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Allergy to Fruits and Vegetables in Pollen-sensitive Patients: Allergen Characterization by IgE Immunoblotting and Peroxidase Staining

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Allergies to several plant foods, i.e. apples, nuts, stone fruits, celery and spices, are highly associated with pollen allergies. The observed cross-reactivities are due to structural similarities between food and pollen allergens. Using horseradish peroxidase (HRP) as the marker enzyme we have further developed and optimized a sensitive and specific immunoblotting procedure for the detection of human IgE antibodies specific to food and pollen proteins separated by SDS-PAGE and immobilized on nitrocellulose blots. Our optimization studies involved four colour substrates and six buffer systems. The best results were obtained when 0.3% Tween 20 and no protein was used for membrane blocking, whereas blocking with proteins yielded high backgrounds. Immuno-detection amplification was accomplished by the following incubation steps: (a) sample (human serum); (b) secondary antibody; (c) biotinylated tertiary antibody; and (d) streptavidin-HRP. Staining was performed with 3,3',5,5'-tetramethylbenzidine which is an Ames test-negative, alternative substrate for HRP. The quality of several commercially available anti-IgE antibodies was examined. For some of these reagents very high non-specific binding to food and pollen proteins occurred. The application of our method to apple and birch pollen allergen characterization is described. Results of IgE- and IgG-immunoblotting and immuno-detection of allergens following two-dimensional-electrophoresis, as well as cross-reactivity studies by means of immunoblot inhibition, are presented.

Keywords: Apple, birch pollen, cross-reactivity, food allergy, IgE, immunoblotting, peroxidase staining.

INTRODUCTION

Allergy to Vegetable Foodstuffs

Immediate-type allergic reactions to vegetable foodstuffs are mediated by allergen-specific IgE antibodies. In adults, fresh fruits, vegetables, nuts and spices are the most frequent stimulators of this type of food allergy, causing mainly oropharyngeal symptoms (Dreborg, 1988; Ortolani *et al.*, 1988; Aulepp & Vieths, 1992). Further-

more, this type of food hypersensitivity is highly associated with the occurrence of pollen allergies, especially to birch and mugwort pollinosis. Up to 70–90% of the patients with birch pollen allergy are sensitive to apples, stone-fruits, nuts, and/or other fruits and vegetables (Eriksson *et al.*, 1982; Dreborg, 1988; Möller, 1989). Allergy to celery, carrots, spices and/or other vegetables is very common in patients with mugwort pollinosis (Wüthrich & Dietschi, 1985; Thiel *et al.*, 1986; Vallier *et al.*, 1988, Wüthrich *et al.*, 1990). The observed 'cross allergies' which usually exceed the taxonomic borders between food plant families are due to the existence of structural similarities between food and pollen allergens (Halmeupuro *et al.*, 1984; Halmeupuro & Løwenstein, 1985; Calkhoven *et al.*, 1987; Vallier *et al.*, 1988; Ebner *et al.*, 1991; Vieths *et al.*, 1992b). However, most of the food allergens are thought to be proteins or glycoproteins of low molecular weights between 10 and 70 kD (Taylor *et al.*, 1987; Sampson, 1989) and isoelectric points (*pI*s) at acidic pH. Up to now, many food allergens remain unidentified.

However, in all immunochemical studies, the fact that less than 0.001% of the antibodies belong to the IgE class in healthy adults has to be considered. In other words, mean serum concentration of IgG is 13.5 mg/ml whereas the corresponding IgE level is approximately 5×10^{-5} mg/ml (Roitt *et al.*, 1987). In addition, sera of allergic and non-allergic persons often contain IgG antibodies specific to food and pollen proteins.

Allergen Characterization by Protein Immunoblotting

The immunoblotting procedure can be divided into six steps (Harlow & Lane, 1988): (1) sample preparation; followed by (2) resolution of the sample by gel electrophoresis; (3) transfer of the separated proteins to a membrane support (most nitrocellulose, NC); (4) blocking non-specific binding sites of the membrane; (5) addition of the antibody (i.e. a serum sample of an allergic patient); and (6) immuno-detection of the bound antibodies. Separation of allergenic extracts by gel electrophoresis with subsequent immuno-detection of 'allergenic' proteins which bind specific IgE antibodies from patients's sera is a very powerful method of allergen characterization and standardization (Tovey & Baldo, 1987; Baldo & Tovey, 1989). SDS-PAGE and isoelectric focusing (IEF) as well as two-dimensional electrophoresis have been applied for this purpose, under native, dissociating and non-dissociating conditions. In addition, a wide variety of blocking solutions, incubation buffers and immuno-detection systems have been applied (Towbin & Gordon, 1984; Stott, 1989; Timmons & Dunbar, 1990).

Although we have performed native IEF-PAGE immunoblotting, in accordance with Baldo & Tovey (1989) we prefer SDS-PAGE immunoblotting under dissociating and reducing conditions. The latter circumvents the problem of isoallergens which is apparent with IEF, and therefore results are more easy to interpret. In our studies of plant allergens we were not able to observe drastic losses of immunoreactivity of the allergens which might be caused by the SDS-PAGE procedure, although the procedure undoubtedly reduces immunological activity of many proteins. This may be due to the existence of sequential epitopes, renaturation effects on the surface of the NC-membrane, or there is often enough residual activity left to be picked up by the sensitive detection methods used (Tovey *et al.*, 1987).

The aim of our present study was to develop a highly sensitive and specific immunoblotting procedure for the detection of IgE-binding compounds from fruits, vegetables and pollens using enzyme-labelled reagents, and to apply the optimized procedure for allergen characterization. Firstly, we therefore performed systematic optimization experiments for the single steps of the immunoblotting procedure.

Although staining with alkaline phosphatase (AP) was found to be more sensitive than with horseradish peroxidase (HRP) (Towbin & Gordon, 1984; Harlow & Lane,

1988), HRP has several advantages: conjugates of HRP are relatively inexpensive, easy to prepare, and in indirect immunoblotting assays 0.1–1 ng of target protein are detectable (Grafin & Bers, 1989). Therefore, and because of our experience in applying HRP conjugates (mainly in ELISA techniques), we used a peroxidase-based immunodetection system.

Systems for Peroxidase Activity Staining

In general, colorimetric substrates for HRP immunoblotting are leuco dyes which are oxidized during reduction of peroxide and which become intensely coloured and insoluble on oxidation. The most common HRP substrates are 4-chloro-1-naphthol (CN), 3-amino-9-ethylcarbazole (AEC) and 3,3'-diaminobenzidine (DAB); the latter can be used with metal enhancement by Ni or Co salts, resulting in an approximately 10-fold higher sensitivity (Harlow & Lane, 1988; Garfin & Bers, 1989). Furthermore, the reduction of nitroblue tetrazolium (NBT) with NADH to formazan can be mediated by peroxidase reaction with phenol as a substrate (Taketa, 1987). Finally, only very few authors (i.e. Koch *et al.*, 1985) have used 3,3',5,5'-tetramethylbenzidine (TMB) which is a soluble non-carcinogenic substrate for HRP ELISAs (Bos *et al.*, 1981) in immunoblotting: addition of dioctylsodiumsulphosuccinate which acts as a stabilizing agent to the staining solution yields insoluble blue-green precipitates.

CN is a very common but insensitive substrate, whereas AEC is slightly more sensitive (Harlow & Lane, 1988). Very inconsistent data are reported for the sensitivity of the other substrates: DAB with metal enhancement is recommended for fast and sensitive staining (Harlow & Lane, 1988; Holzhauer, 1988; Garfin & Bers, 1989). Taketa (1987) found the formazan method to be the most sensitive. Staining with TMB is reported to be more sensitive than using DAB without metal enhancement (Amersham, 1990). Because of these contradictions, we performed dot-blot optimization assays for all of the named colour substrates with the exception of AEC. The assayed staining solutions and buffer systems were selected on the basis of the literature data.

MATERIALS AND METHODS

Patient Sera

The individual sera used were collected at Deutsche Klinik für Diagnostik, Wiesbaden, Germany (Dr Cl. Thiel) and at Rehabilitationsklinik Borkum Riff der BfA, Borkum, Germany (Dr H. Aulepp). The criterion for selection was a pollen allergy of clinical relevance to one or more pollen species (birch, mugwort, grass), based on a positive history, skin tests and radioallergosorbens tests (RAST). (RAST: semi-quantitative non-competitive immunoassay for the determination of allergen-specific IgE in human sera. RAST-class 0=no specific IgE, RAST class 4=very high content of specific IgE.) RAST classes of ≥ 2 were read as positive. Individual and pool sera from non-allergic subjects were used as negative controls. Additional human pool sera from patients allergic to pollen were obtained from Allergopharma (Reinbek, Germany): birch pollen pool WA 2/50, $n=39$; birch pollen pool Wn 13/32, $n=7$; herb pollen pool Mo 2/150, $n=7$; grass pollen pool 18/100, $n=11$. The pool sera had RAST classes of 3 and 4 indicating high specific IgE levels for the named pollen allergens.

Allergenic Extracts

Pollen extracts were prepared by Allergopharma using a conventional buffer extraction procedure followed by diafiltration and lyophilisation (Wahl, 1988). Because some allergens of fruits and vegetables are highly labile, food extracts were prepared from

uncooked material by a low-temperature method developed in our laboratory. The method yields extracts of high immunochemical activity (Vieths *et al.*, 1992a).

Reagents

If not otherwise mentioned, all reagents were of analytical grade.

Fluka (Neu-Ulm, Germany): DAB, DONS, NBT, Pyronin Y, Triton X 100, Tween-20. Merck (Darmstadt, Germany): agar, amido black, bovine serum albumin (BSA), bromophenol blue, nickel chloride, sodium dodecyl sulphate (SDS). Roth (Karlsruhe, Germany): rothiphorese acrylamide stock solution (30%). Pharmacia (Freiburg, Germany) low molecular-weight marker proteins. Serva (Heidelberg, Germany): CN, β -mercaptoethanol, NADH, Servalyt 3-10 T, 4-9 T, *N,N,N',N'*-tetra-methylethylenediamine (TEMED), TMB, tris-hydroxymethyl-aminomethane (Tris). Sigma (Deisenhofen, Germany): 6-aminohexanoic acid (EACA), Nonidet P 40 (NP 40).

Immunochemicals

(a) *Optimized Procedure.* Rabbit polyclonal anti-human IgE, goat polyclonal affinity-purified biotinylated anti-rabbit IgG (DAKO, Copenhagen, Denmark), streptavidin-HRP (Medac, Hamburg, Germany).

(b) *Other Immunochemicals.* Rabbit polyclonal anti-human IgG-HRP and anti-human IgE-HRP (DAKO, Copenhagen, Denmark), sheep polyclonal anti-IgE-HRP (Binding Site, Birmingham, UK), goat polyclonal affinity-purified anti-IgE-HRP (Medac, Hamburg, Germany FRG).

Dot-blot Assay

The systems for peroxidase activity staining and membrane blocking were optimized by means of a simple dot-blot procedure. For this purpose, the detection of birch pollen-specific IgG antibodies in the birch pollen serum pool WA 2/50 was used as a model system. A dilution series of birch pollen extract (1:5, 1:10, 1:20, 1:50, 1:100) was prepared, and 2.5 μ l of each dilution were dotted onto a sheet of NC paper (0.2 μ m, Schleicher and Schüll, Dassel, Germany) and dried for 30 min. The membrane was soaked in the blocking solution of the current immuno-detection system for 2 $\times$ 15 min and incubated with diluted human pool serum (1:600) for 2 h. After three washes the dot blots were incubated with anti-IgG-HRP (diluted 1:2000 in incubation buffer) for 2 h, washed again and stained.

In total, six buffer systems and four immunostaining techniques were compared. All of the six buffer systems contained NaCl at a concentration of 0.15 M with the exception of system F, where 0.5 M-NaCl was used. *System A:* blocking solution and incubation buffer: 3% (w/v) BSA in 0.05 M-Tris-HCl, pH 7.4 (TBS), washing buffer: TBS; *system B:* blocking solution and incubation buffer: 0.1% (w/v) BSA and 0.05% (v/v) Tween 20 in 0.01 M-potassium phosphate buffer, pH 7.4 (PBS), washing buffer: 0.05% (v/v) Tween 20 in PBS; *system C:* blocking solution and incubation buffer: 3% BSA (w/v) in PBS, washing buffer: 0.5% (v/v) Triton X100 and 0.1% (v/v) NP40 in PBS; *system D:* blocking solution and incubation buffer: 0.5% (v/v) Tween 20 in PBS, washing buffer: 0.05% Tween 20 in PBS; *system E:* blocking solution 0.5% (v/v) Tween 20 in TBS, incubation buffer: 0.1% (v/v) Tween 20 in TBS, washing buffer: 0.05% Tween 20 in TBS; *system F:* PBS (0.5 M-NaCl), blocking solution and incuba-

tion buffer: 0.1% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS, washing buffer: 0.05% (v/v) Tween 20 in PBS.

Staining Solutions

1. **CN:** 3 ml of CN solution (90 mg in 30 ml of methanol) were diluted with 45 ml PBS and 20 μ l H_2O_2 were added. Staining of the dot blot was performed in the dark for 30–50 min until the background increased, and stopped by several washes with distilled H_2O .
2. **DAB with metal enhancement:** 12 mg DAB were dissolved in 18 ml 0.05 M-Tris buffer, pH 7.6; 2 ml $NiCl_2$ (0.3% in 0.05 M-Tris buffer (w/v)) were added and the solution was filtered. After addition of H_2O_2 (20 μ l, 30%), the blots were developed in the dark until background staining occurred (approx. 30 min). The staining was stopped by several washes with distilled H_2O .
3. **NBT/NADH (formazan method):** 20 mg of NADH were dissolved in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.0, 4 ml phenol (1.0 mg/ml in distilled H_2O), 1 ml NBT (3.0 mg/ml in dimethyl formamide) and 10 μ l H_2O_2 (30%) were added and the mixture was stirred. The dot blots were developed for 20 min in the dark.
4. **TMB/DONS:** prior to staining, the blots were incubated with 0.01 M-Tris buffer, pH 6.0, for 1 min, and 24 mg TMB and 80 mg DONS in 10 ml ethanol (96%) were added to 30 ml 0.15 M citrate-phosphate buffer, pH 5.0 (9.4 g citric acid and 18.2 g $NaHPO_4 \cdot 2H_2O$ in H_2O , final volume 1000 ml). H_2O_2 (20 μ l, 30%) were added, the blots were stained for 5–15 min in the dark and stopped by several washes with distilled H_2O . For fixation, the blots were immersed in a solution of 100 mg DONS/13 ml ethanol, made up to 50 ml with distilled H_2O , for 10 min.

In general, the stained NC sheets were dried between pieces of filter paper.

Discontinuous Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (DISC-SDS-PAGE)

The vertical DISC-SDS-PAGE was performed according to the method of Lämmli (1970) using a 5% (w/v) acrylamide stacking gel and a 15% (w/v) resolving gel. Samples were mixed with sample buffer 1:1 (v:v) and kept in boiling water for 3–5 min. The sample buffer contained Tris-HCl (0.5 M, pH 6.8), 5% β -mercaptoethanol (v/v), 5.0 ml glycerol, 20 ml SDS solution (10%, w/v), 0.01 g bromophenol blue and 150 μ l Pyronin Y solution (1%, w/v) in a final volume of 50 ml. The stacking gel buffer was 0.5 M-Tris-HCl, pH 6.8, and the resolving gel buffer contained 1.5 M-Tris-HCl, pH 8.8. Slab gels (170 $\times$ 180 $\times$ 1.0 mm) were run in a twin vertical electrophoresis apparatus (Renner, Dannstadt, Germany) at 25 mA/gel for 30 min followed by 35 mA/gel for approx. 2.5 h (until bromophenol blue was just above the bottom of the gel). For immunoblotting, samples of approx. 5–7 μ g of total protein were loaded on the gel (5 mm slot). Optional total protein staining was performed by a Coomassie brilliant blue or a silver staining method.

Two-dimensional (2D) Electrophoresis

Isoelectric focusing (first dimension) was carried out in glass rods (internal diameter 3.0 mm) under native conditions using the twin vertical electrophoresis apparatus (Renner). The PAA gels (4.7%, w/v, acrylamide; 12.0%, v/v, glycerol; and 9.0%, w/v, Servalyt T 3–10) were loaded with 70 μ l of the sample solution and focussed under the following conditions: 100 V (1 h), 200 V (1 h), 400 V (1 h), 600 V (1 h), 800 V (6 min), 1000 V (6 min). The sample solution consisted of 160 μ l of protein sample, 40 μ l

saccharose (50%, w/v), 8 μ l Sevalyt T and 72 μ l of bromophenol blue solution (0.02%, w/v). The cathode buffer was 20 mM-NaOH, and 10 mM-H₃PO₄ served as the anode buffer. The focussed rod gels were equilibrated in buffer containing 3% SDS (w/v) for 10 min, transferred to the top of the DISC-SDS-PAA gel and fixed with agar which was made up with running buffer. The DISC-SDS-PAGE was performed under standard conditions. The gels were stained with Coomassie blue or silver, or processed for immunoblotting.

Protein Blotting

Transfer of (glyco-) proteins from the gel to the NC membrane was carried out with a Novablot semi-dry electroblotting apparatus (Pharmacia, Freiburg, Germany) at 0.8 mA/cm² for 1 h using an anode buffer I of 0.3 M-Tris-HCl, pH 10.4, and an anode buffer II of 25 mM-Tris-HCl, pH 10.4, both containing 20% (v/v) methanol, and a cathode buffer of 40 mM-EACA, pH 7.6, 20% (v/v) methanol. The Pyronin Y dye from the sample buffer served as a blot control. In addition, cut strips of every blot were stained for total protein with amido black (low molecular weight marker and sample). Before blocking, the NC membranes were dried for 30 min.

Peroxidase Immunostaining of Specific IgE and IgG Antibodies in Human Sera (Optimized Procedure)

The dried NC membranes were blocked twice (15 min) in PBS containing 0.3% (v/v) Tween 20. Human sera and all immunochemicals were diluted with PBS containing 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA (incubation buffer). Each incubation step was followed by three washes with 0.05% (v/v) Tween 20 in PBS (5 min). All incubations were made in glass vials (total volume 50 ml) which were rotated by a rolling mechanism. The TMB/DONS method was used for staining.

(a) *Immuno-detection of Specific IgG Antibodies.* Cut NC strips were incubated with 3 ml of human serum (diluted 1:600) overnight followed by incubation with rabbit anti-IgG-HRP (1:2000) and stained.

(b) *Detection of Allergen-specific IgE Antibodies.* The blots were incubated with 3 ml of: (1) patient serum (diluted 1:6) overnight; (2) rabbit anti-IgE (1:4000, 1 h); (3) goat biotinylated anti-rabbit IgG (1:6000, 1 h); (4) streptavidin-HRP (1:20 000, 25 min) and stained.

Immunoblot Inhibition

To 0.5 ml of patient serum, 100 μ g of protein of the allergenic extract under study (inhibitor) were added and pre-incubated for 1 h. The solution was made up with incubation buffer to a final volume of 3 ml and used for probing cut NC strips with separated and blotted proteins of the allergenic extracts. Immuno-detection was performed according to the standard procedure.

RESULTS AND DISCUSSION

Method Optimization

Dot-blot Assays.

Buffers and staining systems. Staining with CN was very insensitive and slow. If Tween 20 was present in one of the buffers used (i.e. system B), the immuno-detection of specific IgG antibodies was totally impossible (Figure 1, lane 1B). The findings of

other authors (Hemmens *et al.*, 1988) that streptavidin–HRP-amplified staining with CN was less sensitive than staining with alkaline phosphatase-labelled anti-IgE may be due to the use of Tween in combination with this substrate for HRP.

Metal-enhanced staining with DAB led to better results, but required long times (approx. 40 min) for development of the 'birch pollen' dots (Figure 1, lanes 2A and 2B). This is not consistent with the literature (Harlow & Lane, 1988) and may be due to the low concentrations of antigen and primary antibody in our assay system.

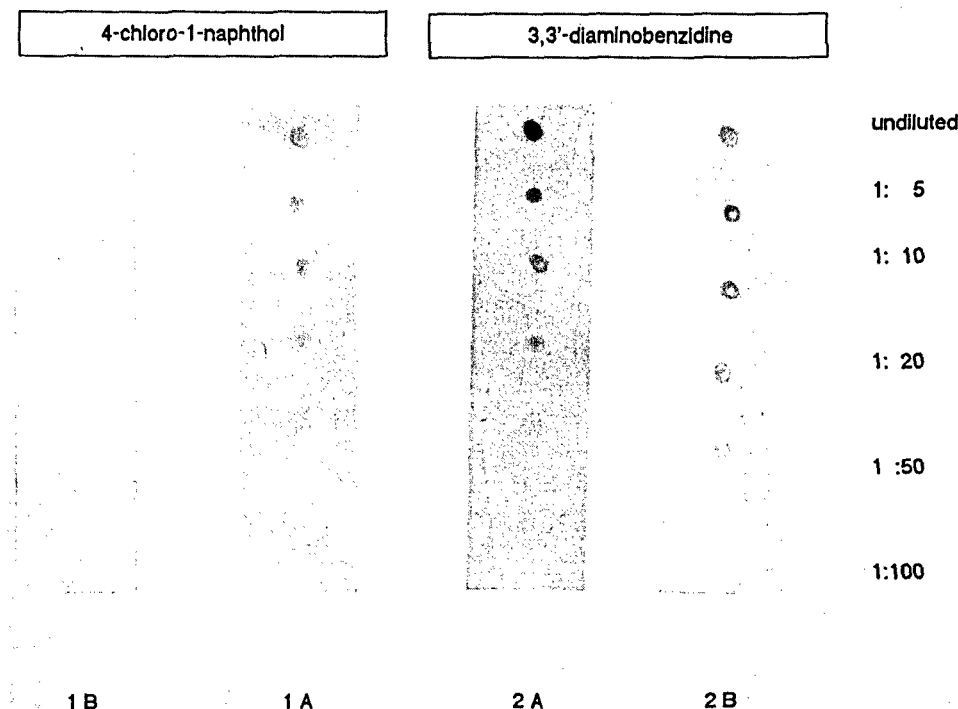


FIG. 1. Dot-blot assay for the optimization of HRP staining. For details see Materials and Methods. Substrates: (1) CN; (2) DAB/NiCl₂. Blocking and incubation buffer/washing buffer: (A) 3% BSA in TBS/TBS; (B) 0.05% Tween 20, 0.1% BSA in PBS/0.05% Tween 20 in PBS.

The formazan method (NBT/NADH) however, revealed sensitivities and staining times comparable to the DAB/NiCl₂ method (results not shown), whereas staining with TMB/DONS was more sensitive and faster than the other methods studied (Figure 2, lane D).

Detergent-free systems caused problems with high background staining (Figure 2, lane A). Blocking solutions with high amounts of BSA (systems A and C) yielded high backgrounds (Figure 1, lane 2A; Figure 2, lane A), possibly due to the occurrence of antibodies specific to BSA in the human pool serum used. These results prompted us to make no further attempts with blocking solutions containing food-relevant proteins (e.g. *Blotto* or gelatine). The best blocking results were obtained when high concentrations of Tween 20 and no proteins were included in the blocking solution (system D; Figure 2, lane D). PBS and TBS gave the same results. Buffers with higher ionic strength (0.5 M-NaCl, system F) did not improve the results. Stronger detergents (Triton X100, NP40, i.e. system C) led to a lower sensitivity which may be due to detachment of proteins from the NC membrane. The buffer and staining system of the

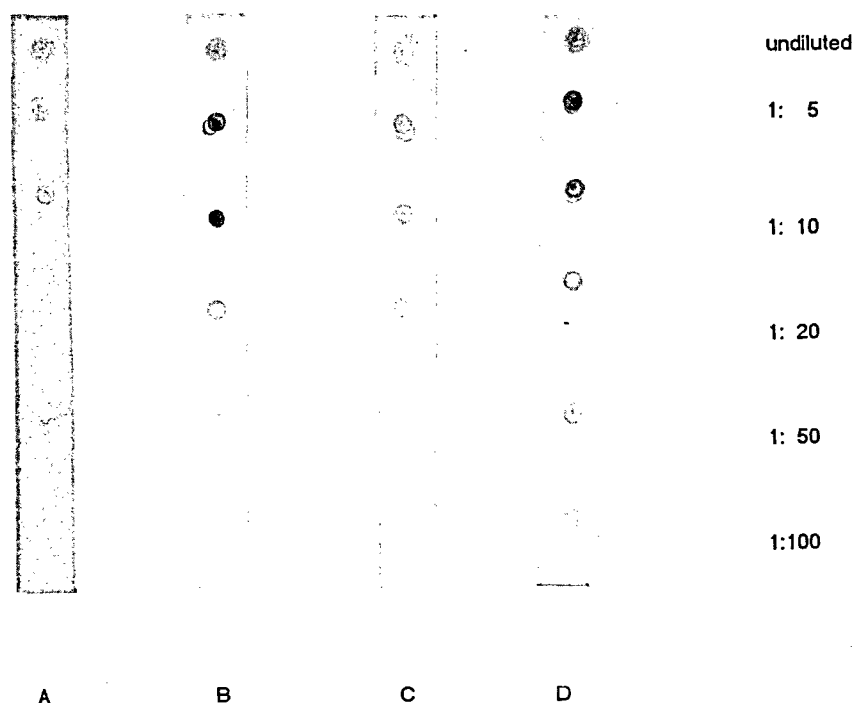


FIG. 2. Dot-blot assay for the optimization of HRP staining. For details see Materials and Methods. Substrate: TMB. Blocking and incubation buffer/washing buffer: (A) 3% BSA in TBS/TBS; (B) 0.05% Tween 20, 0.1% BSA in PBS/0.05% Tween 20 in PBS; (C) 3% BSA in PBS/0.01% Triton X100, 0.1% NP40 in PBS; (D) 0.5% Tween 20 in PBS/0.05% Tween-20 in PBS.

'optimized procedure' was designed with regard to the results of the dot-blot study: TMB/DONS was used for staining and the blocking solution contained high concentrations of Tween 20 and no proteins. As a stabilizing agent, 0.1% (w/v) BSA was added to the incubation buffer.

Non-specific binding of the immunoreagents. All anti-IgE-HRP conjugates used were checked for non-specific binding to food and pollen proteins using incubation buffer instead of serum during the first incubation period. Two of the conjugates showed strong binding to several food and pollen extracts. Figure 3 illustrates some drastic effects with the goat affinity-purified anti-IgE-HRP. Normal working dilutions (1:500–1:10 000) caused strong reactions of the conjugate with birch pollen extract and celery extract but not with olive tree pollen extract. Less than 25 ng of immobilized birch and celery protein led to the false positive results observed. The observed effect may be due to the incorporating of birch pollen and/or celery by the laboratory animals used for immunizations or by lectin-mediated interactions. However, for all of the following immunoblot experiments, only rabbit anti-IgE (DAKO, conjugated and unconjugated, from the same batch) was used.

The Use of Peroxidase-labelled Anti-IgE Antibodies Versus Biotin/Streptavidin-Amplified Detection. Specific IgG antibodies to food and pollen proteins were easily detectable by means of the optimized procedure described. However, the immuno-detection

ALLERGY TO FRUITS AND VEGETABLES

allergenic
extract

undiluted

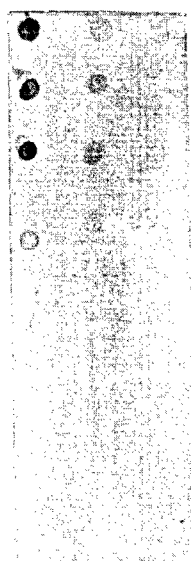
1: 5

1: 10

1: 20

1: 50

1:100



1:500

1:1000

1:10 000

anti-IgE dilution

FIG. 3. Dot-blot assay. Demonstration of non-specific binding of goat affinity-purified anti-IgE-HRP to birch pollen extract (left), celery extract (centre) and olive tree pollen extract (right). Staining was performed with TMB/DONS.

of specific IgE, especially in sera with RAST classes of less than 3 to food allergens revealed sensitivity problems when HRP labelled anti-IgE antibodies were used.

The use of an additional immuno-detection step (biotinylated tertiary antibody) and a biotin/streptavidin-HRP-based detection produced superior detectabilities and lower background levels. Streptavidin should be preferred over avidin because it is not glycosylated and has a more favourable *pI*, both resulting in a lower probability of non-specific interactions (Tijssen, 1985; Harlow & Lane, 1988). Even so, for some allergens (i.e. apple) binding of streptavidin-HRP to some proteins in the low molecular weight range <14 kD occurred, producing single non-specific bands.

Because of its obvious advantages, the amplified immuno-detection was used for all immunoblotting analyses of allergen specific IgE.

Applications

Detection of Apple-specific IgE Antibodies in Human Sera. Immuno-detection after SDS-PAGE and semi-dry-electroblotting was used to map the apple proteins that bind IgE antibodies from birch pollen- and apple-RAST-positive human sera. The antibody response observed showed a high degree of individuality (Figure 4) which seemed to depend on: (a) the sensitization of the individual patient; (b) the apple strain used for extract preparation; and (c) possibly the degree of ripeness of the apple fruit.

By means of our immunoblot studies we were able to detect important IgE binding bands in Granny Smith apples corresponding to molecular weights of approx. 8–16, 18, 35 and 50 kD (Figure 4, right lanes, sera A–F). The 18 kD band, which is recognized by many sera with IgE specific to birch pollen, seems to be of special importance with regard to the phenomenon of 'cross allergy' (Ebner *et al.*, 1991). In comparison to other studies (i.e. Ebner *et al.*, 1991) we found more 'allergenic' bands

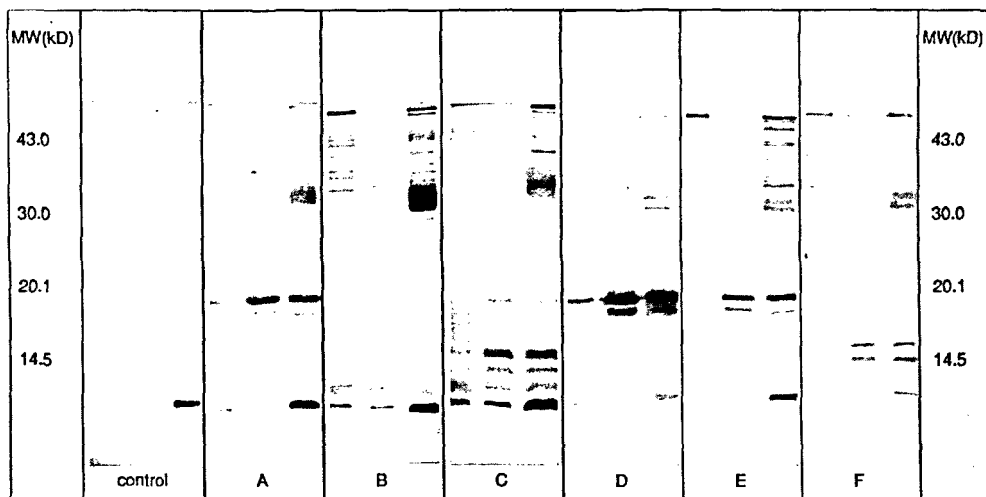


FIG. 4. IgE binding to Granny Smith apple extract (right lanes) and to extracts of mature (central lane) and unripe (left lane) Golden Delicious apple separated by SDS-PAGE and transferred to NC. Serum samples A–F were from patients allergic to birch pollen and apple. Control: incubation buffer was used instead of human serum.

in apple which, in our opinion, is due to the composition of the allergenic extracts used.

A possible influence of the state of maturity on the binding of patient IgE was studied with unripe (Figure 4, left lane) and mature (Figure 4, central lane of individual sera blots) Golden Delicious apples. Immunoblotting using the same amount of apple protein revealed much stronger binding of patient IgE to ripe than to unripe apples. Interestingly, in some cases antibody binding to different proteins in the two apple extracts occurred.

Our results reported in this paper were confirmed by further studies which will be presented elsewhere in more detail.

The quality of our method was examined by several control experiments. In addition to the reagent control (Figure 4, lane 'control') the specificity was checked by: (a) probing the blot with pooled and individual sera of non-allergic patients; and (b) with sera of allergic patients without hypersensitivity to apple (i.e. with grass pollen allergy). In some of these control experiments single bands occurred, but none of the control sera produced a reaction pattern comparable to that of the sera A–F (Figure 4). Furthermore, our immunoblot inhibition studies (see below) support the view that the results obtained are highly specific.

IgG and IgE Antibodies to Food and Pollen Allergens. Up to now, our knowledge of the role of specific IgG antibodies in type I allergic reactions is incomplete. With regard to food and pollen allergy, some important aspects have to be considered. Firstly, specific IgG antibodies to food and pollen proteins can be found in sera of allergic as well as non-allergic subjects. In the case of birch pollen proteins the IgG binding patterns of these two groups differed significantly. Sera of allergic patients contained specific IgG to more birch pollen proteins and showed stronger antibody binding than sera from non-allergic donors (Jarolim *et al.*, 1989). Furthermore, the role of IgG as 'blocking antibody' in connection with allergen immunotherapy (hyposensitization) is discussed

(Golden *et al.*, 1982; Fennerty *et al.*, 1988). Finally, in some cases IgG4 subclass antibodies have been found to be mediators of immediate type allergic reactions (Perelmutter, 1984; Halpern & Scott 1987).

The application of our method to IgE and IgG antibody detection is shown in Figure 5. An extract of Golden Delicious apple served as an example. Birch pollen-specific human pool serum Wn 13/32 (lane 1) as well as seven individual sera from persons with birch pollen allergy (lanes 2–8) and two non-allergic control sera were tested for their antibody binding patterns. All allergic sera showed IgG binding to one or more apple proteins. The importance of the 18 kD compound from apple for IgE binding can clearly be recognized with all sera except one (lane 8).

The IgG reactivities observed differed from the above findings. Although the 18 kD compound reacted with IgG from all sera except one, the 18 kD band was less abundant in relation to other IgG binding proteins (i.e. birch pollen pool, lane 1). Furthermore, IgE and IgG patterns were significantly different. One control serum showed only weak, and the other nearly negligible, IgG binding to apple proteins.

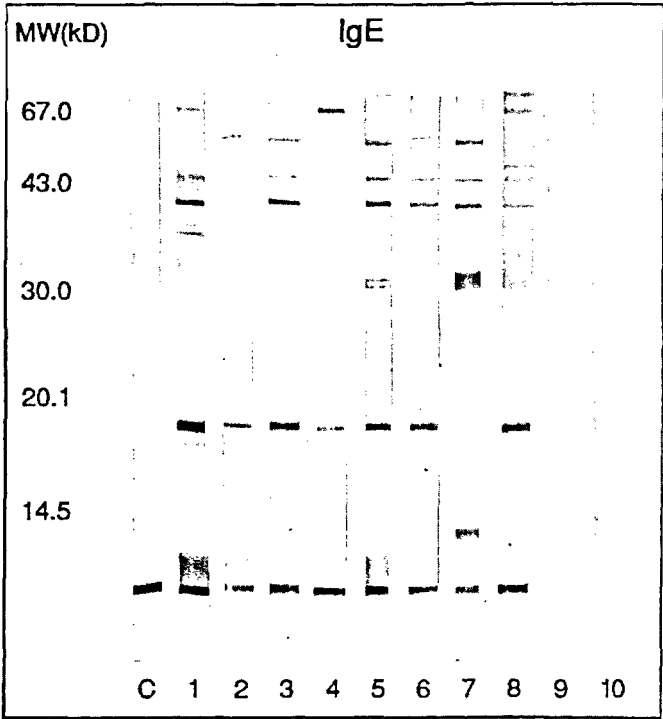
These results are comparable to the findings of Jarolim *et al.* (1989) with birch pollen immunoblots. In the case of Bet v I (the major allergen of birch pollen) it has been speculated that too low levels of specific IgG result in insufficient 'allergen trapping' and therefore may facilitate the induction of IgE antibodies, especially in persons with genetic susceptibility to atopic diseases (Jarolim *et al.*, 1989).

Immunoblot Inhibition Studies. The result of a typical immunoblot inhibition experiment is presented in Figure 6. The birch pollen-specific human pool serum Wn 13/32 was pre-incubated with increasing amounts of protein from birch pollen extract (0–100 μ g). The 'absorbed' serum sample was used for probing SDS-PAGE blots of apple extracts (left lanes: Golden Delicious; right lanes: Granny Smith). It can clearly be seen that some of the 'allergenic' bands from apple extract are quenched with increasing amounts of added pollen protein, thus indicating cross-reactivity between the two allergens. Special attention has to be drawn to the 18 kD band of apple, which probably represents a protein of high homology to Bet v I. (Ebner *et al.*, 1991).

In addition to such cross-reactivity studies the technique can be used to check the specificity of antibody binding in IgE immunoblots. An example for both types of application is shown in Figure 7. Figure 7B presents Golden Delicious immunoblots which were incubated with human pool serum Wn 13/32 without inhibition (lane 3), inhibited with 100 μ g of Golden Delicious protein (lane 4) and with 100 μ g of birch pollen protein (lane 1) in the same experiment. With apple protein, most of the bands were totally inhibited which indicated the specificity of the detection, whereas birch pollen extract quenched several individual bands which therefore could be identified as cross-reacting allergens. Figure 7A illustrates the reverse experiment: separated and blotted birch pollen proteins were incubated with the same pool serum (lane 3) and inhibited with 100 μ g of birch pollen protein (lane 1) and apple protein (lane 4). Although a strong self-inhibition was observed too, the heterologous inhibition with apple extract was nearly negligible. These results support the view that pollens act as the primary immunogens and that the observed cross-reactions may be caused by epitopes of food allergens with a lower association constant to pollen specific IgE.

Immunoblotting following Two-dimensional Electrophoresis. The potential of allergen characterization by a combination of 2D-electrophoresis and immunoblotting could be demonstrated with birch pollen extract. Figure 8A shows the total birch pollen proteins after 2D-PAGE and silver staining. The lane on the left side of the gel contained the low molecular weight marker proteins, separated in the second dimension by SDS-PAGE. In the 17 kD-range the existence of several isoproteins of Bet v I

(A)



(B)

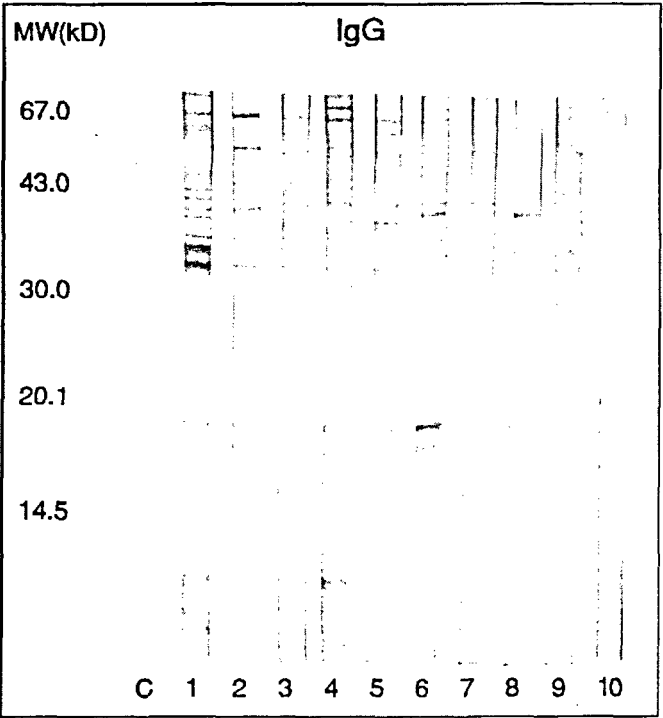


FIG. 5. IgE and IgG binding to extract of mature Golden Delicious apple separated by SDS-PAGE and transferred to NC. Human sera: (1) birch pollen pool Wn 13/32; (2-8) Individual sera from patients allergic to birch pollen and apple; (9-10) non-allergic control sera. Control: incubation buffer was used instead of human serum. (A) IgE binding. (B) IgG binding.

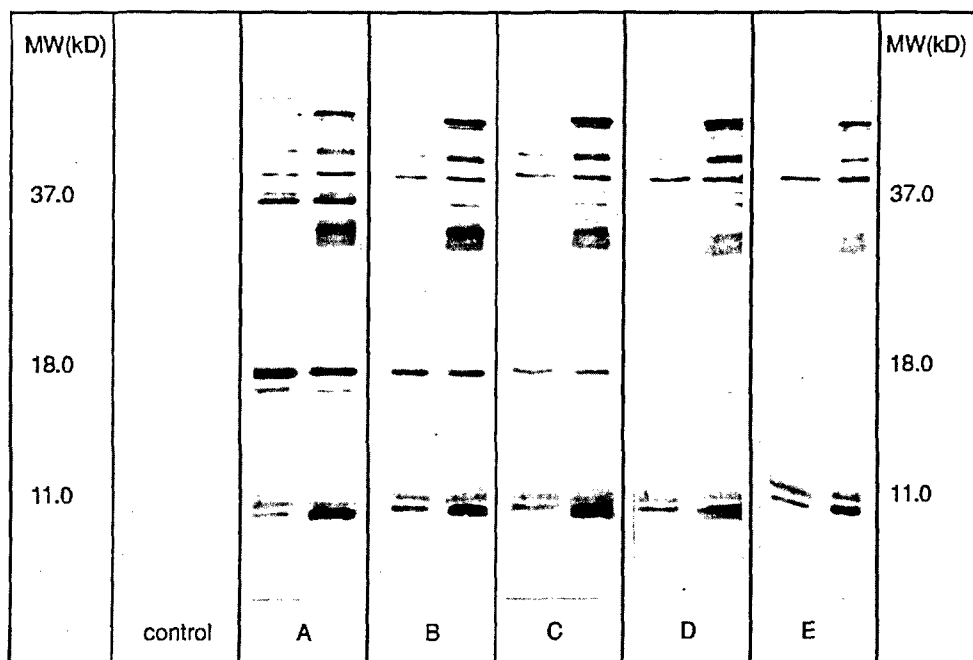


FIG. 6. Immunoblot inhibition experiment. Binding of IgE from human pool serum Wn 13/32 to separated and blotted apple allergens was inhibited by preincubation of serum with: (A) 0 μ g; (B) 5 μ g; (C) 20 μ g; (D) 50 μ g; and (E) 100 μ g of birch pollen protein. Right lanes: Granny Smith immunoblot; left lanes: Golden Delicious immunoblot.

can be recognized. After incubation with the human pool serum WA 2/50, the 2D-immuno-blot analysis revealed that these isoproteins and some other allergens of higher molecular weight bind specific IgE (Figure 8B).

These results confirm the work of other authors (i.e. Ipsen & Løwenstein, 1983; Jarolim *et al.*, 1989): Bet v I was found to consist of 10 spots in the *pI* range of 4.9–5.9.

CONCLUSIONS

The present investigation clearly demonstrates the power of IgE immunoblotting in combination with peroxidase staining for allergen identification and characterization.

In comparison to other studies of plant allergens (Calkhoven *et al.*, 1987; Hemmens *et al.*, 1988; Vallier *et al.*, 1988; van Toorenenbergen *et al.*, 1988; Jarolim *et al.*, 1989) it can be stated that the immunoblot method described produces very sharp bands of high resolution, requires low amounts of food protein for one single immunoblot analysis (5–7 μ g), and can be easily controlled, although only short staining times are necessary. Compared with common working dilutions (1:200–1:500) our detection system requires a very low anti-IgE concentration (1:4000) which lowers the probability of non-specific interactions. In addition, the use of an Ames test-negative substrate for HRP minimizes health risk.

The amplified TMB/DONS method has two overall disadvantages: (a) the immuno-detection procedure involves two more incubation steps which, in comparison to staining with enzyme-labelled secondary antibodies, results in a higher amount of

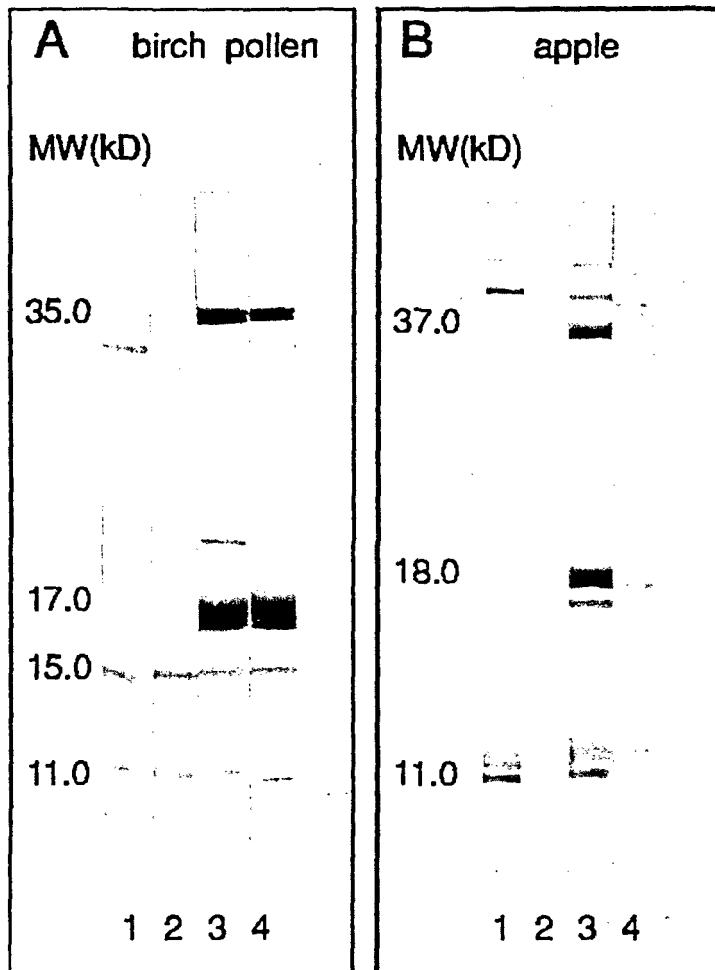


FIG. 7. Immunoblot inhibition experiment. Binding of IgE from human pool serum Wn 13/32 to birch pollen extract (A), and apple extract (B), separated by SDS-PAGE and transferred to NC, was inhibited by preincubation of serum with 100 μ g of protein from allergenic extracts. Lane 1: serum+birch pollen extract; lane 2: control without serum; lane 3: serum without inhibitor; lane 4: serum+apple extract.

laboratory work; and (b) since the coloured reaction product is not stable, the blots have to be documented immediately.

The high specificity of the results was sustained by control experiments with 'allergen-negative' human sera and by means of immunoblot inhibition studies. The latter was found to be a very useful tool for determining immunological cross-reactions of allergens.

Finally, some cautionary remarks:

—in some cases, IgE antibodies to food proteins can occur in sera of healthy persons (as reviewed in Aulepp & Vieths, 1992), which was also observed in our control experiments;

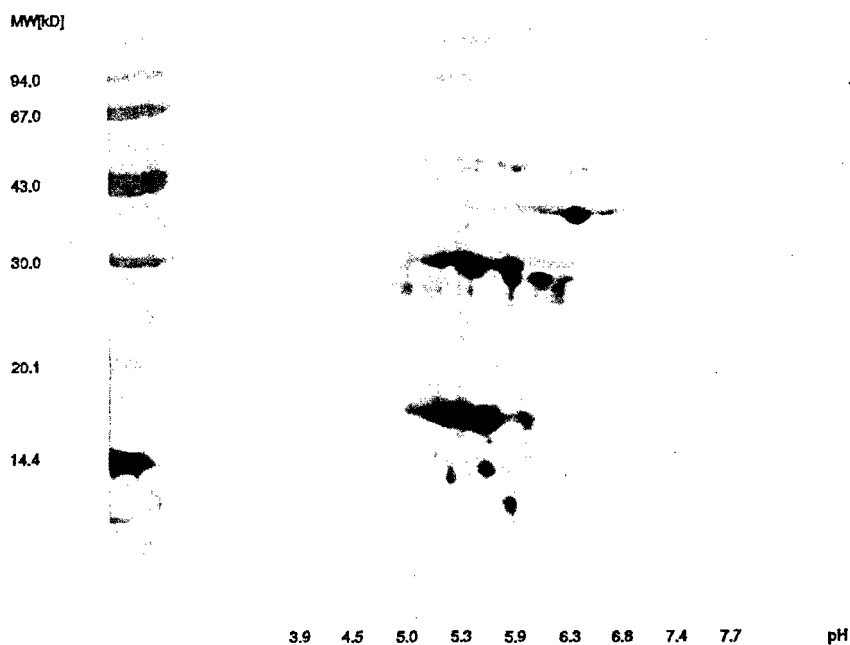


FIG. 8 (A). Two-dimensional electrophoresis of birch pollen extract was combined with immunoblotting. Total birch pollen proteins were made visible by silver staining. The lane on the left side shows the low molecular weight marker proteins.

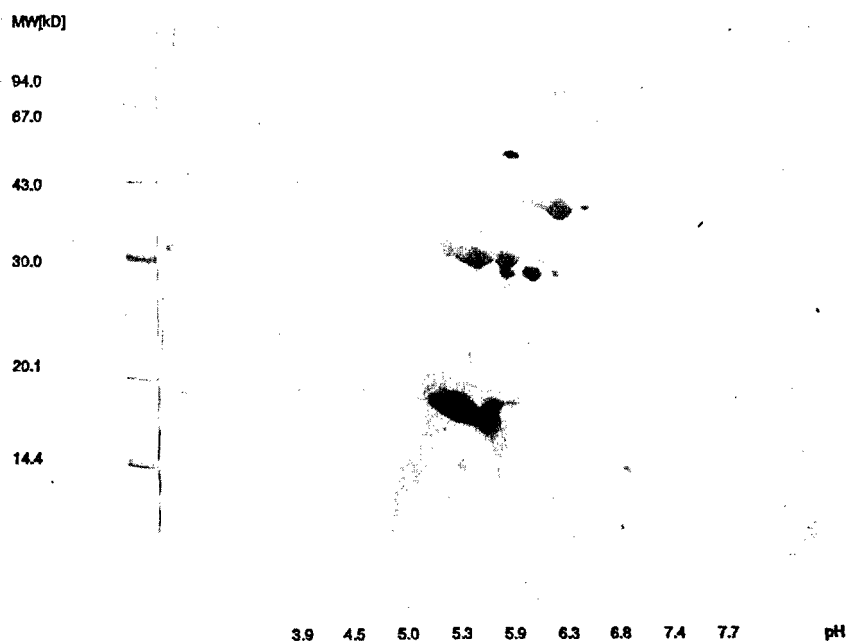


FIG. 8 (B). Binding of IgE from birch pollen-specific human pool serum WA 2/50 to birch pollen proteins after 2-D separation and transfer to NC.

- evident IgE binding to allergenic proteins may arise with sera from patients without symptoms, indicating a so-called 'latent sensitization';
- our immunoblot inhibition studies with apple extracts revealed that single IgE bands of some sera could not be quenched by self-inhibition with the apple extract used for blotting. This finding may be due to the occurrence of 'ubiquitous epitopes' for IgE produced under the dissociating and reducing conditions of SDS-PAGE. Because of their intact tertiary structure, the native proteins used as inhibitors possibly did not present these epitopes to IgE. The fact that these bands sometimes were detectable with apple-negative control sera gives some evidence for this interpretation. From our point of view, the high specificity obtained by inhibition of antibody binding to denatured and blotted proteins using native extracts as inhibitors was surprising. This finding may support the hypothesis that partial renaturation occurs with blotted proteins on NC membranes;
- with regard to the results of other authors our optimization studies revealed that no general procedure for IgE immunoblotting can be recommended. The design of the method seems to be highly dependent on the allergen under study and the detection system used. Critical attention has to be drawn to the quality of commercially available immunoreagents. Non-specific binding has to be carefully checked. In general, with regard to non-specific binding, the amount of blotted allergens as well as the concentration of immunoreagents should be as low as possible.

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