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Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy

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Summary

Background IgE titres tend to rise early after the start of immunotherapy, followed by a decline to pre-immunotherapy levels or lower.

Objectives We were interested to know whether the early increase in IgE antibodies includes new specificities of IgE, and whether these responses persist.

Methods Sera of 64 patients undergoing grass pollen immunotherapy were tested for IgE against four purified grass pollen allergens: Lol p 1, 2, 3, and 5. At least two serum samples were taken, one before the start of therapy and one between 5 and 18 months after the first immunization (mean: 10 months).

Results The mean IgE responses to Lol p 1, 2 and 3 showed a moderate but not significant increase. In contrast, the mean IgE response to Lol p 5 showed a significant decrease of >30%. IgE against total *Lolium perenne* pollen extract moderately increased (>20%), showing that a RAST for total pollen is not always indicative for the development of IgE against its major allergens. For >40% of the patients it was found that IgE against one or more of the four allergens increased, while IgE against the remaining allergen(s) decreased. For 10 sera the ratio of IgE titres against at least two allergens changed by at least a factor of 5. The changes in specific IgE also included conversions from negative (< 0.1 RU) to positive (0.6 to 5.0 RU) for five patients. For two patients, the induction of these 'new' IgE antibodies against major allergens was shown to result in a response that was persistent over several years.

Conclusion Although active induction of new IgE specificities by immunotherapy was not really proven, the observations in this study indicate that monitoring of IgE against purified (major) allergens is necessary to evaluate changes in specific IgE in a reliable way.

Keywords: immunotherapy, grass pollen, IgE-induction, major allergens

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Introduction

Hyposensitization therapy of allergic rhinitis has been shown to cause an increase in allergen specific IgE, early

after the first allergen injections [1–3]. This increase is reported to be transient: after a few months IgE levels usually decline to pre-therapy or slightly lower levels. It is not clear whether the early increase is explained by higher production of IgE with already existing epitope-specificity, by the induction of IgE with new specificities, or by a combination of both. In most immunotherapy (IT) studies,

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IgE responses were monitored against whole allergen extracts. In this way, possible new specificities and changes in the relative importance of single allergens remain undetected. Only in a few cases was IgE against single allergens studied [3–5]. By means of immunoblotting, induction of 'new' IgE antibodies against minor allergens from birch and grass pollen was demonstrated [6,7].

To find out whether induction of IgE against major allergens or shifts in the relative importance of individual allergens occur, a group of sera of patients receiving grass pollen immunotherapy was studied. IgE antibodies were measured against four purified grass pollen allergens, Lol p 1, 2, 3, and 5, before and in the course of IT.

Materials and methods

Human subjects

All sera were obtained from patients ($n = 64$) receiving grass pollen IT. Four different groups were included. The first group contained sera from 10 patients (K1–10) involved in a double-blind study in Poland [8]. Serum samples were taken at the start of therapy (October/November) and preseasonally (April) in the first year of therapy. The second group contained 10 patients (S1–10) from the Netherlands who were followed during several years of IT. Serum samples were taken at four time-points in three consecutive years: January/February, May, July and October. In some cases an additional sample was analysed, taken in the sixth pollen season (July), following the start of therapy.

The third group contained sera from 30 patients (D1–30) from the Netherlands. Serum samples were taken at the start of therapy and 11–15 months later (mean: 13 months). The final group of sera was from 14 patients (B1–14) from the Netherlands. Serum samples were taken at the start of therapy, and at variable times afterwards (5 to 18 months; mean: 10 months).

Treatment was performed with Purethal^(R)-grasses (HAL Allergen Laboratories, Haarlem, The Netherlands) for patients K1–10, with Alutard Grassmix-5 SQ 293 (ALK, Hørsholm, Denmark) for patients D1–30 and B1–14, and with Pharmacia Grassmix-4 (Pharmacia, Uppsala, Sweden) for patients S1–10.

Allergens

Grass (*Lolium perenne*) pollen was obtained from Diephuis Laboratories (Groningen, The Netherlands). Extraction was performed as described previously [9]. Purified Lol p 1, Lol p 2, and Lol p 3 were obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, Maryland, USA): NIH research reagents A611–901–185, A612–901–

185, and A612–901–185 respectively. Purified Lol p 5 was obtained by monoclonal antibody (4B1) affinity-purification as described elsewhere [9]. The source of recombinant Dac g 2 was a crude *E. coli* lysate containing a β -galactosidase-Dac g 2 fusion protein [10,11]. Purified Lol p 1, 2, 3 and 5 were coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) for application in RAST (100 μ g allergen on 100 mg of Sepharose).

Radio allergosorbent test (RAST)

RAST was performed as described previously [12]. Results were expressed in RAST units (RU). A standard curve was made by measuring the response obtained with a reference serum with known total IgE in a two-site assay with Sepharose-coupled sheep-antihuman IgE and ¹²⁵I-labelled anti-IgE. Results ≤ 0.1 RU were regarded as negative.

Depletion for IgG

Depletion for IgG antibodies was performed by a 4-h incubation (end-over-end at room temperature) of 100 μ l serum with 100 mg Sepharose-coupled Protein G (Pharmacia) in a final volume of 1 ml (PBS/0.3% BSA/0.1% Tween 20). As a negative control Sepharose-coupled glycine was used, instead of Protein G-Sepharose. Total IgG and total IgE were tested in the unbound fraction to monitor the specificity of the depletion. In all cases depletion was effective: $<5\%$ IgG was left in the supernatant. Total IgE did not significantly change by the procedure ($<10\%$).

Immunoblotting

Immunoblotting was performed with crude *E. coli* lysates (recombinant Dac g 2) separated by SDS-PAGE, as described previously [11].

Statistical analysis

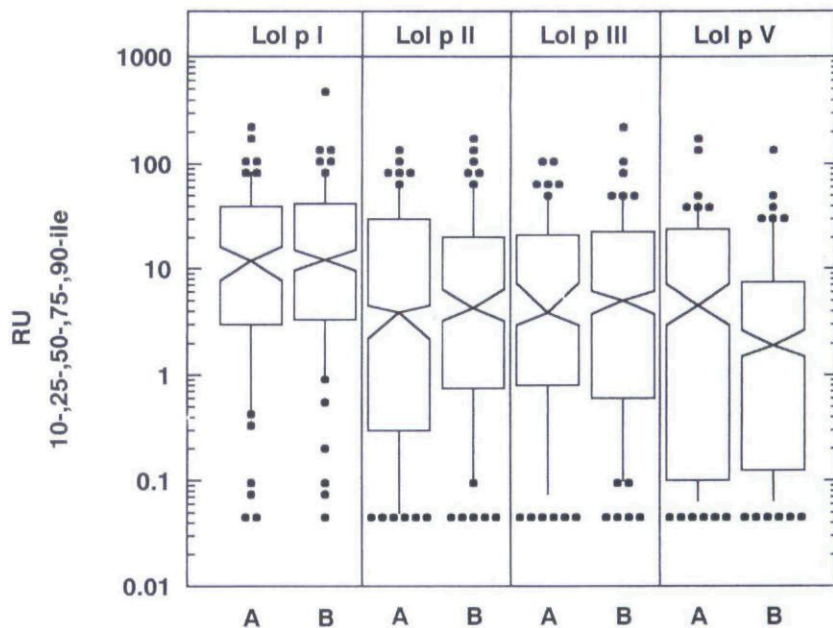
Pre- and post-immune serum IgE levels against Lol p 1, 2, 3 and 5 were compared by means of a Student's *t*-test (paired; two-tailed).

Results

IgE against Lol p 1, 2, 3 and 5

All pre- and post-immune sera ($n = 64$) were tested in a RAST for Lol p 1, 2, 3 and 5 (Fig. 1). The mean of the time interval between the two sample collections was 10 months. For Lol p 1, 2, and 3 a moderate increase of the mean IgE response was observed: 8.1 to 10 RU, 2.7 to 3.3 RU, and 3.0 to 3.3 RU, respectively. In contrast, for Lol p 5 a decrease

Fig. 1. Comparison of pre- and post-immune antibody titres. The median IgE responses against Lol p 1, 2, 3 and 5 are shown at the start of therapy (A) and in the course of therapy (B). Results are expressed in RU. In addition, the 10- (lower dots), 25- (lower box), 75- (upper box), and 90-percentiles (upper dots) are shown.



was found (2.2 to 1.5 RU). In a paired *t*-test only the decrease of Lol p 5-specific IgE was shown to be significant. The mean of post-immune/pre-immune IgE ratios (0.68) had a 95% confidence interval (CI) of 0.52–0.87.

In a sub-selection of the samples, taken within a half year after start of the therapy ($n=24$), the mean IgE responses to Lol p 1, 2 and 3 showed a significant increase. The mean IgE ratios (post/pre) in this case were: 1.5 (1.0 < 95% CI < 2.3), 1.8 (1.1 < 95% CI < 3.0), and 1.8 (1.1 < 95% CI < 3.0), respectively. In these patients the response to Lol p 5 did not change significantly (ratio 1.1; 0.7 < 95% CI < 1.6). For serum samples taken >6 months after the first immunization ($n = 40$), the only significant change was a decrease for IgE to Lol p 5 (mean ratio: 0.50; 0.4 < 95% CI < 0.7).

For 53/64 patients a RAST for total *Lolium perenne* pollen was performed. The mean response increased from 18.0 RU to 22.1 RU (mean ratio: 1.2; 1.1 < 95% CI < 1.4). Within this group again a moderate, but non-significant, increase was observed for Lol p 1, 2 and 3, whereas the response to Lol p 5 showed a significant decrease (mean ratio: 0.68; 0.52 < 95% CI < 0.89).

Depletion for IgG antibodies

For nine patients who demonstrated a decrease of specific IgE against Lol p 5, post-immune sera were depleted for IgG antibodies with Protein G-Sepharose. The depleted sera were tested in a RAST for Lol p 5. After IgG removal, four out of nine sera (B6, S1, S2 and S6) showed a significant increase (25%, 29%, 58% and 27%, respectively) of IgE-binding to Lol p 5; for the other five this was not found (<5%).

Individual IgE antibody responses

The development of specific IgE among individual sera differed. In 16 sera IgE against all four allergens decreased. Only two of these sera were taken within 6 months after the start of IT. For 14 sera, IgE against all four allergens increased. In this case serum samples were taken less than 6 months after initiation of the therapy, again with the exception of only two sera. For five patients no significant changes (<10%) in IgE titres were found. One of these patients showed no binding to any of the purified allergens (RAST for crude *Lolium perenne* extract: 1.3 RU). For the remaining patients ($n = 29$) the development of IgE titres against Lol p 1, 2, 3 and 5 was not consistent: an increase for one or more allergens was accompanied by a decrease for the remaining allergen(s) (Fig. 2). For 10 patients intra-serum ratios of IgE against two or more of the purified allergens changed by a factor of 5–400 (mean: 72).

Induction of IgE with new specificities

Five patients showed a conversion from negative (≤ 0.1 RU) to positive (0.6 to 5.0 RU) in the RAST for one or more of the grass pollen allergens (shown for two patients in Fig. 2(a/b)). This was observed twice for each of the four allergens. In some cases an existing but weak IgE response increased up to ten-fold (shown for two patients in Fig. 2(c/d)). Induction or increase of IgE was confirmed on immunoblot with recombinant Dac g 2 for several patients (Fig. 3).

Persistence of newly induced IgE

Patients S1–10 were followed during several years of IT. At

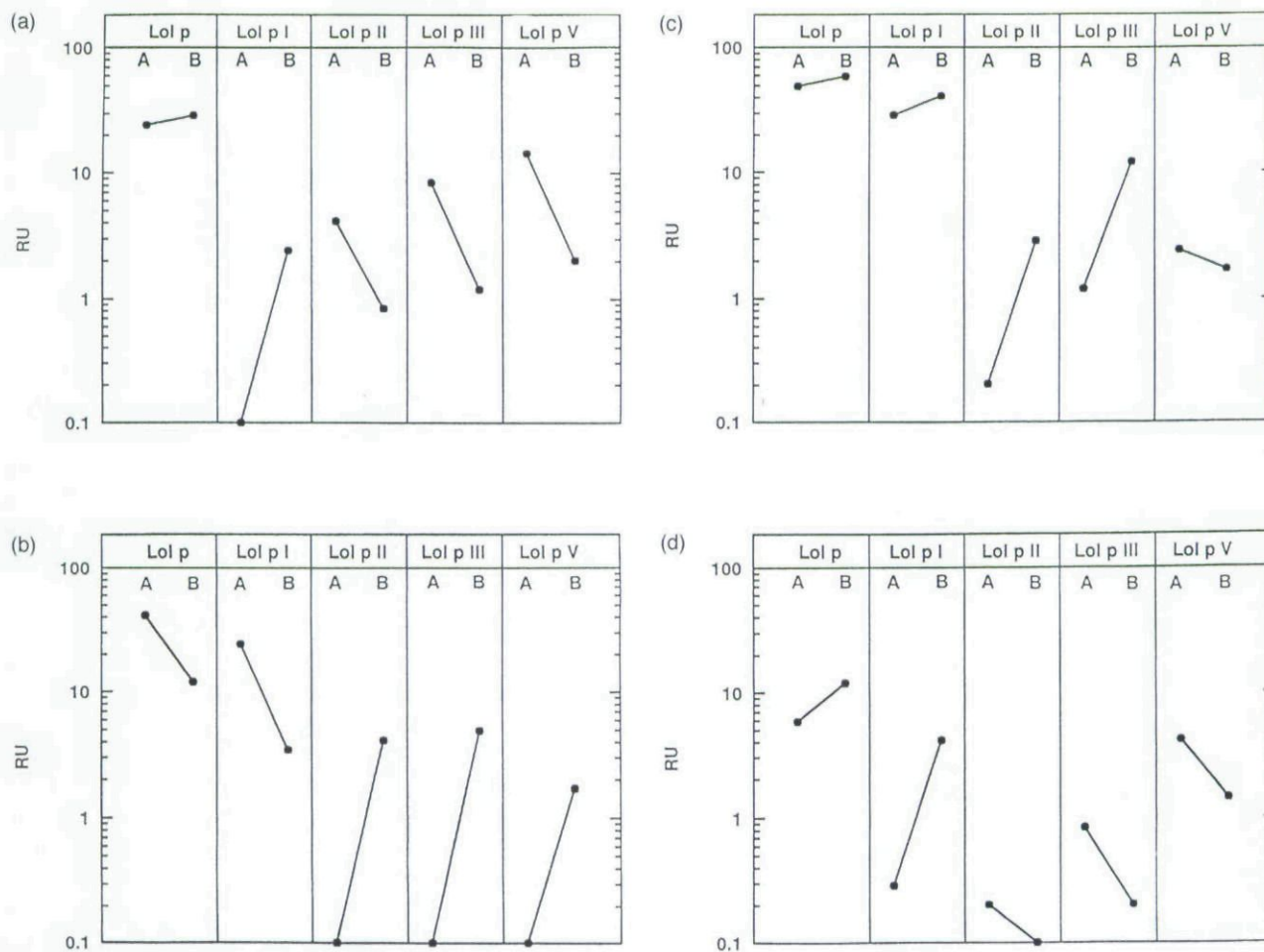


Fig. 2. Variability in the changes observed in IgE patterns. IgE titres against total *Lolium perenne* pollen, and the four purified allergens were measured in a RAST, both at the start of therapy (A) and in the course of therapy (B). Results are expressed in RU. Changes are shown for four patients: S6 (graph a), K15 (b), D27 (c) and B15 (d).

the start of therapy patient S6 had a clear IgE response against Lol p 2, 3 and 5 (≥ 4.5 RU), but only a weak response to the major grass pollen allergen Lol p 1 (< 0.3 RU). During 3 yr of therapy IgE titres against the former three allergens decreased to a 10-fold lower level. In contrast, IgE against Lol p 1 increased by a factor 4 (when titres in January/February were compared) (Fig. 4a). A second patient (S4) was negative in the RAST for Lol p 2 and 3 (≤ 0.1 RU), and almost negative for Lol p 5 (0.15 RU) at the start of IT.

In the three following pollen seasons (1984–1986) specific IgE against Lol p 2 and 5 was detected, every year at a higher level (Fig. 4b). Three years later (1989) an additional (weak) IgE response against Lol p 3 (0.3 RU) was found. By this time IgE levels against Lol p 2 and 5 had increased to 2.7 and 2.1 RU, respectively. This is > 10 times higher than at the start of the therapy. Another patient (S5) showed an increase of specific IgE against all four allergens, as judged

by RAST values found for serum samples taken in January/February (Fig. 4c). In contrast to this, a continuous decrease of specific IgE was observed for patient S3 (Fig. 4d).

Discussion

In this study, the mean IgE responses against total *Lolium perenne* pollen, of patients receiving grass pollen immunotherapy, were shown to increase in the first year of treatment ($n = 53$). When analysis of IgE responses against four purified allergens was performed for these 53 patients, a pattern similar to the complete panel of 64 patients was found: a (non-significant) trend towards an increase for Lol p 1, 2 and 3, and a decrease for Lol p 5. This illustrates that measurement of specific IgE against a crude allergen source does not always give reliable information about the IgE response to its major allergens, in this case Lol p 5.

When serum samples, taken early after the start of therapy

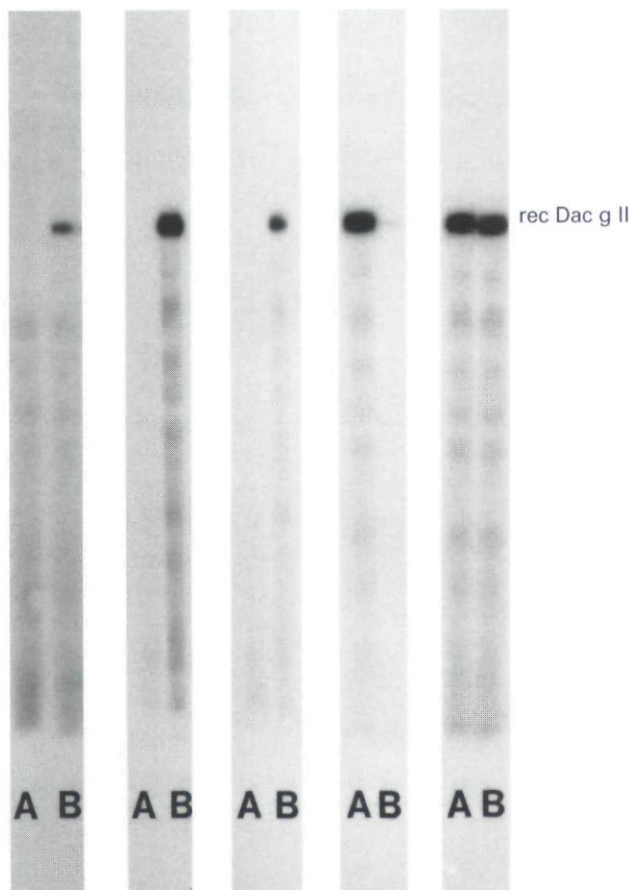


Fig. 3. Recombinant Dac g 2 immunoblot analysis. Binding of IgE from pre-immune (A) and post-immune (B) sera is shown for patients B10, K5, K15, B7 and B12 (from left to right). B10, K5 and K15 are examples of IgE-induction (Lol p 2 RAST increase (RU): 2.0 to 5.8, 0.4 to 4.1, and 0.1 to 4.5, respectively). For B7 the anti-Dac g 2 response has markedly decreased (RAST for Lol p 2: 14.5 to 3.6 RU). Patient B12 is an example for whom no change in reactivity was detected (Lol p 2 RAST: 3.9 to 3.8 RU).

(≤ 6 months) were analysed separately, an increase of IgE against Lol p 1, 2 and 3 was observed. In the group of serum samples taken at a later time point (> 6 months) no significant changes were found in these responses. This fits the generally accepted picture of a transient rise of IgE during the first months of IT [1–3]. For IgE responses to Lol p 5 the early rise was not found, and a decrease of the mean IgE titre was found at later time points.

To investigate whether Lol p 5-specific IgG was blocking the binding of IgE in the Lol p 5-RAST, nine sera were depleted for IgG. In four out of nine cases interference by IgG could (partially) explain the observed apparent decrease in Lol p 5-specific IgE. For the other sera this was not found, suggesting that IgE against Lol p 5 is really down-regulated in these patients. Since the IgG depletion was only

performed for a small selection of sera, we cannot determine whether the decrease in Lol p 5-specific IgE, observed for the whole group of patients ($n = 64$), would still be significant after IgG removal. Furthermore, it is not clear why the immune response to Lol p 5 is different from the responses to the other three allergens. Perhaps Lol p 5 is less efficiently adsorbed in depot-materials, resulting in a higher exposure to this allergen, and thereby an earlier induction of (blocking) IgG or down-regulation of IgE.

It could be argued that the sera originated from too diversely treated patients to be statistically analysed as one group. When the four groups were analysed separately, similar trends were observed: IgE responses to Lol p 1, 2 and 3 remained unchanged or slightly increased, whereas IgE against Lol p 5 decreased. The ratios of post/pre-immune IgE responses against Lol p 5 for groups B, D, K and S were 0.8, 0.4, 0.7 and 0.9, respectively. Due to the low number of observations these figures were, however, not significant.

The changes observed in individual IgE patterns against the four purified grass pollen allergens illustrate that measurement of IgE against total pollen is not a very reliable parameter for monitoring specific IgE in time (e.g. during IT). It was for example found that the IgE level against *Lolium perenne* pollen decreased by a factor of 4, while IgE titres against several of its major allergenic components (Lol p 2, 3 and 5) simultaneously increased by at least a factor of 20 (Fig. 2b). In several cases the opposite was observed: increase of IgE against total pollen, accompanied by a decrease of IgE against one or more of the purified allergens (Fig. 2a, c and d).

Appearance of IgE with specificities not detected before IT is certainly not a desired effect of the treatment. This is especially true if the 'new' IgE antibodies are directed to a major allergen like Lol p 1 or Lol p 5. In this study conversion from negative to positive was observed in about 8% (5/64) of the patients. This phenomenon was also observed in samples taken > 1 yr after the start of the therapy, indicating that it is not just a part of the transient increase of specific IgE early in the course of the treatment. The persistent character of newly induced IgE specificities was confirmed for two patients that were monitored during several years of therapy (Fig. 4).

Of course the observations in this study do not prove direct involvement of immunotherapy in the induction of new IgE antibodies. They could also have appeared 'spontaneously' by natural exposition. Whether these IgE antibodies have clinical relevance is also not clear. Investigating the development of specific IgE responses towards individual allergens in a placebo-controlled trial would be the best way to answer these questions. The main implication from this study is that measurement of IgE against crude allergen extracts can mask serologically important

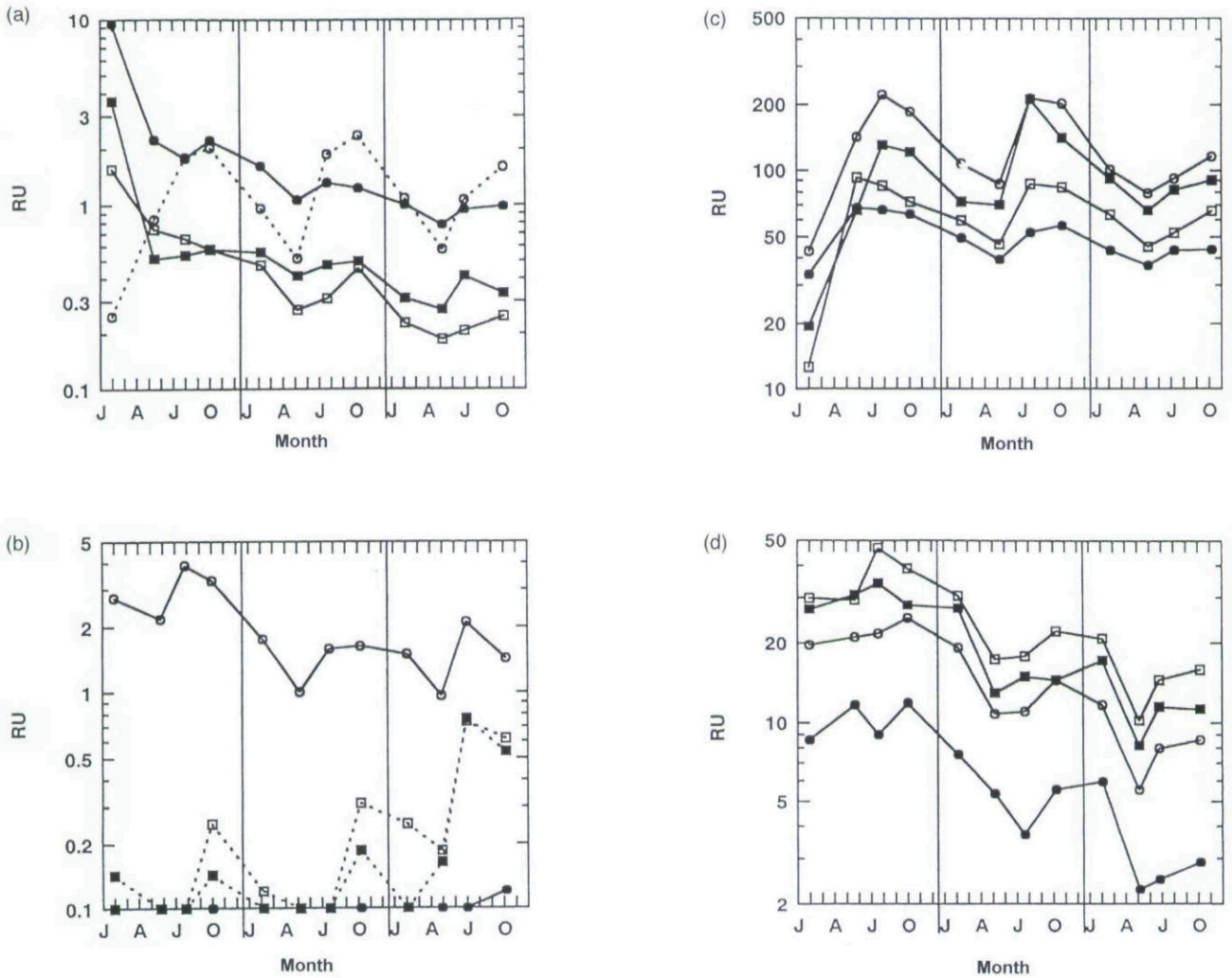


Fig. 4. Development of specific IgE over 3 yr of IT. Specific IgE against Lol p 1, 2, 3 and 5 was followed over a period of 3 yr (1984–1986). Samples were taken in January/February, May, July, and October. Results are expressed in RU. This long-term follow-up is shown for four patients: S6 (graph a), S4 (b), S5 (c) and S3 (d). ○ = Lol p I; □ = Lol p II; ● = Lol p III; ■ = Lol p V.

changes in specific IgE titres, not only in the sera of patients undergoing immunotherapy.

Acknowledgements

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