

When the epitopes are closely spaced both arms of the IgG antibody will bind to antigen in the ELISA. The equations are

$$TB = \frac{B}{2K'(TG - (q+2)B)} + B,$$

where TB is the total antibody concentration, B is the concentration of complex bound with two arms, K' is the functional affinity of the two arm reaction, TG is the epitope concentration and q is the number of epitopes which are sterically hindered by the attachment of one antibody molecule. In this study a value of two was chosen for the parameter q (Griswold, 1987a).

Soluble phase

In the soluble-phase Farr assay with a univalent antigen, the antibody concentration is given by the equation (Griswold, 1987b)

$$TB = \frac{F}{K(1-F)} + F(TG),$$

where TB is the antibody concentration, F is the fraction of antigen bound, K is the intrinsic binding constant, and TG is the epitope concentration.

The binding between multivalent antigen with repeating identical epitopes and antibody has been studied by Otterness and Karush (1982). In their notation, the functional binding constant K' is given by the relationship

$$K' = \frac{G^m B^n}{(B^n - G^m B^n)(G^m - G^m B^n)},$$

where B^n is the concentration of antibody molecules with n sites per molecule, G^m is the concentration of antigen molecules with m sites per molecule and $G^m B^n$ is the concentration of antigen-antibody complex. In accordance with their derivation, we let

$$P = 1 - \frac{G^m B^n}{G^m}.$$

Solving for B^n gives the expression

$$B^n = \frac{1-P}{K'P} + (1-P)G^m.$$

This expression provides an equation for computing the antibody concentration as a function of the antigen concentration (G^m), the functional affinity (K') and the fraction of unbound antigen (P).

IDENTIFICATION AND CHARACTERIZATION OF IMPORTANT ALLERGENS FROM MUGWORT POLLEN BY IEF, SDS-PAGE AND IMMUNOBLOTTING

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Abstract—Mugwort (*Artemisia vulgaris* L.) pollen allergens, separated by SDS-PAGE or IEF, were identified after transfer to NCM by incubation with a panel of sera from 16 patients with clinical mugwort pollen allergy, followed by [¹²⁵I]anti-IgE and autoradiography. Of the at least 23 components separated by SDS-PAGE in a 15% polyacrylamide gel, at least 15 components with mol. wts 12,000–100,000 bound IgE from the panel of patient sera. A component of mol. wt 22,000 bound IgE from at least 94% of the patient sera tested and for all but three sera this component also bound the greatest quantity of IgE. Five other components with mol. wts 12,000, 17,000, 29,000, 39,000 and 42,000 bound IgE from 75–94% of the patient sera. After separation by IEF, at least 28 protein bands were detected in the pI region 3.5–7.2 and at least seven bands were found in the region 8.6–9.3. At least 11 bands in the pI range 4.2–7.3 and at least five bands in the pI region 8.5–9.2 bound IgE from the panel of patient sera. The most intense radiostaining was observed with a component having a pI of 4.35, which bound IgE from 31% of the patient sera. Immunoblotting of the SDS-PAGE and IEF gels using specific rabbit antisera and human sera against three important mugwort pollen allergens, denoted Ag 9, Ag 12 and Ag 13, was performed to determine the mol. wt and pI of these allergens which had earlier only been identified in CIE/CRIE. The results revealed that Ag 13 had a mol. wt of 61,000 and a pI of 4.35. Ag 12 had a mol. wt of 22,000 and Ag 9 had pIs in the region 4.55–5.55 (six isoforms). Ag 9 did not bind IgE after SDS-PAGE and was thus not identified in the SDS-PAGE pattern, and Ag 12 failed to be detected in the NCM after transfer from IEF gels. By crossed immunoelectrofocusing, Ag 12 was found to consist of several isoforms predominantly located in the pI region 3.5–5.1. The immunoblotting analysis also revealed that the glycoprotein allergen *Art v* II was not detected after transfer from either SDS-PAGE or IEF gels. In conclusion, immunoblotting analysis of SDS-PAGE and IEF gels are useful methods for characterization of mugwort pollen extract, but it should be noted that some important allergens which are easily identified in CIE/CRIE may fail to be detected by these methods.

INTRODUCTION

Pollen of mugwort (*Artemisia vulgaris* L.) is one of the main causes of allergic reactions in late summer in Europe (Charpin *et al.*, 1974; Spiekma *et al.*, 1980), and extracts of this pollen are widely used for the diagnosis of mugwort pollen allergy. Using CIE/CRIE, it has been demonstrated that mugwort pollen extracts contain 8–11 different allergens (Ipsen *et al.*, 1985; Simestad Paulsen *et al.*, 1985; Diener *et al.*, 1988) and one or two of these allergens have been defined as major allergens (Ipsen *et al.*, 1985;

Diener *et al.*, 1988). As information on physicochemical parameters such as mol. wt and pI is not obtained in CIE/CRIE, such information is lacking for most of the allergens identified in this system. Dreborg *et al.* (1987) have identified at least 10 IgE-binding bands with apparent mol. wts in the range 10,000–90,000 in mugwort pollen extract, using SDS-PAGE followed by immunoblotting with a patient serum pool. The major allergenic band in this system was identified in the mol. wt region 15,000–20,000 (approximate mol. wt 18,000), but as no attempt was made in this study to compare the results from SDS-PAGE/immunoblotting with results from CRIE, it is not clear whether the major IgE-binding band identified by Dreborg *et al.* is identical to any of the major allergens earlier identified in CRIE. Two important mugwort pollens allergens, *Art v* I and *Art v* II, have recently been isolated and the mol. wt and pI of these two allergens have been determined (de la Hoz *et al.*, 1990; Nilsen and Simestad Paulsen, 1990). *Art v* II is identical to an allergen earlier identified in CIE/CRIE which was denoted AG 7 by Simestad Paulsen *et al.* (1985) and Ag 3 by Ipsen *et al.* (1985). Which of the allergens in the CRIE pattern is identical to *Art v* I remains to be established.

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Abbreviations: CIE, crossed immunoelectrophoresis; CRIE, crossed radioimmunoelectrophoresis; R(R)IE, rocket (radio)immunoelectrophoresis; Ag, antigen; IgE, immunoglobulin E; RAST, radioallergosorbent test; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; %T, polyacrylamide gel concentration defined as percentage total monomer (acrylamide + bisacrylamide); %C, percentage bisacrylamide (as percentage of total monomer); pI, isoelectric point; NCM, nitrocellulose membrane; CBB, Coomassie Brilliant Blue; PAS, periodic acid Schiff; tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; Con A, concanavalin A.

The aim of this study was to characterize a mugwort pollen extract by SDS-PAGE and IEF combined with immunoblotting, to obtain information on mol. wts and *pI* of the IgE-binding components. By using specific rabbit antisera and human patient sera containing high amounts of specific IgE against selected mugwort pollen allergens, we wanted to determine the mol. wts and *pI* of three important mugwort pollen allergens, Ag 9, Ag 12 and Ag 13,* which had earlier only been identified in CIE/CRIE (Smestad Paulsen *et al.*, 1985; Ipsen *et al.*, 1985).

MATERIALS AND METHODS

Pollen

Artemisia vulgaris L. pollen containing less than 0.5% (w/w) foreign pollen was obtained from Allergon AB (Engelholm, Sweden, batch no. 010182101).

Rabbit antibodies

Rabbit antibodies against *Art v* II (anti-*Art v* II) were prepared by immunization with immunoprecipitates of the allergen obtained by CIE essentially as described by Harboe and Closs (1983). Rabbit antibodies against Ag 12 (anti-Ag 12) were kindly provided by Dr H. Ipsen, ALK-Research, Hørsholm, Denmark.

Human sera

A serum pool was prepared from equal volumes of sera from 64 individuals having RAST-class 3 against mugwort pollen.

Sixteen patient sera from mugwort allergic patients were kindly provided by Dr A. Bryhni Frostad, Policlinic of Allergy, Oslo, Norway. The patient inclusion criteria were: symptoms of allergy in the mugwort pollen season, positive skin prick test (mean weal diameter obtained with Spectralgen mugwort pollen extract, 100,000 BU (Nycomed AS, Oslo, Norway), equal to or larger than mean weal diameter obtained with histamine HCl 10 mg/ml) and RAST-class 2 or greater against mugwort pollen.

An individual patient serum exhibiting high binding of specific IgE to Ag 9 and another exhibiting high binding of specific IgE against Ag 12 and Ag 13 in CRIE were also used in this study.

Mugwort pollen extract

Mugwort pollen extract was prepared essentially as previously described (Nilsen and Smestad Paulsen, 1986). The pollen (150 g) was extracted 1:20 (w/v) in phosphate-NaCl buffer (20 mM phosphate-55 mM NaCl-2% *n*-butanol; pH 7.20) containing 5 mM thiourea, for 1 hr at 4°C. After removal of the pollen by filtration, the filtrate was dialysed (mol. wt cut-off

12,000) for a total of 100 hr against nine changes of distilled water (containing 5 mM thiourea for the first 24 hr) and lyophilized. The lyophilized extract (5.3 g dry wt) contained 26.1% protein as determined by the modified Lowry assay (Bensadoun and Weinstein, 1976) with bovine serum albumin as standard.

SDS-PAGE

SDS-PAGE was performed in 0.75 mm × 5 cm × 8 cm homogeneous slab gels (4%T, 2.7°C in the stacking gel and 15%T, 2.7°C in the separation gel) using the BioRad (Richmond, CA, U.S.A.) Mini Protean II dual slab cell and the discontinuous buffer system of Laemmli (1970). Samples containing 20 mg/ml mugwort pollen extract (5.2 mg protein/ml) were heated at 100°C for 5 min in the sample buffer of Laemmli (1970), and electrophoresis was performed at 200 V for about 60 min. The LMW Electrophoresis Calibration Kit, mol. wt 14,400–94,000 (Pharmacia Uppsala, Sweden) was included for mol. wt determination. The gel was stained for protein with CBB, for carbohydrate with the PAS reagent (Zacharius *et al.*, 1969) or used for immunoblotting as described below. Densitometric scanning of the CBB-stained gel was performed at 550 nm using the Shimadzu (Kyoto, Japan) CS-9000 dual-wavelength flying-spot scanner.

Analytical IEF

Analytical IEF was performed in 2-mm-thick polyacrylamide gel (5%T, 3%C) containing 5% Ampholine (pH 3.5–10) (Pharmacia LKB, Bromma, Sweden) using the LKB 2117 Multiphor (Pharmacia LKB). Prefocusing of the gel was performed at 30 W for 20 min. Samples containing 20 mg/ml mugwort pollen extract (5.2 mg protein/ml) were applied and the electrofocusing was performed at 30 W for 1.5 hr with 1 M NaOH and 1 M H₃PO₄ as the cathodic and anodic electrode solutions, respectively. The Isoelectric Focusing Calibration Kit, pH 3–10 (Pharmacia), was included for *pI* determination. One part of the gel was fixed with TCA-sulphosalicylic acid and stained with CBB as described by Winter *et al.* (1977) or with the PAS reagent. The rest of the gel was used for crossed immuno-IEF or for immunoblotting as described below. Densitometric scanning of the CBB-stained gel was performed as described under SDS-PAGE.

Crossed immuno-IEF

In crossed immuno-IEF, IEF in the first dimension was performed as described above. Immediately after focusing, the gel strip was transferred to a 1% agarose gel by the "laying on" technique described by Söderholm *et al.* (1975). Second-dimension electrophoresis was performed towards the anode at 10 V/cm for 1 hr followed by 2 V/cm for 15 hr into 1% agarose gel containing 3–6 µl/cm² anti-Ag 12 antiserum. Other electrophoresis conditions were as described earlier (Nilsen and Smestad Paulsen, 1986). The gel was stained with CBB.

Immunoblotting

Blotting of separated antigens from SDS-PAGE or IEF gels to NCM (Trans-Blot[®] nitrocellulose membrane 0.45 µm, BioRad) was performed by electrophoretic transfer. Blotting of SDS-PAGE gels was performed at 100 V for 1 hr in 25 mM Tris, 192 mM glycine and 20% methanol (pH 8.3) (Towbin *et al.*, 1979), using the Mini-Trans-Blot[®] (BioRad), according to the manufacturer's instructions. Blotting of IEF gels was performed at 70 V for 5 hr in 0.7% acetic acid, using the Trans-Blot cell (BioRad), according to the manufacturer's instructions. After transfer, one part of the NCM was stained with 0.15% Amido Black in 25% methanol-10% acetic acid and destained in 25% methanol-10% acetic acid (Sutton *et al.*, 1982). The blotted gel was stained with CBB to evaluate the effectiveness of transfer.

Binding of specific rabbit IgG or human IgE to separated mugwort pollen components transferred to NCM was detected essentially as described by Bengtsson *et al.* (1985). After incubation of the NCM for 1 hr at 37°C in blocking buffer (0.5% Tween 20-0.3% Dextran 70-0.9% NaCl in 50 mM phosphate buffer; pH 7.4) to block unoccupied protein binding sites (Batteiger *et al.*, 1982), the NCM was cut into 5-mm strips. Each strip was incubated for 15–20 hr with patient serum (1:3, 1:5 or 1:10), rabbit anti-Ag 12 antiserum (1:1000) or rabbit anti-*Art v* II antiserum (1:10), washed, incubated for 4 hr with [¹²⁵I]anti-human IgE (300,000 cpm/ml) (Phadebas RAST[®], Pharmacia) or β-galactosidase anti-rabbit IgG (1:10,000) (MIAB, Uppsala, Sweden) and washed again. All dilutions were made in blocking buffer. Strips incubated with [¹²⁵I]anti-human IgE were dried and subjected to autoradiography at -80°C for 1–7 days. Strips incubated with β-galactosidase anti-rabbit IgG were treated with a chromogenic substrate solution containing 1.5 ml 2-naphthyl-β-D-galactopyranoside (3.3 mg/ml, Sigma Chemical Co., St Louis, MO) in methanol and 1.0 ml Fast Blue B-salt (20 mg/ml, Sigma) in substrate buffer (50 mM phosphate-0.9% NaCl-1 mM MgCl₂; pH 7.5), diluted to 10 ml with substrate buffer (Bengtsson *et al.*, 1985).

Dot-immunoblot

Samples of mugwort pollen extract (20 mg/ml in Tris-HCl; pH 6.80) were heated at 100°C for (1) 2 min, (2) 5 min, (3) 5 min in the presence of 5% mercaptoethanol, (4) 5 min in the presence of 2% SDS or (5) 5 min in the presence of 5% 2-mercaptoethanol and 2% SDS. Five µl of each sample and 5 µl of an untreated control were applied as dots on NCM and allowed to air-dry for 45 min. Binding of Ag 9-specific IgE to the dots was detected as described for immunoblotting using a patient serum containing high amounts of IgE against Ag 9 (diluted 1:7.5 in blocking buffer).

RESULTS

SDS-PAGE, transfer to NCM and detection of IgE-binding components

SDS-PAGE was performed with an extract dialysed with cut-off 12,000. This extract gave fewer problems with streaking and other artefacts in the SDS-PAGE gel than an extract dialysed at cut-off 3500. The same number of bands were detected with both extracts, but components in the mol. wt region 12,000–15,000 were present in larger amounts when SDS-PAGE was performed with the extract dialysed at 3500.

SDS-PAGE of mugwort pollen extract in 15% homogeneous polyacrylamide gel, followed by staining of the gel with CBB, gave at least 23 bands in the mol. wt region 10,000–94,000 (Fig. 1c), the most prominent being the three bands with mol. wts of about 63,000, 22,000 and 12,500. Scanning of the CBB-stained gel (results not shown) indicated that these three bands constituted about 9, 50 and 12% of the total protein in the extract, respectively. The apparent mol. wt of the diffuse band with mol. wt 22,000 in the 15% gel was significantly affected by the gel concentration. Thus, the apparent mol. wt of this band varied from about 30,000 in a 10% gel to about 21,000 in a 18% gel. The mol. wt of the band with mol. wt 63,000 in the 15% gel was not significantly affected by the gel concentration, but in some experiments this band was resolved into two bands with mol. wts of about 61,000 and 64,000. PAS staining of the 15% gel (Fig. 1b) demonstrated the presence of at least three bands containing carbohydrate, the most prominent being a diffuse band with mol. wt about 22,000. In addition, two bands with mol. wts about 19,000 and >100,000, though difficult to see in the photograph, were clearly visible in the original gel.

Mugwort pollen proteins separated by SDS-PAGE were transferred to NCM by electrophoresis. Staining of the NCM with Amido Black demonstrated that all the protein bands detected in the CBB-stained gel were present in the NCM after transfer (Fig. 1e). The transfer was, however, not complete for all protein bands as CBB staining of the blotted gel (Fig. 1d) revealed that about 20% of the band with mol. wt 22,000 and about 5% of two bands with mol. wts 39,000 and 63,000, remained in the gel after transfer. Increasing the transfer time from 1 to 3 hr or performing electrophoresis at 30 V for 18 hr did not improve this result. Exclusion of methanol or addition of 0.1% SDS to the transfer buffer as suggested by Gershoni and Palade (1982) and Sutton *et al.* (1982), respectively, led to elution of more protein from the gel, but at the same time the binding capacity of the NCM seemed to decrease and fewer bands were detected in the NCM after transfer.

Incubation of the NCM with human serum pool and [¹²⁵I]anti-IgE (Fig. 1f) revealed the presence of at least 15 IgE-binding bands in the mol. wt region

*The antigen numbers used in this presentation refer to the designations used by Smestad Paulsen *et al.* (1985). Ag 9, Ag 12 and Ag 13 correspond to the allergens denoted Ag 4, Ag 20 and Ag 11, respectively, by Ipsen *et al.* (1985) (Ipsen, personal communication).

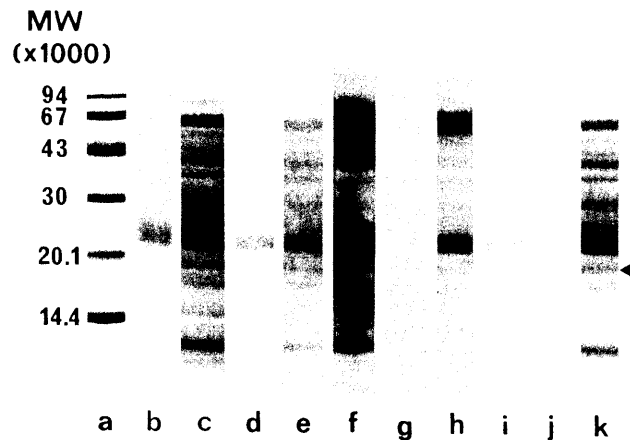


Fig. 1. SDS-PAGE (15%T, 2.7%C) of mugwort pollen extract and immunoblotting to detect IgE-binding components and selected allergens earlier identified in CIE/CRIE. (a) Mol. wt references in CBB-stained gel; (b) mugwort pollen extract (200 μ g, 52 μ g protein) stained with PAS; (c) and (k) mugwort pollen extract (150 μ g, 39 μ g protein) stained with CBB; (d) CBB-stained gel after transfer to NCM; (e) NCM stained with Amido Black after transfer; (f) autoradiograph of NCM incubated with a pool of sera from 64 individuals having RAST-class 3 against mugwort pollen extract; (g), (h) autoradiographs of NCM strips incubated with human sera containing high amounts of specific IgE against Ag 9 (g) or against Ag 13 and Ag 12 (h); (i), (j) NCM strips incubated with rabbit antisera against Ag 12 (i) or against *Art v* II (j). Binding of human IgE was detected by incubation with [^{125} I]anti-IgE followed by autoradiography for 20–30 hr. Binding of rabbit IgG was detected by incubation with β -galactosidase anti-rabbit IgG followed by a chromogenic substrate. Arrow in (k) points to the band earlier identified as *Art v* II (Nilsen and Smestad Paulsen, 1990).

12,000–80,000. The two bands exhibiting the most intense radiostaining had mol. wts of about 61,000 and 22,000. The diffuse IgE-binding band with mol. wt 22,000 was in some experiments resolved into two IgE-binding bands with mol. wts about 21,500 and 23,000.

To assess the relative importance of the IgE-binding bands, immunoblotting of the SDS-PAGE gel was performed with sera from 16 patients with clinical mugwort allergy. The results (Table 1) showed that the panel of patient sera contained specific IgE against at least 15 components in the mugwort pollen extract. A component with mol. wt of about 22,000 bound IgE from at least 94% of the sera tested, and for all but three sera this component bound the greatest quantity of IgE as demonstrated by the intensity of radiostaining. Two other components of mol. wts 39,000 and 42,000 which always appeared together as a doublet, also bound IgE from 94% of the patient sera tested, but the intensity of radiostaining of these bands was significantly lower. Three components with mol. wts 12,000, 17,000 and 29,000 bound IgE from 75% of the patients, and for some sera the component with mol. wt 12,000 exhibited rather strong radiostaining. Rather strong radiostaining was also observed with a component of mol. wt

61,000. This component bound IgE from only five (31%) of the sera tested, but with all these sera (patients 1, 3, 6, 8 and 15) the intensity of radiostaining of the 61,000 band was either stronger or only slightly weaker than the radiostaining intensity of the 22,000 component (Table 1). No radiostaining of any of the components separated by SDS-PAGE was observed when immunoblotting was performed with serum from a non-allergic individual.

IEF, transfer to NCM and detection of IgE-binding components

IEF in a pH gradient 3.5–10, followed by staining of the gel with CBB, gave at least 35 bands, of which at least 28 bands were localized in the pI region 3.5–7.2 and at least seven bands were found in the pI region 8.6–9.3 (Fig. 2c). Scanning of the gel (results not shown) revealed that about 85% of the protein in mugwort pollen extract was localized in the pI region 4.2–5.2. Staining of the IEF gel with the PAS reagent demonstrated that at least 19 bands in the pI region 3.5–6.0 contained carbohydrate (Fig. 2b). The major part of the carbohydrate-containing material was localized in the pI region 4.2–5.2.

Transfer of the mugwort pollen components separated by IEF to NCM and staining of the NCM with

Table 1. Binding of IgE^a from individual sera ($n = 16$) to mugwort pollen components separated by SDS-PAGE and transferred to NCM

Patient no.	MW of IgE-binding components (x 1000)														
	>100	80	61	57	48	42	39	34	29	22	17	16	15	14	12
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															
Frequency (%) ^b	12.5	19	31	44	12.5	94	94	25	75	94	75	19	19	12.5	75

^a The intensity of radiostaining of the individual components is indicated by:

■ = very strong radiostaining, ▨ = strong radiostaining, ▩ = medium radiostaining, □ = weak radiostaining

^b Determined as the number of patient sera containing IgE against the individual component in % of the total number of patient sera.

Amido Black (Fig. 2e) demonstrated that all the bands visible in the CBB-stained gel could be detected in the NCM after transfer. However, CBB staining of the blotted gel (Fig. 2d) demonstrated that about 30% of most of the protein bands in the pI region 4.4–5.6 remained in the gel after transfer. Incubation of the NCM with human serum pool and [^{125} I]anti-IgE and autoradiography (Fig. 2g) revealed at least 11 IgE-binding bands in the pI range 4.2–7.3 and at least four IgE-binding bands with pI in the region 8.5–9.1. A band with pI 4.35 exhibited the most intense radiostaining.

Incubation of the NCM with sera from 16 individual patients with clinical mugwort allergy showed that the panel of patient sera contained specific IgE against 16 components (Fig. 3). About 63% of the patient sera contained IgE against at least four of the five bands with pIs 4.55, 4.70, 4.80, 5.05 and 5.40. The most intense radiostaining was observed with a band with pI 4.35 which bound IgE from 31% of the patient sera tested. No radiostaining of any band was observed when the NCM was incubated with serum from a non-allergic individual (Fig. 3c).

Identification of selected mugwort pollen allergens by immunoblotting of IEF or SDS-PAGE gels

To determine the mol. wts and pI of three important mugwort pollen allergens earlier identified only in the CIE/CRIE system, immunoblotting of SDS-PAGE and IEF gels was performed using a rabbit antiserum against Ag 12, a patient serum exhibiting high binding of specific IgE to Ag 12 and Ag 13 and a patient serum containing high amounts of specific IgE against Ag 9. In addition, a mono-specific rabbit antiserum against the glycoprotein

allergen *Art v* II, previously denoted Ag 7 (Nilsen and Smestad Paulsen, 1990) was used to investigate whether this allergen was detectable in the NCM after transfer from SDS-PAGE and IEF gels. The antigen numbers used in this presentation refer to the designations used by Smestad Paulsen *et al.* (1985). Ag 9, Ag 12 and Ag 13 correspond to the allergens denoted Ag 4, Ag 20 and Ag 11, respectively, by Ipsen *et al.* (1985) (Ipsen, personal communication).

Immunoblotting of the SDS-PAGE gel using the rabbit antiserum against Ag 12 (Fig. 1i) revealed a diffuse band with mol. wt of about 22,000, or possibly two bands with mol. wts 21,500 and 23,000, as the dominant band(s). Incubation with the patient serum containing specific IgE against Ag 13 and Ag 12 resulted in IgE-binding to two bands with apparent mol. wts of 61,000 and about 22,000, respectively (Fig. 1h). These results indicate that Ag 12 has a mol. wt of about 22,000, whereas the mol. wt of Ag 13 is about 61,000. Additional evidence for the assignment of mol. wts of these two allergens was obtained when two patient sera which were found to contain high amounts of specific IgE mainly against the 22,000 band (patient 7) or the 61,000 band (patient no. 1) in SDS-PAGE/immunoblotting (Table 1) were tested in CRIE. The two patient sera were found to contain specific IgE mainly against Ag 12 and Ag 13, respectively (results not shown).

Immunoblotting of the SDS-PAGE gel using the patient serum containing high amounts of specific IgE against Ag 9 did not reveal any band after autoradiography (Fig. 1g), indicating that this allergen was unable to bind IgE in the patient serum after SDS-PAGE and transfer to NCM. To investigate whether the failure of Ag 9 to bind IgE was caused

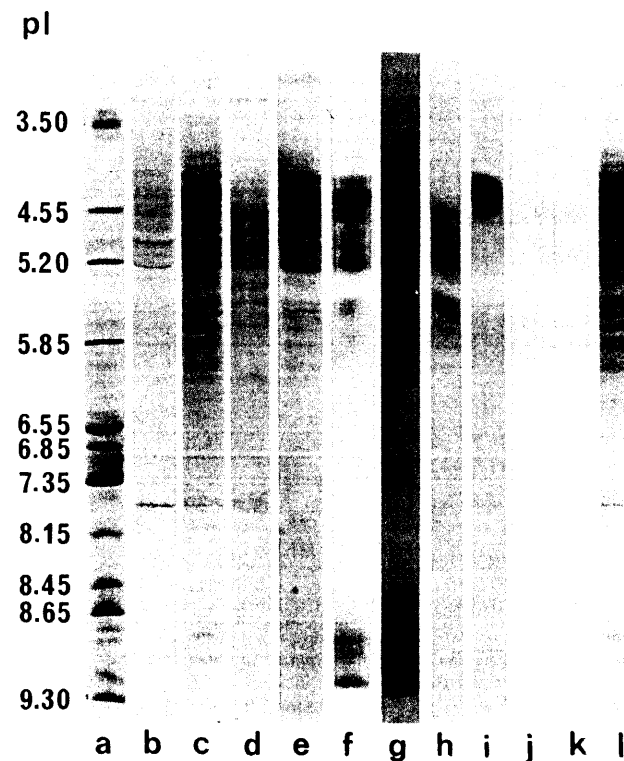


Fig. 2. IEF (pH gradient 3–10) of mugwort pollen extract and immunoblotting to detect IgE-binding components and selected allergens earlier identified in CIE/CRIE. (a) *pI* references in CBB-stained gel; (b) mugwort pollen extract (500 µg, 130 µg protein) in gel stained with PAS; (c) and (d) mugwort pollen extract (400 µg, 100 µg protein) in CBB-stained gel before transfer to NCM; (e) CBB-stained gel after transfer to NCM; (f) NCM with separated mugwort pollen components stained with Amido Black; (g) autoradiographs (1 and 3 days, respectively) of NCM incubated with a pool of sera from 64 individuals having RAST-class 3 against mugwort pollen; (h), (i) autoradiographs of NCM strips incubated with human sera containing high amounts of specific IgE against Ag 9 (h) or against Ag 12 and Ag 13 (i); (j), (k) NCM strips incubated with rabbit antisera against Ag 12 (j) or against *Art v* II (k). Binding of human IgE and rabbit IgG was detected as described in Fig. 1.

by the pretreatment of the samples before SDS-PAGE, samples of crude extract were treated by boiling for 2 or 5 min in the absence or presence of SDS and/or mercaptoethanol and compared with untreated extract in dot-immunoblot using the patient serum containing high amounts of specific IgE against Ag 9. The results (not shown) demonstrated that the ability of the crude extract to bind IgE from this patient serum was reduced to less than 10% after only 2 min boiling in the absence of SDS and mercaptoethanol. Boiling for 5 min in the absence or presence of SDS and/or mercaptoethanol completely destroyed the ability of the crude extract to bind IgE from this serum.

No bands were detected when immunoblotting was performed using the rabbit anti-*Art v* II antiserum (Fig. 1j). The mol. wt of *Art v* II has earlier been found to be about 20,000 (Nilsen and Smestad

Paulsen, 1990), but this value is slightly dependent upon the gel concentration, and in the 15% gel used in this study, the band corresponding to *Art v* II has a mol. wt closer to 19,000 (see arrow in Fig. 1k). Close examination of the Amido Black stained NCM in Fig. 1e revealed that the 19,000 band corresponding to *Art v* II was present in the NCM after transfer, and the failure to detect this band with the mono-specific rabbit antiserum thus indicates that *Art v* II is not able to bind rabbit IgG after transfer from SDS-PAGE gels. The results in Table 1 indicate also that the IgE-binding ability of *Art v* II is destroyed or at least significantly reduced in the SDS-PAGE/immunoblotting procedure. Five of the 16 patient sera tested in SDS-PAGE/immunoblotting (Table 1) had earlier been found to contain specific IgE against *Art v* II when tested in R(R)IE with the purified allergen (Nilsen and Smestad Paulsen, 1990),

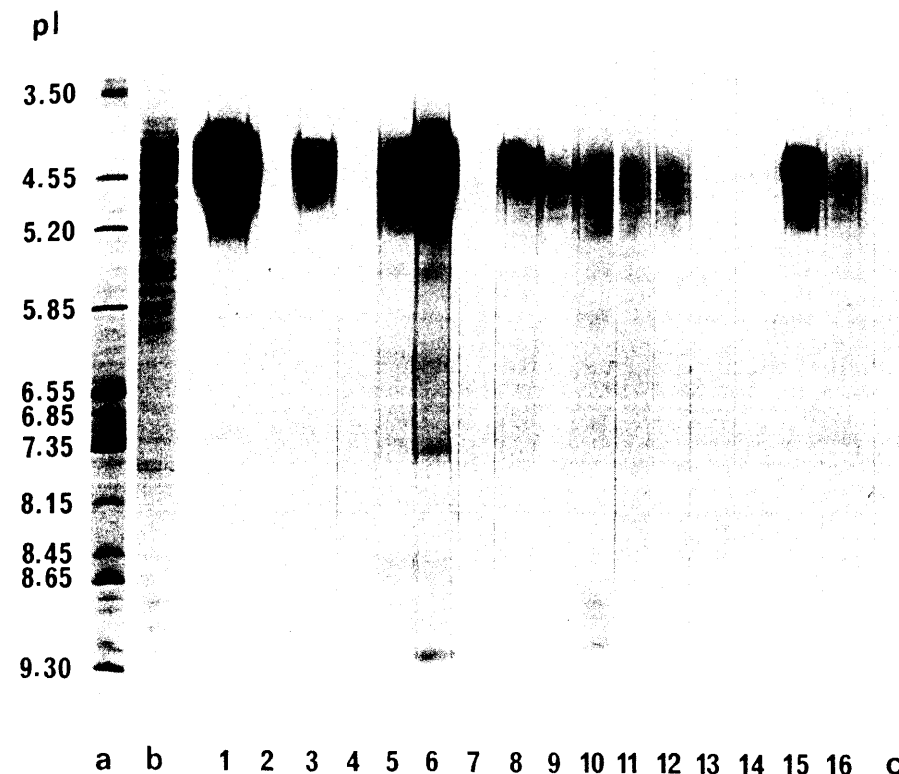


Fig. 3. Identification of IgE-binding components in mugwort pollen extract after separation by IEF and immunoblotting using a panel of patient sera from patients with clinical mugwort allergy. (a), (b) CBB-stained gel showing separation of *pI* references (a) and mugwort pollen extract (b); (1–16) autoradiographs (2 days) of NCM strips incubated with individual patient sera from mugwort allergic patients followed by [¹²⁵I]anti-IgE; (c) autoradiograph (2 days) of NCM strip incubated with serum from a non-allergic individual followed by [¹²⁵I]anti-IgE.

but none of these sera (patient sera nos 1, 2, 6, 9 and 15) demonstrated IgE binding to the band with mol. wt about 19,000 after SDS-PAGE (Table 1).

To determine the *pI* of these allergens, NCM strips containing mugwort pollen antigens transferred from IEF gels were examined by incubation with the same human sera and rabbit antisera as described above. The results demonstrated that the patient serum containing IgE against Ag 9 detected at least six (eight) components with *pI*s 4.55, 4.70, 4.80, 5.05, (5.20), 5.40, 5.55 and (5.75) (Fig. 2h), indicating that Ag 9 is present in mugwort pollen extract in several isoallergenic forms. No bands were detected when the NCM was incubated with the rabbit antiserum against Ag 12 (Fig. 2j). When the results in Fig. 3 are compared with those of Table 1 it is also clear that several of the individual patient sera which gave strong radiostaining of the 22,000 band of Ag 12 after separation by SDS-PAGE, gave only weak radiostaining or no radiostaining at all of components separated by IEF (e.g. patients 4, 7 and 14). These

results indicate that Ag 12 is not detectable in the NCM after transfer from IEF gels. As the *pI* of Ag 12 could not be determined by immunoblotting, crossed immuno-IEF of mugwort pollen extract using rabbit anti-Ag 12 in the antibody-containing gel was performed, and the results (Fig. 4) indicate that Ag 12 also consists of several isoforms. The major part of the Ag 12 molecules seem to have *pI*s in the region 3.5–5.1 with a peak value of 4.3, but isoforms with *pI*s in the region 5.5–7.2 and possibly 8.4–8.6 could also be detected in smaller amounts. As Ag 12 does not seem to bind IgE after transfer from IEF gels, it was possible to determine the *pI* of Ag 13 by immunoblotting of the IEF gel using the patient serum containing specific IgE against both Ag 12 and Ag 13. The result of this experiment is given in Fig. 2i and indicates that Ag 13 consists of three isoforms with *pI*s 4.20, 4.35 and 4.50, with the *pI* 4.35 component as the major IgE-binding component. This result was supported by the results in Fig. 3, which show that the same five patient sera that gave

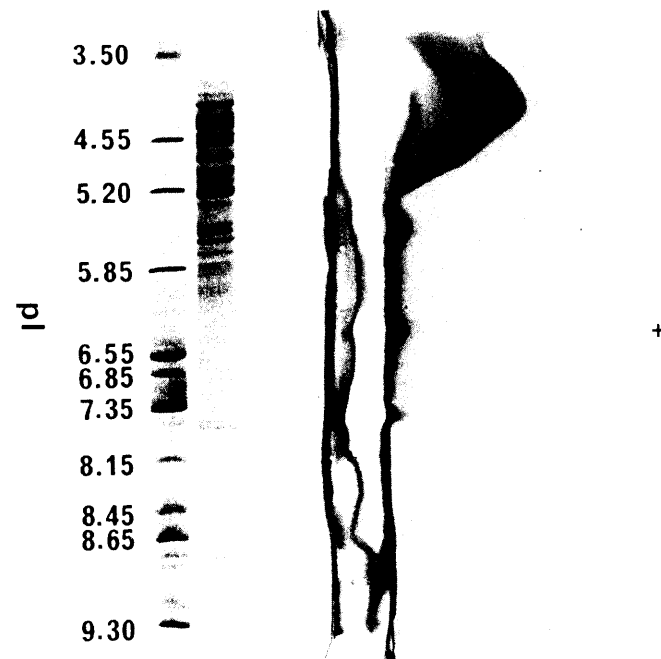


Fig. 4. Crossed immuno-IEF of 300 μ g mugwort pollen extract (78 μ g protein) against anti-Ag 12 rabbit antiserum (6 μ l/cm²). The corresponding IEF separation patterns of mugwort pollen extract and pI references are shown at the bottom. The anode during immunoelectrophoresis was positioned as indicated.

strong radiostaining of the 61,000 band of Ag 13 in Fig. 3 (patient sera nos 1, 3, 6, 8 and 15) also gave strong radiostaining of the pI 4.35 component. *Art v* II did not seem to be detectable in the NCM after transfer from IEF gels, as no bands were detected when the NCM was incubated with the monospecific antiserum against *Art v* II (Fig. 2k).

DISCUSSION

In this study SDS-PAGE and IEF combined with immunoblotting have been applied to characterize an allergen extract of mugwort pollen. Of the at least 23 components separated by SDS-PAGE and transferred to NCM, at least 15 bound IgE both from a pool of sera from 64 individuals with RAST-class 3 against mugwort pollen and from a panel of 16 patient sera with clinical mugwort pollen allergy. The IgE-binding components were found in the mol. wt range 12,000–100,000. This is a larger number of IgE-binding components than has earlier been detected by CRIE of mugwort pollen extract (Smestad Paulsen *et al.*, 1985; Ipsen *et al.*, 1985; Dreborg *et al.*, 1987; Diener *et al.*, 1988). The greater number of IgE-binding components identified in SDS-PAGE may be explained by the possibility of dissociation of some of the allergens into IgE-binding subunits of different mol. wts.

A notable limitation to the use of SDS-PAGE/immunoblotting for the characterization and standardization of mugwort pollen extracts is the observation that some important allergens may fail to be detected by this method. Thus, neither Ag 9 nor *Art v* II seemed to bind human IgE after transfer from SDS-PAGE gels. The loss of IgE-binding ability under the denaturing conditions used for SDS-PAGE indicates that the native protein conformation is essential for the binding of human IgE to both *Art v* II and Ag 9. Electrophoretic transfer to NCM and subsequent washing of the NCM does not seem to be sufficient to renature these allergens. In the case of *Art v* II, this is not surprising, as amino acid analysis of the purified allergen has revealed that *Art v* II may contain at least seven intra- or interchain disulphide bridges (Nilsen and Smestad Paulsen, 1990), which may complicate the restoration of the native conformation. In the case of Ag 9, removal of SDS is not likely to be sufficient to renature this allergen as boiling of the sample, even in the absence of SDS, had a major effect on its IgE-binding ability.

Limitations to the use of IEF/immunoblotting for characterization of allergen extracts of mugwort pollen were also noted in this study. At least 35 protein components with pIs ranging from 3.60 to 9.30 were separated by this method, but about 85% of the protein material was found within the relatively

narrow pI range 4.20–5.20. IgE binding was detected to at least 11 components in the pI region 4.20–7.25 and at least five components in the pI region 8.50–9.15. Several of the important mugwort pollen allergens exist, however, in multiple isoallergenic forms in overlapping pI regions, which makes it difficult to assign any one band in the IEF pattern to a particular allergen. Thus, both Ag 9 (pIs 4.55–5.55) and Ag 12 (major isoforms in the pI range 3.5–5.1) contained isoforms in the same pI region as has earlier been demonstrated for the isoforms of *Art v* II (pI region 4.10–4.65) (Nilsen and Smestad Paulsen, 1990). Of the four important mugwort pollen allergens investigated in this study, only Ag 13 had a relatively discrete pI, with a dominant band at 4.35 and only two weak bands at 4.20 and 4.50.

Neither Ag 12 nor *Art v* II was detected in the NCM after electrophoretic transfer from the IEF gel. The reason for the failure to detect these allergens may be that these allergens are no longer able to bind rabbit IgG or human IgE after the exposure to 0.7% acetic acid during the electrophoretic transfer from IEF gels to NCM. It is also possible that these allergens were not present in the NCM after transfer either because they were not eluted from the gel or because the ampholines in the IEF gel prevented the binding of these two allergens to the NCM by competing with the binding sites on the membrane.

Immunoblotting of SDS-PAGE and IEF gels using specific rabbit antisera and human sera containing antibodies against three important allergens in mugwort pollen provided information about mol. wt and pI and some other physicochemical parameters of these allergens which had earlier been identified only in CIE/CRIE. Ag 12 was found to consist of several isoforms with pIs mainly in the region 3.5–5.1. This may explain the asymmetric precipitation pattern of this allergen in CIE/CRIE (Ipsen *et al.*, 1985). In SDS-PAGE Ag 12 was identified as a diffuse band (or possibly two bands) with mol. wt about 22,000 in a 15% gel. Not only was Ag 12 the most prominent band in the CBB-stained gel, it was also the most prominent band on the autoradiographs after immunoblotting of the SDS-PAGE gel with a panel of 16 patient sera (Table 1). Both in terms of relative radiostaining intensity and frequency of recognition by individual patient sera, this component would be considered the major IgE-binding component in mugwort pollen extract. This correlates well with the results obtained in the study of Ipsen *et al.* (1985). PAS staining of the SDS-PAGE gel indicated that the 22,000 band of Ag 12 is a glycoprotein, which may explain why the apparent mol. wt of this band decreased with increasing gel concentration (Leach *et al.*, 1980). The glycoprotein nature of Ag 12 may also explain why this allergen gives a broad and diffuse immunoprecipitate in CIE/CRIE (Ipsen *et al.*, 1985).

Dreborg *et al.* (1987) concluded that a component in the mol. wt region 15,000–20,000 (approx. mol. wt

18,000) was the dominant allergen in mugwort pollen extract. It is unlikely that this component is identical to Ag 12 as experiments using the same electrophoresis conditions as described by these authors (7.5–20% gradient gel) gave a mol. wt of about 25,000 for Ag 12. The component with mol. wt about 17,000 which bound IgE from 75% of the patient sera in the present study (Table 1) may, however, correspond to the major IgE-binding component identified by Dreborg *et al.* The difference in relative radiostaining intensity of the individual bands in these two studies may be due to differences in extract preparation, patient serum pools and conditions used for SDS-PAGE/immunoblotting.

Identification of Ag 13 in the SDS-PAGE and IEF patterns revealed that this allergen had a mol. wt of about 61,000 and a pI of about 4.35. Recently, de la Hoz *et al.* (1990) have reported the isolation of a mugwort pollen allergen, *Art v* I, with a pI of 4.4 and a mol. wt of 60,000 in SDS-PAGE. It seems likely that Ag 13 is identical to the purified *Art v* I. In our study, five (31%) of the 16 individual patient sera tested contained specific IgE against the bands corresponding to Ag 13 and at least four of these patient sera exhibited strong radiostaining of this allergen (Table 1, Fig. 3). This result is in agreement with the results of Ipsen *et al.* (1985), but not in agreement with the results of de la Hoz *et al.* (1990), who found that at least 70% of mugwort allergic patients had specific IgE against *Art v* I. This discrepancy may perhaps be explained by differences in patient selection and/or the different methods used to determine the patient reactivity in these studies.

Identification of Ag 9 in the IEF pattern revealed a marked pI heterogeneity for this allergen, which may explain why this allergen gives a broad and sometimes slightly asymmetric precipitate in CIE/CRIE. As Ag 9 did not bind IgE after SDS-PAGE and gave several bands after IEF in a pI region where other mugwort pollen allergens may contribute to the IgE binding, the determination of the number of patient sera containing specific IgE against this allergen was difficult using these methods. However, the observation that at least 63% of the patient sera contained specific IgE against at least four of the six bands demonstrated to correspond to isoforms of Ag 9 is in reasonable agreement with the results of Ipsen *et al.* (1985).

In conclusion, the results in this study have shown that immunoblotting combined with SDS-PAGE or IEF are useful methods for the characterization of mugwort pollen extracts. It should, however, be noted that none of these methods gives a complete picture of the IgE-binding components in this extract. Some important mugwort pollen allergens which are easily identified in CIE/CRIE failed to be detected by immunoblotting of SDS-PAGE and/or gels. The results in this study have nevertheless provided information about the mol. wt and pI of some important mugwort pollen allergens earlier identified only in CIE/CRIE.

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ANTI-IDIOTYPIC AND ANTI-ANTI-IDIOTYPIC ANTIBODIES AS HIGHLY SPECIFIC TOOLS IN EPITOPE AND ACTIVE SITE STUDIES ON HUMAN INTERFERON- γ

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Abstract—Immunization of rabbits with F(ab')₂ fragments of different monoclonal antibodies directed against human interferon- γ yielded antisera with anti-idiotypic characteristics. Isolation of the anti-idiotypic fraction, resulting in a highly specific antiserum, allowed us to prove that out of six competing monoclonal antibodies directed against human interferon- γ , only two really recognize the same epitope. The other monoclonal antibodies compete on the basis of steric hindrance, which is not surprising, because of the large difference in M_r between interferon- γ and an immunoglobulin.

The anti-idiotypes provided us also with a tool to study isolated epitopes on the human interferon- γ molecule, a task which was previously not practicable. Exploration of the biological properties of these anti-idiotypes allows us to determine whether the investigated epitopes are involved in receptor binding.

The production of an anti-anti-idiotypic antiserum not only proved that we generated real internal images, but also that these images conserved all of their properties, although with a decreased affinity in comparison with the original monoclonal antibody. As the former is a polyclonal antiserum, directed against a single epitope of the human interferon- γ molecule, competition experiments yielded additional information on the relative position of three epitopes recognized by inhibiting monoclonal antibodies. These antisera will possibly open new ways for the affinity purification of interferon- γ and perhaps for the treatment of autoimmune diseases.

INTRODUCTION

Since Jerne (1984) described the "network hypothesis", anti-idiotypes have become of great interest in almost all fields of immunology. Their influence on the regulation of the immune response has been studied extensively, and they have been used in receptor studies and as vaccines. Most of these experiments are based on the existence of "internal images".

A classification of anti-idiotypes according to their interaction with the idiotypic was proposed and recently reviewed by Köhler *et al.* (1989). If this interaction is antigen inhibitable, they were called Ab2 β , otherwise they were referred to as Ab2 α ; anti-idiotypic antibodies which are inhibited by the antigen as a result of steric hindrance represent a third group, namely Ab2 γ .

The internal images are found in the Ab2 β group, and they represent an interesting tool in epitope studies, especially when small antigens are involved. A strategy often followed in epitope mapping is the production of monoclonal antibodies (mAb) directed against the native protein or against synthetic peptide fragments, followed by several kinds of competition experiments. The results of such investigations are

often subject to criticism because of the possibility of steric hindrance when molecules with a large difference in M_r are involved. This is particularly true for most of the cytokines and other regulatory proteins, as the majority of them have an $M_r \leq 15,000$, i.e. at least 10 times lower than that of immunoglobulins. Therefore the information on the epitopes provided by these studies is not reliable, it is very limited and is not specific enough, especially when one isolated epitope is the aim of the study.

An elegant alternative is provided by the use of anti-idiotypic antibodies, as we report in this paper. Anti-idiotypic antisera are relatively easy to prepare and their use can avoid the problem of steric hindrance. As they offer us the possibility to study one isolated epitope at a time, they will also provide additional information on the importance of this epitope in relation to the active site or the receptor binding site of the antigen. In our study we used eight different mAbs directed against human interferon- γ (HuIFN- γ), six of which block the biological activity and compete for binding to HuIFN- γ . Using anti-idiotypic antisera we were able to demonstrate that only two out of these six really recognize the same epitope, and that there are at least three completely different epitopes, present on HuIFN- γ which are recognized by inhibiting mAbs.

The anti-idiotypes themselves may in turn be used as an immunogen, leading to the production of anti-anti-idiotypes (Ab3). In this way we were able to

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Abbreviations: HuIFN, human interferon; Ig, immunoglobulin; MEM, minimum essential medium.