern was constructed from several experiments by varying the ratio antigen:rabbit antisera. Figure 4(c) shows an X-ray film from a CRIE analysis of an individual patient serum where the intensity of radiostaining illustrates the degree of specific IgE binding.

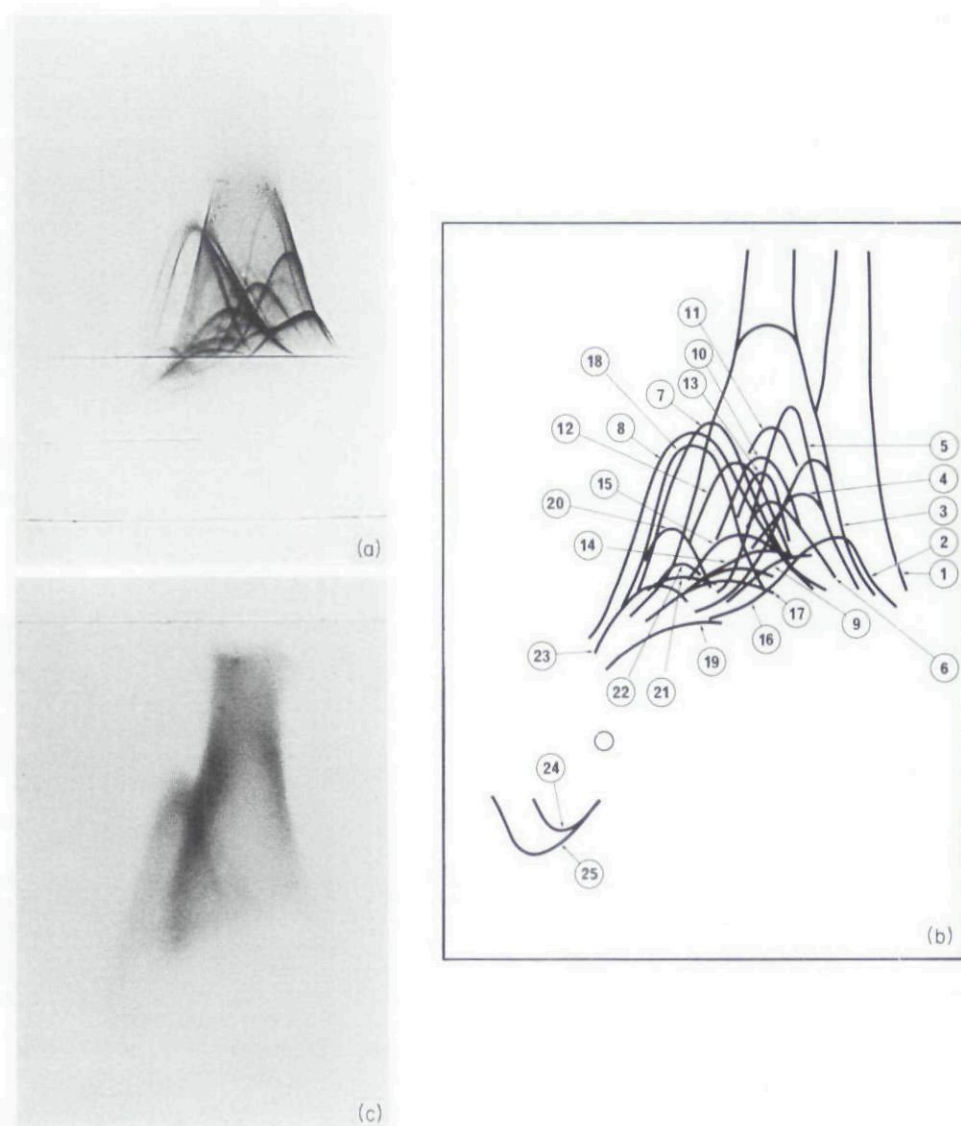


**Fig. 4.** (a) Crossed immunoelectrophoresis of the birch pollen extract against hyperimmune rabbit antisera. The anode is to the right in the first dimension and at the top in the second dimension. (b) Schematic representation of the immune precipitates (a and c) arbitrarily numbered 1–25 from the anode to the cathode. (c) An X-ray film from a CRIE experiment with serum from a birch pollen sensitive patient.

Figure 5 demonstrates the CIE analysis of a heat-treated birch pollen extract. After heat treatment of 15 min at 100°C the antigenicity of all birch components except antigen 3 was completely destroyed, indicating that antigen 3 is a thermostable protein.

The protein-stained pattern of the oak pollen extract is shown in Fig. 6(a) and a schematic drawing of the CIE reference pattern of the oak pollen extract in Fig. 6(b). There were nineteen immunoprecipitates detected in the oak pollen extract and these were arbitrarily numbered from the anode to the cathode. Antigens 2, 10 and 18 were only visible after incubation with patient serum and autoradiography. Figure 6(c)

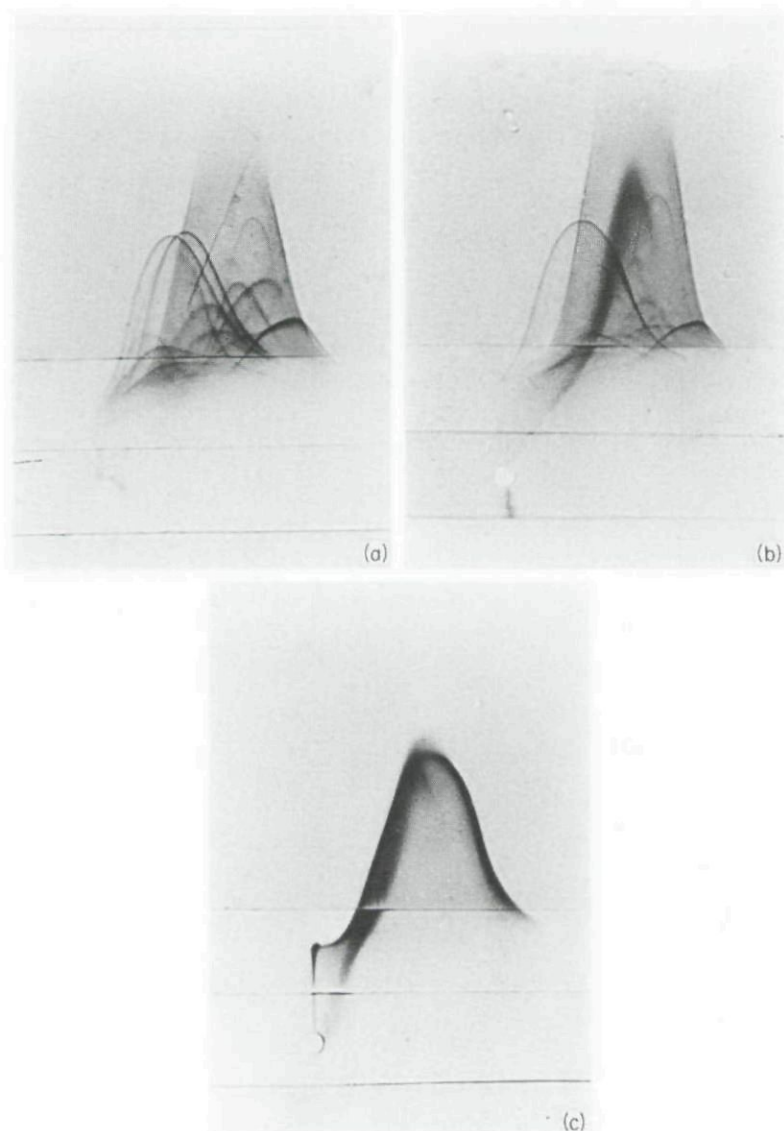


**Fig. 5.** Crossed immunoelectrophoresis analysis of the birch pollen extract after heat treatment (protein stained immunoprecipitates, 11  $\mu$ g protein applied). (a) CIE pattern of the untreated extract; (b) heat-treated for 2 hr at 55°C; (c) heat-treated for 15 min at 100°C. Anode to the right in the first dimension and at top in the second dimension.

illustrates an example of the CRIE analysis of the specific IgE binding to oak antigens for an individual patient serum.

All but one of the children showed IgE antibodies against birch antigens 2 and 3 but antigen 3 gave the strongest radiostaining (Fig. 7). The only child without visible radiostaining was the only one who did not improve by IT according to self-evaluation. This child also had the lowest B-IgE. All children showed radiostaining against oak antigen 4 (Fig. 7). The sum of CRIE scores of the different allergens before and after IT is visualized in Fig. 8.

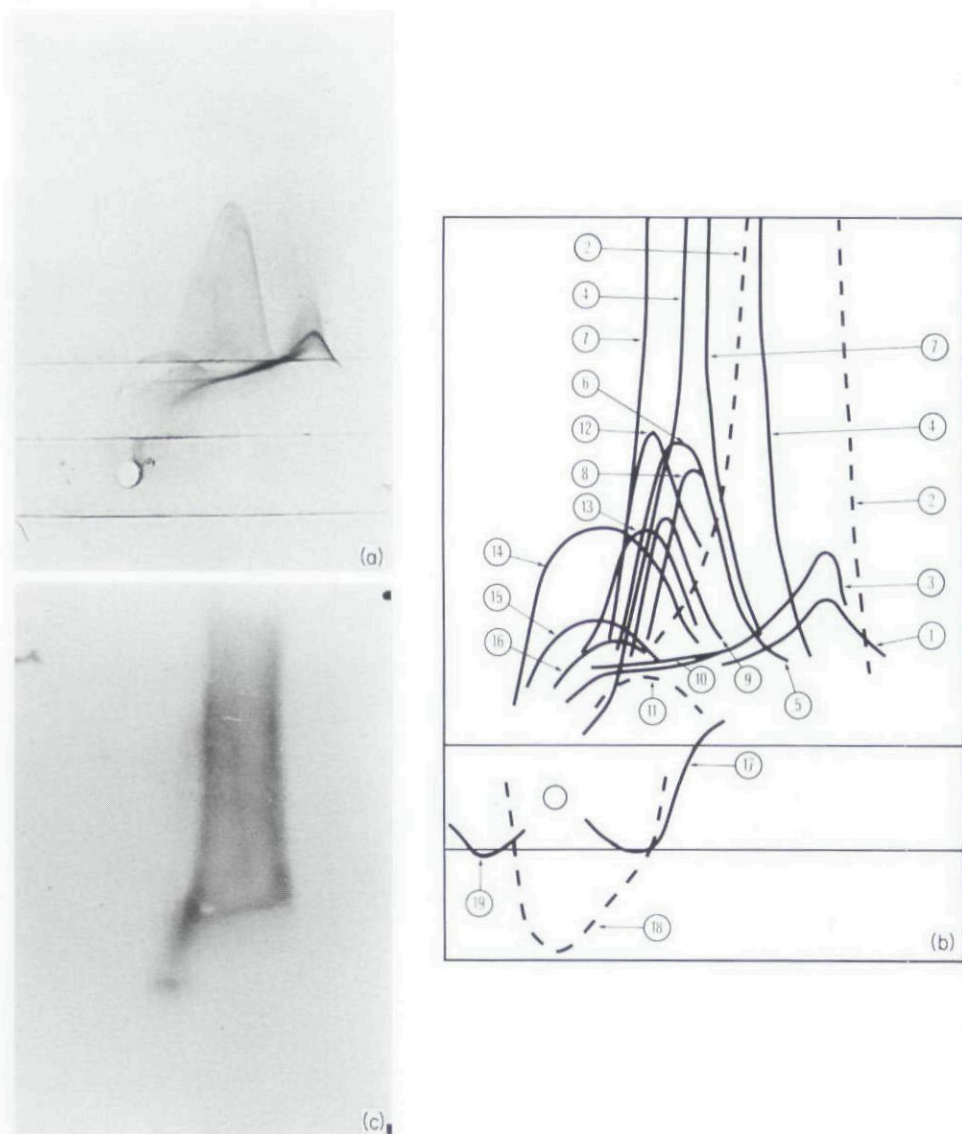


**Fig. 6.** (a) Crossed immunoelectrophoresis of oak pollen extract against hyperimmune rabbit antisera. Anode to right in the first dimension and at the top in the second dimension. (b) Schematic drawing of the immunoprecipitates (a and c) in oak arbitrarily numbered 1-18. (—) Visible protein-stained precipitates, (---) precipitates visible only after radiostaining. (c) An X-ray film from a CRIE experiment with serum from an oak pollen sensitive patient.

In the B group the IgE-CRIE score for birch and oak allergens correlated with the improvement in symptom score from 1979 to 1982 ( $P < 0.01$ ), i.e. a high level before IT gave a good improvement, with the strongest correlations for birch antigens 3, 6 and 15, and for oak antigen 4. This was not seen in the M group (differences between groups  $P < 0.01$ ). In the whole group of children the decrease of the total IgE-CRIE points during IT correlated with the improvement to 1982 ( $P < 0.05$  for both birch and oak)

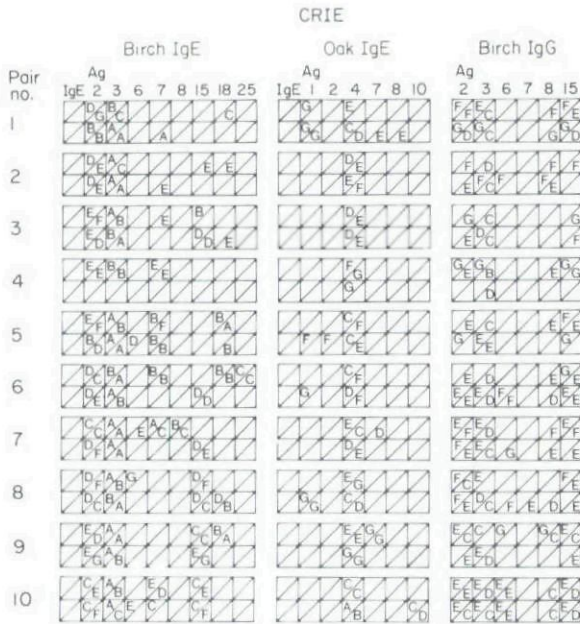


**Fig. 7.** Allergogram showing visible IgE- and IgG-CRIE radiostaining of allergens in birch and oak before (upper left) and after (lower right) IT for individual patients. The upper patient in each pair was treated with the tree mixture and the lower patient with the birch preparation. The intensity of the radiostaining is shown as CRIE-classes (A-G).

similarly in both groups. The ratio between IgG and IgE scores for individual birch allergens did not correlate with changes in, or levels of, symptoms scores.

After IT eight of the fourteen children who were below 13 years of age before the start of IT had developed new IgE specificities against four birch allergens, i.e. antigens 6, 7, 15 and 18 (Fig. 7). None of the six children who were 13 years of age or more before starting IT developed such 'new sensitivities'. In addition, two of the children developing 'new sensitivities' against birch allergens also developed new IgE antibodies against oak allergens, i.e. antigens 7 and 8 (Fig. 7). The development of 'new sensitivities' was not connected with less improvement. Radiostaining against new antigens were common in birch IgG-CRIE but antibodies were only found against the antigens 2, 3, 6, 7, 8 and 15 (Fig. 7). It was not possible to distinguish between the B and the M groups in any of the CRIE patterns before or after IT.

## Discussion

No statistically significant differences were found between the B and M groups in the antibody response as measured *in vitro* with total IgE, B-IgE, A-IgE, O-IgE, B-IgG or CRIE in this study, despite the M group showing less symptoms in the third and the fourth season.

The initial increases followed by decreases in total serum IgE levels and in tree-specific IgE antibodies during IT, and the increase of specific IgG antibodies, agree with previous reports [12]. The correlation in the B group between the increase of total serum IgE levels after 3 months of IT and the improvement after 2.5 years of treatment is in accordance with a previous study [13]. In the B group there was also a statistically

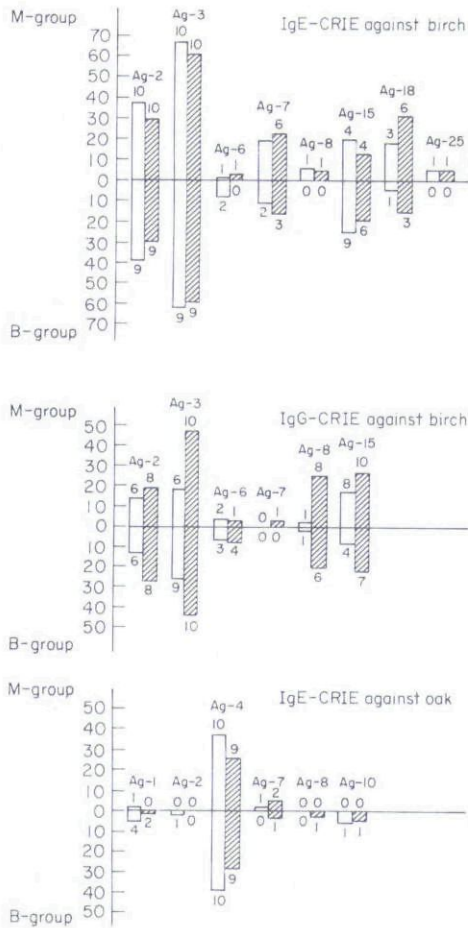


Fig. 8. The sum of IgE and IgG CRIE scores to different allergens before IT (■) and after IT (□) in the two treatment groups. The figures in the bars illustrate the number of patients responding with antibodies.

significant correlation between B-IgE, A-IgE, O-IgE and B-IgG after 2 weeks and after 3 months of IT, and the clinical improvement.

During the first 3 months of IT, birch-specific IgE increased while the conjunctival sensitivity decreased. The correlation was not statistically significant. This indicates that there is no direct connection between the levels of IgE antibodies in serum against an allergen preparation and the sensitivity against the same preparation in the shock organ.

It is well known that the initial increase of specific IgG correlates with the doses given during rush IT [12]. However, there were no significant correlations between the doses given during the rush phase of IT and the outcome of IT, probably since the doses were increased considerably during maintenance IT as the children became less sensitive. The ratio between B-IgG and B-IgE at the beginning of IT was not useful for prediction. However, this ratio correlated well with symptom scores after 2.5 years of treatment [14] although there were outliers with a high quotient and only moderate improvement in symptom scores.

Although none of the tree-specific antibody levels before IT correlated with the improvement, there was a significant correlation between the CRIE scores and the outcome of the therapy in the B group but not in the M group. It is possible that frequent analyses of IgG subclasses during the first months of IT would have yielded more information, especially if the sera had been analysed with CRIE [15].

The immune system of the rabbit identifies at least twenty-five antigens in birch pollen and nineteen in oak pollen. In accordance with other studies the children in this trial developed antibodies against only eight birch pollen antigens. Antigen 3 in our CIE reference system seemed to coincide with the previously reported most important birch allergen, which is a thermostable component with a molecular weight around 20 000 daltons, 10 000–17 000 daltons or 17 000 daltons [16]. The IgE response in patients against the oak antigens has not been reported previously and in our study the children developed specific IgE antibody binding against six of the oak antigens.

Several studies have shown that IT with a preparation against which the person is not allergic may result in the development of IgE antibodies against the injected allergens [17–19]. Conversely, several studies show that IT does not result in 'new sensitivities' in adult patients with clinical indication for IT [14, 20–22]. These adult patients had been exposed to the allergens for a long period so had time prior to IT to develop IgE antibodies directed against all determinants to which they were capable of developing such antibodies according to their genetic code. This is supported by our findings that none of the children at least 13 years old developed 'new sensitivities'. Conversely, in two studies [14, 22] children below 13 years also failed to develop 'new sensitivities' but these children were not treated with pollen allergen preparations. In contrast, in the present study the majority of the children below 13 years developed 'new sensitivities'. These younger children had not been exposed to birch pollen as long as the adults and the older children, and had probably not had the time to develop all IgE antibodies. It is possible that the 'new sensitivities' are a result of IT, but it is more probable that these 'new sensitivities' would have developed even without IT. It was not possible to correlate the development of 'new sensitivities' with the outcome of IT as measured with *in-vivo* data. Thus, the eight children improved despite the new IgE antibodies. It would be interesting to follow young children with pollinosis not given IT.

In conclusion, no immunochemical differences with certain clinical relevance were found between the two treatment groups. 'New sensitivities' developed in 60% of the children younger than 13 years, apparently without clinical implications.

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