

Allergenicity and cross-reactivity of rye grass pollen extracts revealed by monoclonal antibodies *

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Using monoclonal antibodies the immunogenic and allergenic characteristics of rye Group I v redefined by SDS-PAGE analysis and immunoblotting. The purified rye Group I from NIH possess major component of approximately 34 000 Da against which most of the sera from grass-sensitive pati and none from non-atopic volunteers contain specific IgE antibodies. Monoclonal antibodies to rye Gr I were raised and used to purify the antigen and to verify the cross-reactivity between grass extracts. TI monoclonal antibodies studied recognized different components of timothy grass and 2 of them recogni kentucky june grass but none recognized components of ragweed extracts.

Key words: *Allergenicity; Cross-reactivity; Rye grass; Specific IgE; Monoclonal antibody*

Introduction

IgE-mediated allergic diseases are caused by mucous membrane exposure to allergenic material such as dust, animal danders, pollens, etc. Among these, pollens of grass plants which are among the most prolific of the plant families with respect to numbers and species are important. Rye grass (*Lolium perenne*) is widely distributed, particularly in our area and the identification, isolation and characterization of allergenic fractions of rye grass pollen have been achieved in the last decade. Immunochemically distinct groups of antigens, classified as groups I, II, III and IV, were found and some of them could exist in more than one isoal-

lergenic form (Johnson and Marsh, 1965, 1966; Marsh et al., 1970). Their allergenic proper were assessed by direct skin testing in g pollen-sensitive patients; most of them (> 8 had a skin sensitivity to rye Group I (Ma 1975). Immunochemically, rye Group I compo is a protein with a molecular weight estimate 27 000 (Marsh, 1975). Substantial allergenic cr reactivity between several grass pollens was served in neutralization of Prausnitz-Kustner p sive allergic serum transfer experiments or by munodiffusion (Marsh, 1970) and cross-immu electrophoresis (Lowenstein, 1978) against ral antiserum to purified rye grass fractions and nally by RAST inhibition (Foucard et al., 1972 appears that many grass pollens such as rye gi and kentucky june grass possess common a genic determinants. On the other hand, timo grass shares less common determinants with grass than do other grasses and possesses a uni determinant not displayed by other grass poll (Bernstein et al., 1976).

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This work aims to redefine the major antigenic components of rye grass by SDS-PAGE analysis and to determine their allergenicity by immunoblotting with human specific (sp) IgE antibodies (Ab) from sensitive patients. Monoclonal (Mc) Ab against rye Group I were raised using a purified preparation of rye pollens for immunization. These Mc Ab were then used for purification of rye Group I and for assessment of the cross-reactivity between rye Group I antigens and other grass pollens.

Materials and methods

Serum and reagents

Serum was obtained from patients with clinical symptoms of allergic rhinitis to grass pollen and with a reactive skin test to rye grass extracts and from non-atopic normal volunteers with no history of allergic rhinitis and negative skin test.

Crude extracts were obtained from commercial sources: rye grass from Omega (Montréal, Canada) and Pharmacia (Piscataway, NJ), Kentucky blue grass and timothy grass from Pharmacia. Purified rye Group I and ragweed (AgE-B&E-C) were obtained from National Institute of Health (NIH, Bethesda, MD). Goat anti-human IgG, rabbit anti-human IgE and rabbit anti-mouse IgG coupled to peroxidase were purchased from Cappel Laboratories (Cochranville, PA). Purified monoclonal antibodies against human IgE myeloma (P.S.) have been previously produced in our laboratory and stored at -80°C at a concentration of 1 mg/ml until use. These monoclonal anti-human IgE antibodies react strongly with other IgE myeloma (IgE κ , UCB, Belgium), and was used successfully on affinity column to remove completely serum IgE from patients with hyper-IgE syndrome.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE slab gel was performed using a Laemmli discontinuous buffer system (1970). Briefly, a linear gradient (7.5–15%) of polyacrylamide was prepared in the presence of 0.375 M Tris-HCl, 0.1% SDS at pH 8.8. The stacking gel (5%) was prepared in 0.125 M Tris-HCl, 0.1% SDS

at pH 6.8. Samples were boiled for 5 min in the presence of 0.0625 M Tris-HCl, 2% SDS, 2.5% β -mercaptoethanol and 10% glycerol at pH 6.8. The following standards were used to estimate molecular weights: phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α -lactalbumin (14 400). Electrophoresis was performed at 4°C for 8 h at 20 mA.

Immunoblotting

Immunoblotting was carried out according to previously published techniques (Towbin et al., 1979; Brunette, 1981). Briefly, proteins resolved on 7.5–15% SDS-PAGE were electrotransferred onto nitrocellulose paper (Schleicher and Schüll; 0.45 μm pore size) with an electroblot transfer system (Hoffer Scientific Instrument) for 16 h at 4°C and 500 mA. The nitrocellulose papers were then cut into strips and saturated with bovine serum albumin (3%). The presence of sp IgE and IgG Ab against components of pollen extracts was determined by incubating the strips with serum (diluted 1:5 in PBS) from subjects for 1 h at 22°C and then for 16 h at 4°C . After washes, the strips were incubated for 3 h with peroxidase-conjugated rabbit anti-human IgE (1/500) or goat anti-human IgG (1/2000). The cross-reactivity between grass pollens was studied by incubating the strips with Mc anti-rye Group I Ab for 1 h at 22°C and for 16 h at 4°C followed by peroxidase-labelled mouse anti-IgG antiserum (1/2000). Antigen-antibody complexes were detected using 4-chloro-1-naphthol and hydrogen peroxide and the reaction was stopped by washing the strips with distilled water.

Production of monoclonal antibodies against rye grass Group I

BALB/c female mice were immunized on days 1, 28 and 90 with 100 μg of purified rye Group I (NIH) emulsified in complete Freund's adjuvant in multiple subcutaneous injections and 5 days prior to fusion, with 100 μg of antigen given intravenously. Monoclonal anti-rye Group I B cell hybridomas were made from the fusion of SP2/0-Ag 14 myeloma cells and spleen cells from immunized mice. The cell fusion was performed as

described (Köhler and Milstein, 1975) by using 50% polyethylene glycol 4000. Hybrids were grown in HAT selection medium over peritoneal macrophage feeder layers. Anti-rye Group I antibody producing hybridomas were identified by radioimmunoassay. Clones were obtained by 2 rounds of limiting dilutions. Hybrid cells (5×10^6) were then propagated in the peritoneal cavity of BALB/c female mice after prior injection with pristane (0.5 ml). The ascitic fluid of the mice was harvested 10–14 days later, and antibodies purified by passing the fluid through a protein A-Sepharose 4B column. Antibodies were then eluted and stored at -80°C until use.

Purification of rye grass allergen Group I

Fifty ml of crude rye grass extract (Omega) were dialyzed against phosphate-buffered saline, concentrated to 5 ml and passed through a column containing 5 ml of Sepharose CL-4B coupled to 10 mg of purified monoclonal antibody. The column was washed out, absorbed material eluted with 10 ml of 3 M sodium thiocyanate pH 6, dialyzed and finally concentrated to 1 mg protein/ml. The purity of the eluted material was assessed by SDS-PAGE analysis and its allergenic activity was checked by skin testing in grass-sensitive patients. This purified antigen was subsequently used to measure sp IgE and IgG Ab levels in an enzyme-linked immunosorbent assay (ELISA).

Specific IgG and IgE

Serum sp IgG and IgE Ab were measured by ELISA (Voller et al., 1978). Briefly, 96-well microtitre plates were coated with purified rye Group I material (200 ng/well), saturated with 3% bovine serum albumin and then incubated with different dilutions of the serum under test. After washes, specific IgG Ab levels were determined by adding peroxidase-labelled anti-human IgG (1/1000) into the wells for 2 h followed by *o*-phenylenediamine (OPD) and hydrogen peroxide (H_2O_2). The sp IgE Ab levels were determined by addition of purified monoclonal anti-human IgE antibodies (1/500) for 18 h, followed by peroxidase-labelled anti-mouse IgG (1/1000) for 2 h and finally by OPD and H_2O_2 . All incubations were performed at room temperature. The reaction was stopped 30 min later by addition of 4 M H_2SO_4 and the

absorbance at 492 nm was measured with automatic spectrophotometer (Titertek). The were expressed in units (absorbance $\times$ (1/tion)).

Results

SDS-PAGE analysis of rye grass pollen

Under Laemmli conditions, SDS-PAGE analysis (Fig. 1) shows that purified rye Group I NIH possesses a major band of approximately 34 000 Da and 4 minor diffuse bands (14–30 000 Da). The crude rye grass preparation under study also possess this major band; a number of other proteins which have a molecular weight ranging from 14 000–80 000. Since PAGE analysis run under reducing conditions generate subunits from higher molecular weights, experiments were done under both reducing and reducing conditions. The protein file under both conditions is identical (not shown). Therefore, subsequent experiments were carried out only under reducing conditions.

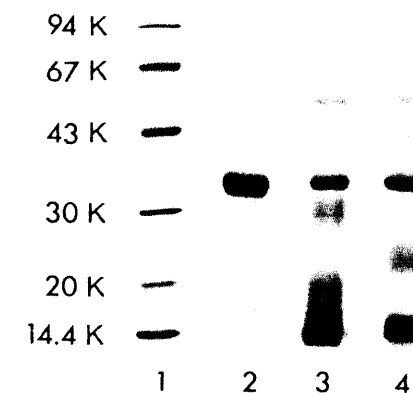


Fig. 1. Coomassie blue-stained SDS slab gel (7.5–15% under reducing conditions; markers (1): purified rye Group I from NIH at 20 μg /well (2); crude rye grass from Pharmacia (3) and from Omega (4) at 20 μg /well.

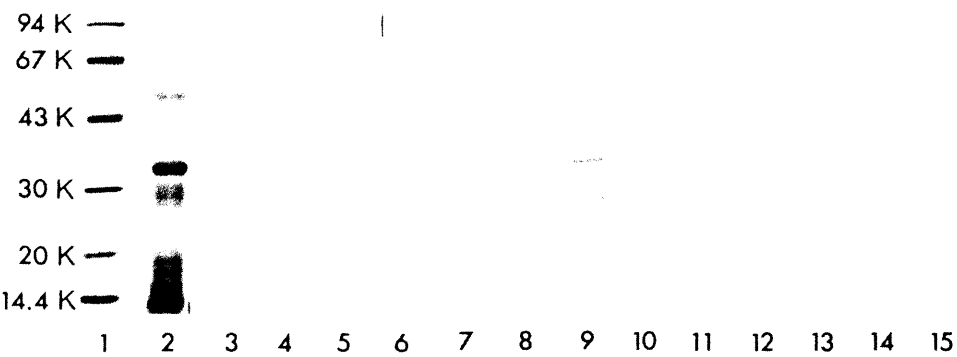


Fig. 2. Immunoblot profile of serum IgE from grass-sensitive patients (6-15) and non-atopic subjects (4-5) against components of rye grass (Omega): markers (1); rye grass extracts stained with Coomassie blue (2); immunoblot with mouse serum as negative control (3).

Allergenicity of rye grass components by immunoblotting

Most of the sera (7/10) (nos. 6-12) from grass-sensitive patients and none from normal volunteers contain specific IgE Ab directed against the 34000 Da component of rye grass (Fig. 2) at levels detectable by immunoblot. However, all sera tested (nos. 4-15) from allergic and non-atopic subjects contain specific IgG Ab which react with most components of rye grass including the 34000 Da component (Fig. 3).

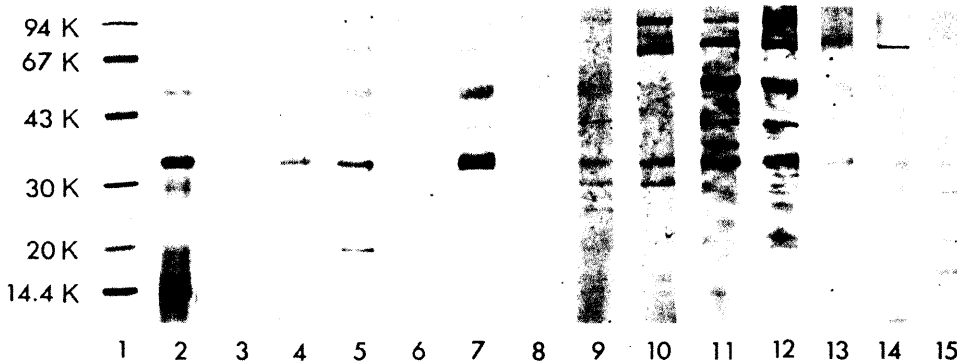


Fig. 3. Immunoblot profile of serum IgG against components of rye grass allergen (same legend as in Fig. 2).

Production of monoclonal antibodies to rye Group I

Monoclonal antibodies against rye Group I were raised using the purified rye Group I from NIH for immunization. Three clones were selected: 290A-167, 348A-6 and 539A-6. The isotypes of these antibodies were: IgG2b, IgG1, and IgG2a respectively (results not shown). These 3 monoclonal antibodies recognize the major component of rye Group I in the 2 crude preparations studied (Fig. 4).

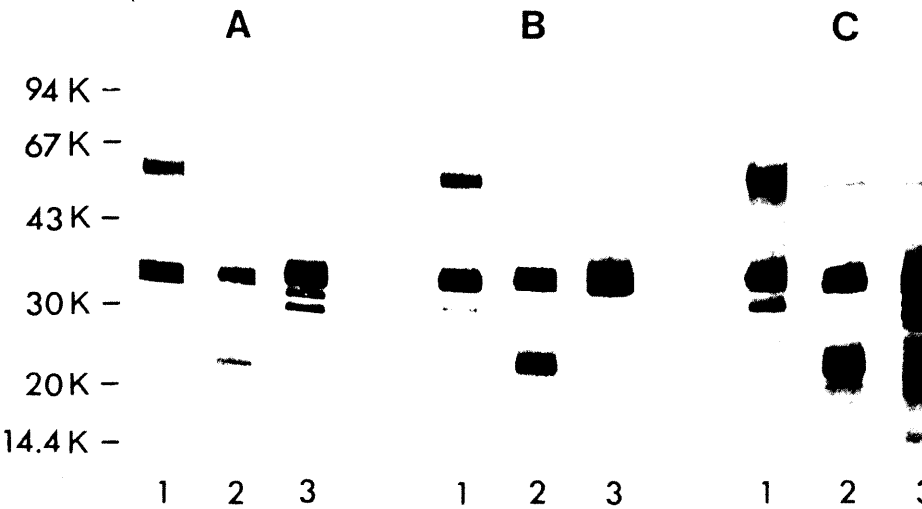


Fig. 4. Immunoblot profile of 3 monoclonal antibodies (A = 290A-167; B = 348A-6; C = 539A-6) against rye Group I from (1); crude rye grass extracts from Omega (2) and Pharmacia (3).

Purification of rye Group I

One of these purified Mc Ab (290A-167 coupled to Sepharose CL-4B and used to purify rye Group I from a crude preparation (On The purified allergen had, on SDS-PAGE an

TABLE I
SERUM LEVELS OF SPECIFIC IgE AND IgG ANTIBODIES DIRECTED AGAINST PURIFIED RYE GROUP I

Subjects	no.	Serum-specific Ab levels (u)	
		IgE	IgG
Non-atopic	4	0.066	158.4
	5	0.080	289.4
Atopic	6	0.966	186.2
	7	3.488	374.2
	8	2.417	189.2
	9	3.234	293.2
	10	2.548	298.6
	11	3.630	157
	12	3.120	347.8
	13	0.220	162
	14	0.176	205.8
	15	0.112	177

Fig. 5. Silver nitrate-stained SDS slab gel (7.5-15%) run under reducing conditions: markers (1); rye Group I purified by affinity chromatography with 5 µg/well (2) and 10 µg/well (3) respectively.

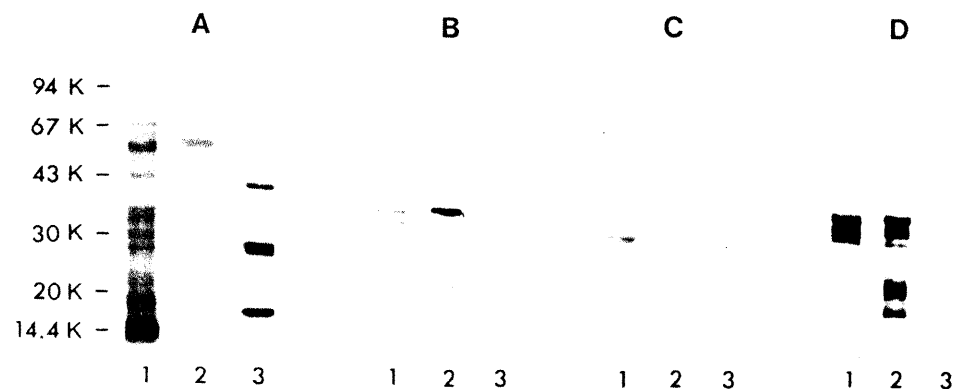


Fig. 6. Cross-reactivity between Mc anti-rye Group I Ab and grass pollens: (1 = timothy grass; 2 = kentucky june grass; 3 = ragweed AgE-B&E-C). *A* = components of the pollens studied by SDS-PAGE analysis and stained with Coomassie blue. *B*, *C*, *D* = patterns of reaction of 3 monoclonal antibodies (290-A-167, 348-A-6, and 539-A-6 respectively) against the same allergens as in *A*.

(Fig. 5), 1 major band (34000 Da) and 1 minor band (23000 Da). Skin tests with this purified material in sensitive patients showed a skin reaction at least equal to that of crude extracts (results not shown).

Serum-specific IgE and IgG as determined by ELISA

The levels of specific IgE and IgG antibodies against purified rye Group I were determined by ELISA in the sera under study. Significant levels of specific IgE Ab could be measured in all 10 sera from allergic patients (Table I) but they were higher in the 7 sera positive by immunoblot than they were in the remaining three. These three were still higher than the values obtained from the 2 non-atopic subjects (0.066, 0.080) (Table I). The specific IgE Ab levels measured in another group of 10 non-atopic donors range from 0 to 0.1 (results not shown). In contrast, high levels of sp IgG Ab could be found in all sera tested (sensitive or non-sensitive patients).

Cross-reactivity between various grass pollen extracts

The 3 monoclonal anti-rye Group I antibodies recognize different individual components of

timothy grass (Fig. 6*B* 1, 6*C* 1, 6*D* 1) and 2 of them recognize kentucky june grass (Fig. 6*B* 2, 6*D* 2). None recognize components of ragweed allergens (AgE-B&E-C).

Discussion

The immunogenic and allergenic properties of the 4 immunochemically different groups of rye grass extracts have been investigated in large numbers of grass pollen-sensitive individuals (Marsh, 1975). Rye Group I, the most important one, is protein in nature and skin reactive in over 85% of grass-sensitive patients. Its molecular weight was estimated to be about 27000 by starch gel electrophoresis (Marsh, 1975). In our hands, the SDS-PAGE analysis under Laemmli conditions indicated a molecular weight of about 34000 for rye Group I using different preparations of rye grass extracts. Comparable results were also obtained using Thomas and Kornberg (1975) rather than Laemmli buffer. Rye Group I is a glycoprotein and its carbohydrate moiety represents 5% of the macromolecule. This may affect the determination of the molecular weight but does not seem to be sufficient to explain the discrepancy between

previous findings and our results. Furthermore, these results were confirmed by Dr. A.K.M. Ekramoddoullah (Winnipeg, personal communication). Our study does not suggest generation of subunits by β -mercaptoethanol. It could be argued that boiling could generate protein subunits or denature the proteins. This study cannot rule out completely these possibilities. On another hand, the proteins resolved on SDS-PAGE run under reducing conditions do not lose their allergenicity since the 34000 Da component (rye Group I) could be recognized using immunoblotting by human specific IgE Ab from 7 out of 10 atopic subjects. In ELISA the 3 patients that did not react in immunoblot had levels of sp IgE Ab lower than the other but still higher than the non-atopic group.

This observed greater sensitivity of ELISA over immunoblot using rye Group I material purified by immunosorbent column with monoclonal anti-rye Group I antibodies could be explained by the fact that human serum used was diluted 1:5. The purity of this purified material was confirmed by SDS-PAGE analysis using silver nitrate stain which is 1000-fold more sensitive than Coomassie blue stain. The allergenicity of the purified material was not lost in the process of purification since it maintained its ability to induce skin reactivity in sensitive subjects.

As previously reported with rabbit antiserum (Lowenstein, 1978), our study confirms the cross-reactivity between rye Group I and certain other grasses, namely kentucky june grass and timothy grass. The specificity was demonstrated by the lack of reaction between monoclonal Ab and unrelated pollen extract such as ragweed.

The present study has redefined the immuno-

genic and allergenic characteristics of rye Group I by SDS-PAGE analysis and immunoblot and confirmed the cross-reactivity between rye grasses and other grasses using Mc anti-rye Group I Ab. Furthermore, purification of allergenic components by Mc Ab is now possible which opens the door to better material for diagnostic or therapeutic purposes.

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