

Isolation of a Kentucky Blue Grass Pollen Allergen Using a Murine Monoclonal Antibody Immunosorbent¹

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Abstract. We have previously reported the production of murine monoclonal antibodies to the retentate fraction of Kentucky Blue Grass pollen (KBG-R). In the present study, one of these monoclonal antibodies (Mab 27) was used in a combination of methods employing Western and immunoblot analysis to establish its reactivity to various antigenic components present in KBG-R. Thus, Mab 27 reacted predominantly with an antigen having a MW of 30 kD and to a lesser extent with other antigenic components with MW of 35 and 17 kD. Fractions of KBG-R obtained by preparative isoelectrofocusing, revealed on analysis by ELISA, that Mab 27 reacted with several components differing in charge. From these observations it was concluded that KBG-R contained a group of related antigens detectable with Mab 27 and differing in size and charge. A reverse immunosorbent, prepared by coupling Mab 27 to CNBr-activated Sepharose 4B, was used to absorb KBG-R. Bound material was recovered by acid elution and designated as Ag 27. SDS-PAGE analysis revealed that Ag 27 consisted of at least two components with molecular weights of 30 and 17 kD. Analysis by RAST using sera from humans allergic to KBG indicated that Ag 27 was highly allergenic.

Introduction

The availability of monoclonal antibodies produced by the hybridoma technology [1] has opened new avenues for the use of monospecific antibodies for: (i) the isolation of individual allergenic components in a pure state in a single step by the use of reverse immunosorbents [2]; (ii) the standardization of allergenic preparations used for immunotherapy [3], and (iii) the antigenic analysis of grass pollen allergens [4]. In principle, a wide spectrum of individual highly purified allergenic constituents of different pollens and other environmental allergens, could become more readily available. Thus it can be envisaged that each allergic patient would be treated with only the constituents to which they are allergic rather than injecting, as in the current practice, crude extracts containing also constituents to which the individual may not be allergic and to which they may actually become sensitized in the course of treatment [5, 6].

We have previously produced monoclonal antibodies to the heterogeneous extract of KBG pollen [7] and in the present study, one of the monoclonal antibodies, designated as Mab 27, was utilized in the form of a reversed immunosorbent to isolate an antigen, designated as Ag 27, from Kentucky Blue grass pollen. Ag 27 isolated by affinity chromatography was found to be highly allergenic and consisted of at least two components having molecular weights of 30 and 17 kD.

Materials and Methods

Preparation of KBG-R and Rye-R

The nondialyzable components of Kentucky Blue grass (*Poa pratensis*) and Rye grass (*Lolium perenne*) pollens (Hollister-Stier Laboratory, Mississauga, Ont., Canada) were prepared by extracting defatted pollen with distilled water, at room temperature, followed by dialysis through spectropor membrane tubing (MW cut off = 6,000-8,000; Spectrum Medical Industries, Inc., Los Angeles, Calif., USA). The retentate fractions were designated as KBG-R and Rye-R, respectively.

Kentucky Blue Grass Pollen Allergen

Rye Grass Pollen Allergens, Gr-I, Gr-II and Gr-III

These rye grass pollen allergens Gr-I, Gr-II and Gr-III were generously supplied by the National Institutes of Health (Bethesda, Md., USA). A preparation of Gr-I allergen was also kindly supplied by Dr. David Marsh, Department of Medicine, Johns Hopkins University, Baltimore, Md., USA.

Production and Purification of Monoclonal Antibody Mab 27

Mab 27 was one of the murine monoclonal antibodies produced against KBG-R [7]. The methods used for the fusion and screening were described elsewhere [4]. Briefly, BALB/c female mice (Tanco Breeding Laboratories, Houston, Tex., USA) were immunized with KBG-R. The immune spleen cells were fused with P3 × 20 myeloma cells. Monoclonal cell lines were produced by the limiting dilution method. Clones of hybridoma cells secreting antibody to components present in KBG-R were expanded in vitro and then propagated in the peritoneum of C2B6F1/J (BALB/cJ × C57B1/6J) female mice (The Jackson Laboratory, Bar Harbor, Me., USA). The ascitic fluids containing the monoclonal antibodies were collected by puncturing the abdominal wall with a 20-gauge needle and allowing the fluid to leak out.

The monoclonal antibody was purified by the method of Bruck et al. [8]. For this purpose, DEAE-Affigel Blue (Bio-Rad Laboratories, Richmond, Calif., USA) was washed according to the manufacturer's instructions and packed into a glass column (1 × 50 cm) and equilibrated with 0.02 M Tris-HCl, pH 7.2. The ascitic fluid was dialyzed against the Tris-HCl buffer, then clarified by centrifugation before applying to the column. The column was washed with 3 bed volumes of the equilibrating buffer, followed by 3 bed volumes of equilibrating buffer containing 25 mM NaCl. The fraction containing the monoclonal antibody was then eluted with the equilibrating buffer containing 50 mM NaCl. The isotypicity of the monoclonal antibody was determined by ELISA as described previously [4].

Preparation of Reversed Immunosorbent

Activated Sepharose 4B (Pharmacia Fine Chemicals, Montreal, Que., Canada) (4.3 g) was washed with ice-cold aqueous solution of HCl (10⁻³ M). The purified monoclonal antibody, Mab 27 at a concentration of 3 mg/ml, was dissolved in 14 ml of coupling buffer (0.1 M NaHCO₃, pH 8.3 containing 0.5 M NaCl) and mixed with the gel in a stoppered plastic centrifuge tube. This suspension was rotated end-over-end for 2 h at room temperature.

The excess antibody was washed away with coupling buffer. From a measurement of optical density of the combined washings, it was estimated that 3.5 mg of the antibody was coupled to 1 ml of the gel. Any remaining active groups were blocked by incubating the gel for 1 h at room temperature with 0.1 M Tris-HCl buffer, pH 8. The gel was then washed 3 times, alternating with acidic and basic buffers. Each cycle consisted of a wash at pH 4 (0.1 M acetate buffer containing 0.5 M NaCl) followed by a wash at pH 8.0 (0.1 M Tris-buffer containing 0.5 M NaCl). The reversed immunosorbent, thus obtained, was then washed with 0.2 M glycine-HCl buffer, pH 2.8, followed by PBS (0.54 g KH₂PO₄, 2.14 g Na₂HPO₄, 7.75 g NaCl, 0.5 g NaN₃, H₂O to make 1 liter, pH 7.2).

Affinity Chromatography

A solution of KBG-R (100 mg dissolved in 5 ml PBS) was mixed with the reversed immunosorbent (19 ml) at room temperature for 3 h by gentle shaking. The gel was then degassed, packed into a

glass column and extensively washed with PBS. The material which had bound to the immunosorbent column was recovered by elution with 0.2 M glycine-HCl buffer, pH 2.8. The eluted material was designated as Ag 27.

Preparative Isoelectrofocusing

One hundred milligrams of KBG-R was fractionated by preparative isoelectrofocusing as described previously [9]. Briefly, Ultrodex (LKB-Products AB, Stockholm-Bromma, Sweden) was swollen in a solution of KBG-R containing ampholyte buffer, pH range 3.5-10 (LKB). The gel was poured onto a horizontal glass tray (11 × 30 × 0.8 cm, Bio-Rad). Paper wicks presoaked with a solution of H₂SO₄ (0.2 M) for the anode or with a solution of ethylenediamine (0.4 M) for the cathode, were laid on top of the gel at either end. The electrophoresis was carried out for 96 h in the Bio-Rad electrophoresis cell, Model 1415. The electrophoresis cell was cooled by circulating water from a refrigerated bath maintained at 4°C.

After isoelectrofocusing, the gel was divided into 38 equal sections with the aid of a gel divider (Bio-Rad). Each gel section was poured into a PEGG elution column (LKB) and eluted with water (5 ml). The absorbance of the eluates at 280 nm was measured with a Perkin-Elmer UV-VIS spectrophotometer (Coleman 139, Coleman Instrument Division, Maywood, Ill.). The pH of each eluted fraction was also measured. The ability of each fraction to bind Mab 27 was examined by ELISA.

Analytical Isoelectrofocusing on Polyacrylamide Gel

Isoelectrofocusing was carried out in the Bio-Rad electrophoresis cell (Model 300A) as described previously [10].

The Radioallergosorbent Test (RAST)

The RAST was performed with disposable flexible polyvinylchloride microtiter plates as described before [11]. Plates were coated with either KBG-R (100 µg/well) or Ag 27 (10 µg/well). A pool of six sera obtained from grass allergic patients and ¹²⁵I-labelled mouse monoclonal antihuman IgE antibody were used in this test. Mouse monoclonal antibody (clone No. CIA/E/4.15) to human IgE was kindly supplied by Dr. A. Saxon of UCLA, Los Angeles, Calif.

Polyacrylamide Gel-Electrophoresis

(i) SDS-PAGE was carried out in the protein slab cell apparatus (Bio-Rad) with 1.5-mm-thick polyacrylamide gels utilizing the Laemmli buffer system [12]. Samples were incubated in the sample buffer overnight at room temperature prior to electrophoresis. The gel was stained with Coomassie Blue according to the method of Fairbanks et al. [13] or with silver stain according to the procedure of Marshall [14].

(ii) Western Blotting: Proteins separated by SDS-PAGE were electrophoretically transferred from the gel onto nitrocellulose membrane [15] with the Bio-Rad transblot cell according to the manufacturer's instructions. To block any further binding of proteins to unreacted sites, the membrane was incubated overnight with a solution of gelatin (3% in TBS (20 mM Tris, 500 mM NaCl, pH 7.5)).

(iii) Immunoblotting: Detection of proteins on the nitrocellulose was achieved by immunoblotting as follows. After the blocking step, the membrane was immersed in a solution of a purified monoclonal antibody preparation (15 µg protein/ml) in TBS containing

¹ Part of this work was presented at the Annual Meeting of the American Academy of Allergy, New York 1985.

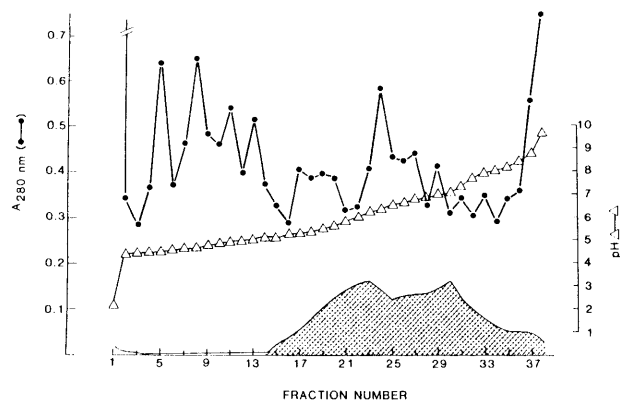


Fig. 1. Preparative isoelectrofocusing of KBG-R (100 mg) on Ultrodex (Ampholine: pH 3.5-10; 96 h). Fractions within the shaded area bound Mab 27 in ELISA.

gelatin (1%) and gently agitated overnight at room temperature. The membrane was washed with TBS, then incubated with the conjugate of goat antimouse IgG and horseradish peroxidase (Bio-Rad), diluted 3,000-fold with TBS containing gelatin (1%), for 1 h at room temperature and then washed with TBS. Solutions of the substrate 4-chloronaphthol and H_2O_2 were added to initiate the reactions which developed as a colored band(s) on the membrane. The immunoprint of the immobilized antigen-antibody complex, thus obtained, was immediately photographed for a permanent record.

High-Performance Liquid Chromatography

Ag 27, isolated by reversed immunosorbent, was fractionated on a Waters I-60 protein column utilizing the Waters HPLC system (Waters Associates, Inc. Milford, Mass., USA). The column was equilibrated with 0.05 M phosphate buffer, pH 7.0. Fraction volumes of 0.5 ml were collected. The ability of the HPLC fractions to bind Mab 27 was analyzed by a direct immunoblotting procedure. Thus, the HPLC fractions were lyophilized and redissolved in 0.05 ml of water. A volume of 5 μ l of each concentrated HPLC fraction was applied onto the nitrocellulose membrane (Bio-Rad). The membrane was blocked with gelatin (3% in TBS) and processed as described under 'Immunoblotting'.

Enzyme-Linked Immunoassay (ELISA)

The ELISA was performed with Costar serocenter 96-well EIA plates (Cat. No. 3590, 205 Broadway, Cambridge, Mass., USA) as described elsewhere [4] with the following modifications. One hundred microliters of the various preparative isoelectrofocused fractions of KBG-R diluted 10-fold with coating buffer (1.59 g of Na_2CO_3 , 2.93 g of $NaHCO_3$ and 0.2 g of NaN_3 made up to 1 liter with distilled water, pH 9.6) was placed into each well and the plates were kept at 4°C in a moist chamber for 18 h. The solution of the various fractions was then withdrawn and the wells were washed with PBS. The capacity of the wells to further absorb proteins was blocked by adding a solution of 3% gelatin (200 μ l/well) followed by incubation at room temperature for 1 h. The plates

were washed three times with PBS (200 μ l/well). One hundred microliters of Mab 27 (in ascites form) diluted 1,000-fold with assay buffer, AB (PBS containing 0.5% BSA, 5% Tween 20) was added to each well and kept overnight at 4°C, the fluids were then withdrawn and the wells washed 3 times with AB. A conjugate of goat antimouse IgG and alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo., USA) diluted 1,000-fold with AB was added and incubated for 1 h at room temperature. After washing, substrate was added, the color reaction was allowed to develop for 40 min and then stopped by the addition of a solution of 3 M NaOH in a volume of 50 μ l. The optical density was measured at 405 nm using the ELISA automatic reader (Model MR 580, Dynatech, Dynatech Instruments, Inc. Torrance, Calif., USA).

Results

Characterization and Specificity of Monoclonal Antibody, Mab 27

Mab 27 was of the IgG-1 isotype and possessed kappa light chains. In the SDS-PAGE analysis of Mab 27 (data not shown) one band corresponding to the heavy chain and two bands corresponding to the light chain were observed on the stained gel.

The specificity of Mab 27 was examined for its ability to bind various antigens of KBG-R separated by isoelectrofocusing. The profile of the absorbance of the fractions at 280 nm (Fig. 1) indicated that KBG-R was resolved into many components. A large number of components, with pI values ranging from 5.3 to 10 had a reactivity towards Mab 27.

The specificity of Mab 27 was also evaluated in terms of its reactivity towards the various antigens

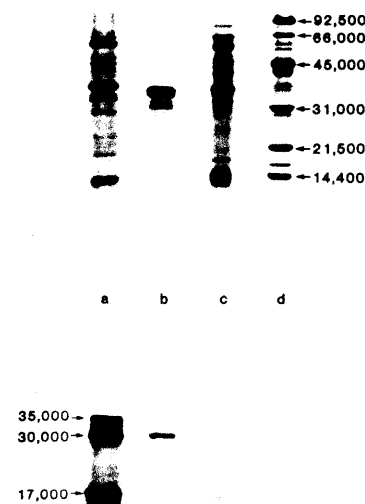


Fig. 2. Specificity of monoclonal antibody Mab 27 as demonstrated by Western and immunoblot analysis. Top panel: gel stained with Coomassie Brilliant Blue following SDS-PAGE of the samples, lanes: (a) KBG-R, 166 μ g; (b) Rye Gr-I (NIH), 20 μ g; (c) Rye-R, 166 μ g (d) Bio-Rad low molecular weight protein markers, 2.5 μ g each. Bottom panel: the corresponding immunoblot obtained by electrotransfer of the separated proteins from the unstained gels onto nitrocellulose membranes; components which bound Mab 27 were visualized by immunoblot as described in 'Methods'.

present in KBG-R, by a combination of SDS-PAGE, Western and immunoblot methods. As shown in figure 2, KBG-R was resolved by SDS-PAGE into multiple components as demonstrated by the stained gels (lane a, top panel), whereas in the corresponding immunoblot developed with Mab 27 (bottom panel) three components were detected. The molecular size of these components was deduced in a separate experiment (data not shown) by comparing the immunoprint of KBG-R developed with Mab 27 with the immunoprint developed with monoclonal antibody Mab 12, which has been shown to detect a 57-kD component of KBG-R [16]. With reference to the po-

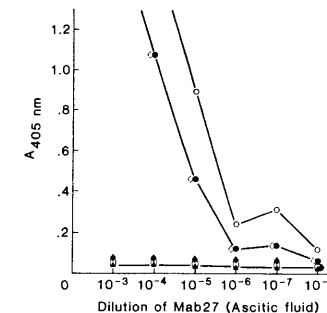


Fig. 3. Specificity of Mab 27 as demonstrated by ELISA. $\circ$ — $\circ$ = KBG-R (10 μ g/well); $\bullet$ — $\bullet$ = Rye Gr-I, NIH (1 μ g/well); $\diamond$ — $\diamond$ = Rye-Gr-I, DM (1 μ g/well); $\blacklozenge$ — $\blacklozenge$ = Gr-II, NIH (1 μ g/well); $\blacktriangle$ — $\blacktriangle$ = Gr-III, NIH (1 μ g/well); $\square$ — $\square$ = HMBA (1 μ g/well). The quantity of each antigen used for coating the wells was more than the amount required (which was established in preliminary experiments) for maximum ELISA value.

sition of this antigenic component detectable by Mab 12, an estimate of the molecular weights of the three components of KBG-R detected by Mab 27 was made. Thus, Mab 27 was found to react predominantly with an antigen having a molecular weight of 30 kD and to a lesser extent with 35- and 17-kD components.

Binding of Mab 27 to Rye Grass Pollen Allergen, Gr-I

Using polyspecific antisera we have demonstrated cross-reactivity among various grass pollen allergens [17]. A cross-reactivity between KBG and Rye grass pollens has been recently demonstrated by the observation that a monoclonal antibody, Mab 12, which was produced by immunization of mice with KBG-R, detected also a purified Rye grass pollen allergen, HMBA [16]. Therefore in the present study, the specificity of Mab 27 was examined in terms of its ability to bind purified Rye grass pollen allergens. As shown in figure 3, Mab 27 bound strongly to the Rye Gr-I allergens (obtained from the NIH). Mab 27 also bound strongly to the Rye Gr-II (NIH) and Gr-III (NIH) allergens. Mab 27 did not bind to Rye HMBA.

On further analysis of Gr-I allergen (NIH) by SDS-PAGE, Western and immunoblot, it was revealed that Mab 27 reacted predominantly to a 30-kD

Table I. Binding of human IgE antibody to AG 27 as demonstrated by direct RAST

Allergen used for coating the microtiter wells	Amount of allergen per well ¹ μg	Human IgE antibody bound ² %
Ag 27	10	10.8
KBG-R	100	12.6

¹ The quantity of each allergen used for coating the wells was more than the amount required (which was established in preliminary experiments) for maximum RAST binding.

² 100 μl of a pool serum from grass pollen allergic individuals was added to each allergen-coated well. The extent to which the IgE antibodies had bound to the allergen-coated wells was determined by the addition of ¹²⁵I-labelled mouse monoclonal antibody (120,000 cpm) to human IgE and was expressed as the percent of the total radiolabelled anti-IgE added. The allergen-coated wells, incubated with serum from a nonallergic individual, bound only 0.1% of the radiolabelled antibody.

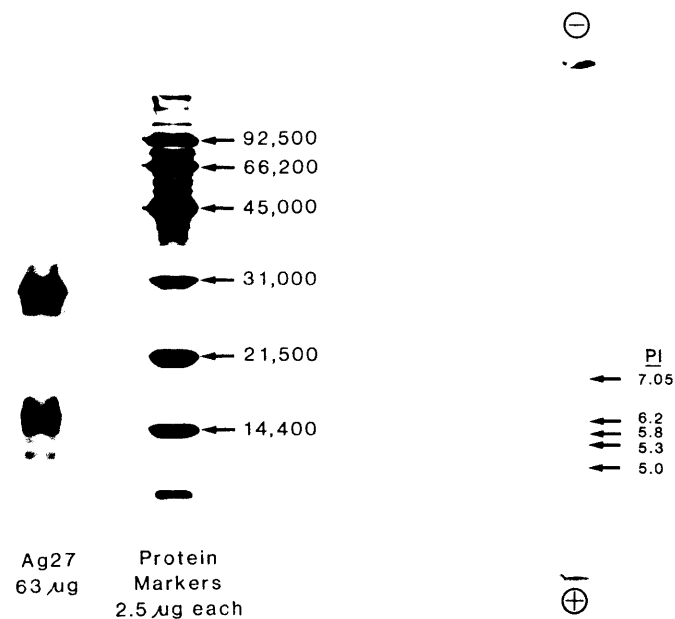


Fig. 4. SDS-PAGE of Ag 27. Proteins separated in the gel were visualized with silver stain as described in the 'Methods' section.

component of Gr-I allergen (fig. 2, lane b). Two major (i.e. intensely stained) bands, corresponding to a size of 35- and 34-kD and two minor bands corresponding to a size of 31- and 30-kD were observed on the stained gels following SDS-PAGE analysis of Gr-I allergen. The 30-kD component reacting with Mab 27 was regarded as one of the minor components of Gr-I allergen.

The analysis of Rye-I (Marsh) (data not shown) also revealed that Mab 27 reacted with a 30-kD component. On the other hand one major protein band corresponding to 34-kD and two minor bands corresponding to 31- and 30-kD of Rye-I were detected on the stained gels.

Since Mab 27 reacted to a 30-kD component of Rye-Gr-I allergen, it was of interest to examine the activity of Mab 27 towards the antigenic components of Rye-R. As shown in figure 2, lane c, Mab 27 bound to 16- and 14-kD components of Rye-R.

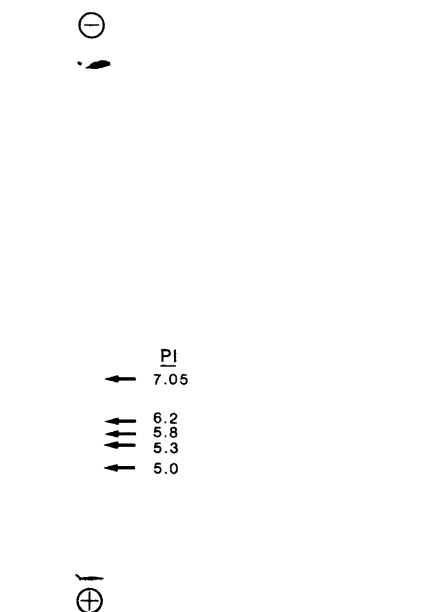


Fig. 5. Analytical isoelectrofocusing of Ag 27 (100 μg) on polyacrylamide gel (Ampholine: pH 3.5-10; 22 h).

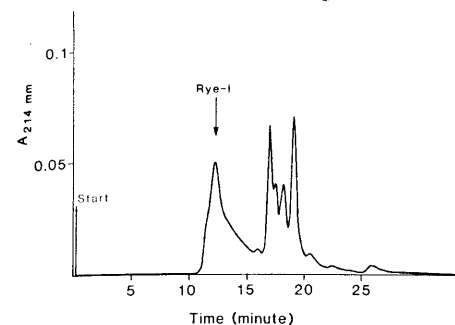


Fig. 6. Separation of Ag 27 (10 μg) on Waters protein column I-60 equilibrated with 0.05 M phosphate buffer, pH 7.0. Flow rate 0.5 ml/min. Fractions were collected at 1-min intervals.

Immunochemical and Physicochemical Properties of Ag 27

Ag 27 isolated from KBG-R with the Mab 27 immunosorbent was shown by direct RAST (table I) to be allergenically active. The analysis of Ag 27 by SDS-PAGE using either reducing or nonreducing conditions, revealed that Ag 27 consisted of two components, one with a molecular weight of 17 kD the other 30 kD (fig. 4). Ag 27 was also shown to consist of several components differing in their pI values which ranged from 5 to 7.05 (fig. 5).

Ag 27 was also shown to consist of several components which could be separated according to size by HPLC (fig. 6). Each isolated fraction was demonstrated by direct immunoblot to bind to Mab 27. The material recovered in the fractions which corresponded to the first peak gave the most intense coloration on the immunoblot. Moreover, this fraction had also the same elution volume as that of Rye grass Gr-I (Marsh) allergen chromatographed under identical conditions. It would thus appear that Ag 27 contains a major protein component which is similar to Rye grass Gr-I allergen.

Discussion

In this study, we have demonstrated that a monoclonal antibody, which was produced by utilizing a heterogeneous extract of Kentucky Blue Grass Pollen as the immunogen, can be used specifically to isolate antigenic components with allergenic activity directly

from the KBG-pollen extract. The efficacy of this procedure demonstrates that useful monoclonal antibodies can be produced to the various allergens without their prior isolation from a grass pollen extract.

The data presented in this study clearly demonstrated that Mab 27 recognized several components present in Kentucky Blue Grass pollen extract. Since the clone producing Mab 27 was obtained by the limiting dilution method, the possibility that Mab 27 might be the product of more than one clone is unlikely. Therefore, the binding of Mab 27 to various components in KBG-R was interpreted to indicate that they possessed identical or similar epitopes. The observation that, on the Western and immunoblot analyses of KBG-R, Mab 27 gave a stronger reaction with the 30-kD component compared to the 35- and 17-kD components of KBG-R suggests that the 30-kD component (i) is present in KBG-R in an amount which is higher than the amount of the 35- and 17-kD components and/or (ii) has a higher epitope density than that of the 35- or the 17-kDa component. Although these three components could be easily detected in KBG-R by Mab 27 only two of these components, i.e. 30 and 17 kDa could be detected by SDS-PAGE (fig. 4) in Ag 27 which had been isolated from KBG-R by the reversed immunosorbent prepared with Mab 27. The reason for the failure of detection of the 35-kDa component in Ag 27 by SDS-PAGE is not clear. However, the HPLC profile of Ag 27 clearly showed that Ag 27 consisted of more than two components which bound Mab 27 on direct immunoblot suggesting that one of the components might represent a 35-kD component.

In view of our recent findings that monoclonal antibody, Mab 12, which had been prepared against components present in KBG-R bound also the purified Rye grass pollen allergen, HMBA [16], the ability of Mab 27 to bind purified Rye grass pollen allergens was therefore examined. Although Mab 27 bound neither HMBA, Gr-II nor Gr-III allergens, this monoclonal antibody bound strongly the Rye grass pollen Gr-I allergen. The analyses of Rye Gr-I allergens by SDS-PAGE, Western and immunoblot methods revealed that Mab 27 bound a 30-kD component. On the basis of the intensity of the stained band on SDS-PAGE, the 30-kD component appeared to be a minor constituent present in the Gr-I allergen.

The finding that Mab 27 detected a 30-kD component in purified Rye grass Gr-I allergen but failed to

detect this component in Rye-R suggests that the 30-kD component is present in Rye-R in trace amounts and is detectable only after its enrichment in the isolated Rye Gr-I allergen. It was, however, interesting to note that Mab 27 bound the 16 and 14-kD components present in Rye-R.

Clearly, Ag 27 represents a group of antigenic components capable of binding Mab 27. The relationship of the components which comprise Ag 27 and their role(s) in (i) inducing the allergic state, (ii) eliciting allergic reactions, and (iii) stimulating the formation of blocking antibodies in allergic individuals remain to be elucidated.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada.

The authors would like thank R.T. Cook and P. Warkentin for their capable technical assistance and the excellent secretarial assistance of J. Gilmour is gratefully acknowledged.

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Received: August 16, 1985

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