

Allergenic Relationships of Two Different Antigens of Kentucky Bluegrass Pollen

S. Chakrabarty, A. K. M. Ekiamoddoullah, F. T. Kisil, A. H. Sehon

Medical Research Council Group for Allergy Research, Department of Immunology, Faculty of Medicine, University of Manitoba, Winnipeg, Man., Canada

Abstract. Two purified allergens, allergen C and KBG-1, isolated from Kentucky bluegrass (KBG) pollen, were found to be antigenically distinct with respect to rabbit precipitating antisera produced separately to each allergen. On the other hand, allergen C and KBG-1 appeared to be allergenically identical in terms of the specificities evaluated with murine reagenic antisera produced to each of these allergens; whereas they appeared to be only partially identical when evaluated with respect to the IgE antibodies present in a pool of human sera from individuals allergic to KBG pollen. Therefore, not all of the allergenic determinants recognized by the human IgE antibodies were detected by the murine reagenic antisera. This study serves to emphasize that for a meaningful evaluation of allergenic specificities any antisera prepared in animals and intended for the standardization of allergens must recognize allergenic specificities which are similar or identical to those to which the human IgE antibodies are directed.

Introduction

Allergens may be defined in terms of their capability to provoke IgE-mediated type I reactions in allergic individuals. Most allergens characterized so far fall within molecular size in the range of 20,000-40,000 daltons; the lower size limit of allergens is determined by the molecular structure which must be sufficiently complex to be immunogenic for the induction of IgE antibody formation and the upper size limit is determined by the ability of the allergens to penetrate mucosal membranes [5]. Whether or not a substance is potential-

ly recognized as an allergen is governed by the genetic predisposition of certain individuals to form IgE antibodies following exposure to low doses of the substance [14]. Although allergens were at one time regarded as 'special' antigens which could induce the formation of IgE antibodies, there is no evidence to suggest that in a natural course of induction of an allergic condition, the determinants which are responsible for the induction of IgE antibodies are different from those determinants which are responsible for the induction of IgG antibodies. In fact, there is some evidence to suggest that the IgG and IgE antibodies in ragweed-sensitive

individuals may be recognizing the same determinants on (some) components present in the ragweed pollen aqueous extract [13].

In a preceding study on allergens of Kentucky bluegrass (KBG) pollen [4], it was found that the determinants of allergen C recognized by murine IgE antibodies were different from the determinants recognized by rabbit IgG antibodies. These observations were not unexpected since the conditions for immunization were selected to favour the production of reaginic antibodies on one hand and precipitating antisera on the other in two different species of animals. The present study was undertaken to investigate the immunochemical specificity(ies) and the relationship(s) of two purified allergens of KBG pollen, allergen C and KBG-1 [7, 8]. These allergenic preparations were examined for their ability to be recognized by IgE antibodies present in murine and human antisera and by precipitating antibodies present in rabbit antisera.

Materials and Methods

Production of Murine Reaginic Antisera

Murine reaginic antisera to the non-dialyzable components, i.e., retentate (R) of the aqueous extract of KBG pollen (KBG aq. ext.), allergen C, and KBG-1, were produced in A/HeJ mice (Jackson Laboratories, Bar Harbor, Me.) using procedures as described previously [10]. An amount of 100 µg of each allergen adsorbed onto freshly prepared aluminum hydroxide gel was administered intraperitoneally into the mice. The antisera was obtained from the blood taken from the mice 1 month after the immunization.

Production of Rabbit Precipitating Antisera

A rabbit precipitating antiserum to allergen C was produced as described previously [4]. A rabbit precipitating antiserum to KBG-1 was obtained after multiple intradermal immunization of rabbits

with doses of 100–500 µg of KBG-1 emulsified with Freund's complete adjuvant administered over a period of approximately 3 months.

Isolation of Allergen C and KBG-1

Allergen C was isolated from the retentate fraction, R, of KBG aq. ext. by preparative isoelectrofocussing and gel filtration [4]. KBG-1 (previously referred to as C-1-2-6) was isolated from the dialysate fraction of KBG aq. ext. by combination of procedures employing gel filtration, ion-exchange chromatography, and preparative isoelectrofocussing [8].

Analysis of Allergic Cross-Reactivity

The allergenic cross-reactivity of allergen C and KBG-1 was evaluated in terms of their ability to (1) elicit passive cutaneous anaphylactic (PCA) reactions in rats sensitized with murine reaginic antiserum to R, allergen C, and KBG-1 [9, 10]; (2) neutralize the PCA reactions [9, 10] elicitable with these murine reaginic sera, and (3) inhibit radioallergosorbent test (RAST) employing human IgE antibodies reactive with these allergens.

RAST Inhibition Method

Allergosorbent discs (solid-phase allergen) were prepared by coupling allergen C or KBG-1 to cyanogen bromide activated cellulose discs, and the RAST was performed essentially as described by Ceska et al. [1]. To make the most economical use of the purified allergens, it was first determined what minimum amount of allergen could be used for the preparation of the allergosorbent discs capable of binding the maximum quantity of IgE antibodies present in a volume of 50 µl of a pool of six human allergic sera obtained from individuals sensitive to KBG pollen. This amount was found to be of the order of 50–100 µg. Thus, for the preparation of solid-phase allergen C and KBG-1, an allergen in the amount of 100 µg was incubated with an activated cellulose disc. For the inhibition procedure, allergen C or KBG-1 were mixed with a volume of 50 µl of the pool of human allergic sera prior to addition to each of the solid-phase allergens.

Radiolabelling of Allergens and KBG-1 with ¹²⁵I

Allergens C and KBG-1 were labelled with ¹²⁵I according to the procedure of Hunter and

Greenwood [12]. 1 mCi of carrier-free Na ¹²⁵I (Amersham, Oakville, Ont.) was diluted with phosphate-buffered saline to a final volume of 0.1 ml and added to a 0.1-ml solution of phosphate-buffered saline containing 200 µg of allergen C or KBG-1. Next, a volume of 0.1 ml of a solution of chloramine T (1 mg/ml in 0.1 M phosphate buffer, pH 7.4) was added followed by immediate mixing. The reaction mixtures were allowed to stand at room temperature for 150 s after which time a volume of 0.1 ml of a solution of sodium metabisulphite (2 mg/ml in 0.1 M phosphate buffer pH 7.4) was added to stop the reaction. To remove free ¹²⁵I the mixtures were dialyzed extensively against distilled water through a Spectrapor membrane (molecular cut-off size of 3,500; Spectrum Medical Industries, Los Angeles, Calif.).

Radioimmunoassay Analysis of Antigenic Cross-Reactivity

Antigenic cross-reactivity between allergen C and KBG-1 was evaluated on the basis of the ability of one allergen to inhibit the combination of the other ¹²⁵I-labelled allergen with a rabbit precipitating antiserum to the latter allergen. For this purpose, a titration curve was first established for each of the ¹²⁵I-labelled allergens with their homologous rabbit antiserum. A constant volume of 10 µl of ¹²⁵I-labelled allergen C or KBG-1 (approximately 100,000 cpm) was added separately to volumes of 200 µl of the homologous antiserum diluted serially in phosphate-buffered saline containing normal rabbit serum to the extent of 10% (v/v). These mixtures were shaken at 200 rpm on a continuous horizontal shaker at room temperature for 2 h after which time 210 µl of 66% saturated solution of (NH₄)₂SO₄ was added to each tube and allowed to stand at 4 °C overnight. The precipitates formed were washed twice with 33% saturated (NH₄)₂SO₄ and recovered by centrifugation at 1,800 rpm for 15 min (International Centrifuge, head No. 259, model PR-1). The radioactivity of the precipitates were measured with a Beckman Gamma 300 System counter. A titration curve was established for each allergen and its homologous antiserum by plotting the radioactivity (cpm) of the precipitates versus the dilution of the antisera. None of the labelled allergens coprecipitated with the normal rabbit serum employed for preparation of the dilutions of the antisera.

For the antigenic cross-reactivity studies, the antisera were employed at a dilution which corresponded to the midpoint of the respective titration curves. The degree of inhibition to binding of labelled allergen with its homologous antibody in the presence of varying amounts of one of the allergens provided an indication of the extent of antigenic cross-reactivity of the test allergen relative to that employed in the homologous antigen-antibody system. For these studies, unlabelled allergens in various amounts were mixed with an antiserum (at a fixed dilution of 1:100) and gently shaken for 2 h at room temperature before the addition of ¹²⁵I-labelled allergen (10 µl, approximately 100,000 cpm). The antigen-antibody complexes were subsequently precipitated with (NH₄)₂SO₄ as described. The reduction in the level of radioactive allergen precipitated in the presence of an unlabelled allergen was expressed as percent inhibition of the maximal level precipitable in this system, i.e., % inhibition = (A-B)/A × 100; A = cpm in the absence of inhibitor; B = cpm in the presence of inhibitor.

Table I. PCA titres elicited in rats passively sensitized interdermally with murine reaginic antisera to R, allergens C, and KBG-1 on challenge with R, allergen C, and KBG-1

Murine reaginic sera	PCA titres on challenge with		
	R	allergen C	KBG-1
Anti-R	1,280	320	640
Anti-Allergen C	1,280	640	640
Anti-KBG-1	1,280	320	640
Anti-OA	nil	nil	nil

Hooded rats were passively sensitized by intradermal injections of a serially diluted murine reaginic antisera, listed in the first column. 24 h later, 2 of the rats sensitized in an identical manner were challenged with one of the allergenic preparations at a dose of 500 µg in Evans blue dye (0.25%), injected intravenously. The PCA titre of the murine reaginic anti-OA sera determined with 1 mg OA (dry weight) was 1,280.

Results

Analysis of Allergic Cross-Reactivity

Elicitation of PCA. Allergen C and KBG-1 elicited comparable PCA titers regardless of whether the rats had been sensitized with the murine reagenic antiserum to allergen C or KBG-1 (table I). From these observations it may be concluded that these two allergens shared a common immunogenic specificity which induced the formation of IgE antibodies with similar specificities. Thus, the allergenic specificities were regarded as similar, if not identical to each other. The PCA titers elicited with R were consistently higher, albeit only marginally, than those elicited with allergen C or KBG-1. Neither allergen C nor KBG-1 nor

R was capable of eliciting PCA reactions in animals sensitized with a murine reagenic anti-ovalbumin (OA) antiserum.

Neutralization of PCA. Each allergen at a concentration of 500 $\mu\text{g}/\text{ml}$ could neutralize the PCA reactions elicitable with its homologous murine reagenic antisera (table II). The results of the cross-neutralization experiments revealed that doses of allergen C and KBG-1 of the order of 200–500 $\mu\text{g}/\text{ml}$ could neutralize the PCA reactions normally elicitable with R. R was used as the challenging allergen instead of the homologous allergen because (1) it was readily available; (2) it contained both allergen C and KBG-1, and (3) it always elicited PCA titers higher than the titres obtained with the homologous allergens. The specificity of the

Table II. Evaluation of the ability of allergen C and KBG-1 to cross-neutralize murine reagenic sera produced to these two allergens

Allergen-murine reagenic antiserum system	Concentration of allergen incubated with the murine reagenic antisera				
	5 $\mu\text{g}/\text{ml}$	50 $\mu\text{g}/\text{ml}$	200 $\mu\text{g}/\text{ml}$	500 $\mu\text{g}/\text{ml}$	1 mg/ml
Allergen C-anti-C	+	+	+	-	-
Allergen C-anti-KBG-1	+	+	+	-	-
Allergen KBG-1-anti-KBG-1	+	+	+	-	-
Allergen KBG-1-anti-C	+	+	-	-	-
Allergen C-anti-OA	+	+	+	+	+
Allergen KBG-1-anti-OA	+	+	+	+	+

The PCA of the murine anti-allergen C and the murine anti-KBG-1 antisera established in rats with R at a dose of 1 mg (dry weight) was 1,280. For the neutralization experiments, these antisera were used at a final dilution of 1:400. For positive control, the reagenic antisera at a final dilution of 1:400, in the absence of allergens, were used for the intradermal sensitization of rats. The PCA reactions, if any, were elicited by challenging the animals with a saline solution containing 1 mg R (dry weight) in Evans blue dye (0.25%). All the controls exhibited PCA reactions.

The titre of the murine anti-OA reagenic sera was 1,280 as determined with a challenging dose of 1 mg OA (dry weight). This antiserum was used at a final dilution of 1:400 in the test for specificity of PCA neutralization with allergens C and KBG-1.

+ = Positive PCA reaction; - = absence of PCA reaction.

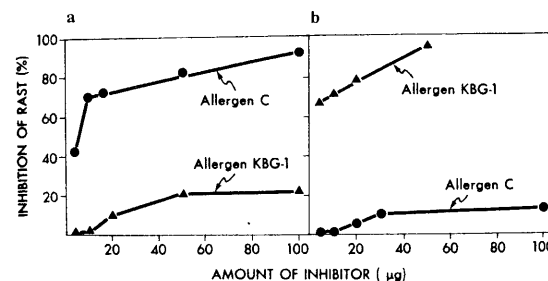


Fig. 1. Inhibition of RAST by allergens C and KBG-1 employing solid-phase allergen C **a** and solid-phase allergen KBG-1 **b**. A pool of human sera from individuals allergic to KBG pollen was employed in these studies.

neutralizations was demonstrated by the fact that allergen C and KBG-1 had no discernable effect on the ability of a murine reagenic antiserum to OA to elicit PCA reactions on challenge with OA.

Inhibition of RAST. KBG-1 was found to inhibit the binding of human IgE antibodies to solid-phase allergen C to the extent of 20%, and allergen C could inhibit the binding of human IgE antibodies to solid-phase KBG-1 to the extent of 16% (fig. 1). These results suggested that the two allergen shared an allergenic specificity(ies) in common with each other in addition to possessing distinct non-cross-reactive allergenic specificities. An alternate interpretation of the results is to suggest that the cross-inhibitions of the order of 16–20% were due to the contamination of one allergen by the other. If this latter situation was true, then one should find the degree of inhibition of RAST to continue to rise with increasing amounts of the respective allergen (inhibitor) on addition to the appropriate solid-phase allergen-human allergic serum system which clearly was not the case.

Analysis of Antigenic Cross-Reactivity
Radioimmunoassay Analysis. The inhibition curves for the binding of ^{125}I -la-

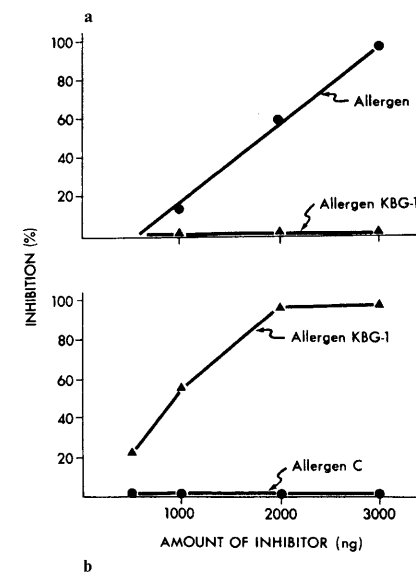


Fig. 2. Radioimmunoassay analysis of antigenic cross-reactivity. Increasing amounts of unlabelled allergen was employed to inhibit the precipitation obtainable with a fixed amount of ^{125}I -labelled allergen and its homologous rabbit antiserum (final dilution 1:100). **a** Inhibition of the rabbit anti-allergen C + labelled allergen C system. **b** Inhibition of the rabbit anti-KBG-1 + labelled allergen KBG-1 system.

belled allergen C and KBG-1 with their homologous rabbit antisera are illustrated in figure 2. The precipitation of ^{125}I -labelled allergen C by the rabbit antiserum to allergen C was completely inhibitable by the homologous antigen employed at a level of 3,000 ng. Allergen KBG-1 was completely ineffective in inhibiting the precipitation of allergen C in this system. With the ^{125}I -labelled KBG-1 and rabbit anti-KBG-1 system, KBG-1 in an amount of 2,000 ng could completely inhibit the precipitation of the labelled homologous allergen with the rabbit anti-KBG-1 serum. Allergen C could not inhibit the precipitation of labelled KBG-1 in this system. Thus, on the basis of these observations, it can be concluded that allergen C and KBG-1 each possessed distinctly different antigenic determinants that were not recognized by the heterologous rabbit antisera.

Discussion

Allergen C and KBG-1 were isolated by different protocols and possessed widely different pI values (9.7 and ~ 4.7, respectively). Allergen C appeared to consist of a single antigenic component. KBG-1 consisted of two antigenic components with closely related pI values (4.5 and 4.9). It is not known if the allergenic activity of KBG-1 is associated with one or both components. Nevertheless, it is reasonable to suggest that at least one of these components must carry both the allergenic and antigenic specificities of KBG-1.

Allergen C and KBG-1 were found to be different antigens. However, they appeared to be allergenically identical with respect to the specificities detected with murine IgE

antibodies produced to each allergen in separate groups of mice. This conclusion was made on the basis of observations that allergen C could completely neutralize the IgE antibodies produced to KBG-1, and allergen KBG-1 could completely neutralize the IgE antibodies produced to allergen C substantiated earlier findings made in this laboratory that one or two discrete components detected by analytical isoelectrofocussing) isolated from a heterogenous mixture of components present in R could completely neutralize the PCA reactions elicitable with the R-anti-R murine system [3]. It would appear that many of the components present in KBG aq. ext. possess an allergenic specificity(ies) in common with each other, and this immunodominant determinant is responsible for eliciting a murine IgE antibody response which is restricted in specificity.

The PCA titres elicited with R on challenge of animals sensitized with the murine reaginic antisera to allergen C or KBG-1 were consistently higher than the titres elicited on challenge with the homologous allergens. To account for this observation it may be suggested that the number of different allergenic antibody specificities was larger for the R-anti-R system than that for any one of the purified allergens and its homologous reaginic antiserum. However, this would appear to be unlikely in view of the observations which so far have consistently indicated that the murine IgE antibody response appears to be restricted to an immunodominant allergenic determinant [3, 4, 8]. It is more tenable to suggest that R was more efficient than the purified allergens in cross-linking the IgE antibodies on mast cells and triggering them for their release of mediators.

A somewhat different interpretation re-

garding the allergenic relationships of allergen C and KBG-1 was made on the basis of allergenic specificities detectable with IgE antibodies present in human allergic sera. In this situation the two allergens appeared to cross-react to a limited degree, i.e., each allergen possessed also unique allergenic specificity(ies). These results are not entirely unexpected since the human sera were obtained from what must be regarded as an outbred population of donors, and it is reasonable to expect that the pool of human antisera could potentially recognize a greater number of allergenic specificities of allergens C and KBG-1, in comparison with a restricted number of allergenic specificity(ies) recognized by the murine reaginic antisera obtained from an inbred strain of mice. The results of these studies serve to illustrate some of the drawbacks of using an animal model system where some of the allergenic determinants recognized by human IgE antibodies apparently escape detection in the murine models. For use of murine reaginic antisera in the standardization of allergenic potency and for a meaningful interpretation of allergenic specificity(ies), it is clear that the animal models must recognize those determinants to which humans respond by forming IgE antibodies [11].

Radioimmunoassay analysis of allergen C and KBG-1 employing rabbit precipitating antisera produced separately to each of these allergens revealed that these two allergens were antigenically unique. Thus, the allergenic determinants on allergen C and KBG-1 recognized by the murine IgE antibodies appeared to be quite different from the antigenic determinants recognized by the rabbit precipitating antibodies. The results of the present studies support the con-

clusion made in an earlier report from this laboratory [4] which suggested that the murine IgE and rabbit precipitating antibodies were recognizing different determinants on allergen C. Similar observations have been reported for two protein allergen isolated from *Ascaris suum* which were found to be allergenically similar when tested with murine IgE antibodies; however, they were found to be antigenically unique when tested with a rabbit precipitating antiserum [6]. In another relevant study, group II antigens of perennial ryegrass pollen were found to share allergenic determinants with group I and III antigens in that they were recognized by IgE antibodies to group II antigens [11]. By contrast, a rabbit antiserum reactive with group II antigens was specific for this group of antigens and did not recognize antigenic determinants present on group I or group III antigens. Observations of this nature emphasize the requirement that antisera prepared in animals and intended for use in the identification and standardization of allergens must detect those allergenic determinants recognized also by human IgE antibodies. The production of monoclonal antibodies with the desired specificity may one day meet this need. Until such time, evaluations of allergenic specificity and potency should be made with reference to the IgE antibodies present in human allergic sera.

Acknowledgements

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Correspondence to: Dr. F.T. Kisil,
Medical Research Council Group for
Allergy Research, Faculty of Medicine,
Department of Immunology,
University of Manitoba,
Winnipeg, Man. R3E 0W3 (Canada)