

Mouse Monoclonal IgE Antibodies Specific for Ragweed Pollen Antigens

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ABSTRACT

In order to obtain monoclonal IgE antibody specific for the major allergens in ragweed pollen extracts, hybridomas were constructed from spleens of mice immunized with ragweed antigen E (AgE). Two hybridomas were selected for thorough study, both secreting antibodies of the IgE class. Large quantities of IgE antibodies were isolated by affinity purification using Sepharose 4B conjugated with ragweed pollen proteins (Fraction A). Both MABs were found to bind to a high molecular weight and heterogeneous population of proteins, but not to monomeric AgE as demonstrated by protein blot analysis. It is suspected that both MABs react with low affinity with AgE determinants and binding could be demonstrated only with aggregated forms of AgE. Although the specific antigens with which they react are unknown, these monoclonal IgE antibodies should be useful reagents, complementing the previously obtained monoclonal anti-dinitrophenyl IgE antibody, for studying various aspects of the mouse and human IgE antibody systems.

INTRODUCTION

IG E IS THE MAJOR CLASS of antibody that mediates immediate hypersensitivity reactions. The mouse animal model has been used extensively for studying the regulation of IgE antibody responses and the nature of immediate hypersensitivity reactions^(1,2) and mouse monoclonal IgE antibodies have already proven to be very useful reagents in these studies.

We have previously constructed a mouse hybridoma cell line secreting monoclonal IgE specific for the hapten, 2,4-dinitrophenyl (DNP)^{3, (3)}. This cell line has allowed the preparation of a large quantity of homogeneous IgE antibody and has made possible the study of various aspects of the rodent IgE system.⁽⁴⁻¹⁹⁾ While conducting some of these studies, it became obvious that monoclonal IgE of another specificity, and of comparable quality, would be highly advantageous.

One potential application of an IgE-secreting hybridoma is in the study of pollen allergens. If one uses a pollen protein extract as the immunogen and constructs and selects for hybridomas secreting antibodies of the IgE class, one may be able to obtain monoclonal antibodies (MABs)

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ABBREVIATIONS: AgE: antigen E; alum: aluminum hydroxide gel; DNP: dinitrophenyl; Fr.A: ragweed pollen fraction A; GAME: goat anti-mouse IgE; GARG: goat anti-rabbit IgG; IgE: immunoglobulin E; MAB: monoclonal antibody; PAGE: polyacrylamide gel electrophoresis; PCA: passive cutaneous anaphylaxis; RAME: rabbit anti-mouse IgE; RIA: radioimmunoassay; RPP: ragweed pollen protein.

useful for identifying various allergens. These monoclonal IgE antibodies can then be used for purifying large quantities of specific allergens. A second, and perhaps more important, application pertains to the utilization of such IgE monoclonals in studies designed to further dissect regulatory mechanisms controlling the IgE system and the molecular basis of IgE-induced cellular events.

Therefore, in order to generate monoclonal IgE antibodies specific for a determinant other than DNP and for the development of a general protocol for studying pollen allergens, we have constructed hybridomas secreting IgE specific for a prototype antigen, namely the major allergen(s) in ragweed pollen.

MATERIALS AND METHODS

Reagents

Ragweed antigen E (AgE) and ragweed pollen extract Fraction A (Fr.A)⁽²⁰⁾ were kindly provided by Dr. Dan Wilson of Miles Laboratories. Mouse monoclonal anti-DNP IgE, rabbit anti-mouse IgE (RAME), goat anti-mouse IgE (GAME) and goat anti-rabbit IgG (GARG) antibodies and DNP₁₆-BSA and DNP₂₈-BSA were prepared as described previously.⁽³⁾ Rabbit antiserum specific for mouse γ_1 - or γ_2 -heavy chain and for κ - or λ -light chain were from Litton-Bionetics, Kensington, MD. ¹²⁵I-GARG and ¹²⁵I-GAME were prepared by the chloramine-T method⁽²¹⁾ yielding specific activities of 30–40 μ Ci/ μ g.

Preparation of hybridomas

1. Adoptive Transfer: Following the type of protocol originally described by us several years ago,⁽³⁾ donors for the adoptive transfer were (BALB/c $\times$ A/J)F₁ (CAF₁) female mice that had been immunized three times over a period of one year with AgE (5 μ g; initially in 4 mg of aluminum hydroxide gel (alum) and subsequently in saline). They were boosted with 5 μ g of AgE in 2 mg of alum 7 days prior to the adoptive transfer. Spleen cells from these donors were transferred to sublethally irradiated (650 rads) syngeneic recipients (35 $\times$ 10⁶ cells/recipient). All recipients were boosted with 5 μ g of AgE in 2 mg of alum shortly after cell transfer, and seven days later their spleens were removed for fusion.

2. Construction of Hybridomas: The procedure used to obtain hybridomas was essentially as described.⁽³⁾

3. Screening of Hybridomas: Hybridomas secreting antibodies of the IgE class were detected by radioimmunoassay (RIA), and further confirmed by the activity of the antibodies in passive cutaneous anaphylaxis (PCA) in rats. Both methods are described below.

4. Subcloning of Hybridoma Cells: Positive clones were subcloned by limiting dilutions at 1 cell/well, screened as outlined above and further expanded.

5. Preparation of Ascites Fluids: Hybridoma cells were injected into CAF₁ mice (1 $\times$ 10⁶ cells/mouse) previously injected with 0.5 ml of pristane at least one week earlier. The tumor cells were allowed to grow and ascites fluid was collected after approximately two weeks. The supernatants were stored at -70°C .

6. Purification of IgE Antibodies: To the hybridoma ascites fluid was added dropwise 1.5 volumes of cold saturated solution of ammonium sulfate (pH 7.0), to obtain a final concentration of 60%-saturated ammonium sulfate. The mixture was stirred at 4°C for 30 min and centrifuged. The pellet was dissolved in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2 (PBS) and dialyzed against PBS overnight. The solution was then applied to a column of ragweed Fraction A protein-conjugated Sepharose 4B (prepared by reacting 10 mg of proteins with 1 g of cyanogen bromide-activated Sepharose 4B). After extensive washing with PBS, the bound protein was eluted with 0.1 M glycine-HCl, pH 2.3 and the eluates were neutralized immediately with 1/20 volume of 0.5 M Tris-HCl, pH 8.5. The eluted protein was dialyzed against PBS and stored at -70°C .

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Solid-phase radioimmunoassay (RIA)

The procedure is essentially as described.⁽³⁾ Briefly, 96-well Flex-vinyl U-bottom microtiter plates were coated with protein antigens by applying to each well 100 μ l solution of Fr.A (100 μ g/ml), AgE (50 μ g/ml), or DNP₂₈-BSA (20 μ g/ml). Coating of unattached sites in the wells with fetal calf serum, incubations with test samples and then with rabbit anti-mouse IgE (RAME) or other class-specific reagents and, finally, with ¹²⁵I-labeled goat anti-rabbit IgG antibodies (GARG) were all performed as described.⁽³⁾

SDS-polyacrylamide gel electrophoresis (PAGE) and protein blotting

SDS-PAGE analyses were conducted using the Laemmli system.⁽²²⁾ For protein blot analysis, the proteins were transferred to a nitrocellulose filter using a Bio-Rad electro-blot apparatus (Trans-blot) at 10 volts for 16 h in 0.025 M Tris-HCl, pH 8.3/0.192 M glycine/0.1% SDS/20% methanol.⁽²³⁾ The filter was soaked in PBS, 0.5% newborn calf serum, 0.5% Triton X-100 (PNT) for 15 min, rinsed with the same buffer and incubated in 20 ml PNT containing 2 μ g/ml of affinity-purified IgE for 2 h at room temperature. The filter was washed five times with 50 ml PBS, 0.5% Triton X-100 (PT) and then incubated with 20 ml PNT containing ¹²⁵I-labeled GAME (200,000 cpm/ml) for 2 h at room temperature. Following five washes with 50 ml PT over 30 min, the filter was air dried and autoradiographed (Kodak XAR-5 film).

Passive cutaneous anaphylaxis (PCA)

The PCA assay was performed as described.⁽²⁴⁾ The antigen used for challenge was Fr.A (3 mg/ml). Titers are presented as reciprocals of the highest dilutions yielding positive reactions.

RESULTS

Determination of the heavy chain isotype of the MABs

In this study, we have obtained two clones (26.6 and 35.11), both of which are stable cell lines and high secretors; about 2–5 mg of specific antibody protein could be isolated from 1 ml of ascites fluid. As discussed below, the specific antigenic determinants with which these MABs react are unknown and, therefore, they are designated anti-ragweed pollen protein (anti-RPP) MAB. Both MABs were identified as belonging to the IgE class by the following criteria.

1. *Mediation of Passive Cutaneous Anaphylaxis Reaction:* PCA titers in rats of 320 and 80,000 were obtained for confluent culture supernatants and ascites fluids, respectively, using ragweed pollen protein Fraction A (Fr.A) as the antigen. It is to be noted that these titers are somewhat lower than those obtained with the anti-DNP monoclonal IgE;⁽³⁾ we think this most likely reflects a low concentration of the specific antigen(s) in Fr.A.

2. *Reactivity with IgE Isotype- and Light Chain-Specific Antibodies:* The reactivity of both MAb 26.6 and 35.11 with polyclonal antibodies specific for the IgE class was determined in a solid-phase RIA in which the MABs were allowed to bind to either Fr.A or AgE adsorbed on a solid phase and detected by specific anti-mouse antibodies. As shown in Table 1, both MAb 26.6 and 35.11 reacted strongly with RAME antibodies, comparable to the anti-DNP IgE MAB (26.82) (samples 1, 3, and 5). The specificity of RAME for IgE was confirmed by its lack of reactivity to IgG₁ (sample 8). Table 1 also shows the lack of significant binding of MAb 26.6 and 35.11 to γ_1 and γ_{2b} class-specific antibodies (samples 1, 3, and 5 vs. 8). Finally, both MAb 26.6 and 35.11 were determined to be of the κ light chain isotype as they reacted preferentially to the κ -specific, and not λ -specific, antibodies, as shown in Table 1. Although not shown, both γ_{2b} -specific and λ -specific antibody reagents have been demonstrated to react selectively with antibodies of the γ_{2b} heavy chain class and the λ light chain class, respectively.

TABLE 1. CHARACTERIZATION OF HEAVY-CHAIN AND LIGHT-CHAIN ISOTYPES AND ANTIGEN SPECIFICITIES OF ANTI-RAGWEED POLLEN PROTEIN (RPP) MABS, AND COMPARISONS TO IgE ANTI-DNP MAB ϵ -DNP-26.82, BY RADIOIMMUNOASSAY.

Sample	MAB ^a Class/specificity (designation)	Solid-phase antigen	Radioimmunoassay indicator reagents ^b				
			Heavy- and light-chain-specific developing antibodies				
			Anti- ϵ	Anti- γ_1	Anti- γ_{2b}	Anti- κ	Anti- λ
1	IgE Anti-RPP (26.6)	Fr.A	12,135	475	0	3,299	0
2	IgE Anti-RPP (26.6)	AgE	2,451	—	—	—	—
3	IgE Anti-RPP (35.11)	Fr.A	10,966	674	291	2,822	0
4	IgE Anti-RPP (35.11)	AgE	1,691	—	—	—	—
5	IgE Anti-DNP (26.82) ^c	DNP-BSA	7,943	0	0	1,287	0
6	IgE Anti-DNP (26.82)	Fr.A	347	—	—	—	—
7	IgE Anti-DNP (26.82)	AgE	682	—	—	—	—
8	IgG ₁ Anti-DNP (109.3) ^c	DNP-BSA	0	7,691	425	2,259	0

^aConcentration of the MAB used: samples 1, 3, 5, 6, and 8, 10 ng/ml; samples 2, 4, and 7, 100 ng/ml.

^bNumbers presented are cpm bound per well with background counts subtracted.

^cLight chain isotype for both anti-DNP IgE and anti-DNP IgG₁ is κ .⁽³⁾

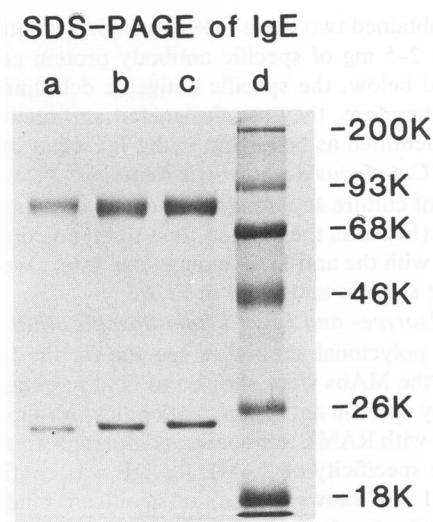


FIG. 1. SDS-polyacrylamide gel electrophoretic analysis of affinity-purified monoclonal IgE. Lane a: anti-RPP MAb 26.6; lane b: anti-RPP MAb 35.11; lane c: anti-DNP MAb 26.82; lane d: molecular weight markers. Sizes of the markers are indicated at the right-hand side. Proteins were electrophoresed under reducing conditions in a 10% polyacrylamide gel in Laemmli's buffer system and stained with Coomassie Blue.

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3. *Biochemical Characterization:* As shown in Fig. 1, the mobility of the heavy chain of MAb 26.6 and 35.11 was found to be identical to that of the anti-DNP IgE MAb (26.82), with an apparent mw of 82 KD. It has been previously demonstrated that mouse IgE heavy chain has a mobility different from that of μ and γ chains on SDS-PAGE.⁽³⁾

Antigen specificity

Although purified antigen E was used as the immunogen, Fr.A was employed as the antigen for screening positive clones in order to conserve the consumption of the more precious, and limited, AgE. Both MAb 26.6 and 35.11 were then affinity-purified with Fr.A-conjugated Sepharose 4B which was found to have high capacity for the antibodies: more than 40 mg of homogeneous antibodies could be obtained from a 10 ml column prepared with 30 mg of Fr.A protein. The specificity of the antibodies for a component(s) in Fr.A was further demonstrated in a RIA (Table 1), wherein the MABs were detected based on their binding to a solid phase coated with Fr.A, and in which anti-DNP IgE did not show any significant binding (sample 6 vs. 1 and 3).

However, we have so far failed to demonstrate the specificity of these MABs for AgE. First, although both 26.6 and 35.11 showed binding to AgE-coated wells in the RIA (samples 2 and 4), and is significantly higher than the background binding manifested by the anti-DNP IgE MAb (sample 7), the binding was not as high as that to Fr.A (samples 1 and 3). Second, when either Fr.A or AgE was applied to columns of Sepharose 4B conjugated with MAb 26.6 or 35.11, very little protein was bound.

To determine the antigen recognized by the MABs, protein-blot analyses were performed. Fr.A, AgE and DNP₁₆-BSA were subjected to SDS-PAGE and electrotransferred to a nitrocellulose filter. The proteins on the filter were then allowed to react with MAb 35.11 and the bound MAb 35.11 was detected with ¹²⁵I-labeled GAME. As a specificity control, the same procedure was repeated with the anti-DNP IgE MAb in place of MAb 35.11. As shown in fig. 2, MAb 35.11 did not bind to AgE (lane a) nor did the anti-DNP IgE MAb (lane d). However, MAb 35.11 did react with certain components in Fr.A, which electrophoresed as a smear at the high molecular weight region of the gel (lane b). The specificity of this reactivity was demonstrated by the lack of such

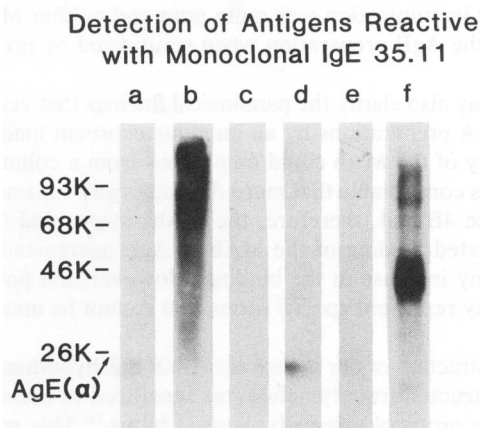


FIG. 2. Detection of antigens reactive with the monoclonal IgE 35.11 by protein-blot analysis. Proteins (nonreduced) were electrophoresed in a 10% polyacrylamide gel in Laemmli's buffer system, electrotransferred to nitrocellulose filter and the blot was developed with ¹²⁵I-labeled IgE. Proteins electrophoresed: lanes a and d: AgE (20 μ g); lanes b and e: Fr.A (200 μ g); lanes c and f: DNP₁₆-BSA (1 μ g). ¹²⁵I-labeled IgE used: lanes a–c: anti-RPP IgE (35.11); lanes d–f, anti-DNP IgE (26.82). Autoradiograph exposure time: lanes a–e; 22 h, lane f: 8 h. Molecular sizes are indicated at the left-hand side, the position of antigen E α -subunit is also indicated.

binding by anti-DNP IgE MAb (lane e) which did, however, bind DNP-BSA specifically (lane f compared to lane c).

Similar observations were made when ^{125}I -labeled AgE was reacted with MAb 26.6- or 35.11-conjugated Sepharose 4B and the bound protein eluted and electrophoresed; again, proteins reacting with the MAb appeared as a smear similar to that seen in the protein blots (data not shown).

DISCUSSION

This report describes the preparation and characterization of mouse monoclonal IgE antibodies specific for ragweed pollen antigens. Two hybridoma lines were established, both secreting MAb of the IgE class based on the following criteria: (1) ability to elicit passive cutaneous anaphylaxis reactions in rats; (2) reactivity with polyclonal antibodies specific for mouse IgE heavy (ϵ) chains (Table 1); and (3) identical mobilities of the heavy chains in SDS-PAGE with that of the previously established anti-DNP IgE MAb (Fig. 1).⁽³⁾

Both MAbs were shown to react specifically with component(s) in Fr.A based on: (1) binding capacities in RIA (Table 1); and (2) elicitation of PCA reactions with Fr.A. as the challenging antigen. Furthermore, both MAbs could be purified with Fr.A-conjugated Sepharose 4B. However, we have not been able to establish their reactivity to the immunizing antigen—AgE. Although both MAbs bind to AgE-coated wells in RIA, binding of AgE to the MAbs conjugated to solid supports could not be demonstrated. When resorting to protein blot analysis, we found that MAb 35.11 indeed did not recognize AgE electrophoresed and transferred to nitrocellulose but rather recognized a heterogeneous population of proteins with characteristics of aggregates, which were, however, not detected in the AgE sample (Fig. 2).

Our explanation for the findings is that both MAbs are low-affinity antibodies specific for AgE and binding can be demonstrated only with aggregated forms of AgE. Thus, our interpretation of the smear pattern in the protein blot is that it represents aggregates of AgE in the Fr.A preparation, which were probably formed during the extraction of ragweed pollen protein and were subsequently removed in the purification steps to obtain AgE. We think the possibility that both MAbs recognize a contaminating antigen(s) in the AgE preparation is less likely, since the AgE preparation used for immunization was quite pure and neither MAb displays specific reactivity with any protein in the AgE preparation when scrutinized by protein blot analyses (Fig. 2, lane a).

Our explanation may also clarify the paradoxical findings that very little protein could be isolated from the AgE or Fr.A preparations by an immunoabsorbent made of either MAb 26.6 or 35.11, while a large quantity of the MAb could be purified from a column prepared with Fr.A coupled to Sepharose 4B. It is conceivable that more AgE aggregates formed as a result of the conjugation of Fr.A to Sepharose 4B and, therefore, the MAbs might bind to this adsorbent with a higher affinity. We have tested binding of the MAb to AgE aggregated by chemical crosslinkers and have not detected any increase in the binding. However, the postulated AgE aggregates in the Fr.A preparation may represent special forms that cannot be mimicked by the chemically cross-linked AgE.

Similar to the construction of our mouse anti-DNP IgE hybridoma line, the hybridomas reported here were also constructed from lymphocytes sensitized to immunogen and then expanded, by the adoptive transfer protocol originally devised by us.⁽³⁾ This protocol is particularly useful in generating spleen cell populations with a high frequency of IgE-secreting cells. The advantage of this protocol in generating IgE hybridomas as well as hybridomas of any class in general has been documented following our initial report.^(25,26)

In the last few years, several laboratories have reported mouse MAb of the IgE class.^(26–30) With the exception of one anti-ovalbumin IgE MAb,⁽²⁷⁾ all others are specific for the hapten DNP. The study presented here describes monoclonal IgE of yet another specificity—in particular, one that is directed to antigenic determinants in allergenic extracts involved in a major allergic disorder

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of humans—and should therefore be a useful complementary reagent for studies of both the mouse and human IgE systems.

MAB 35.11 has been found to mediate activation of basophils which were first sensitized with the MAb and subsequently challenged with the Fr.A antigen.⁽¹⁵⁾ Therefore, it will be a useful reagent to use in conjunction with the anti-DNP IgE MAb (26.82) for various studies on the activation of basophils and mast cells. It is pertinent to mention that mouse anti-DNP IgE MAb (26.82) has been shown to bind to IgE receptors on human basophils⁽³¹⁾ and, furthermore, to sensitize these cells for antigen (DNP-conjugated human serum albumin)-induced histamine release. Anti-RPP IgE MAb described herein should benefit studies of activation of human mast cells and basophils to challenges by ragweed pollen proteins, a situation that is highly relevant to human allergy.

Fc receptors for IgE (FcR ϵ) on lymphocytes have drawn significant attention in recent years.⁽³²⁾ FcR ϵ ⁺ lymphocytes have been demonstrated to play a significant role in regulation of IgE responses,^(1,33) and mouse anti-DNP IgE MAb has contributed significantly to many of these studies.^(4,34-36) Anti-RPP IgE MAb has already proven to be a useful reagent in our laboratory in corroborating results obtained with anti-DNP IgE MAb.^(4,37) Furthermore, our mouse IgE MAb has recently been shown to bind to FcR ϵ on human lymphocytes,^(38,39) demonstrating that these mouse IgE MAb will be useful for studying regulation of IgE synthesis in the human system as well.

Finally, rabbit antiserum to 35.11 has been obtained and, after absorption with normal mouse serum and affinity purification with anti-DNP IgE (26.82), provided an ϵ -chain specific antibody reagent free of any idiotype-specific antibodies. this reagent will be ideal to use for the detection of mouse IgE in sera, culture fluids, or on cell surfaces.

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