

Comparison of IgE and IgG antibody responses of atopic individuals with sensitization to tree and grass pollens

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Sera of atopic individuals with predominant sensitization to either tree pollen (TAs) or tree and grass pollens (TGAs) as well as of nonatopic subjects (NAs) were analyzed for IgE, IgG, and IgG4 antibodies specific for grass pollen allergens. Of 600 atopic individuals with serum IgE antibodies specific for birch pollen allergens, 54% also had serum IgE antibodies specific for grass pollen. The mean titers of IgG antibodies specific for grass pollen proteins were about 10 times higher in the sera of TGAs than those in the TAs and NAs. SDS–PAGE immunoblotting analysis of grass pollen proteins using sera of TGAs, TAs, and NAs with respect to the binding of these proteins with IgE and IgG antibodies in these sera exhibited a similar pattern of variation. Quantitation by enzyme immunoassay of the antibody binding to a recombinant grass pollen allergen, rKBG8.3, further demonstrated that elevated IgG antibody levels in TGAs are mainly due to a broader range of specificities, and not to high specific binding to the individual protein. Statistically significant correlation was found between IgE and IgG4 antibodies specific for the Kentucky bluegrass (KBG) extract, but not for the isolated recombinant allergen. These results indicate that the grass pollens elicit a complex array of antibody specificities in both atopics and nonatopics, and that the profile of antibodies specific to the pollen extract and pure allergens differs, suggesting that single grass allergens may be inadequate for replacing grass pollen extracts for immunotherapy.

Key words: ELISA; IgE; IgG4; immunoblotting; immunotherapy; pollen allergies; recombinant allergen.

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A distinctive and remarkable feature of human atopic disorders is the elevation of serum IgE antibodies in response to a group of environmental antigens, referred to as allergens. In genetically predisposed atopic individuals, persistent exposure to allergen(s), through either natural exposure or immunotherapy, leads to synthesis of both allergen-specific IgE and IgG4 antibodies (1–5). Studies *in vitro* using human PBMC cultures demonstrated that addition of recombinant interleukin (rIL)-4 to these cultures led to

a proportional increase in IgE and IgG4 antibodies compared to other isotypes (6–9). The increase of IgG4 with that of IgE is of relevance since the IgG4 antibodies have been implicated in blocking the accessibility of soluble allergens to IgE-coated mast cells (6, 7, 10, 11). The induction and persistence of IgE antibodies to diverse allergens and the potential of the specific IgG4 antibodies to elicit *in vivo* neutralization of allergens depends on the recognition of shared specificity for allergenic epitopes by the individual's IgE antibodies.

Previously, in our laboratory, the molecular cloning and characterization of cDNAs and proteins of allergens of a number of plant pollens corresponding to known epitopes of grass allergens suggested cross-reactivities among allergens of various pollens (12–14). Moreover, extensive cross-reactivities were

Abbreviations used in this paper: EIA, enzyme immunoassay; KBG, Kentucky bluegrass; IFN- γ , interferon-gamma; IL-4, interleukin-4; NA(s), nonatopic(s); TA(s), tree-atopic(s); TBS, Tris-buffered saline; TBST, Tris-buffered saline added 0.05% Tween 20; TGA(s); tree and grass atopic(s).

Definitions: tree/grass atopics – individuals with specific IgE for both tree and grass pollen allergens; tree-atopics – individuals with specific IgE for tree pollen, but not for grass pollen.

recently reported for a group of plant allergens, the profilins (15). In an effort to shed light on antibody responses to related antigens involved in the development of the atopic condition and to understand the basis of the effectiveness of specific therapies, we undertook a comprehensive study of the allergen-specific antibody responses of IgE and IgG4 isotypes in selected individuals of a large pollen-allergic population. In this study, we have used the recombinant allergen, rKBG8.3, which constitutes the conserved fragment of the *Poa p* IX allergens (16–18), and the proteins present in the Kentucky bluegrass (KBG) pollen extract to examine the magnitude and isotypes of specific antibodies produced in a defined group of allergic individuals who were predominantly allergic to either tree pollen (TAs) or to several aeroallergens including both tree and grass pollen (TGAs). Our results demonstrate that the antibody response to the grass pollen extract is more complex in TGAs than in TAs and nonatopic subjects (NAs). Moreover, the relationship between antibodies of IgE and IgG4 isotypes specific to the extract and pure allergens differs.

Material and methods

Preparation of KBG pollen extract

The protein extracts of KBG pollen were prepared as described previously (16). Briefly, a 1% (w/v) suspension of pollen, *Poa pratensis* (Hollister-Stier, Ontario, Canada) in Tris-buffered saline (TBS) (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl) containing 0.01% NaN₃ and 1 mM phenyl methyl sulfonyl fluoride (Sigma), was incubated with shaking overnight at 4°C. Pollen grains were removed by filtration (Whatman 3MM), and the pollen extract was further concentrated $\times 10$ by ultrafiltration (YM5, Amicon, MA, USA) and stored in aliquots at -20°C . The protein concentration was determined by the Bio-Rad protein assay.

Human sera

Individual sera of patients in Norway were kindly provided by Nycomed Allergen Laboratory, Oslo, Norway. The sera were originally analyzed by RAST for allergen-specific IgE antibodies, as well as by PRIST for total IgE, as described by the manufacturer. These analyses were performed after requests by physicians. The use of these sera for extended examinations was approved by health authorities in Norway. Sera from 20 individuals 1) with no history of allergy, and 2) with normal serum concentration of total IgE antibodies and no detectable IgE antibodies specific for a panel of different allergens, as assayed by PRIST and RAST, respectively, were included as nonatopic controls.

SDS-PAGE and immunoblotting

SDS-PAGE was performed in a discontinuous system according to Laemmli (19) in a Fisher minislab gel apparatus. Samples were prepared in loading buffer; 0.06 M Tris-HCl buffer, pH 6.8, containing 2% SDS and 5% 2-ME. Western blotting was performed by PolyBlot (American Bionetics, Inc, CA, USA) using a discontinuous buffer system as described by the manufacturer. The membranes were subsequently blocked by incubation in TBS/1% gelatin for at least 6 h, and then incubated overnight with human serum diluted 1:100 in TBST/0.1% gelatin. Antibody binding was detected by incubation overnight with either AP-conjugated goat anti-human IgE at 1:1000 (Tago, CA, USA), or AP-conjugated goat antihuman IgE at 1:2000 (Zymed, CA, USA), or alkaline phosphatase (AP)-conjugated goat antihuman IgG4 at 1:500 (Zymed, CA, USA). All conjugated antibodies were diluted in TBST with 0.1% gelatin. The membranes were washed five times, 4 min each, in TBST between each incubation step. The enzymatic reaction was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate and was stopped by incubating the membranes in 50 mM Tris-HCl, pH 8.0, 100 mM EDTA.

Enzyme immunoassay

Proteins, 10 $\mu\text{g}/\text{ml}$ of PBS, were coated to wells of microtiter plates (Nunc, Immunoplate, Denmark) overnight. The wells were further blocked by PBS/1% gelatin for 8 h. After washing five times with PBS 0.05% Tween 20 (PBST), the wells were incubated with human serum diluted at 1:30 to 1:100 with PBST/0.1% gelatin (PBSTG) overnight. After washing with PBST, the wells received AP-conjugated secondary antibodies of either goat anti-human IgE 1:1000 (TAGO, CA, USA), or goat antihuman IgG, Fc 1:2000 (Caltag, CA, USA), or goat antihuman IgG4, 1:500 (Zymed, CA, USA), and the plates were incubated overnight. After washing with PBST, the color reaction was developed by adding pNPP (para-nitrophenyl phosphate), 1 mg/ml 0.1 M Tris-HCl buffer containing NaCl and MgCl₂, and measured at 405 nm in a Titertek Multiscan spectrophotometer (Flow Laboratories, McLean, VA, USA). All washing steps were performed with the Nunc "ELISA hand washer", and the incubations were at room temperature.

Statistical analysis

Statistical analysis was performed with Student's *t*-test and *Biometrical tables for statisticians* by E. S. Pearson and H. O. Hartly (3rd. ed., 1966). The cor-

relation coefficient was calculated employing Sigma plot software. Agreement analysis was carried out as described (20, 21). Briefly, for each pair of results, the difference between the results was plotted against the mean value of the two results. The coefficient of agreement and the standard error of mean for the replotted values as well as the corresponding correlation coefficients were calculated. A P -value > 0.05 for this correlation coefficient was the major criterion for accepting the hypothesis that the two methods were significantly associated.

Results

Sera of TGAs contain higher total IgE antibodies than TAs

A total of 4825 sera collected during spring (March–May) 1991 in Norway were analyzed for total IgE antibody and birch pollen extract-specific IgE antibody levels by PRIST and RAST, respectively. Of these, 811 (16.8%) were found to be RAST-t3 positive with a score of > 1 . Further analysis of 600 of the 811 t3-positive sera for RAST-g6 (timothy) demonstrated that 54% (322) were positive (RAST score > 1); these subjects were referred to as tree and grass atopics (TGAs), and the remainder as tree atopics (TAs). The distribution of subjects into individual RAST class in terms of tree and grass pollen reactivity is illustrated in Fig. 1. These results indicated that almost half of the TAs also had been sensitized to grass pollens. The concentration of total IgE antibodies in the sera which contained specific IgE antibodies for both tree and grass pollen varied between 5 and 1000 IU/ml (1 IU = 2.4 ng), with a mean value of 358 U/ml, and 47% of these sera gave

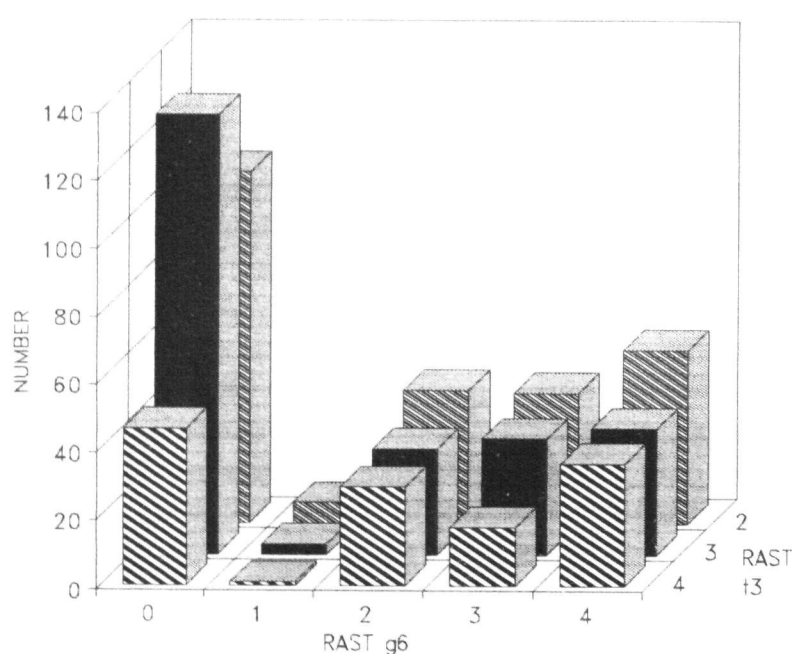


Fig. 1. Distribution of 811 human sera with positive RAST for birch pollen with respect to levels of IgE antibodies specific for grass and tree pollen extracts, as quantitated by RAST-g6 and -t3, respectively.

a positive RAST score for other allergens. The IgE antibody levels in birch-allergic individuals varied between 1 and 1000 IU/ml, with a mean value of 131 IU/ml. These results indicated that the TGAs possessed higher IgE antibody levels than those of TAs.

IgE and IgG antibodies specific for grass pollen allergens differed in sera of TGAs and TAs

To examine further specific IgE and IgG antibody levels, two groups of 30 sera each, representing the high IgE responders in the TA and TGA populations, were selected. The distribution of IgE antibodies to birch pollen according to RAST-t3 analysis in these TGAs and TAs is shown in Fig. 2A. The two groups differed considerably ($P < 0.001$) with respect to total IgE antibodies in serum (Fig. 2B); the TA group had a mean value of 97 UI/ml, which was at the upper normal limit (100 IU/ml), whereas the mean value in the TGA group was 450 IU/ml. The analysis of KBG-specific IgE antibodies confirmed the overall results of the RAST-g6 (timothy) analysis (Fig. 2C). No statistically significant difference in IgE antibodies specific for KBG was found

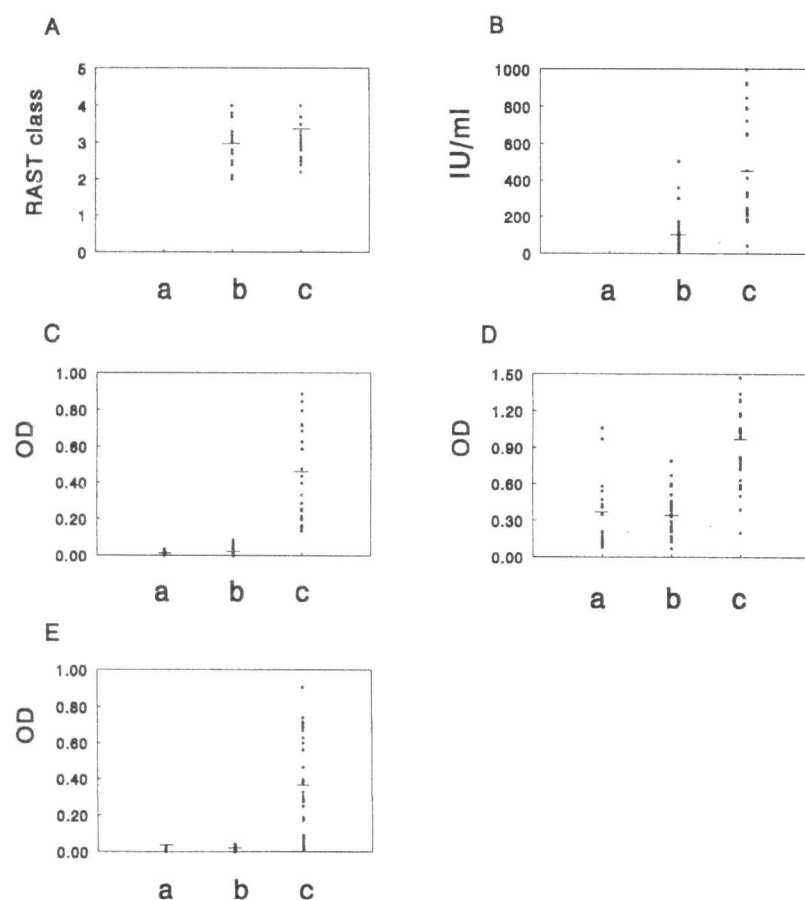


Fig. 2. Concentration of total and specific antibodies in sera of 20 nonatopics (a), 30 tree-atopics (b), and 30 tree/grass-atopics (c). A) IgE antibodies specific for birch pollen assayed by RAST-t3; B) total IgE antibodies assayed by PRIST; C) IgE antibodies specific for KBG pollen extract assayed by EIA; D) IgG antibodies specific for KBG pollen extract assayed by EIA; E) IgG4 antibodies specific for KBG pollen extract assayed by EIA. Nonatopics were all negative in RAST-t3 and had total IgE less than 100 IU/ml. Mean values within each group are indicated.

between NAs and TAs, while TGAs differed significantly from NAs and TAs ($P < 0.001$). Moreover, the NAs and the TAs did not show significantly different distribution of IgG antibodies binding to KBG pollen extract, whereas the level of specific IgG antibodies in the TGA group was elevated; $P < 0.001$ (Fig. 2D). According to the titration curves for IgG binding (data not shown), the mean value of the TGAs corresponded to a $\times 10$ higher concentration than the mean of either NAs or TAs. However, it is to be noted that the two groups of atopics had partly overlapping distribution of the total content of grass pollen-specific IgG antibodies in serum. The binding of IgG4 antibodies in the above sera to the grass pollen proteins was also examined by enzyme immunoassay (EIA), and the results are shown in Fig. 2E. The relative binding of IgG4 antibodies to the KBG pollen proteins among different groups was similar to that of the IgE antibodies; the binding of KBG pollen proteins to IgG4 antibody in sera of TGAs was significantly higher than those of the other groups ($P < 0.001$).

The IgE binding to KBG pollen extract was compared to the corresponding binding of IgG as well as IgG4 in the TGA group (Fig. 3A and B). A statistically significant correlation was found both between IgE and IgG, $R_{\text{val}} = 0.349$; $P < 0.05$; and IgE and IgG4, $R_{\text{val}} = 0.476$; $P < 0.01$. However, further examination by agreement analysis indicated poor agreement for the correlation between IgE and IgG (coeff. agree. = 0.52, SEM = 0.075, and $P < 0.0001$), while there was significant agreement between IgE and IgG4 (coeff. agree. = 0.08, SEM = 0.057, and $P = 0.16$). We concluded from these results that the responses of individuals to allergen extracts are different at the level of different isotypes, and that the levels of specific IgE antibodies are weakly associated with the total IgG, but are strongly associated with IgG4 antibodies.

Antibody levels specific for individual proteins in KBG pollen extract differ between TGAs and TAs

Twelve sera from each group were further analyzed for antibody binding to the individual proteins in

KBG pollen extract by SDS-PAGE immunoblotting. Most of the sera of the TGAs contained IgE and IgG antibodies binding to all the major allergens in KBG; groups I, IV, V, and IX (18) (Fig. 4A and B). The IgG antibody levels in sera of TAs and NAs demonstrated almost the same qualitative variation of specificities as the TGA group (Fig. 4C and D). The IgE binding of sera in these two groups was completely negative (results not shown). Titration of the IgG binding in one of the positive sera demonstrated that the immunoblot system could discriminate within a relative concentration range of at least 30-fold (Fig. 4E). The variation in intensity of Ig binding to the individual protein bands in the immunoblots demonstrated that the antibody response to the different proteins varied considerably among NAs and atopic individuals.

Antibody levels specific to a recombinant grass pollen allergen in TGAs and TAs

The availability of the recombinant polypeptide, rKBG8.3, enabled quantitation of IgE and IgG, as well as IgG4, antibodies binding to rKBG8.3, which was produced as a soluble nonfused molecule (22). The concentration of IgG which bound to this allergen varied considerably in all three groups of individuals with respect to the distribution and mean values of the immunoglobulin. Moreover, quantitatively, both NAs and TAs had a very similar IgG response to rKBG8.3 (Fig. 5A). Titration curves (data not shown) showed the levels of specific IgG antibodies to vary considerably; as high as 100-fold differences were seen among the groups, and the IgG concentration in sera of TGAs was only 1.5 times higher than that of the TA group. The distribution of IgG4 antibody binding to rKBG8.3 for all three groups of individuals is shown in Fig. 5B; TGAs were significantly different from both TAs and NAs ($P < 0.001$), and 10–20 times higher concentrations of antibodies were found in the sera of TGAs.

IgE binding to the recombinant *Poa p* IX allergen was compared to the corresponding binding of IgG, as well as IgG4, in the TGA group (Fig. 6A and B). A statistically significant correlation was found between IgE and IgG as well as IgG4, both with $R_{\text{val}} = 0.449$, $P < 0.01$. However, further examination by agreement analysis revealed that there was no significant agreement between any two pairs (coeff. agree.: 0.58, SEM: 0.079, and $P < 0.001$ and coeff. agree.: 0.11, SEM: 0.041, and $P = 0.02$, respectively). We inferred from these results that IgE and IgG antibody responses to individual allergens are complex and are weakly associated at the isotype level.

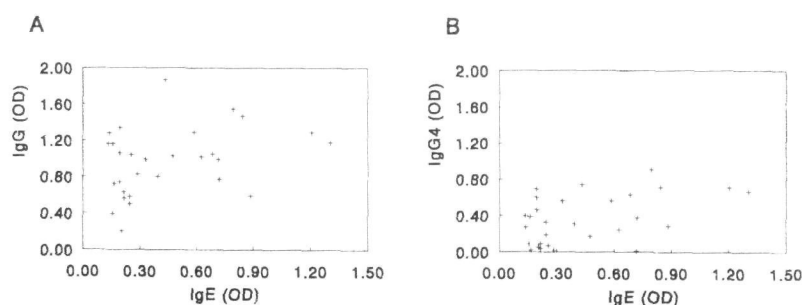


Fig. 3. Correlation analysis ($n = 30$ TGAs) between binding of IgE versus IgG (A) and of IgE versus IgG4 (B) antibodies to KBG pollen extract, as assayed by EIA.

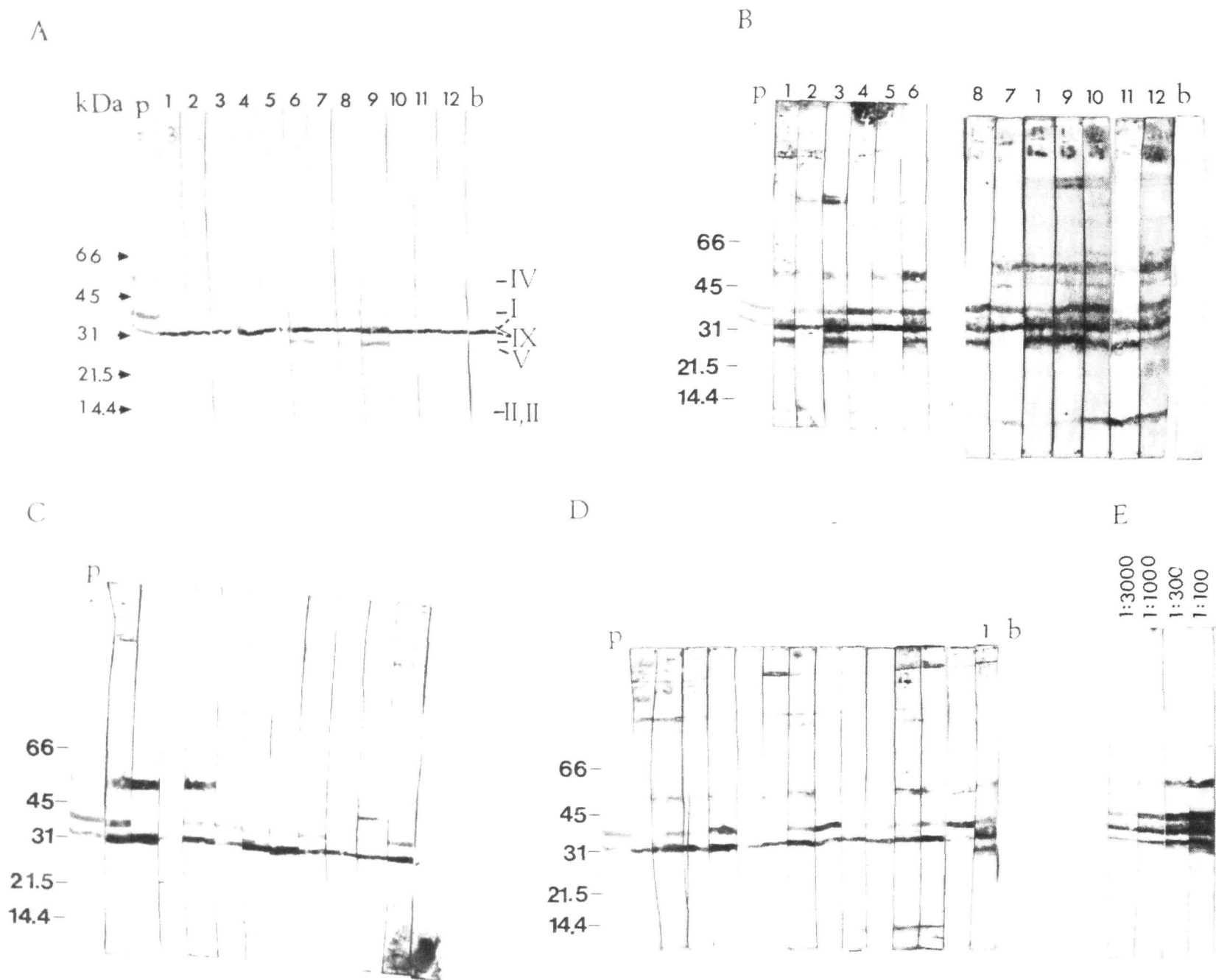


Fig. 4. SDS-PAGE and immunoblotting of individual proteins in KBG pollen extract using serum antibodies of allergic individuals. Binding of IgE (A) and IgG (B) in sera from 12 TGAs. Binding of IgG in 12 tree-atopics (C) and 14 nonatopics (D); (E) titration of IgG binding of tree/grass atopic serum. All sera analyzed 1:100. Molecular weight markers and allergens of groups I–V and IX indicated in A. p: reference strip stained with amido black; b: control strip incubated with buffer instead of serum and developed for relevant conjugated antibodies.

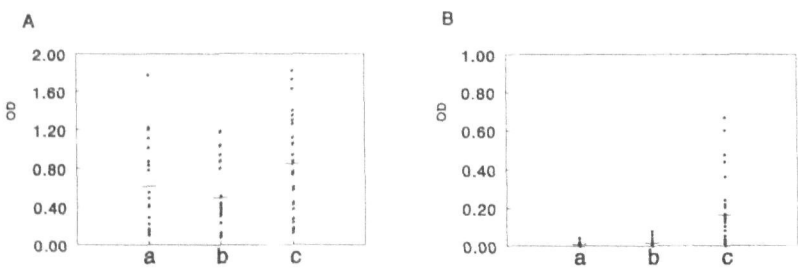


Fig. 5. Binding of IgG (A) and IgG4 (B) antibodies to recombinant *Poa p* IX allergen, rKBG8.3, as assayed in EIA, in nonatopics (a), tree-atopics (b), and tree/grass atopics (c). Mean values within each group are indicated.

Discussion

The last few years have seen monumental progress in our knowledge of the molecular structure of allergens (23). Studies in our laboratory (12–14, 18) and those of others (24–26) have revealed that certain epitopic sequences appear to be conserved

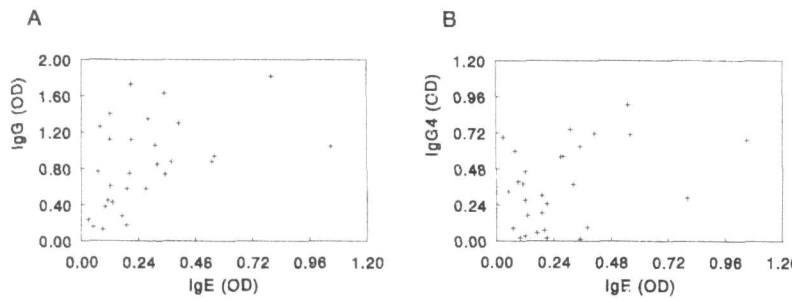


Fig. 6. Correlation analysis ($n = 30$) between binding of IgE versus IgG (A), and IgE versus IgG4 (B) antibodies to the recombinant *Poa p* IX allergen, rKBG8.3, as assayed by EIA.

among diverse allergens, leading to the widespread cross-reactivities at the level of recognition by both T and B cells. Moreover, both atopic and NA individuals are known to produce allergen-specific antibodies of different isotypes (4, 5) and, in the presence of IL-4, B lymphocytes from both atopics and

NAs produce IgE antibodies (6–9). In an effort to shed light on the relationship between different specific antibody isotypes, we investigated in this study the levels of relevant antibodies to grass allergens in groups of atopic individuals pragmatically classified as TGAs and TAs on the basis of their sensitization to either grass and/or tree pollens. Among the examined sera, which were collected during the spring in Norway, i.e., prior to the grass pollen season, there was a high frequency of samples that contained grass pollen-specific IgE antibodies. This is consistent with the previous reports that the IgE antibodies specific for pollen allergens are persistent throughout the year, although their levels decrease after the season (27).

Our results demonstrate that, as expected, the sera of the TGA group not only had IgE antibodies to more than one group of aeroallergens (results not shown), but also had higher levels of total IgE than the TAs (with no detectable IgE to grass pollen). However, the IgE response to birch pollen was similar in both of these groups. This was probably due to the presence of one major allergen, i.e., *Bet v* I, in tree pollens (28, 29). These results suggest that the immune system of atopics can discriminate between different groups of pollens with respect to synthesis of specific IgE antibodies, and that the immune system of the TGA group is less discriminative with respect to the induction of IgE synthesis.

We analyzed the grass pollen-specific IgE antibody binding by the EIA and immunoblotting analysis. A major drawback of these techniques is the possibility that binding of IgG antibodies may block the binding of IgE antibodies to the same molecules (30, 31). To avoid possible interference, we analyzed the sera in 1:30 to 1:100, where antibody binding to the antigens and/or allergens is not saturated. The concomitant strong binding of both IgE and IgG antibodies of the most reactive sera to KBG extract in the EIA and to individual proteins in immunoblotting indicates that these techniques provide sufficient resolution for quantitative detection of binding of antibodies of both classes to the allergens.

With respect to grass pollen-specific IgG antibodies, the TGAs were, as a group, distinct from both the TAs and the NAs, by having approximately 10 times higher concentration of KBG-specific IgG antibodies in serum. In addition, antibodies in the sera of TGAs recognized a larger number of proteins than the other two groups. Although the results of the IgE and IgG systems cannot be compared quantitatively because of different conjugated secondary antibodies, the relative intensity of each individual band developed for IgE and IgG antibodies, respectively, indicated correlation between the responses of the two antibody isotypes to the individual proteins. However, it is to be noted that because the

enzyme-conjugated anti-IgE antibody bound directly to a 31-kDa band, it was impossible to determine whether the IgE antibodies in any of the sera recognized either group IX or group I, or both proteins; thus, this band was excluded in interpretation of these results. Clearly, except for the allergenic proteins of M_r 28–29 000, all potential allergenic molecules had generated an IgG response within each group. The degree of binding to the individual protein bands indicated that the IgG response to the individual protein was similar in the TGA, TA, and NA groups. These results agree with the previous report (30) and with the results of IgG binding to the rKBG8.3 polypeptide.

A positive correlation between IgE and IgG4 antibody response to allergens has previously been reported (6–9) from *in vitro* studies; however, the *in vivo* results are not so clearcut. An association between these two isotypes was demonstrated for the KBG pollen extract in this study. This observation is consistent with our present knowledge of class switch, whereby IL-4 induces synthesis of both IgE and IgG4 antibodies (2, 4, 5). Surprisingly, however, there was a poor relationship between these isotypes specific to rKBG8.3 allergen. The reason for this discrepancy is as yet unclear.

Taken together, the results of all these studies suggest that antibody levels in atopic individuals represent a complex pattern and that the atopic subjects respond differentially, not only to tree and grass pollens, but also to individual allergenic molecules. The total and specific antibody levels in the two groups of atopic individuals, as defined in this study, suggest that the overall response to pollens is governed by a number of genetic and environmental factors determining both generalized and specific immune responses. The mechanisms underlying these differential antibody responses require detailed investigations of these factors. Furthermore, the demonstration that the antibody responses in TGAs appear to be more complex than in TAs has implications from the immunotherapeutic standpoint. Bousquet et al. have shown that immunotherapy is more effective in patients who are monosensitized than in those who are polysensitized (32, 33). Our results suggest that TAs who do not develop sensitization to grass allergens would probably benefit from immunotherapy with tree pollen extract or the single major allergen, *Bet v* 1, extract. However, TGAs exhibiting complex antibody profiles to grass are less likely to benefit from such immunotherapy.

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