

Antigen- and Isotype-Specific Immune Responses to a Recombinant Antigen-Allergen Chimeric (RAAC) Protein¹

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ABSTRACT. Ag-specific IgE and IgG antibody responses to a recombinant Ag-allergen chimeric (RAAC) protein were examined in B6D2F1 mice. The RAAC protein consisted of the truncated β -galactosidase (β -gal), linked at its C terminus to a polypeptide representing the conserved region of the recombinant Kentucky Bluegrass allergen encoded by the cDNA clone KBG8.3(rKBG8.3). Immunization of the mice with the RAAC protein in dextran sulfate as adjuvant led to the differential production of antibodies to the two constituents of RAAC protein with respect to their isotypic classes. Most of the antibody responses to both sets of determinants on the fusion protein were IgG1. In addition, the allergenic polypeptide of RAAC protein induced IgE antibodies, whereas the β -gal elicited IgG2a antibodies. The same pattern of antibody isotypes was produced when the individual components, rKBG8.3 and β -gal were separately used for immunization with dextran sulfate. On the other hand, immunization of mice with either RAAC or the β -gal in CFA induced primarily a IgG response, and no IgE antibodies; however, under the same conditions of immunization rKBG8.3 induced IgG1 antibodies and also low levels of IgE antibodies. In contrast, the RAAC and the β -gal induced IgG2a antibodies, whereas rKBG8.3 induced no detectable IgG2a antibodies. Furthermore, high titers of IgE antibodies were induced by the rKBG8.3 and not by the RAAC protein in dextran sulfate after the mice had been immunized twice with the same polypeptide in CFA. It is inferred from these results that the induction of isotype-specific immune responses in the animals with the same genetic background is dependent upon the Ag in question as well as the adjuvant; the latter, however, influences the magnitude but does not determine the isotype of the immune responses. *Journal of Immunology*, 1993, 151: 791.

Allergens represent a class of Ag which, at extremely low doses, are capable of inducing the synthesis of IgE antibodies in genetically predisposed individuals, referred to as atopic individuals. Comparative studies of MHC proteins of atopic versus non-atopic individuals, and of experimental animals, indicate that several MHC loci are involved in the development of the specific IgE immune response (1–4). Some non-MHC

genes are also believed to be involved in the generalized IgE immune responses in atopic individuals and may play a role in production of allergen-specific IgE antibodies (5–7).

It is generally accepted that T cells play a critical role in the induction of specific primary and secondary IgE immune responses (8–10). As with other T-dependent Ag, allergens are processed by APC; the resulting allergenic/antigenic peptides are bound to MHC class-II molecules, and then presented to the TCRs of T cells (11). In recent years, the functional analysis of two subsets of CD4⁺ T helper cells, Th1 and Th2, has revealed major differences in the mechanisms underlying IgE antibody production (12–14); whereas Th1 cells produce IFN- γ , IL-2, and lymphotoxin; Th2 cells produce IL-4, IL-5, and IL-6. Furthermore, the effects of IL-4 and IFN- γ are reciprocal. Thus, in mice, whereas IL-4 promotes the switch of B cells to IgE antibody producing cells, IFN- γ favors the switch to IgG2a

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antibody-producing B cells, and inhibits the synthesis of IL-4 (14).

A number of studies in mice have suggested that the structure of the antigenic epitope may play a role in the recognition and proliferation of Th1 and Th2 type of cells leading to differential Ab production (15–18). Similarly, although cloned human T cells cultured in vitro produce IL-4 when stimulated with the appropriate purified allergens, they secrete INF- γ when stimulated with nonallergenic Ag (19, 20). However, the role of Ag or APC and other signal(s) triggering the differentiation of the Th0 cells to the functionally distinct Th1 and Th2 cells remain to be defined.

To investigate the possible effects of different Ag on IgE and IgG antibody production, and to test the possibility of modulating an IgE antibody response to an allergen by linking it to a nonallergenic Ag, we examined in this study the immunogenicity of a RAAC protein, consisting of a truncated β -galactosidase (β -gal⁴) and a cloned allergenic polypeptide, rKBG8.3, that represents a conserved region of the *Poa p* IX allergens of Kentucky Bluegrass (KBG) pollen. The *Poa p* IX polypeptides represent one of the major group of allergens of KBG pollen (21–24). The analysis of antibody responses to the RAAC protein and its individual constituents revealed that the two components of the chimeric molecule induced different Ig isotypes in comparison with the two immunogenic constituents when immunized separately with different adjuvants.

Materials and Methods

Synthesis of RAAC protein

A cDNA clone, referred to as KBG8.3, was isolated from a λ gt11 cDNA library (21–24); the corresponding polypeptide represents the common C-terminal region of all cloned *Poa p* IX allergens. The cDNA insert of KBG8.3 was purified and ligated to the plasmid vector, pWR590.1. This vector has *Escherichia coli* Lac Z gene coding for a truncated β -gal and is endowed with high efficiency of expression (25, 26). The recombinant plasmid DNA was transformed into *E. coli* strain JM105 by the CaCl₂ method. The transformants were screened for high level of expression of the fusion protein and for binding with human IgE antibodies. The orientation and frame of the cDNA insert in the vector of the positive clones was confirmed by restriction analysis and DNA sequencing. The truncated β -gal was produced by transformation of pWR590.1 vector plasmid into JM105 cells as described above. The RAAC protein and the β -gal fragment were produced, purified and stored at -20°C by methods described before (23, 25).

Synthesis of rKBG8.3

The KBG8.3 DNA insert in the above recombinant pWR590.1 plasmid was isolated by digestion with *Eco*RI and purified by agarose gel filtration as described elsewhere (27). The rKBG8.3 protein was produced by employing pGEX vector system according to the method described before (28). Briefly, the insert was ligated in the modified pGEX 2T vector and then transformed into JM105 cells. The fusion protein was expressed with IPTG induction and purified with glutathione agarose beads (Sigma, St. Louis, MO). Thrombin was used to cleave rKBG8.3 from the fusion protein when it was bound to the glutathione agarose beads and the rKBG8.3 was harvested by pelleting the beads.

SDS-PAGE and immunoblotting

Proteins isolated from the IgE binding clones were analyzed by a 10% SDS-PAGE gel and stained with 0.1% Coomassie Blue in a solution of 10% acetic acid and 20% methanol in water. The gel-separated proteins were also electrotransferred onto nitrocellulose membranes. The membranes were blocked with 2% bovine serum albumin in PBS buffer, and then probed with antibodies raised in mice against proteins of KBG pollen extract and β -gal. After three washes with PBS-Tween buffer, the antibodies bound to the polypeptides on the membrane were detected with alkaline phosphatase-conjugated goat anti-mouse IgG1 (Zymed, CA) and its substrate solution consisting of *P*-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Immunization of mice

Female 6 to 8 wk old B6D2F1 mice (Jackson Laboratories, Bar Harbor, MI) were used for immunization. Groups of three mice each were immunized s.c. in the nuchal region with different doses of RAAC, β -gal and rKBG8.3 proteins, in dextran sulfate (Sigma) or CFA (Sigma) as adjuvant. Before immunization, the mice were bled, the sera were isolated and used as negative controls. Each mouse was primed with 5 μ g of the RAAC protein, or of β -gal. Three different doses of rKBG8.3 (5, 50, and 100 μ g) were used for different experiments. As adjuvant, 2.5 mg DS in 200 μ l saline or equal volume of CFA was used for the first immunization. After 23 days, the mice were boosted with the same amount of the protein in 1 mg DS in 200 μ l of saline or in equal volume of CFA. The animals were bled 7 days after the second immunization. Initially the antibody titers of individual mice was determined; as little variation in titers (<30%) was observed among individual sera, subsequently equal aliquots of individual sera from each group were pooled and the serum pools were stored at -20°C for future analyses. Antibodies were raised against the extract

⁴ Abbreviations used in this paper: β -gal, truncated β -galactosidase; DS, dextran sulphate; KBG, Kentucky Bluegrass; PCA, passive cutaneous anaphylaxis; *Poa p* IX, *Poa pratensis* group IX protein; RAAC, recombinant Ag-allergen chimera; rKBG8.3, cleaved recombinant protein encoded by cDNA KBG8.3.

of KBG pollen and the native β -galactosidase by immunization of the mice with the respective Ag in DS, as described before (26, 27). Each experiment was repeated at least twice.

For the studies aimed at the modulation of IgE antibody production, B6D2F1 mice were immunized twice with RAAC protein or rKBG8.3 in CFA, as described above, for establishment of an immune response to RAAC protein or to rKBG8.3. The mice were then boosted with the same Ag in DS for three times after an interval of three weeks. The sera were collected seven days after each immunization, and pooled as described above. All sera were analyzed on the same day for determination of the titers of their different antibodies.

Cross-inhibition of rKBG8.3 and RAAC protein in binding to anti-KBG antibodies

The degree of conservation of the epitopes of rKBG8.3 in RAAC was determined by reciprocal cross-inhibition of the binding of each of these molecules with respect to murine antibodies of all Ig classes to KBG pollen extract. Firstly, the titer of antibodies in antisera to KBG pollen extract was determined by ELISA using rKBG8.3- or RAAC-coated microplates (Nunc, Denmark). For this purpose, each well was filled with 100 μ l bicarbonate buffer, pH 9.6 containing 0.1 μ g of each of these two proteins and maintained at room temperature overnight. To block any remaining binding sites of the ELISA plates, the wells were filled with 200 μ l of a 2% solution of BSA in PBS buffer and the plates were incubated at room temperature for 2 h, and washed three times with PBS containing 0.2% Tween-20. One hundred μ l in PBS of serially diluted antisera against KBG pollen extract was added to the wells and incubated at room temperature for 2 h. The plates were washed three times with PBS-Tween buffer, followed by incubation at room temperature for 2 h with 100 μ l of 1:2000 diluted goat anti-mouse Ig conjugated with alkaline phosphatase (Zymed, CA); finally, the antibody binding was detected by enzymatic color reaction of p-nitrophenyl in 100 μ l alkaline phosphatase buffer (Tris 0.1 M, NaCl 0.1 M, $MgCl_2$ 5 mM, pH 9.6) at 37°C for 30 minutes, and the O.D. values were determined with an ELISA reader (Dynatech, CA) at wave length 405 nm. For the ELISA inhibition assay, the antisera, at a dilution of 1:4000 in PBS, were incubated with different amounts, (1 to 400 μ g per ml) of either rKBG8.3 or RAAC protein, 1 h before their addition into the wells as described before (26, 27).

Detection of specific IgG1 and IgG2a and total antibodies

The KBG pollen allergen and β -gal specific IgG1 and IgG2a antibodies were determined by using ELISA. The wells of microtiter plates (Nunc, Denmark) were coated

separately with 1 μ g KBG pollen extract prepared as described previously (29) and 0.1 μ g β -gal per well in 100 μ l bicarbonate buffer (0.05 M, pH 9.6), overnight at room temperature. After three washes with PBS-Tween buffer and saturation of the plates with 2% BSA in PBS buffer, 100 μ l of serially diluted sera were added to the wells, and the plates were incubated for 2 h at room temperature, washed three times and then incubated with 1:2000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, CA) or goat anti-mouse IgG1 (Zymed, CA). The wells were washed again three times and the enzyme substrate p-nitrophenyl was added in 100 μ l color development buffer and incubated for 30 min at 37°C. The antibody titer was determined by the O.D. values that were threefold higher than the preimmune sera used as negative control in all experiments. Total Ag specific Ig was detected with 1:2000 diluted goat anti-mouse Ig conjugated with alkaline phosphatase (Zymed, CA) as described above.

Detection of specific IgE antibodies

Ag-specific IgE antibody titers were determined by passive cutaneous anaphylaxis (PCA). The backs of three groups of hooded rats, two rats in each group, were intradermally injected with 50 μ l of serially diluted immunized murine sera and the sera of the mice collected before immunization. Forty-eight hours later the rats were challenged by intravenous injection of different Ag dissolved in 1.0 ml of PBS containing 0.5% Evan's blue; the first group was challenged with 1.0 mg per rat of the KBG pollen extract, the second with 1.0 mg per rat of Bermuda pollen extract, and the third with 0.5 mg per rat of β -gal (BRL, Mississauga, Ont). The PCA titer of each serum was taken as the highest dilution of the serum giving a clearly visible skin reaction (>5 mm in diameter).

Results

The RAAC protein

The RAAC protein was analyzed by SDS-PAGE electrophoresis followed by western blotting. As expected, the protein was detected in the lysate of the transformants with the recombinant plasmid and recognized by antiserum raised against either native β -gal or the KBG pollen extract, whereas the lysate of the parent plasmid transformed cells containing the truncated β -gal was only recognized by anti- β -gal sera. These results are demonstrated in Figure 1. The rKBG8.3 was also synthesized in *E. coli* and recognized by the anti-KBG pollen sera but not by the anti- β -gal sera (data not shown).

Antibodies induced with the RAAC protein in DS

Immunization of B6D2F1 mice with the RAAC protein in DS led to induction of both IgG and IgE antibody responses. For differentiation of the two repertoires of specific

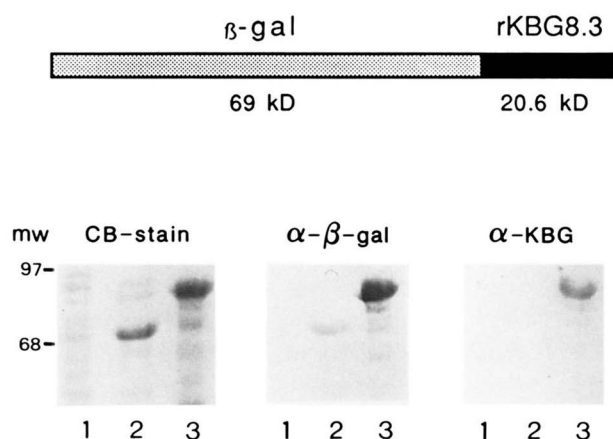


FIGURE 1. Schematic diagram of the recombinant Ag-allergen chimeric (RAAC) protein. (Top) The protein consists of a 69-kDa fragment of a common bacterial Ag, β -gal, and a 20.6KD allergenic peptide of *Poa p IX* allergens from grass pollen. (Bottom) SDS-PAGE electrophoretogram of the RAAC protein stained with Coomassie Blue (CB), and an immunoblot of the gel developed with the pooled antisera to β -gal (α - β -gal) or antiserum to KBG pollen proteins (α -KBG), as primary antibodies. Lanes 1, 2 and 3 contained the protein lysates from the nontransformed control, pWR590-transformed (self-ligated), and pWR590-rKBG8.3 (cDNA) transformed bacteria.

antibodies induced with the RAAC protein, native KBG pollen extract and β -gal were used as coating Ag in ELISA and PCA assays. Figure 2 showed the binding of antisera with both KBG pollen proteins and β -gal. The titer of total specific antibodies against β -gal was slightly higher than that of the antibodies against KBG pollen allergens. The preimmune sera used as negative control showed no detectable antibodies to either of the Ag at the lowest dilution of the sera in Figure 2. These results indicated that both the components of the RAAC protein are highly immunogenic.

Analysis of isotypes and subclasses of immunoglobulins induced with the RAAC protein revealed different spectra of antibodies to β -gal and KBG pollen allergens. There was no significant difference in the total specific antibodies, nor in specific IgG1 antibodies (Fig. 3a). However, striking difference in the titers of IgG2a and IgE antibodies was found between the repertoires of β -gal and KBG specific antibodies. The IgG2a antibodies in the sera to β -gal was about 1000-fold higher than that to KBG allergens (Fig. 3b). Conversely, the IgE antibodies induced with the RAAC protein were found to be specific mainly to *Poa p IX* polypeptide (Fig. 3c). These results indicate that the β -gal and the rKBG8.3 of RAAC differed in their capacity to induce differential IgE and IgG2a antibodies.

Conservation of antibody binding abilities between RAAC protein and rKBG8.3

The antisera to KBG pollen extract recognized both RAAC protein and the rKBG8.3, and titers of the antibodies in

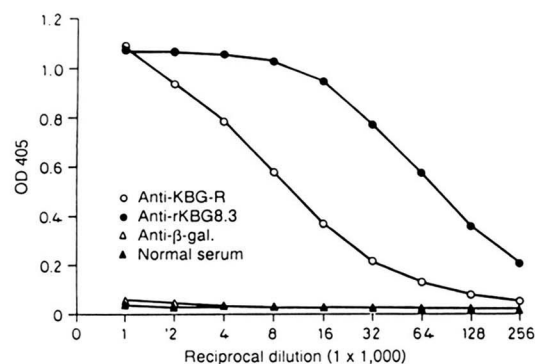


FIGURE 2. Specific immunoglobulins against KBG pollen allergens or β -gal induced with RAAC protein. The wells of the microtiter plates were coated with either native β -gal (●-●) or KBG pollen allergens (○-○), and the specific antibodies in the pooled antisera to RAAC protein were detected by ELISA assay. The preimmune sera tested with β -gal (△-△) and KBG pollen proteins (▲-▲) were used as negative controls. The experiment was repeated twice with the same results and the data from one experiment is shown.

response to these proteins are similar, i.e., 1:64000. The apparent inhibition of anti-KBG sera to bind to the RAAC protein or to the rKBG8.3, by the rKBG8.3 or the RAAC protein, respectively, was observed even at a concentration of 1 μ g/ml, but for complete inhibition, a total of 400 μ g of protein in 1 ml of 1:4000 diluted sera was required (data not shown). The results of cross-inhibition with the concentration of inhibitor at 400 μ g/ml, and positive and negative controls, are shown in Figure 4. As each protein inhibited itself completely, the RAAC protein and the rKBG8.3 was able to abolish the binding of the antisera to each other, respectively. Like the noninhibited antisera, the bacterial protein at 400 μ g/ml had no noticeable effect on the antisera to bind to the RAAC protein and the rKBG8.3. It is inferred from these results that the protein encoded by the cDNA, KBG8.3, when synthesized in the different forms, retained its antibody binding abilities.

Antibodies induced with rKBG8.3 and the truncated β -gal in DS

The antibodies induced with rKBG8.3 and the truncated β -gal are shown in Table I. In this experiment, the corresponding native Ag, KBG pollen extract and β -gal, were used as coating Ag. The rKBG8.3 and the truncated β -gal were highly immunogenic; each induced high titers of total antibodies and IgG1 antibodies. However, a marked difference was observed between IgE antibodies, and IgG2a antibodies induced by the two Ag. The rKBG8.3 at an amount of 5.0 μ g per mouse induced IgE antibodies up to the titer of 1280; β -gal induced IgE titer only up to 80. In contrast, whereas β -gal induced high titers of IgG2a antibodies, rKBG8.3 induced no detectable IgG2a titer even at 1:20 dilution of the antisera. Similarly, the rKBG8.3

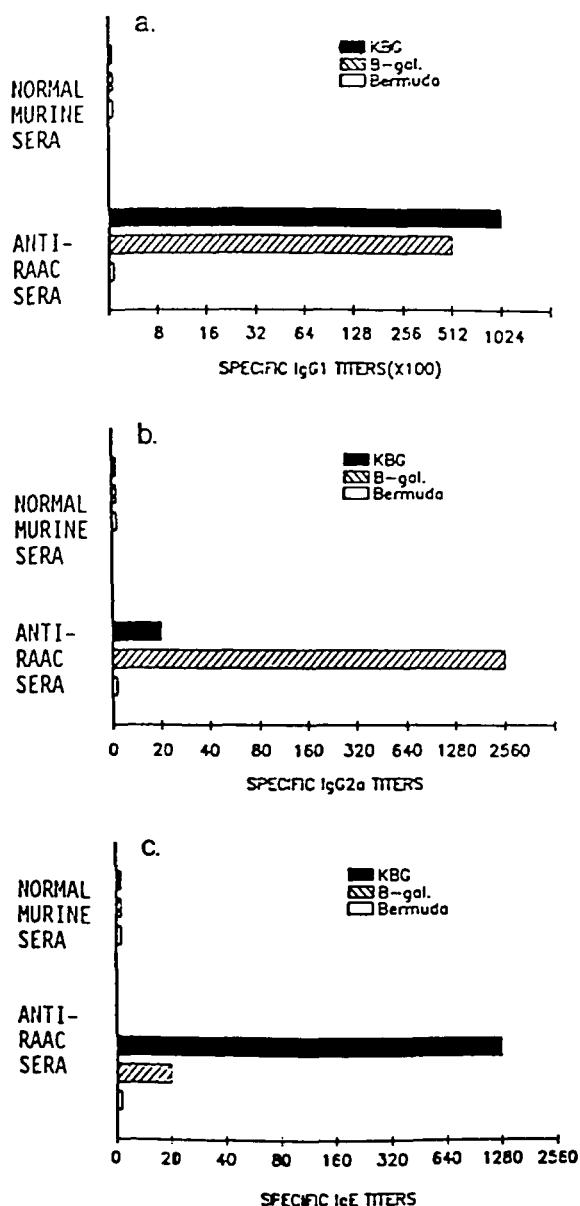


FIGURE 3. Detection of IgG1, IgG2a and IgE immunoglobulins specific to β -gal and to *Poa p IX* pollen allergens in pooled sera of a group of three mice immunized with the RAAC protein in conjunction with DS used as adjuvant. The IgG1 (a) and IgG2a (b) immunoglobulins were detected by ELISA employing alkaline phosphatase-conjugated anti-murine subclass Ig antibodies. The titers of IgE (c) antibodies were determined by PCA assay. Bermuda grass extract was used as negative control. The experiment was repeated twice with same results; the results of one experiment is shown.

immunized at doses of 50 μ g and 100 μ g per mouse induced high titers of IgG1 and IgE antibodies but low titers of IgG2a antibodies. From these results, it is concluded that independently the two Ag induced the same pattern of total and IgG1 antibodies as that of the RAAC protein; however their ability to induce IgE and IgG2a antibodies were significantly different.

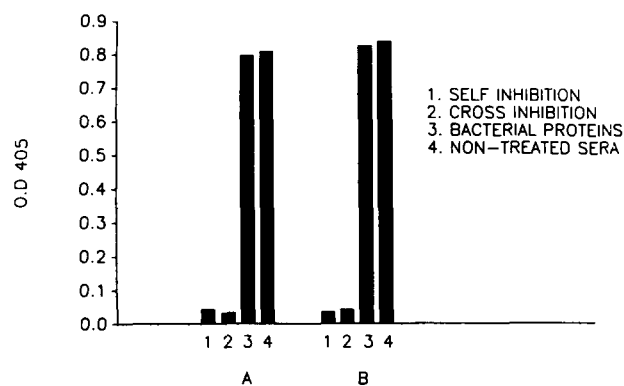


FIGURE 4. Cross-inhibition of the RAAC protein and the cleaved rKBG8.3 binding to anti-KBG pollen protein extract. The RAAC protein and the free rKBG8.3 were coated in triplicate wells. The sera were incubated with the different Ag before addition into the wells. Among the Ag, the self and bacterial proteins were used as positive and negative controls.

Table I
Specific antibodies induced after immunization with rKBG8.3 allergen and β -gal in dextran sulphate^a

Ag Used in Immunization	Antibody Titres		
	IgG1	IgG2a	IgE ^b
rKBG8.3 ^c (5 μ g)	655,360	NIL	1,280
rKBG8.3 (50 μ g)	2,621,440	320	1,280
rKBG8.3 (100 μ g)	1,310,720	160	1,280
Truncated β -gal ^d	2,621,440	10,240	80
Normal serum	NIL	NIL	NIL

^a Groups of three mice each were used for each treatment. The sera were pooled for the analysis of the antibody titers by ELISA or PCA. The experiment was repeated twice with similar results; the data from one experiment is shown.

^b The IgE titers were determined by the PCA assay; the IgG1, and IgG2a antibody titers were determined by ELISA.

^c The Ag used for coating ELISA plates was KBG pollen extract. Each mouse in the first group was immunized with 5 μ g of Ag; the two other groups received 50 and 100 μ g of the Ag, as indicated.

^d Native β -gal was used as coating Ag, and each mouse in this group was immunized with 5 μ g of Ag.

Antibodies induced with RAAC protein and the rKBG8.3 and the truncated β -gal in CFA

When mice were immunized with the RAAC protein in CFA as adjuvant, high titers of total antibodies, IgG1 and IgG2a antibodies to both KBG pollen allergens and β -gal were induced. However, IgE antibodies were not found in the sera to either components of the RAAC protein. Similar pattern of antibody isotypes was also found in the sera of the mice immunized with the truncated β -gal in CFA. In contrast to the above findings, the rKBG8.3 in CFA induced no detectable IgG2a antibodies, although similar high titers of total and IgG1 antibodies were induced. Moreover, rKBG8.3 in CFA induced detectable IgE antibodies as determined by PCA assay. These results, summarized in Table II, indicate that CFA as an adjuvant enhanced IgG2a antibody and inhibited IgE antibody production to β -gal;

Table II
Specific antibodies induced after immunization with RAAC protein and its individual components in CFA^a

Ag Used in Immunization	Antibody Titres		
	IgG1	IgG2a	IgE ^b
RAAC PROTEIN ^c			
<i>Poa p</i> IX specific	20,480	20,480	NIL
β -gal specific	40,960	40,960	NIL
rKBG8.3	327,680	NIL	40
β -gal	327,680	163,840	NIL
Normal serum	20	NIL	NIL

^a Groups of three mice were used for each treatment. The sera were pooled for the analysis of the antibody titers by ELISA or PCA. The experiment was repeated twice with similar results; the data from one experiment is shown.

^b The IgE titers were determined by the PCA assay, the IgG1 and IgG2a antibody titers were determined by ELISA.

^c The two repertoires of the antibody specificities induced with the RAAC protein were determined with different coating Ag.

however, it did not influence the isotype of antibody production to rKBG8.3 allergen.

Modulation of IgE antibody production by immunization of mice with RAAC protein in CFA

To investigate the possibility of inducing IgE antibodies in mice immunized with the RAAC protein in CFA, the mice were subsequently immunized with either constituents of RAAC protein in DS. The results are shown in Figure 5. The group of mice, after immunization with RAAC protein in CFA, showed no detectable IgE antibodies to KBG pollen allergens. The mice were then challenged with the same protein but in DS; the IgE antibodies were found at titer 1:20 of the serum dilution. Further injection of the protein in DS increased the IgE titer slightly to 1:80, and then it was stable even after third immunization. The total, IgG1 and IgG2a antibody titers to KBG allergens and to β -gal, and specific IgE antibodies to β -gal were also determined after each immunization by ELISA and PCA assays, respectively. The results showed no significant changes in total, IgG1 and IgG2a antibody titers to both Ag. Furthermore, there were no detectable IgE antibodies to β -gal (data not shown). The mice immunized with the rKBG8.3 in CFA twice showed high IgE antibody production after further challenges with the allergenic polypeptide in DS. The IgE titer increased from 1:40 to 1:640 after the first immunization of mice with the rKBG8.3 in DS, and remained at this level even after the second immunization. It is inferred from these results that it is possible to modulate antibody responses to an allergen by linking it with a nonallergenic Ag and by immunizing the chimeric protein in an appropriate adjuvant.

Discussion

The RAAC protein consisted of two different Ag; the N-terminal half of β -gal (residue 1–590) and the C-terminal half of *Poa p* IX allergen (residue 122–303). The β -gal, a

common bacterial Ag, has been used to investigate the interactions between B and T cells (30–32). In mice, it was found that whereas C-terminal fragments of β -gal provided T-cell help, the N-terminal fragments favored suppression (31). To the best of our knowledge there is no evidence that this Ag induces IgE antibodies and causes immediate hypersensitivity in humans. In mice, however, β -gal induced a very low titer of IgE response (27); this response was very transient in nature in that no β -gal-specific IgE titers were seen after 42 days. On the other hand, the *Poa p* IX allergens have been shown to represent a major group of grass pollen allergens and >90% of patients allergic to grass pollens possess IgE antibodies specific to these allergens in their sera (24, 33). In contrast to β -gal, the KBG-specific IgE antibodies were persistent; no reduction of IgE titer was observed even after 63 days (27). Thus, the RAAC protein provides an excellent model to study the role of antigenic peptides in specific immune responses, and allows a test of the possibility of modulating IgE responses to the allergen by linking it with a nonallergenic Ag.

We have previously reported that there is no cross-reactivity between KBG pollen Ag and β -gal (26, 27). In this study, immunization of mice with the RAAC protein clearly demonstrated that both components of the RAAC protein possessed epitopes capable of stimulating immune responses and are being recognized by their respective antibodies. Whether the epitopes in the two components of the RAAC protein differ in their abilities to stimulate different antibody isotypes was examined by analysis of the specific isotypic antibodies induced by the individual Ag. Marked difference in their ability to induce either specific IgE or IgG2a antibodies corresponding to the two constituents of the RAAC protein implies that one component induced predominantly IgE antibodies, i.e., a Th2 type immune response, whereas the other induced mainly IgG2a antibodies, i.e., a Th1 type response. Interestingly, both components of RAAC induced similar titers of specific IgG1 antibodies. Recent evidence including the studies with IL-4 “knock-out” mice suggest that the late IgG1 responses are IL-4 dependent (34–36). The finding that most of the antibody responses to both sets of determinants on the fusion protein, when DS is used as adjuvant, are of IgG1 isotype, is inconsistent with a Th1-associated antibody response typically characterized by a high IgG2a:IgG1 ratio. Our data, therefore, suggest that IFN- γ is not the primary cytokine produced in response to the β -gal moiety of fusion protein, when DS is used as adjuvant. It may be that relatively little IL-4 or IFN- γ is delivered to the β -gal-specific B cells under this condition.

Furthermore, substantial differences between the constituent Ag of RAAC protein in terms of their abilities to induce the antibody isotypes under the same experimental conditions suggest that the structure of these two Ag determine the pattern of the immune responses in the animals

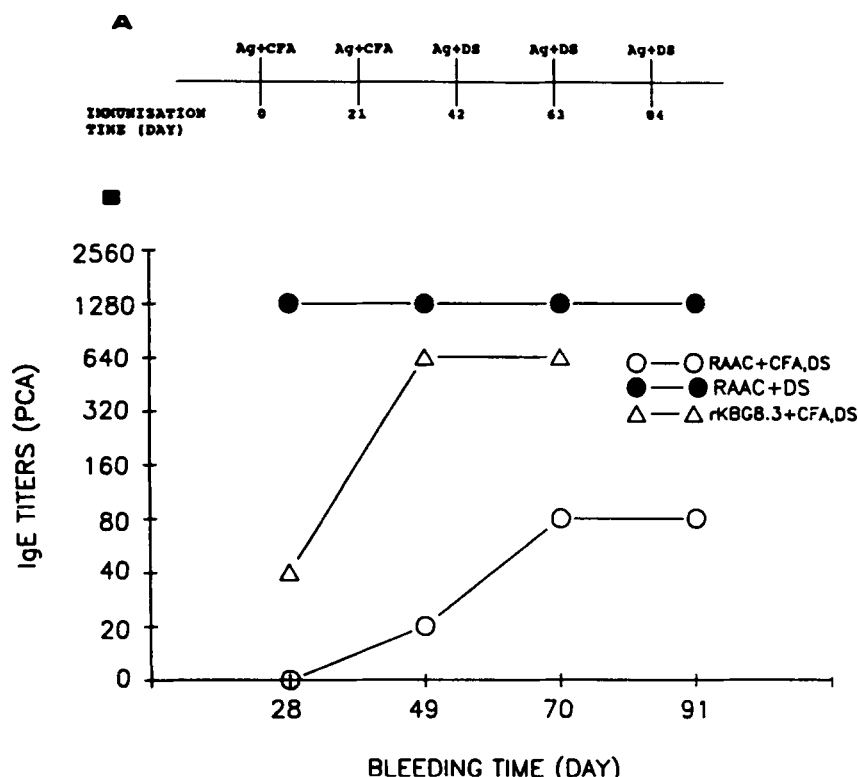


FIGURE 5. Modulation of IgE antibody production. The mice were first immunized twice with either the RAAC protein or the free rKBG8.3 in CFA, and then challenged with the same protein but in DS, at three week intervals. The sera were collected 7 days after each injection, pooled, and tested for the IgE antibody titers by PCA assay. A representative experiment is shown.

with the same genetic background. These results support the recent findings that in the same individual some T-cell clones are of Th1 type with specificities predominantly to the bacterial Ag, and others are of Th2 type with specificities mainly to the allergenic proteins (15, 16). Furthermore, the patterns of immune responses induced by the two components of the RAAC protein and the individual components immunized separately were essentially the same. These results strengthen the notion that distinct epitopes on an Ag may differentially regulate the immune response (31).

Interestingly, the rKBG8.3 immunized with DS showed no dose dependent induction of specific IgE antibodies, which is not in agreement with the previous findings that the low doses of the Ag preferentially induced IgE antibodies, whereas high doses mainly induced IgG antibodies (37, 38). However, high doses of rKBG8.3 induced also a low titer of IgG2a antibodies. The β -gal was also found to induce a low titer of IgE antibodies when immunized with DS. Thus, under certain conditions antibodies of all isotypes may be produced upon a given Ag stimulation.

It has been reported that there exists a hierarchy among linked epitopes with respect to induction of immune responses (31, 39). The RAAC protein injected in conjunction with DS in this report, however, showed no such hierarchy between the two components. The discrepancy may be partly because of (i) the fact that the constituent proteins of RAAC comprise multiple epitopes, and/or (ii) that the adjuvant, DS, may play a role in enhancing the IgE immune

responses and inhibiting the IgG2a responses. Thus, the truncated β -gal could not fully express its ability to elicit the Th1 type response.

Unlike the RAAC protein in DS, the RAAC protein in CFA used for immunization induced high titers of IgG2a but virtually no IgE antibodies to either of the components. These data suggest that the isotypes of antibodies to a given Ag are determined by the type of adjuvant used in immunization. However, the results from the immunization with two individual components, the rKBG8.3 and the truncated β -gal, suggest that the adjuvant can only influence but does not determine the isotype of immune responses to different Ag. This conclusion is consistent with recent reports that some polypeptides induce IgE antibodies independent of the adjuvant used (40, 41). The reason that high titers of IgG2a antibodies were induced also to the allergenic components by the RAAC protein was presumably a result of the fact that β -gal possesses the structure favoring the Th1 type response, and CFA enhanced this property. The allergenic *Poa p* IX polypeptide having been linked to the β -gal also led to the induction of a Th1 response.

The precise mechanism underlying the phenomenon described here is as yet unclear. Other than the differences in the antigenic structure *per se*, the individual components are more soluble, and therefore are processed and presented by B cells. Although the RAAC protein was administered in soluble form its fate *in vivo* remains unknown. Furthermore, macrophages or some other cell type may partially degrade the RAAC protein into recombinant allergen and

β -gal moieties before B-cell binding, further processing, and presentation to T cells. This, in turn, may allow the cognate T-B interaction for the individual components of RAAC, and lead to the production of β -gal- and KBG-specific T cells that, respectively, produce IFN- γ and IL-4. However, simultaneous immunization, and possibly the number and nature of epitopes on 69kDa- β -gal may result in an altered IL-4:IFN- γ ratio and may, therefore, tilt the ratio of IgE and IgG2a antibody production. Alternatively, B cells for the KBG allergen and/or β -gal in this system may involve memory B cells that have been influenced by past exposure to cross-reacting environmental Ag. Such cells may have undergone previous isotype switches that influence their responses to the RAAC protein. However, this seems unlikely because in the same strain of mice the N-terminal region of the recombinant allergen induced low (<40) PCA titers. It is to be noted that there may exist other possibilities and that these are not mutually exclusive. Characterization of the APC responding to the RAAC protein and its individual components may shed some light.

The original goal of this study, i.e., the modulation of specific IgE antibody production was tested in the mice immunized with the RAAC protein or the rKBG8.3 in CFA before injection of the same protein in DS. Although both the RAAC protein and rKBG8.3 are able to increase the IgE antibody production in DS afterwards, the substantial difference in IgE titers between them indicates that the (i) β -gal has influenced the IgG2a antibody production to the linked *Poa p* IX polypeptides, and (ii) the IgE antibody production is not affected significantly by the preexisting IgG1 antibodies, but is significantly affected by the pre-existence of IgG2a antibodies. In essence, these studies have pointed out the prospect of manipulating isotype-specific immune response in vivo by immunizations with chimeric Ag.

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