

Successful Technique for the Selective Production of Monoclonal Antibodies Against a Major Allergenic Component in Timothy Pollen Extract

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ABSTRACT

Monoclonal antibodies (MAbs) were selectively raised against a major allergenic component of 38 kD in timothy grass pollen (*Phleum pratense*). We used a special prefractionating technique to isolate the 38 kD allergen, because immunizations with crude pollen extract had resulted in a wide variety of antibodies against other determinants, and cross-reactions between different-sized proteins occurred. Pollen extract was separated by Western blotting. Strips of the nitrocellulose membrane containing the allergen were excised, dispersed into allergen bearing particles, and used for immunization of BALB/c mice. Five MAbs were obtained that reacted with the 38 kD allergen. Four of these antibodies (BF 1, DC 9, EB 4, GE 2) exclusively detected the 38 kD protein, while one MAb (EB 6) additionally bound to a 32 kD allergen. Epitope mapping with the 3 IgG 1 antibodies (BF 1, EB 6, DC 9) was performed by ELISA inhibition tests using the purified 38 kD component. The antibodies did not interfere with each other. This confirms that they bind to different sites of the 38 kD molecule. Only a weak inhibitory effect of the MAbs on the binding of patient's IgE was determined, we suppose that the MAbs do not bind to IgE reactive epitopes. For standardization and further characterization of the 38 kD protein by peptide mapping the MAbs will be useful tools.

INTRODUCTION

In allergy research the standardization of crude allergen extracts is required and is important for improving hyposensitization therapy. For this purpose monoclonal antibodies (MAbs) can be used as useful reagents for the quantification of allergenic components (1). We intended to get monoclonal antibodies for the characterization of major allergens of grass group V allergens by epitope mapping and for investigation of their IgE binding structures. The immunization of BALB/c mice with crude timothy pollen extract (*Phleum pratense*) resulted in the production of a wide variety of MAbs (Petersen, unpublished results). This is due to the fact that pollen extracts consist of about 1000 components (2). Furthermore, there is a cross-reactivity between pollen proteins, which makes it difficult to obtain MAbs that only bind to a single allergenic component.

The isolation of proteins from crude mixtures is difficult and often causes loss of epitopes. Knudsen (3), Chiles et al. (4), Young and Lamb (5), and Gratecos et al. (6) successfully isolated nitrocellulose particles containing certain proteins to stimulate the immunosystem. In the field of allergy research Jelke et al. (7) utilized pieces of a nitrocellulose membrane after IEF print as immunogen for raising MABs against an allergen of rye. Wheeler et al. (8) obtained MABs against the birch pollen allergen Bet v I by use of antigen bearing nitrocellulose particles after Western blotting.

We focused our interest on the allergen grass group V. Up to 90 % of the grass pollen allergic patients reveal IgE reactivities against these allergens (9). Matthiesen and Løwenstein (10) identified by Western blotting that the allergenic grass group V consists of 33 kD (Phl p V a) and 29 kD (Phl p V b) components. We (11) determined slightly higher molecular weights of 38 and 32 kD, respectively. Until now no differences in the allergenic reactivity of the two proteins were identified. We decided to raise MABs against the 38 kD allergen.

Since timothy pollen extract contains many proteins with molecular weights of 20 - 40 kD, we decided to perform Western blotting (protein separation by SDS-PAGE followed by blotting onto nitrocellulose membrane) as a one-step prefractionation to isolate the 38 kD allergen for immunization.

MATERIALS AND METHODS

Preparation of Timothy Pollen Extract:

1 g timothy pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1 M NH_4HCO_3 buffer (pH 8.0) and incubated under agitation at room temperature for 30 min. After centrifugation for 30 min. at 17000 x g (4 °C) the supernatant was dialysed against double distilled water. The extract solution was lyophilized and kept at 4 °C until use.

Patients' Poolserum:

The identification of IgE reactive proteins was done with a poolserum consisting of 3 sera of pollinosis patients with a high specific-IgE titre (RAST IV (radioallergosorbent test, Pharmacia, Bromma, Sweden). The poolserum was adsorbed with protein A sepharose (Pharmacia, Bromma, Sweden) in order to avoid blocking effects by human IgG antibodies.

Western Blotting:

Proteins were separated by discontinuous SDS-PAGE (12) with a stacking gel of 5 % T and 0.75 % C and a running polyacrylamide gradient gel of 7.5 % - 20 % T and 0.75 % C. The proteins were transferred to nitrocellulose membrane (0.45 μm , Schleicher & Schüll, Dassel, FRG) by semi-dry blotting (13) for 30 min. at 0.8 mA/cm².

Detection of Proteins and Antigens:

After blotting the nitrocellulose membrane was incubated in 0.1 M Tris buffered saline (pH 7.4) containing 0.05 % (v/v) Tween 20. (This buffer was also used for washing steps and for dilution of the antibodies.) One strip of the membrane was incubated in 1 % (v/v) India ink (Pelikan AG, Hannover, FRG) (14) for protein staining. For the detection of antigens and

allergens the strips were incubated with supernatants of hybridomas and patients' poolserum, respectively. Afterwards the nitrocellulose strips were put into alkaline phosphatase (AP)-conjugated goat-anti-mouse IgG/M (Dianova, Hamburg, FRG) or AP-conjugated goat-anti-human IgE (Medac, Hamburg, FRG). The binding patterns were visualized by a substrate solution of 0.1 M Tris buffered saline (pH 9.5) containing 0.033 % (w/v) nitro blue monotetrazolium chloride (NBT) and 0.017 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Serva, Heidelberg, FRG)(15).

Preparation of the 38 kD Sample for the Immunization:

Pieces of nitrocellulose containing the 38 kD protein were excised from the blotting membrane and solubilized in DMSO. Afterwards the protein bearing nitrocellulose particles were precipitated and dispersed in 0.01 M carbonic buffer (pH 9.6)(3). After washing twice the particles were suspended in 0.9 % (w/w) NaCl solution for immunization.

Production of Monoclonal Antibodies:

BALB/c mice were immunized four times with the suspension of allergen bearing nitrocellulose every 2 weeks. The last booster was done 3 days before fusion using crude timothy pollen extract. For the production of hybridomas (16) spleen cells were fused with cells of the myeloma cell line P3X63Ag8U.1. The screening for antibody producing hybridomas was performed by a dot test using timothy pollen extract as antigen.

Ouchterlony Diffusion Test:

The isotypes of the MAbs were determined by double diffusion test in agarose gel using goat-anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM (Nordic Immunological Laboratories, Tilburg, The Netherlands) as precipitating antibodies.

Antibody Purification and Biotinylation:

The monoclonal antibodies of IgG1 subclass were precipitated by 31 % (w/v) of $(\text{NH}_4)_2\text{SO}_4$ for 2 h and separated afterwards by centrifugation at $10.000 \times g$ for 30 min. at 4 °C. The precipitate was diluted in 0.1 M PBS and affinity purified by protein A sepharose (Pharmacia, Bromma, Sweden). The antibodies were eluted by 0.1 M citric buffer (pH 6.0). After dialysis against PBS the antibodies were freeze-dried and stored at 4 °C until use.

For biotin-conjugation (17) the MAbs were first dialysed against 0.1 M NaHCO_3 . Afterwards 5 μl biotin-NHS (5 μl 70 nM biotinyl-N-hydroxysuccinimid (EY Labs, San Mateo, USA) + 45 μl dimethylformamide (Merck, Darmstadt, FRG)) was added to 1 ml IgG antibody solution (1 mg/ml). After incubation for 4 h at 4 °C under agitation the reaction was stopped by addition of 1 M Tris buffer pH 7.4. Excess of biotin was removed by dialysis and concentrating by microcentrifugation in PBS buffer.

Inhibition Tests:

The inhibition studies were performed according to Mourad et al. (18) with modifications. All steps were performed at 37 °C. Microtiter plates were coated with the 38 kD allergen, which

was affinity purified from timothy pollen extract (method according to the manufacturer's instruction Pharmacia, Bromma, Sweden). 100 µl of 0.2 µg/ml antigen diluted in sodium carbonate buffer (0.05 M, pH 9.6) was incubated for 2 h. After washing with TBS buffer (0.1 M Tris, 0.1 M NaCl, 2.5 mM MgCl₂, pH 7.4) containing 0.1 % Tween-20, each well was blocked with 200 µl TBS-Tween buffer containing 3 % (w/w) bovine serum albumin for 1 h. For epitope mapping the unlabelled MABs were used as inhibitors and diluted in series (5 - 0.001 µg/ml). After mixing with equivalent volumes of the biotinylated antibodies (0.5 µg/ml) 100 µl of each sample was transferred onto the microtiter plate and incubated for 2 h. The binding of the biotinylated antibodies was detected after incubation with AP-conjugated streptavidin (Dianova, Hamburg, FRG) for 2 h and visualized with p-nitrophenylphosphate (Merck, Darmstadt, FRG) as substrate.

To test the competition of patients' IgE by MABs we transferred equivalent volumes of MABs (10 µg/ml) and patients' poolserum (diluted 1:4) onto the microtiter plate after the blocking step and incubated overnight. The binding of the IgE antibodies was detected by AP-conjugated goat-anti-human IgE (Medac, Hamburg, FRG). After incubation for 4 h p-nitrophenylphosphate was used as substrate.

The absorption was measured at 405 nm after 30 min.

The percentage of inhibition was calculated as follows:

$$\left(1 - \left(\frac{\text{O.D. in presence of inhibitor}}{\text{O.D. in absence of inhibitor}} \right) \right) \times 100$$

RESULTS

Production of Monoclonal Antibodies against the 38 kD Protein

For the isolation of the 38 kD allergen we performed a one step prefractionation of timothy pollen extract by Western blotting. Best results were obtained, when SDS-PAGE was done in an acrylamide gradient of T = 5 - 20 % and C = 0.75 %. 30 µg of crude timothy pollen extract was loaded onto the whole gel (0.2 µg/cm). Higher protein amounts were not used to avoid overloading and overlapping of the bands. The whole gel was blotted onto nitrocellulose membrane. After blotting, small strips were cut off from the membrane as reference strips. These strips were used for immunological detection of the 38 kD band by using patients' poolserum. Thus we were able to localize the position of the 38 kD protein

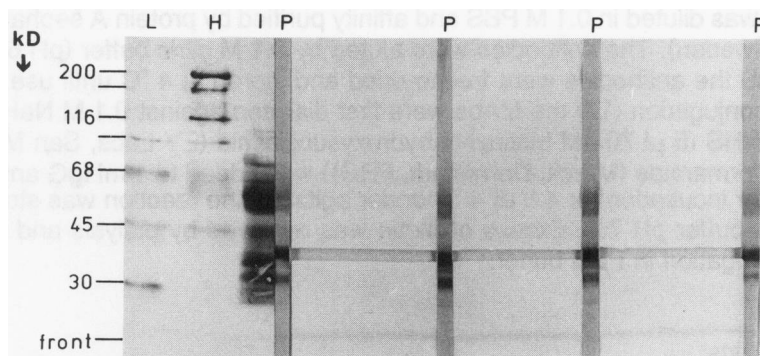


FIGURE 1. Isolation of the 38 kD protein by Western blot for subsequent immunization. L and H = low and high molecular weight markers, I = protein staining with India Ink, P = detection of the 38 kD protein by patients' poolserum.

bands within the rest of the unstained nitrocellulose, too. Those parts of the untreated blotting membrane containing the allergen (isolated from 30 µg crude extract) were cut off and used for immunization (Fig. 1).

Supernatants of proliferating cell colonies were screened for antibodies against timothy pollen components by the dot test. After the hybridomas had been cloned twice by limiting dilution we obtained 5 stable cell lines: BF 1, EB 6, DC9, EB 4, GE 2.

Characterization of the Monoclonal Antibodies

Western blotting was performed in order to find out, if the 5 MAb were selectively directed against the 38 kD protein (Fig. 2).

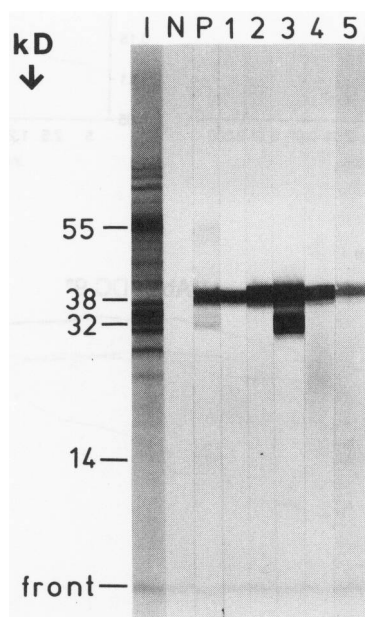


FIGURE 2. Western blot of timothy pollen extract showing the binding patterns of the monoclonal antibodies. I = protein staining with India ink, N = control, P = patients' poolserum, 1 = BF 1, 2 = DC 9, 3 = EB 6, 4 = EB 4, 5 = GE 2.

Fig. 2 demonstrates that all 5 MAbs bind to the 38 kD protein. The intensities of the reactivities are quite different. For the detection with the monoclonal antibodies EB 4 and GE 2 we had to use 8fold amount of supernatant, thus provoking a higher background staining of those strips. The MAb EB 6 detects an additional protein of 32 kD with the same intensity as the 38 kD band. This indicates an identical epitope on the two different-sized proteins. The 32 kD protein seems to be an allergen, too, since it is also detected by patients' serum. The 5 monoclonal antibodies were investigated by Ouchterlony diffusion test. BF 1, EB 6, and DC 9 belong to the IgG 1 isotype, whereas EB 4 and GE 2 are IgM antibodies. Because of the weak reactivities and the difficulties in the antibody purification of EB 4 and GE 2, we concentrated our further investigations on the three IgG 1 antibodies.

Epitope Mapping of the 38 kD Allergen

For epitope mapping we purified the 38 kD allergen from crude timothy pollen extract and tested the IgG 1 antibodies BF 1, EB 6, and DC 9 vice versa with the biotinylated MABs.

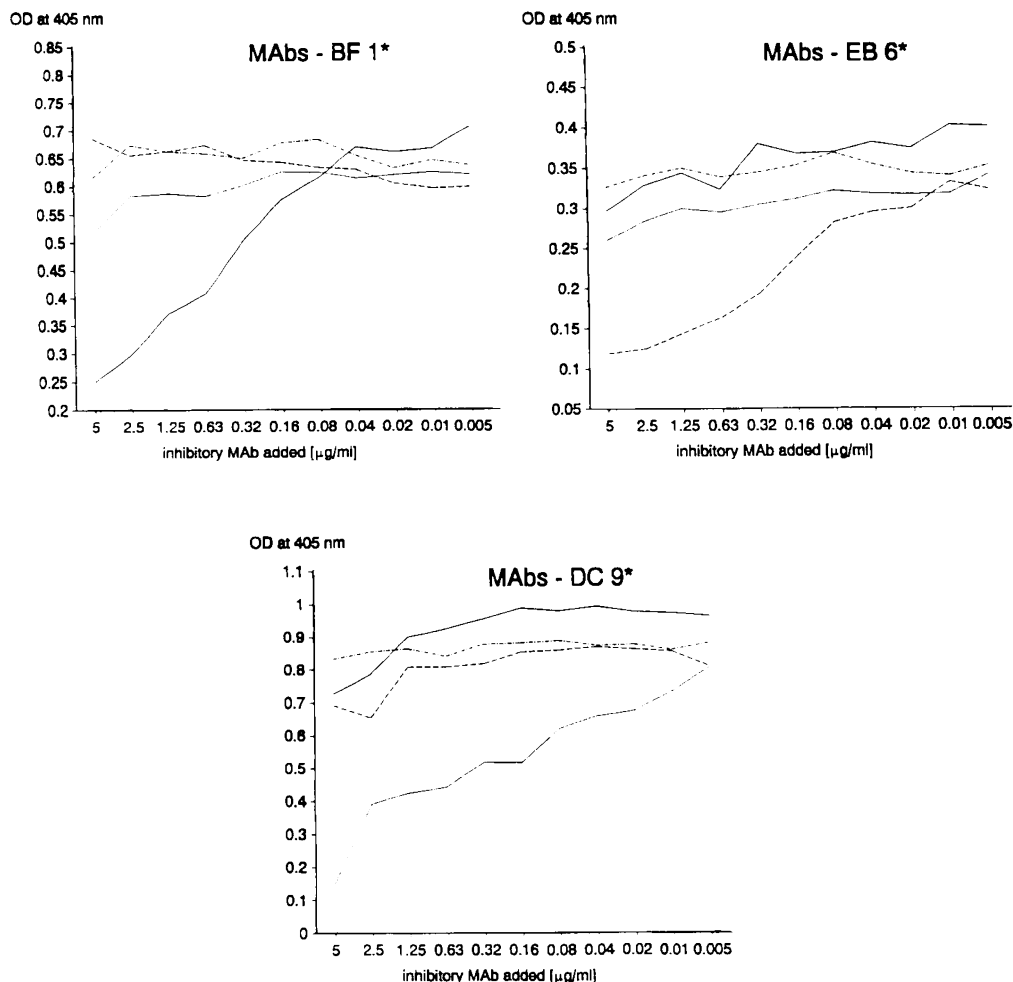


FIGURE 3. Investigation of the 4 monoclonal antibodies for their competitive binding to the 38 kD allergen (Ova 22 is a negative control). BF 1 (—), EB 6 (---), DC 9 (— · —), and Ova 22 (·····). The Binding of the biotinylated antibodies indicated by asterisks were determined. The results are mean values of 3 independent experiments.

Fig. 3 depicts the inhibitory effect of the 3 MABs (as inhibitors) on the biotinylated MABs. In all cases the biotinylated antibodies could be inhibited by themselves (unlabelled form). Thus the biotinylation procedure had no inhibitory effect on the binding of the labelled antibodies. The MABs do not interfere with each other, except that there is a slight inhibitory effect between DC 9 and BF 1 vice versa. For determination of the maximal binding of the biotinylated antibodies we used the MAb Ova 22 (19) raised against ovalbumin as negative control. The binding intensities of the 4 MABs directed against grass pollen varied from 1.0

in the case of DC 9 to 0.4 in the case of EB 6. Therefore, we assume that the 4 MAbs bind to different epitopes on the 38 kD protein.

In order to investigate whether the monoclonal antibodies attach to IgE-binding sites, we tested the IgE-binding to the 38 kD component in the presence and absence of the MAbs. (In previous studies MAb concentrations of 10 µg/ml had shown intensive binding reactivity without an unspecific inhibitory effect caused by the added protein amount itself.)

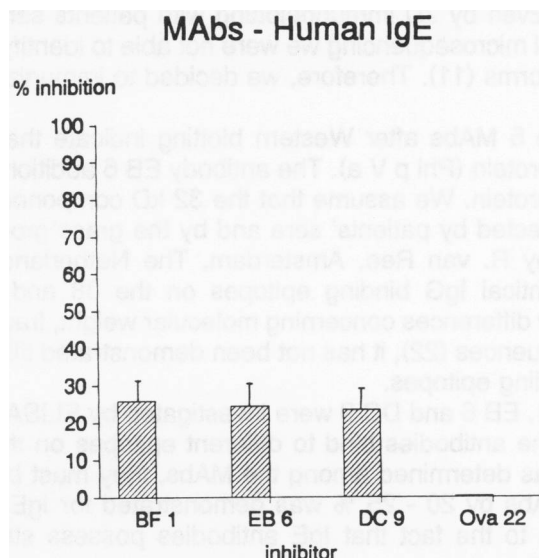


FIGURE 4. Inhibitory effect of the 3 monoclonal antibodies for the IgE binding (patients' poolserum) to the 38 kD allergen. The results are mean values of 3 independent experiments.

Fig. 4 lists the inhibitory effect estimated from 3 determinations. The 3 MAbs BF 1, EB 6, and DC 9 indicate weak inhibitory effects. IgE-binding to the 38 kD protein was inhibited within the range of 20 - 25 %.

DISCUSSION

In order to raise monoclonal antibodies selectively against the 38 kD allergen of timothy pollen extract we prefractionated crude extract by Western blotting, excised the 38 kD protein bands from the nitrocellulose membrane and immunized with the suspended allergen bearing nitrocellulose particles. We obtained 5 MAbs, each of them directed against the 38 kD protein.

This technique proves to be especially useful, when the crude extracts consist of about 1000 different components as in the case of pollen extracts. Prefractionating before immunization was the way of choice in order to get MAbs directed only against a single component. Furthermore, prefractionation makes this method economical, because it reduces the number of samples to be screened to those recognizing the particular antigen.

Western blotting is easy to perform and it is not necessary to purify the immunogen by lengthy chromatographic separations. Since the 38 kD protein (Phl p V a) represents only about 3 % (w/w) of the whole timothy pollen extract (10), this technique can also be used for proteins at low concentrations or components that only provoke weak immunological

reactions. Abou-Zeid et al. (20) showed that a suspension of antigen-bearing nitrocellulose particles stimulates lymphocytes to proliferate. Disadvantages of this method might be the denaturation of the proteins and the possible unaccessability of epitopes on the blotted protein samples. Jelke et al. (7) performed immunization of allergen bearing nitrocellulose particles after prefractionation by IEF-print, thus using immunizing antigens that were not denaturated during the electrophoretical separation. We preferred Western blotting, because a lot of grass pollen proteins are isoallergens showing identical molecular weights but different isoelectric points. Even by 2D immunoblotting with patients sera and monoclonal antibodies and by N-terminal microsequencing we were not able to identify differences in the primary structure of the isoforms (11). Therefore, we decided to immunize with a mix of 38 kD isoallergens.

The binding patterns of the 5 MABs after Western blotting indicate that each of them is directed against the 38 kD protein (Phl p V a). The antibody EB 6 additionally binds with the same intensity to a 32 kD protein. We assume that the 32 kD component is the Phl p V b allergen, since it is also detected by patients' sera and by the grass group V specific MAB 4B1 (21) (kindly donated by R. van Ree, Amsterdam, The Netherlands). These results indicate that there are identical IgG binding epitopes on the 38 and 32 kD allergens. Although both proteins show differences concerning molecular weight, fragmentation by Glu-C digestion and internal sequences (22), it has not been demonstrated till now whether they share any common IgE-binding epitopes.

The 3 IgG 1 antibodies BF 1, EB 6 and DC 9 were investigated by ELISA inhibition tests, in order to find out, whether the antibodies bind to different epitopes on the 38 kD allergen. Since no inhibitory effect was determined among the MABs, they must be different. Only a weak competition of the MABs by 20 - 25 % was demonstrated for IgE-binding sites. But these results might be due to the fact that IgE antibodies possess stronger affinities to epitopes than IgG antibodies.

Further investigations are necessary to characterize the IgE-binding epitopes on the major allergenic 38 kD protein. We intend to isolate the 38 kD protein in larger amounts by affinity chromatography using our MABs. Since the MABs are directed against different sites of the allergen, we intend to identify the IgE-binding epitopes by peptide mapping in double binding assays. Furthermore the MABs are required for screening of cDNA libraries, in order to elucidate the primary structure of the whole 38 kD allergen (23).

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