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## A long-term follow-up study of hyposensitization with immunoblotting

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*The formation of specific IgE, IgG1, and IgG4 antibodies was investigated by immunoblotting during hyposensitization with timothy grass-pollen extract and 6 years later. Until the end of immunotherapy, specific IgG antibody levels increased. Also simultaneously, the number of allergenic components detected by IgG increased. However, this IgG response was similar in responding and nonresponding patients; thus, it did not correlate with the clinical outcome of the therapy. More allergenic compounds were also detected by IgE on immunoblots, but again without correlation to success of therapy. Six years after immunotherapy, the therapeutic effect was still present, although by now the observed immunoglobulin-binding patterns were similar to patterns observed in the same patients' sera collected before the initiation of hyposensitization. Thus, changes of antibody-binding patterns in immunoblot do not relate to the success or failure of immunotherapy. (J ALLERGY CLIN IMMUNOL 1990;85:996-1004.)*

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Hyposensitization has been widely used since it was first recognized in 1911.<sup>1</sup> It has been suggested that "blocking factors" are induced by this kind of immunotherapy that inhibit allergic responses.<sup>2</sup> These blocking factors have been identified as IgG antibodies.<sup>3</sup> Devey et al.<sup>4</sup> and Van der Giessen et al.<sup>5</sup> further characterized these antibodies to be mainly of the IgG4 subclass but also questioned that their biologic function was predominantly the trapping of the allergen. Furthermore, they suggested that specific IgG4 inter-

*Abbreviations used*

|           |                                                           |
|-----------|-----------------------------------------------------------|
| IT:       | Immunotherapy                                             |
| MW:       | Molecular weight                                          |
| SDS-PAGE: | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |

feres with IgE binding to Fc $\epsilon$ -receptors in the presence of allergen.<sup>4, 5</sup>

The rise of specific IgG4 serum levels during IT has been explained as a result of prolonged immunization.<sup>6</sup> However, attempts to correlate clinical improvement with titers of IgG4 antibodies during IT demonstrated that a direct correlation of the two parameters could only be observed for parenteral allergies.<sup>7, 8</sup> IgG4 antibodies in Hymenoptera allergies were thereby considered to be "blocking antibodies."<sup>9</sup> In regards to inhalant allergens, only a few authors have described positive correlation between clinical improvement and serum IgG levels.<sup>10, 11</sup> Enhanced IgG levels and simultaneously decreased serum IgE have been observed,<sup>12</sup> but no correlation to clinical improvement was found.<sup>13-15</sup> For timothy grass-pollen extract, a high specific IgG4/IgG1 ratio in the early course of IT and elevated persisting serum IgG4 levels were considered to be predictive for unsuccessful IT.<sup>16</sup>

The concept of blocking antibodies directed against the allergen appears to remain controversial.<sup>17-21</sup> The aim of the present study was, therefore, to examine this hypothesis in patients suffering from grass-pollen allergy during a 3-year course of IT and also 6 years after treatment. Immunoblotting was used to analyze specific IgE and IgG subclass antibody levels against the separated allergens. We have recently demonstrated that immunoblotting distinguishes between 17 allergenic polypeptides in extracts of timothy-grass pollen that may be derived from only two or three allergens. (Jarolim E, Matthiesen F, Skov PS, et al. Manuscript submitted.) Indeed, we have never observed enhancement of IgG response that is directed against a single component during hyposensitization, and we found enhanced IgE, as well as IgG1 and IgG4 levels against most detectable allergenic compounds. Occasionally, 6 years after treatment, IgE was still elevated, but IgG was decreased to background levels. We did not find any correlation between clinical outcome and immunoglobulin levels. Based on the immunoblotting results, it also became obvious that the clinical success of IT did not correlate with a specific antibody formation against distinct allergenic components.

## MATERIAL AND METHODS

### Timothy grass-pollen extract

Lyophilized extract of timothy-grass (*Phleum pratense*) pollen was a gift kindly supplied from Allergologisk Laboratorium A/S, Hoersholm, Denmark).

### Serum samples

Sera of 20 patients allergic to grass pollen derived from a double-blind IT study reported in 1981<sup>22</sup> were reexamined in the present study. These sera represented patients of the group called "WPA," hyposensitized with whole pollen extract of timothy grass. Sera had been stored at  $-20^{\circ}\text{C}$  until use. After 6 years, the same patients were reevaluated.<sup>23</sup> Samples from week 0 (before IT), week 12, week 116 (end of IT), and from 6 years after IT with extracts of timothy-grass pollen was stopped were subjected to immunoblotting. Equal volumes of sera from six patients from week 12 were pooled. This serum pool was used in inhibition experiments. In addition, sera from week 12 of three patients allergic to grass pollen and presently undergoing IT with grass-pollen extract were freshly obtained and used for controls in immunoblotting. As a negative control, we used a large pool of sera negative in RAST toward common allergens, as previously described.<sup>24</sup>

### SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli<sup>25</sup> with 16% separation gels and 5% stacking gels in an SE 600 electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, Calif.). Samples were prepared by boiling in Laemmli sample buffer devoid of mercaptoethanol; 2.4 mg of timothy grass-pollen extract was loaded onto one gel (100  $\mu\text{g}$  of timothy grass-pollen extract per 0.5 cm of gel). Molecular weights were determined with prestained molecular weight markers (Amersham International, Buckinghamshire, England). Staining of gels was performed with a silver-stain kit (Bio-Rad Laboratories, Richmond, Calif.).

### Electroblotting

Electroblotting was performed with a semidry electroblotter (JKA-Biotech, Copenhagen, Denmark). Proteins were transferred to nitrocellulose, 0.2  $\mu\text{m}$  pore size (Schleicher & Schuell, Kassel, West Germany) in a discontinuous buffer system (anodic buffer 1: 0.3 mol/L of Tris, 20% (vol/vol) of methanol, pH 10.4; anodic buffer 2: 25 mmol/L of Tris, 20% (vol/vol) of methanol, pH 10.4; cathodic buffer: 25 mmol/L of Tris, 40 mmol/L;  $\epsilon$ -amino caproic acid, 20% (vol/vol) of methanol, pH 9.4) for 1½ hours at 200 mA.

### Immunoblotting

Nitrocellulose was cut into strips of 0.5 by 11.5 cm and blocked in Tris-buffered saline (50 mmol/L of Tris, 150 mmol/L of NaCl, pH 7.4), containing 0.5% (vol/vol) Tween 20 and 3% (wt/vol) powdered milk (blocking solution) for 30 minutes. All following incubation steps were

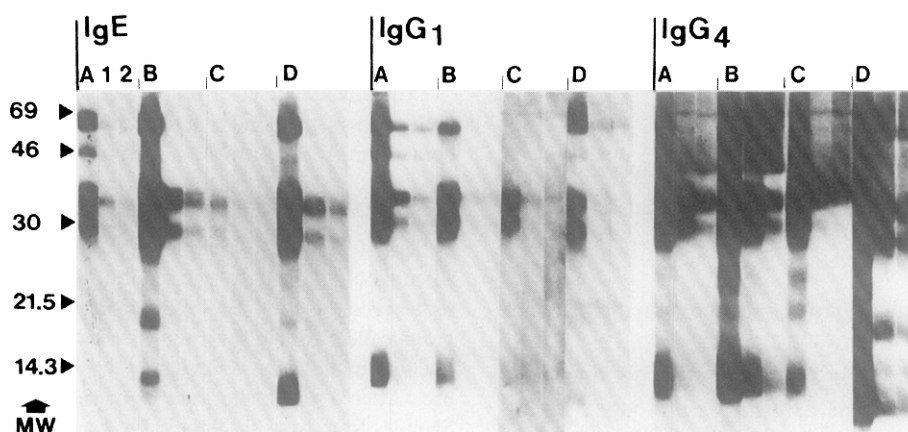


**FIG. 1.** Immunoglobulin-binding patterns of a serum pool consisting of six sera from the present study and three freshly obtained sera. All sera were from week 12 after the start of hyposensitization therapy with grass-pollen extracts. *Lanes A*, serum pool; *lanes B, C, and D*, sera of three different patients. IgE, IgG1, and IgG4 binding after preincubation with 100  $\mu$ g (*lane 1*) and 250  $\mu$ g (*lane 2*) of crude timothy grass-pollen extract; MW standards in kilodaltons.

performed at room temperature on a rocking platform. Washing was performed in Tris-buffered saline containing 0.5% Tween 20 for 30 minutes.

#### Detection of specific IgE by immunoblotting

Serum samples were diluted 1:10 in blocking solution, and 1 ml aliquots were applied to blot strips and incubated overnight. After strips were washed, bound IgE was detected by  $^{125}$ I-labeled rabbit antihuman IgE (Phadebas RAST Kit, Pharmacia, Uppsala, Sweden), 100,000 cpm/ml in blocking solution, overnight.

#### Detection of specific IgG, IgG1, and IgG4 by immunoblotting

Sera were diluted in blocking solution, 1:100, for detection of specific IgG and IgG1, and 1:50 for IgG4, and applied onto strips overnight. After strips were washed, bound IgG1 and IgG4, respectively, were detected by different mouse monoclonal antibodies (Seward Laboratory, London, England). Mouse monoclonal antihuman IgG (BAM 06) was diluted 1:2000; mouse antihuman IgG1 (BAM 15), 1:2000; and mouse antihuman IgG4 (BAM 11), 1:1000 in blocking solution. With mouse anti-IgG2 (BAM 10) and mouse anti-IgG3 (BAM 08), no immunoglobulin binding to timothy grass-pollen antigens could be detected in immunoblot (data not presented). This is in accordance with a previous article.<sup>16</sup> All strips were incubated for 2 hours. After strips were washed, bound mouse IgG was detected by  $^{125}$ I-labeled rabbit antimouse immunoglobulin,  $\gamma$ -chain specific (Dakopatts, Glostrup, Denmark) and purified by immunosorption of Sepharose-coupled polyclonal human IgG and mouse IgG, as described previously.<sup>26</sup> Labeled antibody (100,000 cpm/ml) in blocking solution was added to each strip for 1 hour.

#### Inhibition experiments in immunoblotting with allergen extract

Serum samples were diluted in blocking solution, as described above, and 1 ml aliquots were incubated with 100

or 250  $\mu$ g of timothy grass-pollen extract at 4°C overnight. Then samples were added onto blotting strips, and bound IgE, IgG1, or IgG4 was detected as described above.

#### Autoradiography

Blotted nitrocellulose strips were washed, dried, and exposed to Hyperfilm MP (Amersham International) in exposure cassettes (NETO, Nielsen & Olsen, Copenhagen, Denmark), containing intensifying screens, for 24 hours at -80°C. Films were analyzed with a hand-held digital reflection densitometer (Gretag Ltd., Regensburg, Switzerland). This densitometer is used by professionals of the graphics industry for color-intensity comparisons. It was calibrated to zero on the exposed film, and then several small integral measurements (calibrated at 2 mm each) were undertaken within the autoradiographic band. This hand-held densitometer yields very reproducible results.<sup>27</sup> The statistical analysis was performed with Student's *t* test.

## RESULTS

#### Reliability of immunoglobulin-binding patterns in immunoblotting

In the study we intended to use sera kept refrigerated for 11 years.<sup>22</sup> We therefore studied the binding patterns of IgE, IgG1, and IgG4 to blotted timothy grass-pollen extract in these sera in comparison to sera freshly obtained from other grass pollen-allergic patients. Six sera of the immunotherapy study, pooled in equal parts (serum pool), and three freshly obtained individual sera from patients allergic to grass pollen were subjected to immunoblotting. IgE-, IgG1-, and IgG4-binding patterns of sera and serum pool to timothy grass-pollen allergens are illustrated in Fig. 1. The patterns were comparable with regard to the number of allergenic components recognized. Also, the concentration of specific antibodies as estimated by the intensities of the autoradiographic signals was sim-

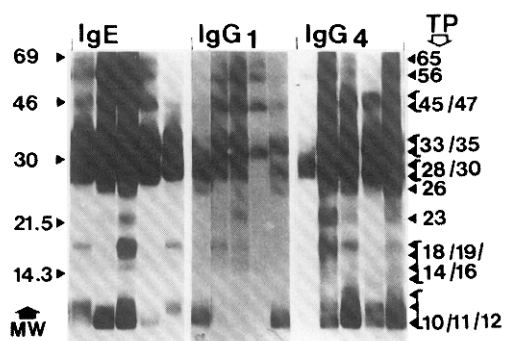


**FIG. 2.** IgE, IgG1, and IgG4 binding of timothy grass-pollen allergens. Three groups of five sera were selected according to their typical IgE-, IgG1-, or IgG4-binding patterns to timothy grass-pollen allergens in immunoblotting; *TP*, timothy grass-pollen allergens in kilodaltons; *MW* standards in kilodaltons.

ilar. Thus, it was feasible to use the stored serum samples.

To examine if the observed immunoglobulin binding directed against separated and eventually denatured allergens was relevant, we used native timothy grass-pollen extract for inhibition experiments, also illustrated in Fig. 1. Binding of IgE, IgG1, and IgG4 was markedly reduced in individual sera and the serum pool in the presence of 100 or 250  $\mu$ g timothy grass-pollen extract. Only IgG4 binding directed against one allergen (65 kd) was not reduced by the applied amounts of timothy grass-pollen extract.

Thus our data demonstrated that the allergenic components on immunoblots are also present in native preparations similar to preparations used for IT.

### Recognition of timothy grass-pollen allergens by IgE, IgG1, and IgG4

We intended next to investigate whether the same allergenic components are detected by IgE as well as by IgG. For this purpose, 15 sera from the IT study were selected, based on their IgE-, IgG1-, or IgG4-binding patterns in the immunoblot (Fig. 2). All timothy grass-pollen major allergens of MWs of 10, 11, 28, 30, 33, 35, 45, and 56 kd, and all timothy grass-pollen intermediate allergens of MWs of 19, 23, 26, and 65 kd, as described in a recent study (Jarolim E, Matthiesen F, Skov PS, et al. Manuscript submitted.), were recognized equally well by IgG1 as by IgG4. Although IgG4 binding was observed against all minor timothy grass-pollen allergens of MWs of 14, 16, 18, and 47 kd, and an intermediate allergen 12 kd, no IgG1 binding to allergens of MWs 12, 14, 16, and 18 kd, and only weak IgG1 binding to allergen MW 11 kd was observed. IgG1 or IgG4 binding was exclusively directed against antigens of timothy-grass pollen that also were capable to bind IgE. The individual patient's IgE, IgG1, or IgG4 antibody levels were not always equally elevated against

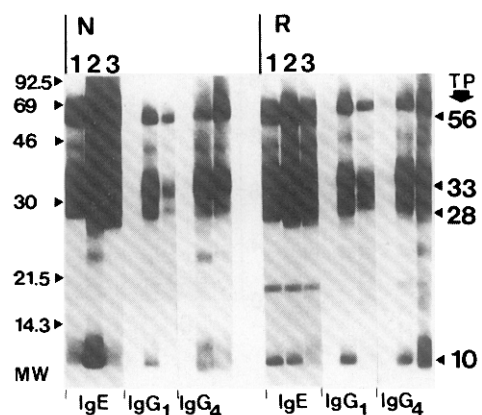


**FIG. 3.** Typical IgE, IgG1, and IgG4-binding patterns of patients undergoing IT with timothy-pollen extract. Immunoglobulin-binding profiles of one responding patient (*R*) and one nonresponding patient (*N*) after hyposensitization therapy are illustrated (for quantitation of all patients' immunoglobulin-responses, see Figs. 5 and 6); *lane 1*, week 0 (before start of IT); *lane 2*, week 12 of IT; *lane 3*, week 116 (end of IT); *MW* markers in kilodaltons; *TP*, major timothy grass-pollen allergens (in kilodaltons).

the different allergens. However, we did not identify any components that were recognized preferentially either by IgE or IgG.

### IgE, IgG1, and IgG4 binding to timothy grass-pollen allergens of patients responding or nonresponding to IT

The aim of our study was to find an antibody-binding profile in immunoblot that would correlate with the clinical outcome of IT. Therefore, patients of the IT study were distributed into two different groups: patients responding or patients not responding to IT. Success of IT was determined based on the results of nasal provocation tests and skin prick tests, as described in a previous article.<sup>28</sup> Nine patients demonstrated marked improvement of test parameters and were classified as responders. A group of only four patients demonstrated no improvement in the provocation tests used. Those patients were classified as nonresponding. However, seven patients demonstrated only moderate improvement during IT. This group of intermediate responders was also included in the screening procedure, but their results were not significantly different from the responders or from the nonresponders and are, therefore, not presented here.

All sera obtained before IT, after 12 weeks of IT, and at the end of IT (week 116) were then tested by immunoblotting. Typical binding patterns of IgE, IgG1, and IgG4 from serum of a nonresponder and a responder are illustrated in Fig. 3 to illustrate the basic findings as outlined in Figs. 4A, 4B, and 5. Titers of IgE, IgG1, and IgG4 increased initially during IT and then declined until the end of IT, except for IgG4 levels, which further increased. Furthermore, during

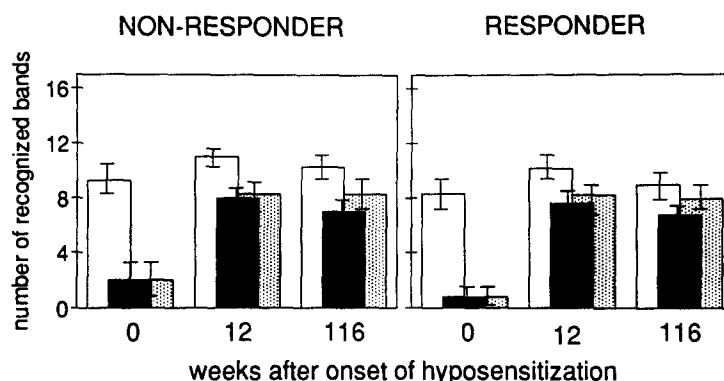


**FIG. 4A.** IgE and IgG1 to timothy pollen. Number of allergens recognized by IgE (open columns), IgG1 (shaded columns), or recognized by IgE as well as IgG1 (black columns). The number of bands on the autoradiographies has been enumerated by eye for each individual patient. Averages ( $\pm$  SEM) are indicated for patients nonresponding ( $N = 4$ ) and responding ( $N = 9$ ) to therapy.

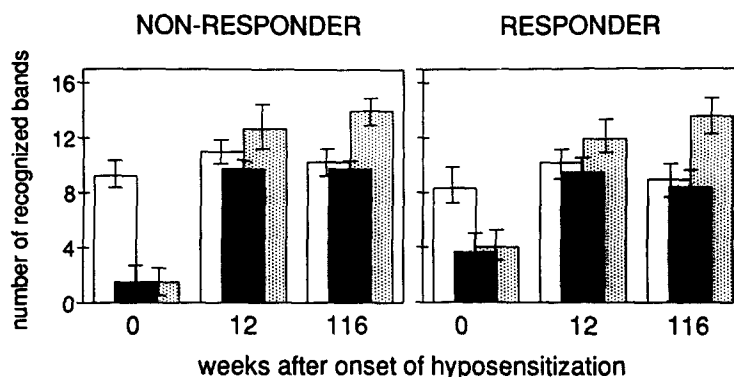


**FIG. 4B.** IgE and IgG4 to timothy pollen. Number of allergens recognized by IgE (open columns), IgG4 (shaded columns), or recognized by IgE as well as IgG4 (black columns). The number of bands on the autoradiographies has been enumerated by eye for each individual patient. Averages ( $\pm$  SEM) are indicated for patients nonresponding ( $N = 4$ ) and responding ( $N = 9$ ) to therapy.

and at the conclusion of IT, antibodies against additional antigenic components were detected. There appeared to be no difference in patterns of immunoglobulin binding between responders and nonresponders (Fig. 3).

We investigated qualitatively whether distinct allergenic compounds would be recognized preferentially by either IgE or IgG, and we studied how many allergenic compounds were recognized by IgE as well as IgG. For this purpose we enumerated the number of bands recognized by IgE, IgG, or by both for each patient (Figs. 4A and 4B). The numbers of bands recognized by IgE and IgG1 or by both are illustrated in Fig. 4A from nonresponders (*left panel*) or responders (*right panel*). Compared to week 0, it is evident that more IgG1 positive bands were detected in sera from week 12 in responders ( $p < 0.01$ ) and nonresponders ( $p < 0.005$ ). The same phenomenon

was observed for bands detected by IgE as well as IgG1, which also increased in this early period (for responders,  $p < 0.005$ ; for nonresponders,  $p < 0.01$ ). We also observed a trend toward detection of more allergenic compounds by IgE (responder,  $p$  not significant; nonresponder,  $p < 0.1$ ) in week 12. In week 116, we observed the same antibody patterns as in week 12 (yielding similar significances, data not presented), suggesting that antibody formation remained at a plateau already reached in week 12.

In Fig. 4B, the results of the number of bands recognized by IgE are illustrated, but in this case, in relation to the patients' IgG4 patterns. As for IgG1, IgG4 is significantly increased early in IT. In nonresponders and responders, more IgG4-binding bands were detected in week 12 than in week 0 ( $p < 0.005$ ). During the same period, significant increases in the number of allergenic compounds binding IgE and

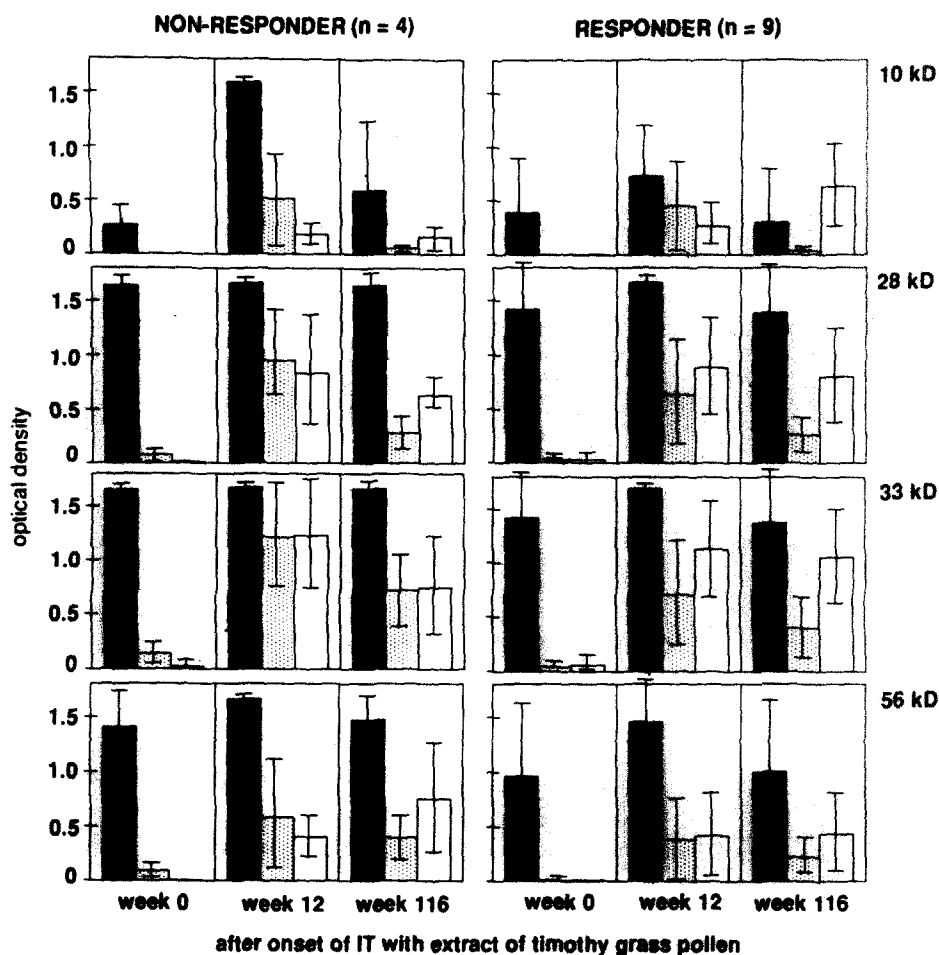


FIG. 5. Relative immunoglobulin-binding levels determined by reflection densitometry; binding intensities of IgE (black columns), IgG1 (shaded columns), and IgG4 (open columns). Four allergens of MW 10, 28, 33, and 56 kd of timothy-grass pollen were examined. Columns represent means  $\pm$  SEM.

IgG4 were observed in both patient groups, but the number of IgG4-binding bands exceeded even the raised number of IgE-binding bands. There appeared to be a trend for a further increase in the number of IgG4-positive bands in week 116. Most importantly, we found no statistically significant differences between responders and nonresponders with the Student's *t* test, suggesting that IT leads to the formation of more IgE, IgG1, and IgG4, independent of the success of therapy.

So far our analysis evaluated only the numbers of allergenic compounds induced by IT. Therefore, we intended to semiquantitatively determine the extent of the antibody response by measuring the signal intensity of individual bands. A selection of four allergically important bands (MWs of 10, 28, 33, and 56 kd) are illustrated in Fig. 5. All antigens examined demonstrated a trend toward more intense staining by IgE in sera of week 12. The intensity of IgE binding

declined again until week 116 (not significant). Only IgE-binding intensities to the 10 kd allergen increased significantly until week 12 in sera of nonresponders ( $p < 0.005$ ), followed by a significant decline until week 116 ( $p < 0.05$ ). However, IgG1 binding directed against all four investigated allergens was significantly increased between week 0 and week 12 (for responders,  $p < 0.05$  to  $0.005$ ; for nonresponders,  $p < 0.1$  to  $0.01$ ) and then decreased in week 116 in both patient groups ( $p < 0.1$  to  $0.01$ ). IgG4 binding before IT was mostly at zero levels but increased significantly against all four allergens until week 12 (responders,  $p < 0.05$  to  $0.005$ ; nonresponders,  $p < 0.01$  to  $0.001$ ). Until the end of IT, no further increase of IgG4-binding intensities was observed, except the trend in the case of the 10 kd allergen in the responder group ( $p < 0.1$ ).

Thus, based on intensity assessment of the immunoblots, it became evident that, during IT, more spe-



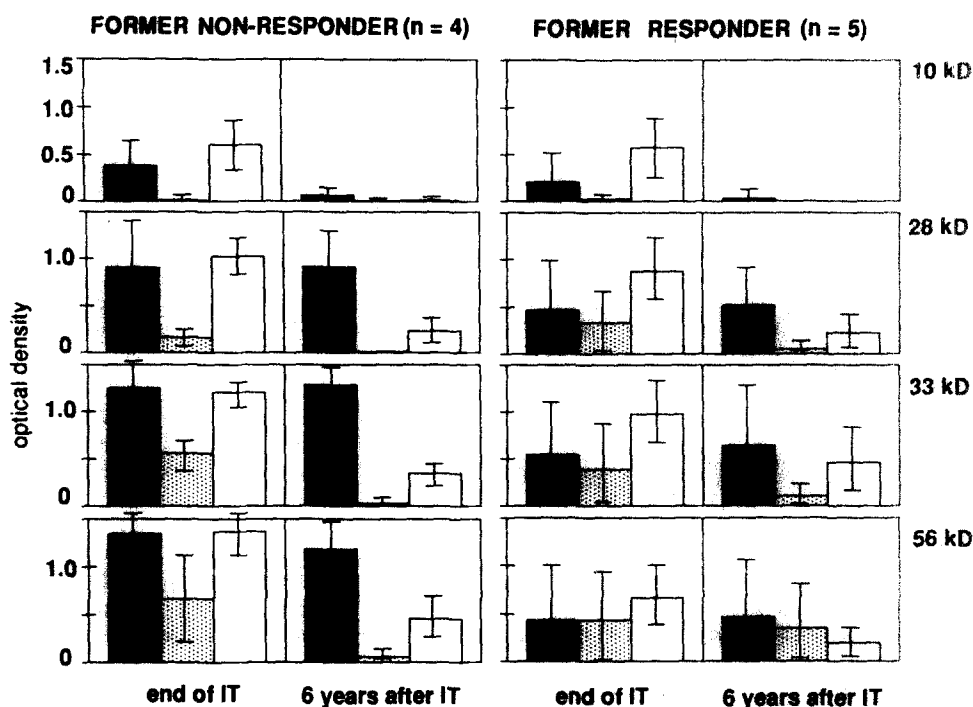


**FIG. 7.** Relative immunoglobulin-binding intensities in sera 6 years after IT of IgE-, (black columns) IgG1-, (shaded columns) and IgG4- (open columns) binding intensities directed against four timothy grass-pollen allergens of major importance. MWs of 10, 28, 33, and 56 kD were determined for serum samples of week 116 (end of IT) and 6 years after IT by reflection densitometry. Columns represent means  $\pm$  SEM.

arated allergens can be directly compared. Deards et al.<sup>29</sup> only investigated IgG antibody profiles during IT with grass-pollen extracts by immunoprecipitation combined with SDS-PAGE analysis. IgG responses were reported to be of a complex nature and to vary between individuals. This is in agreement with our findings, but in the latter study, during the course of IT, IgG was only enhanced against components already recognized by IgG before onset of therapy. In contrast to that finding, we detected an induction of IgG1 and IgG4 against allergens that were not recognized by IgG before IT. Our results were obtained by testing with monoclonal anti-IgG1 and anti-IgG4 antibodies of high specificity.<sup>30</sup> A possible explanation of this discrepancy is that by precipitation techniques, not only free IgG, but also specific IgE in complexes with IgG anti-IgE antibodies<sup>31</sup> might be precipitated by the allergen extract, thus, grass-pollen components that actually might have been recognized via IgE before IT could be precipitated.

The rise of IgG1 and IgG4 levels during IT, and the fact that a rather large proportion of allergens became recognized by IgE as well as IgG, are, to some extent, favorable to the hypothesis of blocking antibodies. However, the nature of our assays, in which we varied serum dilutions and used different revealing antibodies, does not permit a strict com-

parison of signal intensities and actual levels of antibody concentrations. Thus, future studies should focus on the actual protein concentrations rather than determination of relative signals. In the present study, neither relative levels of different immunoglobulin isotypes nor their specificity in immunoblots correlated with the clinical outcome of IT.

This is the first detailed study on sera from patients with follow-up 6 years after termination of IT.<sup>23</sup> All the nonresponding (N = 4) and five out of nine patients responding to IT were still available. None of the patients had relapsed, judged from clinical scores and challenge tests. It was impossible to distinguish between the groups formerly responding or nonresponding. Because of the fact that spontaneous remission is a rather rare event in allergy,<sup>32, 33</sup> the observed improvement in all patients might, nevertheless, represent a beneficial effect of IT. However, specific IgE was elevated back to initial levels, as determined by RAST. In immunoblotting, we also still found elevated levels of specific IgE with an equal distribution in both groups. In contrast to that finding, IgG1 and IgG4 had returned to background levels. Immunoglobulin-binding patterns observed 6 years after IT resembled immunoglobulin-binding patterns before the start of therapy. Nevertheless, all the patients that were available to us had clinically improved.

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