

Induction of Polyclonal and Monoclonal Antibodies with a Single Allergen Separated from Birch Pollen Extract by SDS-PAGE and Nitrocellulose Blots

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Abstract. A Gold staining allergenic band, Bet v 1, was excised from a nitrocellulose blot following SDS-PAGE of birch pollen extract. This was used to raise a polyclonal rabbit antiserum and monoclonal mouse antibodies specific only for the Bet v 1. The method offers practical advantages for the production of antibodies to individual allergens in complex extract mixtures, without laborious purification methods.

Introduction

Rabbit antisera and mouse monoclonal antibodies to clinically significant allergens are invaluable tools for standardisation [1], purification [2] and characterisation [3] of allergen extracts. In addition, detailed analysis of individual epitopes of major allergens with monoclonal antibodies is leading to a greater understanding of the molecular basis of allergenicity [4], and allowing studies of anti-idiotypes in the sera of atopic patients to be carried out [5].

Induction of these antibodies usually requires the use of immunogens purified from complex mixtures by laborious physicochemical means [6] and further serum absorption is often required to obtain antisera with a high degree of specificity for the allergen. Although clones producing monoclonal antibodies of required specificity can be selected following immunisation with crude mixtures, their frequency is sometimes low, and partial purification of the allergen for immunisation is advantageous [6].

A more convenient means of producing allergen-specific antibodies is described. Allergens were separated by SDS-PAGE and then blotted onto nitrocellulose. An appropriate gold-stained band was excised, dissolved and used to raise allergen-specific antibodies.

Methods

Birch Pollen Extract

Birch (*Betula verrucosa*) pollen extract was prepared in a freeze-dried form as described previously for other pollen extracts [6].

Rabbit antisera

(i) A single New Zealand white rabbit was immunised subcutaneously with 2 mg of pollen extract emulsified in Freund's complete adjuvant on day 0, and in Freund's incomplete adjuvant on day 7. On day 28, 1 mg of extract was injected subcutaneously in saline (0.9%). Blood was taken 10 days later and serum prepared.

(ii) A single New Zealand white rabbit was immunised by the injection of solutions of strips of nitrocellulose which had been excised from an SDS-PAGE blot of birch pollen extract. A 17,000M_r band corresponding to an important allergen was identified on these strips as described below and located using gold stain. The strips were dissolved in a minimal volume of dry dimethyl-sulphoxide, and injected subcutaneously with an equal volume of Freund's complete adjuvant as described by Knudsen [7]. Two further injections, in Freund's incomplete adjuvant, were repeated at fortnightly intervals. Four months later, a further injection was given and the rabbit exsanguinated after a further 2 weeks.

Monoclonal Antibodies

Immunisation. Four male mice (C5B6 × Balb C) were similarly immunised with solutions of nitrocellulose strips. One-quarter of a strip was dissolved in 125 µl of dry DMSO, which was mixed with 250 µl of Freund's complete adjuvant and injected subcutaneously into each mouse. The injection was repeated 14 days later but was given in Freund's incomplete adjuvant. Seven days later sera from these animals gave a positive ELISA result against birch pollen ex-

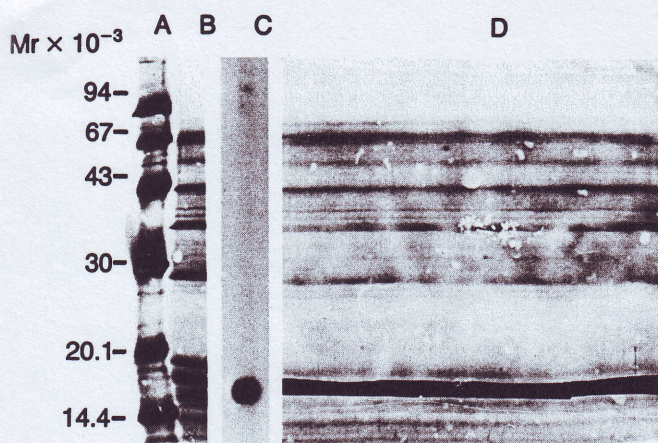


Fig. 1. Identification of band for excision. A Gold-stained markers. B Gold-stained birch extract. C Human birch-specific IgE. D Gold-stained nitrocellulose showing excision of major allergen band.

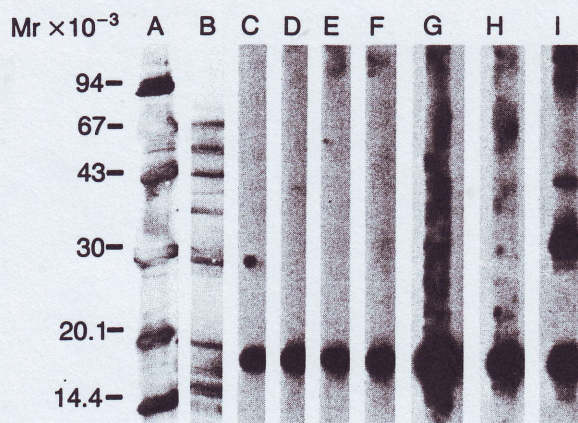


Fig. 2. Reaction of antibodies with allergen of interest in SDS-PAGE immunoblotting. A Gold-stained markers. B Gold-stained birch pollen extract. C-F Monoclonals to 17,000 M_r component. G Rabbit antiserum (1:250) to 17,000 M_r component. H Rabbit antiserum (1:1,250) to 17,000 M_r component. I Rabbit antibody to birch pollen extract.

tract, and were shown by SDS-PAGE immunoblotting to contain antibody specific for the immunising band. Four months after the first injection, whole extract (50 μ g) was injected intraperitoneally in physiological buffered saline (0.5 ml), and the following day a further 12.5 μ g was given intravenously. Spleens were removed for cell fusion 2 days later.

Cell Fusion. Hybridization was carried out as described by Köhler [8] using a non-secreting myeloma cell line SP₂O₈ and a mixed spleen cell preparation from the 2 mice with the highest antibody titres. The fused cells were cultured with a peritoneal macrophage feeder layer in RPMI 1640 HAT medium containing 15% myocloned foetal calf serum (Gibco). Supernatants from plated cells were screened for antibody production to birch pollen extract by ELISA, and cells from positive wells were cloned by limiting dilution. Single clones were tested for specificity by SDS-PAGE, immunoblotting, and CRIE.

¹²⁵I-Labelled Proteins

¹²⁵I-protein A was prepared as described previously [6]. Affinity-purified ¹²⁵I-rabbit anti-mouse IgG was obtained from Sigma.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out on a Bio Rad Protean apparatus using a 12.5% w/v polyacrylamide separating gel and 3% w/v stacking gel [9]. Birch pollen extract protein (75 μ g) was reduced by 1% w/v dithiothreitol and loaded across the top of the gel in a 15-cm wide well. Reduced molecular weight markers were also run.

Immunoblotting

Protein bands in SDS-PAGE gels were transferred electrophoretically onto nitrocellulose. One strip containing the molecular weight markers and one with a profile of the pollen extract were

stained with colloidal gold (Janssen Pharmaceutica). The remaining nitrocellulose prints were saturated in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature to block unoccupied binding sites on the nitrocellulose. These were then cut into 0.5-cm wide strips and each strip placed in individual incubation tubes. Patients' serum, rabbit serum or monoclonal antibody were added in dilutions in PBS containing BSA (1%) and Tween 20 (1%). Tubes were incubated for 5 h at room temperature and mixed by rolling. The strips were washed in PBS/Tween 20 (0.05%) and then incubated with ¹²⁵I-anti-IgE (Pharmacia) ¹²⁵I-Staph-A protein, or ¹²⁵I-anti-mouse IgG overnight at 4°C. After thorough washing, the strips were exposed to pre-flashed X-ray film (Fuji), with intensifying screen (Dupont) for 5 days at -70°C. Results were recorded by photography.

Isoelectrofocusing Immunoblotting

Isoelectrofocusing of birch pollen extract was carried out on Isogel pH 3-10 agarose gels (FMC Bio Products). PI markers (Pharmacia) were also run. Focused proteins were transferred to nitrocellulose sheets by diffusion.

Gold staining, immunodetection and autoradiography were carried out as described above.

ELISA

IgG monoclonal culture supernatants were screened by ELISA. Birch pollen extract at 50 μ g/ml was used for coating the micro-ELISA plates (Dynatech). Detection of bound mouse IgG was performed with alkaline-phosphatase-conjugated rabbit anti-mouse IgG (Sigma) 50 μ l diluted 1:500 into PBS containing 0.5% BSA and Tween 20 (0.05%) and phosphatase substrate (100 μ l at 1 mg/ml *p*-nitrophenyl phosphate (Sigma). The colour intensity was estimated visually or spectrophotometrically at 410 nm on a Mini-reader II (Dynatech).

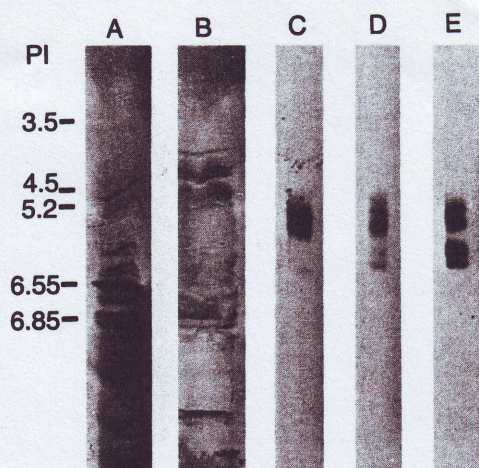


Fig. 3. Isoelectrofocused immunoblots. **A** Gold-stained markers. **B** Gold-stained extract. **C** Human birch-specific IgE. **D** Rabbit antiserum to 17,000 M_r component. **E** Monoclonal to 17,000 M_r component.

Crossed Immunoelectrophoresis (CIE)

CIE was carried out after gel electrophoresis of birch pollen extract by a second-dimension electrophoresis into gel containing rabbit antiserum raised to the excised band. Precipitation peaks were stained with Coomassie blue.

Crossed Radioimmuno-electrophoresis (CRIE)

CRIE was done by overlaying the CIE plate with a serum pool from birch pollen allergic patients. Further incubation was carried out with ^{125}I -anti-human IgE (Pharmacia). Autoradiography was used to visualise the allergenic peak.

Results

A main allergen of birch pollen extract was identified as an approximately 17,000 M_r band by SDS-PAGE IgE immunoblotting (fig. 1). This band was stained with colloidal gold (fig. 1), excised and used to immunise 1 rabbit and 4 mice. When the resulting rabbit antiserum was used to develop nitrocellulose blots of SDS-PAGE-separated extract components, only one band in the allergenic 17,000 M_r region was seen (fig. 2). A rabbit antiserum raised against whole birch pollen extract bound to additional bands (fig. 2). All 4 mice immunised with the cut-out band developed IgG antibodies in their sera that gave a positive ELISA against whole birch extract. The two best antibody producers were boosted and their

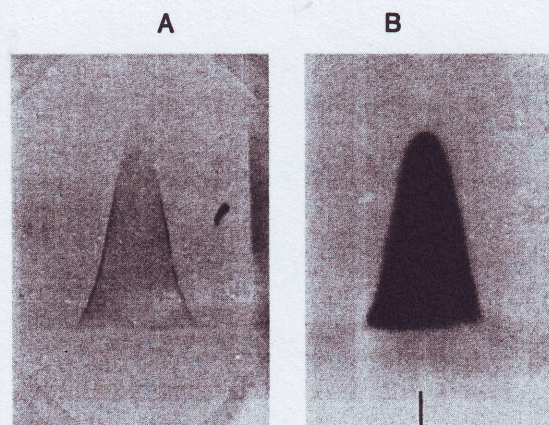


Fig. 4. CIE and CRIE using rabbit antiserum to the 17,000 M_r component. **A** CIE. **B** CRIE – repeat of **A**, developed with human serum from birch pollen-sensitive donors and ^{125}I anti-IgE.

spleen cells pooled for fusion. Twenty-one birch pollen extract-specific clones were selected by ELISA from 120 wells in which cell growth was observed. None produced antibody that reacted to extract components other than that at 17,000 M_r by immunoblotting. The strongest reactions are shown (fig. 2). One hybridoma showing good growth characteristics was grown in suspended culture to produce antibody used in this study.

Isoelectrofocusing of birch pollen extract and transfer to nitrocellulose showed proteins with a broad range of pI values. The main IgE-reactive proteins appeared as two clusters of bands between pIs 5 and 6 (fig. 3). Similar results were seen when the IEF nitrocellulose blots were developed with both rabbit antiserum and monoclonal antibody specific for the 17,000 M_r components (fig. 3). In the case of the monoclonal antibody, the two groups of reactive components resolved clearly into 5 bands.

Crossed immunoelectrophoresis using whole birch pollen extract in the first dimension and rabbit antiserum to the 17,000 M_r component in the second dimension resulted in one clear peak (fig. 4). This peak was shown to be allergenic by its reactivity with human IgE antibody (fig. 4). The serum pool containing the IgE used for immunoblot development was made from the sera of 20 birch-sensitive patients who all reacted with the 17,000 M_r band (data not shown).

Discussion

A gold-staining component of birch pollen extract was identified by immunoblotting as a highly IgE-reactive material, probably Bet v 1, separating at 17,000 M_r on SDS-PAGE under reduced conditions. Immunisation with this material as a band excised from nitrocellulose led to the induction of birch pollen extract-specific rabbit antibody and of monoclonal antibody-producing clones that were specific only for the component used for immunisation; whereas an antiserum raised in a rabbit to the whole pollen extract reacted with a number of components.

The method of raising antibody described here is more generally applicable than that reported by Knudsen [7], who relied on ³⁵S-methionine metabolically labelled antigens and autoradiography to locate the immunogen on the nitrocellulose, or that of Gratecos et al. [10] who reported the preparation of monoclonal antibodies to drosophila proteins using nitrocellulose-bound antigen. They used a relatively complex in vitro immunisation procedure which tended to interfere with hybridoma growth.

The 17,000 M_r component appeared to be heterogeneous with respect to charge, as the rabbit antiserum and monoclonals raised with this component bound to at least five antigens at PIs between 5 and 6. IgE antibody also bound to these bands, and they thus are likely to have been isoallergens of Bet v 1.

This technique provides an easy means of producing antibodies to selected allergens and thus facilitates the preparation of highly specific reagents which can act as invaluable tools in allergy research.

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