

RECOGNITION OF A SITE OF A KENTUCKY BLUEGRASS POLLEN ALLERGEN BY ANTIBODIES IN THE SERA OF ALLERGIC AND NON-ATOPIC HUMANS AND A MURINE MONOCLONAL ANTIBODY¹

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Allergen 27 was isolated from the aqueous extract of Kentucky Bluegrass pollen (KBG-R) with a reversed immunosorbent prepared by coupling murine monoclonal antibody, Mab 27, to Sepharose 4B. Sera of patients allergic to KBG pollen, as well as serum of nonatopic individuals possessing anti-KBG antibodies, inhibited the binding of Mab 27 to either Ag 27 or KBG-R to the extent of 20 to 35% in ELISA. In contrast, sera devoid of antibodies to KBG-R had no inhibitory capacity. In a radioallergosorbent test, it was demonstrated that Mab 27 could inhibit the binding of human IgE antibodies to Ag 27 to the extent of 52%. From these results, it is concluded that Ag 27 contains a determinant recognized by both human IgE and blocking antibodies and a murine Mab.

On the basis of the observations that the specificity of IgE antibodies to the allergenic constituents of pollen varies among allergic patients, it would seem desirable to use for their immunotherapy only those constituents to which the individual patient is allergic. The current form of treatment for pollen allergies consists of repeated injections of a crude aqueous extract of the pollen, and it runs the risk of sensitizing the patients to components of the extract to which they were not originally sensitive (1, 2). Moreover, studies from our laboratory that made use of animal model IgE antibody systems demonstrated that such crude aqueous extracts could be separated into fractions, one of which could suppress the IgE antibody response on immunization with the crude extract, and another fraction that could enhance the formation of the IgE antibodies (3, 4). Therefore to minimize the possibility of the untoward responses it would seem appropriate to treat patients with the purified allergen(s) according to their individual sensitivities.

In view of the diversity and complexity of the allergic constituents present in pollens, the task of isolating and characterizing individual allergenic constituents by using traditional procedures has been considered to be impractical. However, with the advent of monoclonal antibodies

(Mab)³ that can provide an inexhaustible and constant source of antibodies with a unique specificity, the task of identifying, characterizing, and standardizing allergens becomes meaningful. Moreover, the use of Mab in the form of reverse immunosorbents for the isolation of a diverse spectrum of purified allergens is feasible (5-12).

In a recent study (5) we demonstrated that a murine Mab, Mab 27, which was produced by appropriate fusion of spleen cells from mice immunized with a heterogeneous mixture of components present in a retentate fraction of Kentucky Bluegrass pollen (KBG-R), predominantly recognized one component in KBG-R. The consequent preparation of Mab 27 in the form of a reverse immunosorbent was shown to be efficacious for the isolation directly from KBG-R of allergen Ag 27. SDS-PAGE analysis revealed that Ag 27 consisted of at least two components with m.w. of 30,000 and 17,000. Analysis by radioallergosorbent test (RAST) by using sera from humans allergic to KBG indicated that Ag 27 was highly allergenic. This study was undertaken primarily to establish the relationship, if any, between the specificities of the IgE antibodies in the sera of patients allergic to KBG pollen and the murine Mab 27, to allergen Ag 27. The data obtained revealed that Mab 27 and the human IgE antibodies recognized a similar if not identical site on Ag 27. The evidence suggested that this site constituted a major allergenic site on Ag 27.

MATERIALS AND METHODS

Preparation of KBG-R. The non-dialyzable components of KBG (*Poa Pratensis*) pollen (Hollister Strier Laboratory, Mississauga, Ontario, Canada) were prepared by extracting ether defatted pollen with distilled water for 4 hr at room temperature, followed by dialysis through Spectropor membrane tubing (m.w. cut off, 6,000 to 8,000; Spectrum Medical Industries, Inc., Los Angeles, CA). The retentate fraction was designated KBG-R.

Production and purification of Mab 27. Mab 27 was one of the murine Mab produced against KBG-R (5, 10). Mab 27 was of the IgG1 isotype and possessed κ light chains (5). The production and purification of Mab 27 has recently been described (5). Briefly, BALB/c female mice (Tmco Breeding Laboratories, Houston, TX) were immunized with KBG-R in Freund's complete adjuvant. The immune spleen cells were fused with P3X20 myeloma cells. Clones of hybridoma cells secreting Mab 27 were expanded in vitro and then were propagated in the peritoneum of C2B6F₁/J (BALB/CJ $\times$ C57BL/6J) female mice (The Jackson Laboratory, Bar Harbor, ME).

The Mab was purified from the ascitic fluid by affinity chromatography on DEAE-Affigel Blue (Bio-Rad Laboratories, Richmond, CA) according to the method of Bruck et al. (13). Alternatively, the ascitic fluid was fractionated by the FPLC system (Pharmacia, Montreal, Quebec, Canada) by using a mono Q column equilibrated with

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³ Abbreviations used in this paper: Mab, monoclonal antibody; KBG-R, retentate fraction of Kentucky Bluegrass pollen; Ag, antigen; AB, assay buffer; IB, incubation buffer; RAST, radioallergosorbent test.

0.02 M triethanolamine-HCl, pH 7.7. The antibody was eluted by applying a linear gradient of 0.354 M NaCl in the equilibrating buffer.

Isolation of Ag 27. Allergen Ag 27 was isolated from KBG-R by affinity chromatography as described recently (5). Briefly, Mab 27 was coupled to activated Sepharose 4B (Pharmacia). This reversed immunosorbent was mixed with a solution of KBG-R and was gently shaken for 3 hr at room temperature. The gel was packed into a column and was washed extensively with phosphate-buffered saline (PBS) (0.54 g KH_2PO_4 , 2.14 g Na_2HPO_4 , 7.75 g NaCl, 0.5 g NaN_3 , and H_2O to a final vol of one liter, pH 7.2). Ag 27 was then recovered by elution with 4 M guanidine-hydrochloride (Schwartz/Mann Biotech, Cambridge, MA) in 0.25 M sodium acetate, pH 4. The eluted material was immediately neutralized with 2 M Tris-HCl, pH 8.5.

Isolation of anti-KBG-R from the serum of a non-atopic individual. Serum obtained from a non-atopic individual and demonstrated by RAST to be devoid of IgE antibodies to Ag 27 was applied to an immunosorbent column prepared by coupling KBG-R to activated Sepharose 4B (Pharmacia). The column was extensively washed with PBS. The bound antibodies were recovered by elution with a 4 M solution of guanidine hydrochloride in a 0.25 M sodium acetate buffer, pH 4.0. The eluate was immediately neutralized by adding 2 M Tris-HCl, pH 8.5, dialyzed against PBS and concentrated to the original volume of the serum. The protein content of the reconstituted purified antibody was 1 mg/ml as determined by its OD at 280 nm (using $E_{280}^{1\%} = 14$ for human IgG).

Conjugation of biotin to Mab 27. Biotinylation of Mab 27 was performed according to the method of O'Shannessy et al. (14). For this purpose, 10.152 mg of Mab 27 was dialyzed against 0.1 M sodium acetate buffer, pH 5.5, and then was oxidized by the addition of sodium periodate to give a final concentration of 10 mM, and the reaction was allowed to proceed for 20 min at 0°C. The reaction was stopped by the addition of glycerol to a final concentration of 15 mM, and the preparation was dialyzed against 0.1 M sodium acetate buffer, pH 5.5.

The oxidized Mab 27 was placed into a plastic tube, and biotin hydrazide (Sigma Chemical, St. Louis, MO) was added to a final concentration of 10 mM. The coupling reaction was continued for 2 hr at room temperature with continuous shaking of the tube. The mixture was then dialyzed against PBS. The biotinylated Mab 27 was tested for its antibody activity by ELISA.

Enzyme-linked immunosorbent assay (ELISA). The ELISA was performed with the flat bottomed EIA plates (Costar Seroclear, Cambridge, MA) coated with KBG-R (10 $\mu\text{g}/\text{well}$) dissolved in coating buffer (1.59 g of Na_2CO_3 , 2.19 g of NaHCO_3 and 0.2 g of NaN_3 made up to 1 L with distilled water, pH 9.6) overnight at 4°C. Wells were blocked by the addition of 3% gelatin in water at room temperature for 3 hr and then were washed three times with PBS. Volumes of 100 μL of the biotinylated Mab 27 at various dilutions was added to the wells and was kept overnight at 4°C. The conjugate was diluted with assay buffer AB; PBS containing 0.5% bovine serum albumin (BSA) and 5% Tween 20). Plates were washed three times with AB (100 $\mu\text{L}/\text{well}$). A stock solution of an avidin-alkaline phosphatase conjugate (Sigma) was prepared by dissolving 0.25 mg in 1 ml of 0.01 M Tris-HCl, pH 8. This stock solution was diluted 500-fold with AB. The diluted conjugate (100 μL) was added to each well and was kept for 2 hr at room temperature and then was washed three times with AB (100 $\mu\text{L}/\text{well}$). After washing 100 μL of the substrate, *p*-nitrophenol phosphate (Sigma) (1 mg/ml in a diethanolamine buffer, pH 9.6) was added to each well, the color reaction was allowed to develop for 40 min and then was stopped by the addition of a solution of 3 M NaOH in a vol of 50 μL . The OD was measured at 410 nm by using the ELISA automatic reader (Model MR 580; Dynatech, Dynatech Instruments, Inc., Torrance, CA).

Determination of the levels of IgG antibodies in human sera by ELISA. The levels of anti-KBG-R-specific human IgG antibodies were determined by ELISA by using plates coated with KBG-R (10 $\mu\text{g}/\text{well}$). Wells were blocked and were washed as described under ELISA. The sera of atopic and non-atopic individuals and the affinity-purified anti-KBG-R antibody preparation were serially diluted, were added to the antigen-coated wells, and were kept at 4°C overnight. The plates were washed with AB, and goat anti-human IgG-alkaline phosphatase conjugate (Sigma) diluted 1000-fold with AB was then added to the wells. The ELISA was continued as described.

Competitive inhibition of ELISA. ELISA plates were coated with the affinity-purified Ag 27 (1 $\mu\text{g}/\text{well}$) and were blocked with gelatin. The biotinylated Mab 27 preparation was diluted 100-fold with AB and was mixed with an equal volume of one of the competing antibodies in varying amounts (i.e., unlabeled Mab 27, human serum, affinity-purified anti-KBG-R antibodies), and 100 μL of the mixture were added to each well. The plate was kept at 4°C overnight. After washing with AB, avidin-alkaline phosphatase was added to each well, and the ELISA was continued as described above. The

percent of inhibition of the binding of the biotinylated Mab 27 to Ag 27 was calculated according to the formula:

$$\frac{A_0 - A_I}{A_0} \times 100$$

in which A_0 = absorbancy at 410 nm in the absence of inhibitor and A_I = absorbancy at 410 nm in the presence of inhibitor.

Competitive inhibition of RAST. The possibility that murine Mab 27 and human IgE antibodies had a specificity in common with each other for binding to allergen Ag 27 was evaluated by the competitive inhibition of RAST. Disposable flexible polyvinylchloride plates "U"-bottomed wells (Dynatech Laboratories, Inc., Alexandria, VA) were coated with Ag 27 (1 $\mu\text{g}/\text{well}$) as described in ELISA. The buffer IB (PBS containing 0.3% HSA) was used for blocking. A pool of six sera obtained from grass allergic patients was diluted 2.5-fold with IB and was mixed with an equal volume of Mab 27 in varying dilutions. Similarly Mab 12, with a specificity to a KBG pollen antigen unrelated to Ag 27, was used for control. Volumes of 100 μL of the mixtures were added to each well. The extent to which the IgE antibodies bound to the allergosorbent was evaluated by the addition of ^{125}I -labeled mouse anti-human IgE Mab. (Mouse Mab, clone No. CIA/E/4.15, specific for human IgE was kindly supplied by Dr. A. Saxon, UCLA, Los Angeles, CA). The degree of the inhibition of the binding of the IgE antibodies to the allergosorbent was assessed in terms of the corresponding decrease in the uptake of the ^{125}I -anti-IgE antibodies. This was determined in relation to the amount of radioactivity bound to the allergosorbent in absence of the inhibitor, Mab 27, and was expressed as the percent of inhibition of RAST by the formula: % inhibition = $(A - B)/A \times 100$ in which A = cpm in the absence of inhibitor and B = cpm in the presence of inhibitor.

RESULTS

A determinant on allergen Ag 27 is recognized by human IgE and murine Mab 27 as demonstrated by competitive inhibition of RAST. The relationship in specificities between Mab 27 and human IgE antibodies was examined in terms of the extent to which Mab 27 could inhibit the binding of the human antibodies to Ag 27. Thus the addition of increasing amounts of Mab 27 to the allergic serum pool resulted in corresponding increases of the inhibition of the binding of human IgE antibodies to the Ag 27 allergosorbent (Fig. 1). The maximum level of inhibition obtained was of the order of 52%. The specificity of the inhibition was demonstrated by the observation that murine Mab 12, with a specificity to components of KBG-R unrelated to that of Mab 27 (15), was unable to inhibit the binding of human IgE antibodies to Ag 27.

Because the IgE antibodies present in the pool of sera

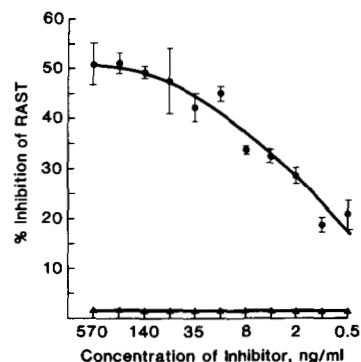


Figure 1. Competitive inhibition of the binding of human IgE antibodies to allergen Ag 27-coated wells by Mab 27 in RAST. A pool of six allergic sera obtained from grass-sensitive patients was diluted 2.5-fold with incubation buffer and was mixed with equal volumes of the inhibitor, Mab 27 (●) or Mab 12 (for control) (▲) in varying dilutions. One hundred microliters of the mixture were added to each allergen-coated well. The extent to which IgE antibodies bound to the wells was determined by the addition of ^{125}I -labeled mouse monoclonal anti-human IgE as described in Materials and Methods. Each point represents the arithmetic mean of triplicate analyses. The SD is represented by the vertical bar.

from grass-allergic individuals were of polyclonal origin and thus were capable of binding to their respective allergenic sites on Ag 27, the high degree of inhibition obtained with the Mab 27 would therefore suggest that the determinant to which Mab 27 bound represents an allergenic site recognized also by the majority of the IgE antibodies present in the human sera.

Competitive inhibition of ELISA. The ability of antibodies present in the sera of grass-allergic patients and non-atopic individuals to compete with murine Mab 27 for binding to the antigenic site(s) on Ag 27 was evaluated by a procedure of competitive inhibition of ELISA. For this purpose, Mab 27 was conjugated with biotin according to the procedure of O'Shannessy et al. (14) in which the biotin is attached to the carbohydrate moiety of the antibody molecule. This results in a biotinylated antibody preparation with an affinity comparable with that of the unconjugated antibody.

The competitive inhibition procedure of ELISA was first established by using unlabeled Mab 27 in different amounts to compete with the binding of the biotinylated Mab 27 to Ag 27-coated plates. A linear inhibition was obtained over a wide range of concentration (57 ng to 1 ng/ml) of the inhibitor (Fig. 2). The failure of Mab 12, with a specificity unrelated to that of Mab 27, to inhibit the binding of the biotinylated Mab 27 to Ag 27 was taken as a demonstration of the specificity of the inhibition of ELISA system obtained with Mab 27.

The results of the competitive inhibition of the binding of Mab 27 to Ag 27 by the human sera are shown in Figure 3. It was evident that the pool of allergic sera could effectively inhibit the capacity of Mab 27 to bind to Ag 27. It was therefore concluded that the pool of sera from human atopsics contained antibodies with specificity directed to the antigenic site recognized also by Mab 27. The assay system that was used could not distinguish whether the inhibition was due to IgE antibodies or antibodies of isotypes other than IgE. In an attempt to resolve this issue, sera from three non-atopic individuals (which were devoid of IgE antibodies to grass pollen allergens on examination by RAST) were evaluated for their ability to compete with Mab 27 for binding to Ag 27 in the ELISA. All three non-atopic sera could inhibit the ELISA system to varying degrees (one of these is illustrated in Fig. 3).

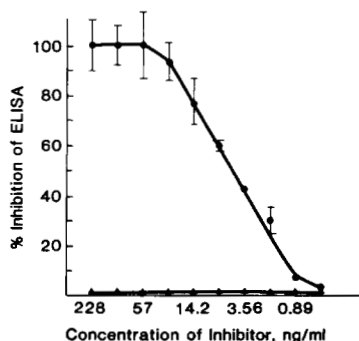


Figure 2. Competitive inhibition of the binding of biotinylated Mab 27 to allergen Ag 27-coated wells by unlabeled Mab 27 in ELISA. Biotin-labeled Mab 27 was diluted 100-fold and was mixed with equal volumes containing different amounts of unlabeled Mab 27 (●) or Mab 12 (▲) (control). One hundred microliters of the mixture were added to each well. Avidin-alkaline phosphatase was added, and the ELISA was continued as described in *Materials and Methods*. Each point represents the arithmetic mean of triplicate analyses. The SD is represented by the vertical bar.

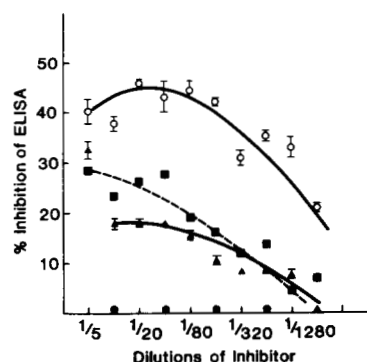


Figure 3. Competitive inhibition of the binding of biotinylated Mab 27 to allergen Ag 27-coated wells by a serum pool from atopic patients, sera from non-atopic individuals, and affinity-purified human anti-KBG-R antibodies in ELISA. The plates were coated with allergen Ag 27 (1 μ g/well). Biotin-labeled Mab 27 was diluted 100-fold and was mixed with equal volumes of serially diluted human allergic serum pool (○), non-atopic human serum containing anti-KBG-R human IgG antibodies (▲), non-atopic human serum lacking anti-KBG-R human IgG antibodies (●), affinity-purified anti-KBG-R antibodies (■) (isolated from the non-atopic human serum (▲) and reconstituted to the original volume of the serum). One hundred microliters of a mixture were added to each well, avidin-alkaline phosphatase was added, and the ELISA was continued as described in *Materials and Methods*. Each point represents the arithmetic mean of triplicate analyses. The SD is represented by the vertical bar.

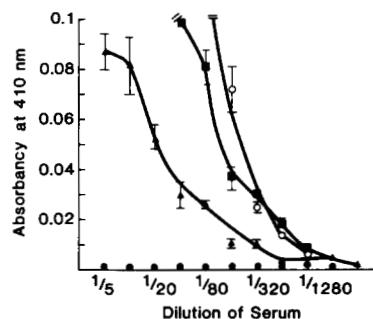


Figure 4. Detection of anti-KBG-R human IgG antibodies by ELISA. Plates were coated with KBG-R (10 μ g/well). One hundred microliters of serial dilutions of: an allergic serum pool (○); non-atopic sera (▲, ●), and a preparation of affinity-purified anti-KBG-R antibodies (■) (isolated from the non-atopic human serum (▲) and reconstituted to the original volume of the serum). The antigen-bound IgG antibodies were detected by adding goat anti-human IgG-alkaline phosphatase and developing the color reaction as described in *Materials and Methods*. Each point represents the arithmetic mean of triplicate analyses. The SD is represented by the vertical bar. The absorbance of <0.001 U was obtained when assay buffer was used in place of the test sample.

The inhibitory capacity of the non-atopic sera was attributed to the anti-KBG-R antibodies that could be specifically isolated from these sera by affinity chromatography and could be demonstrated to have inhibitory activity comparable with that of the original serum. A non-atopic serum that was devoid of antibodies to KBG-R failed to inhibit the competitive ELISA system. This serum was found by a direct ELISA to lack IgG antibodies to KBG-R (Fig. 4). In contrast, the pool of sera from atopic patients, the non-atopic sera, and the affinity-purified anti-KBG-R antibodies that were all capable of competing with Mab 27 for binding to Ag 27, contained IgG antibodies to KBG-R.

DISCUSSION

In a recent study we reported that a useful Mab could be obtained from hybridoma cells that were prepared by appropriate fusion of spleen cells of animals immunized with a heterogeneous mixture of components, i.e., KBG-R (5). The value of this Mab was demonstrated by

the fact that it served not only to identify a highly allergenic component in KBG-R, but it also proved to be efficacious as a reverse immunosorbent for the isolation of the homologous allergen, Ag 27, from KBG-R.

In view of the allergenicity of Ag 27, this study was undertaken to establish the relationship between the specificities of the antibodies present in the sera of KBG allergic patients and the murine Mab 27 that recognizes Ag 27. For this purpose, competitive inhibition assays were used. Thus a close relationship between Mab 27 and the IgE antibodies in the sera of grass allergic patients was established by two separate systems. In the first case, Mab 27 was found to inhibit the binding of the human IgE antibodies to Ag 27. The maximum level of inhibition obtained was of the order of 52%. Considering that the human IgE antibody responses are polyclonal, this high level of inhibition was interpreted to mean that a majority of the human IgE antibodies to Ag 27 were directed to an epitope that was identical or similar to that recognized by Mab 27. Alternately, Mab 27 may have bound to a site adjacent to the epitope recognized by the human IgE antibodies and sterically hindered their binding to Ag 27. The specificity of this inhibition system was demonstrated by the observation that an unrelated murine Mab 12 had no effect on the binding of human IgE antibodies to Ag 27.

The studies of Kahn and Marsh (8) and Esch and Klapper (9) similarly demonstrated that murine Mab produced against rye grass group I antigens could also inhibit the binding of the human IgE antibodies to this antigenic preparation. The maximum degree of inhibition obtained in one of these studies (9) was of the order of 25%. Recently, Olsen and Klapper (7) reported that murine Mab to ragweed antigen E were very effective in blocking the binding of human IgE antibodies to this allergen. It should be noted, however, that Mab produced to allergic components may not necessarily recognize the same epitopes as the human IgE antibodies. To illustrate the case, in one of our studies (11) on the structural analysis of the allergen cytochrome c of rye grass, the antibodies present in the sera of grass allergic individuals or murine Mab that recognized the cytochrome c allergen were examined for their ability to bind to enzymatically derived fragments of the allergen. Each of three Mab was found to recognize a different epitope, and none was recognized by the human IgE antibodies. Differences in the specificities of the antibodies of these two sources was also made by Chapman et al. (6), who found that none of the murine Mab to house dust mite antigen P1 could inhibit the binding of human IgE antibodies to this antigen. These studies indicate that preparation of Mab to epitopes also recognized by human IgE antibodies may require the selection of appropriate strains of mice that have the potential of recognizing certain epitopes. In addition, it would appear to be necessary to screen hybridoma cells for selection of only those that are secreting Mab of the desired specificity.

The assay system based on the competitive inhibition of RAST clearly demonstrated that Mab 27 could effectively inhibit the binding of human IgE antibodies to allergen Ag 27. Conversely, the second assay system that made use of biotinylated Mab 27, was designed to evaluate the ability of antibodies present in sera of grass allergic individuals to compete with Mab 27 for binding

to Ag 27. The results revealed that sera of the atopic patients could partially inhibit Mab 27 from binding to Ag 27. The observations that Mab 27 could completely inhibit the binding of the biotinylated Mab 27 to Ag 27 served to indicate that the specificity of the Mab was not altered by the labeling process. Serum from a non-atopic individual produced an inhibition of the order of 18%. Two additional sera samples from a total of four sera from non-atopic individuals that were examined also displayed a capacity to inhibit to varying degrees the binding of Mab 27 to Ag 27. Because none of these sera had IgE antibodies to Ag 27, it was postulated that the normal sera contained "blocking antibodies". These antibodies could be isolated from the non-atopic sera by affinity chromatography by using an immunosorbent prepared with KBG-R and were shown to be as effective as the whole serum in blocking the binding of the biotinylated Mab 27 to Ag 27. In contrast, a serum from a non-atopic individual that was not able to inhibit the binding of Mab 27 lacked antibodies to Ag 27. The detection of the "blocking antibodies" in the atopic sera is in conformity with the fact that allergic individuals produce antibodies of other isotypes in addition to IgE.

The finding that Mab 27 recognizes a major allergenic site on KBG Ag 27 means that this Mab may be used in lieu of the sera of the grass allergic patients to quantitate and standardize levels of this allergen. The observation that the Mab, the human IgE, and IgG antibodies to Ag 27 recognize a common determinant on Ag 27 suggests that these antibodies may also possess a common idiotype. Thus the Mab could be used to produce the corresponding anti-idiotypic for use in studies on the role of anti-idiotypic antibodies in the regulation of IgE antibody formation to a specific allergenic site.

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