

Development of Monoclonal Mouse Antibodies Specific for Allergenic Components in Ryegrass (*Lolium perenne*) Pollen

I.J. Smart, R.J. Heddle, H. Zola, J. Bradley

Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, Australia

Abstract. The development of monoclonal mouse antibodies against ryegrass (*Lolium perenne*) pollen allergens is described. Hybridoma colonies secreting antibodies specific for allergenic components were detected using an enzyme-linked immunosorbent assay. Positive colonies were cloned and expanded. The pollen components with which the monoclonal antibodies interact were identified and characterised following sodium dodecyl sulphate polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose. In this paper six monoclonal mouse antibodies are described. Three antibodies interact with a single molecule of between 30,000 and 35,000 daltons. One antibody interacts with a component of 16,000 daltons whereas the remaining two antibodies react with more than one component, one reacting with two components at 28,000 and 30,000, and the other with five components having molecular weights between 18,000 and 71,000 daltons.

Introduction

The majority of allergen extracts used in the diagnosis, therapy and research of allergic disease are complex mixtures of biological molecules in which the active allergens often comprise only a small proportion of the material [Marsh, 1974; King, 1976]. Major variations in the composition and allergen content of allergen extracts in clinical use have been recognised for many years and a variety of methods have been proposed for their standardisation [Baer et al., 1970; Foucard et al., 1972; Aas, 1975], but there exists no consensus on the application of these methods. Furthermore, most approaches to allergen standardisation do not quantitate individual allergenic components. Techniques such as quantitative crossed immunoelectrophoresis [Lowenstein, 1978] and radial immunodiffusion using sera raised against purified allergens can be used to quantitate individual components, but represent a substantial investment in time and expertise, and would be difficult to standardise between different laboratories. Recently, the application of monoclonal mouse antibody technology to al-

lergen standardisation has been proposed, together with a description of some antibodies raised against *Dermatophagoides pteronyssinus* extracts [Baldo et al., 1981]. We have developed monoclonal mouse antibodies against allergenic components of ryegrass pollen using methods which are likely to have wide applicability to a variety of allergenic substances.

Materials and Methods

Allergen Extract

Ryegrass (*Lolium perenne*) pollen was obtained from Greer Laboratories (Lenoir, NC., USA) and extracted with 0.1 M Tris, 0.15 M NaCl, pH 8.0 (Tris-saline), as described by Smart and Knox [1980]. This extract was dialysed against water and freeze dried.

Mice

Female Balb/c mice were obtained from the Institute of Medical and Veterinary Science, Adelaide (Australia) and were aged 10-12 weeks at the start of the experiment.

Development of Monoclonal Antibodies

Each mouse was initially immunised subcutaneously with 100-200 µg of allergen extract in phosphate-buffered saline (PBS),

emulsified in Freund's complete adjuvant. At least one further subcutaneous injection of 50–100 µg of allergen extract in PBS, emulsified in Freund's incomplete adjuvant was given at monthly intervals. Spleens were removed for hybridisation 3 days after an intravenous injection of 10–50 µg of allergen extract in PBS, administered 1 month after the last subcutaneous injection.

Myeloma hybridisation was conducted as described by Brooks et al., [1980] using the myeloma line P3-NS1-1 AG4-1 [Köhler et al., 1976]. The cells were cultured in RPMI-1640, 10% fetal calf serum (FCS), hypoxanthine, thymidine and aminopterin (HAT medium) and wells containing growing colonies of hybridoma cells were screened for antibody production using an antigen-binding enzyme-linked immunosorbent assay (ELISA).

Cells from positive wells were cloned at limiting dilutions in RPMI-1640 and 10% FCS, hypoxanthine and thymidine (HT medium). Cloning wells containing more than one colony were discarded. Single colonies were then tested for antibody production by an IgE-binding ELISA.

Enzyme-Linked Immunosorbent Assays

Enzyme-Linked Antibodies. The urease system [Chandler et al., 1982] was used in all enzyme-linked immunosorbent assays. Reactions were graded visually as either negative (bright yellow), positive (purple), or strong positive (dark purple). Immunoglobulin preparations from goat anti-mouse IgG serum and rabbit anti-human IgE (Dako Immunoglobulins, Denmark) were conjugated to urease by the Commonwealth Serum Laboratories (CSL, Australia) customer services. Affinity chromatography was used to absorb the urease-goat anti-mouse IgG (UGAM) with normal rabbit IgG and allergen extract, and to absorb the urease-rabbit anti-human IgE (URAE) with normal goat IgG and allergen extract. Affinity columns were prepared from 2–3 g of CNBr-activated sepharose CL-4B (Pharmacia, Sweden) coupled to 10–20 mg protein using the method described by the manufacturer.

The specificity of the URAE was checked in an ELISA. Wells of a flexible microtitre tray were coated with 50 µl of a 50-µg/ml solution of either rabbit anti-human IgG, IgA, IgM or IgE (Dako Immunoglobulins, Denmark). The wells were washed thrice with saline containing 0.05% (v/v) Tween 20 and 0.5% (w/v) ovalbumin (saline-ova), allowing 10 min between each aspiration. To each well 50 µl of pooled normal human serum was added and incubated for 3 h at room temperature and overnight at 4°C. The wells were again washed thrice and dilutions of URAE added to each well (50 µl/well), and incubated for 60 min at room temperature. After three washes with saline-ova and three washes with distilled water, 50 µl of CSL urease substrate solution was added to each well. Cross-reactions were observed with other immunoglobulins, but at an appropriate dilution a positive reaction occurred only in wells pre-coated with anti-human IgE.

Antigen-Binding ELISA. The wells of microtitre trays were coated with allergen by incubating 50 µl of allergen extract solution (50 µg/ml in PBS) in each well for 3 h at 37°C. Coated trays were stored at 4°C. The wells were washed thrice with saline-ova and 50 µl culture supernatant added to each well. After 3 h of incubation at room temperature and overnight at 4°C the wells were again washed thrice and 50 µl of UGAM (diluted in ELISA buffer (Dulbecco's PBS containing 0.5% ovalbumin and 0.05% Tween 20) was added to each well and incubated for 30 min at room temperature. The wells were washed thrice with saline-ova, and then with distilled water, and 50 µl of CSL urease substrate was added to each

well. Supernatant from cultures of the mouse myeloma line X-63, which secretes an IgG1 molecule, was used as a negative control.

IgE-Binding ELISA. Affinity purified goat anti-mouse immunoglobulin was prepared using an affinity column of sepharose CL-4B coupled to mouse immunoglobulin. Wells of microtitre trays were coated with 50 µl of 50 µg/ml affinity purified goat anti-mouse immunoglobulin in PBS. The wells were washed thrice with saline-ova and 50 µl of supernatant added and incubated for 3 h at 37°C. The wells were again washed thrice and 50 µl of allergen extract (50 µg/ml in ELISA buffer) was added to each well, and incubated at 37°C for 3 h, followed by overnight incubation at 4°C. After three washes 50 µl of pooled serum from human subjects with intense skin reactivity to the allergen extract was added to each well and incubated for 30 min at room temperature. After three further washes, 50 µl of URAE (diluted in ELISA buffer) was added to each well and incubated for a further 30 min at room temperature. The wells were washed thrice in saline-ova, thrice in distilled water and 50 µl urease substrate was added to each well.

A series of controls was used in every IgE-binding assay. These controls were the use of X-63 myeloma cell culture supernatants and, in wells where a known positive hybridoma supernatant was used (a) a serum pool from subjects with high serum IgE but no skin reactivity to ryegrass pollen extract, and (b) replacement of each of the assay components individually by ELISA buffer. All controls were negative indicating that binding of allergen-specific mouse antibody and allergen-specific human IgE were both required to generate a positive reaction, and that no cross-reactivities existed between the immunoglobulins of each of the species used in the assay system.

Sodium Dodecyl Sulphate (SDS) Polyacrylamide Slab Gel Electrophoresis and Electrophoretic Transfer to Nitrocellulose Sheets. Samples of allergen extract (100 µg/sample port) were subjected to electrophoresis in 1.5-mm thick slabs of polyacrylamide gel (12% acrylamide) according to the method of Laemmli [1970]. Upon completion of the electrophoretic run the components within the polyacrylamide gel were transferred to nitrocellulose sheets using the method described by Towbin et al. [1979]. Transfer was conducted in a transfer buffer of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol pH 8.3, using 300 mA for 4–6 h. Initially the voltage drop was 7.5 V/cm. When required, staining was conducted using 0.1% amido black in 45% methanol, 10% acetic acid for 1 min. The sheet was destained in 5% methanol, 7% acetic acid, and then air dried.

Detection of Hybridoma Antibody Specificities

Following transfer, the nitrocellulose sheets were soaked overnight at 4°C in Tris-EDTA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5 ml nonidet P40 [BDH], 0.2% ovalbumin, pH 7.4), containing an extra 2% albumin. The sheet was cut into strips and each strip was incubated with a hybridoma supernatant, diluted to between 1:3 and 1:5 in Tris-EDTA buffer. After 3 h of incubation at room temperature with gentle rocking, the strips were washed five times with Tris-EDTA buffer. Affinity purified, alkaline phosphatase-conjugated goat-anti mouse IgG (E-Y Laboratories) was diluted 1:100 in Tris-EDTA buffer. The strips were incubated in this solution for 1 h at room temperature with rocking. After a further five washes the strips were immersed in substrate solution of 1 mg/ml Naphthol AS-BI phosphate (Sigma) and 1 mg/ml Fast red TR salt (Sigma) in 0.1 M Tris, 0.01% MgCl₂ · 7H₂O, pH 9.2. After 5–10 min at room temperature the strips were rinsed in water and air dried.

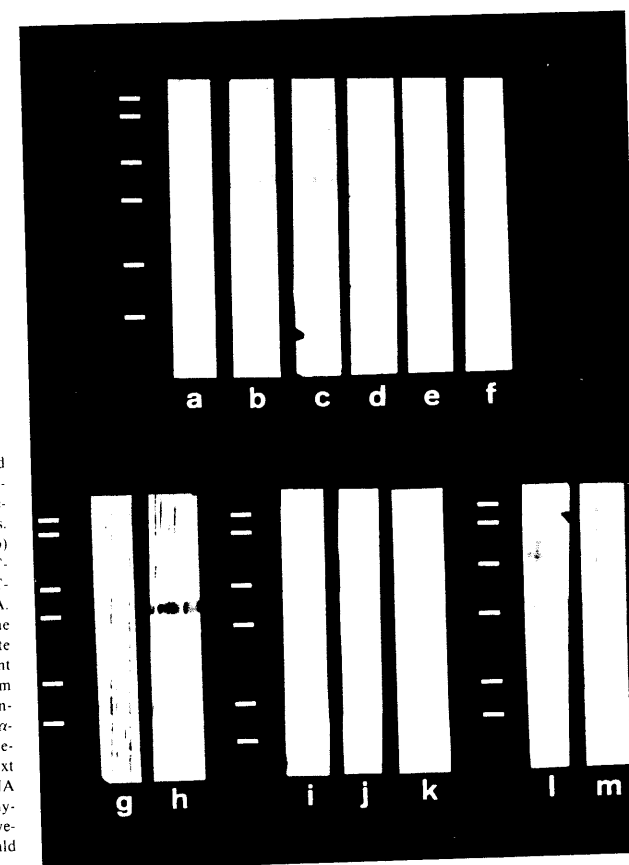


Fig. 1. Characterisation of antigens bound by FMC-A series antibodies following SDS-polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose strips. Supernatants from cultures of (a) X63; (b) FMC-A1; (c) NA; (d) NA; (e) NA; (f) FMC-A5; (g) X-63; (h) NA; (i) FMC-A6; (j) FMC-A7; (k) FMC-A8; (l) FMC-A9, and (m) NA. The horizontal white lines to the left of the groups of strips represent the approximate positions (from top) of the molecular weight markers, phosphorylase (94k), bovine serum albumin (67k), ovalbumin (43k), carbonic anhydrase (30k), trypsin inhibitor (20k) and α -lactalbumin (14.4k). These positions are relevant for all strips to the right up to the next molecular weight marker identification. NA refers to culture supernatants from other hybridomas producing antibody against ryegrass allergens whose monoclonality could not be verified.

Results

IgE-Binding ELISA Activity and Immunochemical Characterisation of Antibodies Produced by the FMC-A Series Hybrids

From two separate hybridisations, a total of 86 hybrids were screened for antibody production by the antigen-binding ELISA. A total of 23 positive hybridoma colonies were identified and, of these, 15 were scaled up to bottle culture and cryopreserved. IgE-binding ELISA analysis of the supernatants of six of these hybrids, together with an immunochemical

characterisation of the antibody produced by each of these hybridomas is presented in table I. Of these antibodies, five were found to be specific for IgE-binding components.

Characterisation of Monoclonal Antibody-Binding Specificities

The reactivity of the monoclonal antibodies with the components of the pollen extract, analysed after electrophoretic transfer to nitrocellulose strips, is presented in figure 1. The approximate molecular weights of the components with which the individual

Table I. IgE-binding ELISA analysis and immunochemical characterisation of FMC-A series hybridoma antibodies

Antibody	Immunoglobulin class	IgE-binding ELISA
FMC-A1	IgG ₁ K	+
FMC-A5	IgG ₁ K	++
FMC-A6	IgM K	+
FMC-A7	IgG ₁ K	++
FMC-A8	IgM K	+
FMC-A9	IgM K	-

Results given as: - = negative; + = positive; ++ = strong positive.

Table II. Approximate molecular weights of FMC-A series hybridoma antibody specificities

Antibody	Approximate molecular weight of antigen bound $\times 10^3$ daltons
FMC-A1	35
FMC-A5	16
FMC-A6	32
FMC-A7	30, 28
FMC-A8	32
FMC-A9	71, 87, 49, 47, 18

monoclonal antibodies reacted is presented in table II. The monoclonal antibodies FMC-A1, FMC-A6 and FMC-A8 reacted with components having molecular weights between 30,000 and 35,000 daltons. FMC-A5 reacted with a component of 16,000 daltons, FMC-A7 reacted with two components of 28,000 and 30,000 daltons, and FMC-A9 reacted with five components with molecular weights between 18,000 and 71,000 daltons.

Discussion

In this paper we have described six monoclonal antibodies which bind components of ryegrass pollen extract. The monoclonal antibodies FMC-A1, FMC-A6 and FMC-A8 reacted with IgE-binding components having molecular weights determined by SDS polyacrylamide gel electrophoresis to be between 30,000 and 35,000 daltons. The components identified by each of these antibodies ran in parallel with a major component identified on the amido-

black-stained strip. This component has a similar relative mobility to antigen Lp1 [Smart and Knox, 1980] and glycoprotein 1 [Howlett and Clarke, 1981a], both of which were concluded to be the group-I allergen [Johnson and Marsh, 1965a, b]. It is therefore likely that these monoclonal antibodies react with one or more of the group-I 'isoallergens' [Johnson and Marsh, 1965b].

The monoclonal antibody FMC-A5 bound a distinct component of approximately 16,000 daltons clearly identified as an allergen by specific IgE binding. The lack of any reactivity with other ryegrass pollen components suggests that it is not an antigenic determinant of the group-I allergens present on a lower molecular weight component as has been described by Marsh [unpublished work cited by Lowenstein, 1978]. It is possible that FMC-A5 reacts with either a group-II or a group-III allergen, both of which have molecular weights of 11,000 daltons [Marsh, 1975]. Further work is required to characterise fully the specificity of this antibody. FMC-A7 reacted with IgE-binding components of molecular weight 28,000 and 30,000 daltons. These components appear to be distinct from the group-I allergen referred to previously.

The remaining antibody FMC-A9 reacted with a variety of ryegrass pollen components including a major component of 71,000 daltons. This major component is very similar in molecular weight to that of glycoprotein 2 (68,000) [Howlett and Clarke, 1981a, b] which was thought to correspond to antigen Lp2 of ryegrass pollen [Smart and Knox, 1980], antigen A of *Dactylus glomerata* [Augustin et al., 1971] and the group-IV allergen [Marsh, 1975]. This monoclonal antibody possibly binds to a cross-reactive carbohydrate determinant which has been identified on glycoproteins 1 and 2 of ryegrass pollen and a variety of other plant glycoconjugates [Howlett and Clarke, 1981b]. Both glycoprotein 2 and the group-IV allergen have been described as allergens although the monoclonal antibody FMC-A9 was negative in the IgE-binding ELISA. However, the present IgE-binding ELISA was only positive using sera from subjects which had strongly positive skin tests to ryegrass pollen. This suggests that monoclonal antibodies against some minor allergens may not be detected in the test. Modifications to the test, such as the use of a monoclonal mouse antibody specific for human IgE, or the use of an amplified ELISA system [Metzger et al., 1981] could be used to increase the sensitivity of the IgE-binding ELISA.

In a recent paper Krilis et al. [1981] described the development of monoclonal antibodies to allergens in house dust mite extracts. We have demonstrated in this paper that monoclonal antibodies specific for pollen allergens can be readily developed. This paper describes an approach to the development of monoclonal antibodies against allergens which is generally applicable to a wide variety of allergen sources. Crude extracts can be used for the immunisation of the mice, in the ELISA system used to identify hybridomas secreting antibodies specific for IgE-binding components of the extracts, and in the gel electrophoretic techniques used to characterise the components of the extract for which the monoclonal antibody is specific. For some minor allergens it may be necessary to undertake some purification procedures to increase the frequency of hybridomas secreting the specific antibody to minor allergens, although this appears unnecessary for ryegrass pollen extracts.

The ELISA was found to be suitable for both the screening of hybridoma supernatants for antibody activity, and for the detection of IgE-binding activity.

The IgE-binding ELISA was designed such that the incidence of hybridoma antibodies specific for IgE-binding components was reflected by a change from a negative response (yellow) to positive (purple). Such responses were easily identified visually. Competitive-inhibition assays were avoided because, although such assays are much simpler, the result would be more difficult to evaluate visually; antibodies to some minor allergenic components may cause little reduction in the intensity of the responses, and screening for positive cultures would be restricted to those specific for determinants which bind human IgE antibody, possibly missing other useful antibodies. The specificity of the IgE-binding ELISA was established in each assay by using a series of controls. Negative reactions were invariably observed if the mouse antibody was replaced by non-allergen-specific mouse IgG1 myeloma protein, or if the human IgE antibody was replaced with serum from subjects with high serum IgE but without skin reactivity to ryegrass pollen extract. In addition, some hybridoma supernatants were positive in the antigen-binding ELISA but were negative in the IgE-binding ELISA. These observations demonstrate that, in the IgE-binding ELISA, there were no cross-reactions between the various reagents used, and that a positive reaction occurred only when a hybridoma antibody bound a component of the allergen extract which also bound

human IgE. The use of the two ELISA systems was designed such that the antigen-binding ELISA could be used in the screening and cloning procedures where large numbers of culture supernatants are screened. The IgE-binding ELISA, which was expensive in terms of the high demand on serum from allergen-sensitive subjects and in the relatively lengthy assay time, could then be used to identify hybridoma antibodies specific for IgE-binding components of the extract after such hybridomas had been screened, cloned, and grown up into small scale bottle culture.

Characterisation of the monoclonal antibody specificities by SDS polyacrylamide gel electrophoresis followed by electrophoretic transfer to nitrocellulose was suitable for these ryegrass pollen components but some antigenic determinants may be destroyed by this process. Other forms of gel electrophoresis followed by electrophoretic transfer may be more suitable for more labile components. Alternatively, well-established techniques of immunoprecipitation of a radiolabelled antigen and detection of this antigen after redissolving and electrophoresis on polyacrylamide gel may be used. The system used in this study was thought to be more generally applicable to allergen extracts.

A further development of the method used in this study would be the use of two-dimensional electrophoresis in polyacrylamide gels followed by electrophoretic transfer to nitrocellulose. This technique would be eminently suitable for the characterisation of the specificities of monoclonal antibodies against allergens considering the variety of allergens which exist as groups of molecules of the same molecular weight, but with different isoelectric points [Johnson and Marsh, 1965b]. This technique would also aid in the characterisation of the FMC-A5 specificity. Two allergen groups with a molecular weight of approximately 11,000 daltons have been identified in ryegrass pollen extracts [Marsh, 1975]. The very different isoelectric points of these allergens would be readily recognisable using two-dimensional electrophoresis.

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Correspondence to: Prof. J. Bradley,
Department of Clinical Immunology,
Flinders Medical Centre,
Sturt Road,
Bedford Park, SA 5042 (Australia)