

Preparation of patient-related allergens for hyposensitization

Qualitative aspects

L. K. POULSEN, I. SØNDERGAARD* & B. WEEKE

Laboratory of Medical Allergology, Medical Dept. TTA, State University Hospital, Copenhagen, and *Chemical Institute, Royal Veterinary and Agricultural High School, Copenhagen, Denmark

An affinity chromatography method for preparation of patient-related antigens from commercially available allergen extracts has been investigated. IgG_{1,2,4} from a patient previously hyposensitized with dog hair and dandruff allergen was bound to protein A-sepharose. Secondly, commercial allergen extract was applied to the immunosorbent, and patient-related antigens were selectively absorbed by the specific antibodies from the patient serum. Finally, immune complexes containing antigen and IgG were eluted. Alternatively, the antigens alone were purified by retaining the IgG on the column by means of a covalent reinforcement of the protein A-IgG-binding. Purified, patient-related antigens were investigated in crossed immunoelectrophoresis and identity to IgG- and IgE-binding antigens (as determined by the CRIE-technique) was suggested. The affinity purified IgG was unaltered with regard to protein A-binding, binding of antigen and affinity for monoclonal antibody to human IgG-subclasses. Further, it was demonstrated that the antigen-binding capacity of IgG in the immune complexes was intact as evidenced by strong affinity to antigens even at low pH. The antigens eluted together with IgG were predominantly found in immune complexes with a molecular weight of > 300 kdalton equivalent to 2 or more molecules of IgG. The possibility of employing a similar method with IgE instead of IgG for preparation of patient-related allergens instead of antigens, is discussed.

Key words: affinity chromatography; allergens; hyposensitization; IgE; IgG; immunotherapy.

Accepted for publication 29 June 1988

CLINICAL ASPECTS

Allergen extracts used for immunotherapy (hyposensitization) of IgE-mediated diseases contain a large number of proteins. The IgE-response of the individual patient is often restricted to a more limited number. Thus, when an extract is used for immunotherapy, antigenic proteins hitherto unrecognized by the immune system are administered to the patient. From a theoretical point of view, it would be an advantage if only the individual, patient-related allergens were injected. This paper describes a model system, in which specific IgG-antibodies - developed during immunotherapy - are used for preparation of an extract matching the individual IgG immune response of the patient. The individual, patient-related antigens can be obtained in the form of immune complexes containing allergen and IgG of the subclasses 1 and 4. It is suggested that this form might be more efficient in suppressing the allergic symptoms. Based on previous works it is hypothesized that IgE from allergic patients could be utilized for preparation of minute amounts of patient-related allergens, with which an immunotherapy regimen can be initiated. This will in turn lead to a formation of IgG_{1,4} with specificities restricted to the actual allergens. By means of methods described in this paper, this IgG can subsequently be used for preparation of larger amounts of patient-related allergens for continuation of immunotherapy.

More than 50 years ago Cooke reported the development of blocking antibodies during immunotherapy of atopic disease (3), this immune response being independent of the reaginic antibody titre (4). Later investigators (15) have shown that these antibodies are of IgG-type, and lately the subclass distribution of the antibodies has been elucidated (6, 7). The role of IgG immune response during immunotherapy still has not been completely clarified, but several works indicate that high titres of IgG correlate to improvement of symptoms during the course of treatment (1, 6, 7). Furthermore, in an animal model it has been demonstrated that when hapten is coupled to autologous IgG, it has the capability to suppress the IgE-synthesis in mice (14). This spurred us to develop a method for preparation of immune complexes between IgG and allergens, to which the individual patient reacts (i.e. *patient-related allergens*) (21). In this study we have used sera from patients who have been hyposensitized with commercial allergen extracts. Thus, the present investigation concerns preparation and characterization of *patient-related antigens* and immune complexes thereof. The utilization of the methods in a total strategy for immunotherapy exclusively with patient-related allergens will be discussed.

MATERIAL AND METHODS

Sera

Sera from two patients (B.F.A. and A.G.) allergic to dog epithelium were applied. Both patients had been hyposensitized for 3 years with dog allergen extract (Alutard, ALK, Copenhagen) and presented titres of allergen-specific IgG₁ and IgG₄ as determined by the IgG-sucrose-RAST of our laboratory (8) (see description of method later). Serum A.G. was sampled during maintenance therapy, while serum B.F.A. was sampled about 4 years after cessation of treatment.

A pool of sera from blood donors from the State University Hospital blood bank was used as normal human serum (NHS). The individual

sera were negative when tested in IgE-RAST against pools of animal dandruffs, pollen and fungi (17).

Antisera

Antiserum against dog allergen extract was raised in rabbits (DAKO, Copenhagen, Denmark), and the IgG-fraction was isolated by means of ammonium sulphate precipitation (12).

Murine monoclonal antibodies against the four human IgG-subclasses were obtained as ascitic fluid from Unipath, Bedford, England (Code Nos. BAM15, BAM10, BAM08 and BAM11) (8). For the IgG-subclass RAST and CRIE, the monoclonals were diluted to 1:1000, 1:1000, 1:64000, and 1:64000, respectively.

Rabbit anti-mouse immunoglobulin was obtained from DAKOPATTS, Copenhagen, Denmark (Code No. Z 109), was purified by positive and negative immunoadsorption and radiolabelled as described (8). ¹²⁵I-labelled rabbit anti-human IgE was obtained from Pharmacia, Uppsala, Sweden.

Allergens

Dog hair and dandruff allergens were obtained from Allergologisk Laboratorium (ALK), Copenhagen, Denmark (Aquagen 553) or kindly donated by Dr. Roland Einarsson of Pharmacia AB, Uppsala, Sweden (Intermediary Product).

To improve detection of the extract components during different chromatographic procedures, allergen extracts were radiolabelled by means of modification of the Chloramin T method (11): To 300 µg of allergen extract in 100 µl of PBS were added 700 µCi of ¹²⁵I (sodium iodide, Amersham, England, Code No. IMS 30) and 50 µl (100 µg) of Chloramin T. After 60 s the reaction was stopped by addition of 50 µl (100 µg) of sodium metabisulphite. Desalination was performed on a Pharmacia PD-10 column (MEDA, Copenhagen, Denmark) in PBS containing 10 mg of Bovine Serum Albumin/ml.

Generally, the radiolabelled allergen extracts were 40–60% precipitable by trichloroacetic acid. To ensure that all extract components had

been labelled, the labelled extract was included in a crossed immunoelectrophoresis (see below). As shown in Fig. 4 a, b the precipitates of the unlabelled extracts could be redetected in the autoradiography.

The radiolabelled extracts included a fraction which showed a retention time longer than that of cytochrome C ($M_w \approx 13$ kdalton) when chromatographed on a Sephadex G-75 gel permeation column (cf. Fig. 3). This fraction was, however, not excluded by the PD-10 desalination, which in our hands excludes $> 95\%$ of low molecular iodination products. Further, the high-retention-time radioactive fraction coincided with a 280 nm absorbing fraction, when unlabelled allergen extract was chromatographed.

Crossed immunoelectrophoresis (CIE)

CIE was performed according to Højby & Axelsson (13) using 1% of agarose (HSA-grade, Litex, Denmark) in tris-barbital-buffer (0.024 M sodium barbital, 0.073 M tris and 0.01 M sodium azide, pH 8.6). In the first dimension 5 μ l of antigen was applied and run for 30 min at 10 V/cm. If labelled antigen was to be analysed, an appropriate volume of radioactive antigen was applied together with 5 μ l of unlabelled antigen. The second dimension was run at 2 V/cm for 18 h using 12 μ l/cm² of rabbit anti-serum against dog allergen extract. The plates were washed, pressed and dried with hot air (13), and stained with Coomassie blue or autoradiographed.

Crossed radioimmuno-electrophoresis (CRIE)

For determination of the IgE antibody response to individual allergens in an extract, the CRIE was used according to Weeke et al. (22): The CIE-plates were dried with cold air and incubated for 3 h with 400 μ l of serum in 7 ml of incubation buffer (phosphate buffer 70 mM, pH 7.0, sodium azide 15 mM), washed 4 times (saline) and incubated overnight with ¹²⁵I-anti-human IgE antibody (200,000 cpm). Finally, the plates were washed, dried with hot air and placed in an X-ray cassette for autoradiography.

IgG-subclass crossed radioimmuno-electrophoresis

IgG-subclass-CRIE was performed according to Søndergaard et al. (19): The CIE-plates were dried with cold air and incubated for 3 h with 360 μ l of serum in 7 ml of incubation buffer (glycine 0.1 M, sodium chloride 0.65 M, Tween 20 10 ml/l, sodium azide 15 mM, pH 9.2, bovine gammaglobulin 0.5% and Normal Rabbit Serum 10% (w/v)), washed 4 times (sodium chloride 0.65 M, Tween 20 1 ml/l, chlorohexidine acetate 20 mg/ml) and incubated for 3 h with monoclonal anti-IgG-subclass antibodies. Again the plates were washed 4 times and incubated overnight with ¹²⁵I-rabbit anti-mouse IgG (400,000 cpm) in 7 ml of incubation buffer. Finally, the plates were washed, dried with hot air and placed in an X-ray cassette for autoradiography. In the IgG-subclass-CRIE and the CRIE, NHS was tested parallel to the patient serum in order to ensure that no serum components bound nonspecifically to the CIE-plates.

IgG-subclass-RAST

This analysis was performed according to the principles described by Djurup et al. (8): To the test tubes ("Maxisorp", polystyrene tubes, 70 $\times$ 11 mm, NUNC, DK-4000 Roskilde, Denmark) were added 500 μ l of the allergen extract diluted to 10 μ g/ml, and the tubes were incubated overnight at room temperature. After coating, the tubes were washed three times (PBS). The sera to be tested were diluted 1:100 in buffer (sodium phosphate 0.1 M, sodium chloride 0.15 M, sodium azide 10 mg/l, EDTA 7.4 g/l, Bovine Gamma Globulin 5 g/l and Normal Rabbit Serum 10% (w/v), pH 7.0), and 500 μ l were added to the tubes and incubated for 3 h at room temperature. Then the tubes were washed three times, and 500 μ l of the relevant anti-IgG-subclass monoclonal antibodies were added and incubated for 3 h. After washing the tubes were incubated overnight with 500 μ l of iodine-labelled rabbit anti-mouse IgG (see below), and on the following day they were washed and counted in a gamma-scintillation counter.

Preparation of immunosorbents

Patient-IgG was covalently coupled to protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) by the following batch-wise procedure modified from Geersten & Marchalonis (9): 140 mg of protein A-Sepharose were allowed to swell for 2 h in 0.5 ml of PBS. The gel was precipitated, and 0.3 ml of serum A.G. was added after removal of the supernatant. After 15 min the gel was washed three times (PBS) and finally once with borate buffer (BB) (0.1 M, pH 8.0). 2 ml of dimethylsuberimidate (DMSI, from Sigma), 5 mg/ml in BB, were added and allowed to react for 30 min. Unreacted groups were hydrolysed by washing the gel thrice in PBS, and non-covalently bound IgG was removed by incubation overnight with glycine (0.1 M, pH 2.4) (9). The following day the gel was washed twice in PBS before addition of allergen extract. Testing of this and other immunosorbents was accomplished by incubating the radiolabelled allergen extract and the sorbent for 2 h. The bound radioactivity was determined after washing the sorbent 3 and 6 times in PBS and 3 times in glycine.

Alternatively, the procedure was performed continuously in a column according to Reeves and coworkers (18). By this method patient serum (0.5 ml) was applied to a protein A-sepharose column which was subsequently washed free from non-IgG components with triethanolamin-HCl (0.2 M, pH 8.5) for 2 h. Then DMSI (46.2 mg in 5 ml triethanolamin) was allowed to recycle for 5 h, and finally unreacted DMSI-groups were hydrolysed with PBS (pH 7.4) and non-covalently bound IgG removed with glycine buffer (0.1 M, pH 3.0).

Chromatography

Equipment: Columns for affinity chromatography were Bio-Rad (10 × 2.5 cm) (Richmond, CA). A peristaltic pump, P-3 from Pharmacia (Uppsala, Sweden) was used for flow control. The absorbance was determined with an UV-recorder (280 nm) Dual Path Monitor UV-2 from Pharmacia. Fractions were collected by means of a Frac-300 collector (Pharmacia).

Buffers: A phosphate buffer (0.02 M, sodium chloride 0.5 M, Thiomersal 0.005 %, pH 7.4) was used for application of samples and washing of the column. Elution was performed by means of the following buffers: acetate (0.1 M, pH 4.2), glycine (0.1 M, pH 3.0) or glycine (0.1 M, pH 2.4).

The principles of preparing patient-related antigens by means of affinity-bound patient-IgG have previously been described (21), and the conditions reported were subject to slight modifications only (see Results).

A Sephadex 75 fine (Pharmacia Fine Chemicals, Uppsala, Sweden) column (28 × 2.6 cm, flow rate 12 ml/h) was used for gel permeation chromatography of allergen extracts in the range of 10–80 kdalton, and a Sephacryl 400 Superfine (Pharmacia) column (90 × 2.6 cm, flow rate 12 ml/h) was used for IgG and immune complexes.

RESULTS

Preparation of patient-IgG-related antigens

To produce patient-related antigens devoid of IgG, a Sepharose-based immunosorbent was prepared. To accomplish this, we made a covalent reinforcement of the linkage between IgG and protein A-Sepharose that would not break when antigens were eluted at low pH.

Fig. 1 illustrates an experiment in which an immunosorbent with covalently coupled patient-IgG is compared with immunosorbents with affinity-bound patient-IgG or NHS-IgG. Also included is protein A-Sepharose itself. From the figure it can be seen that even though the sorbents containing specific IgG showed a higher binding of antigen, both the protein A-Sepharose itself and NHS adsorbed a considerable amount of radioactivity that was not removed by washing three times. Washing six times diminished both the specific and non-specific binding, and thus the radioactivity of sorbents with protein A and NHS still amounted to 30 % and 20 %, respectively, of the covalently bound patient-IgG. When serum B.F.A., which showed lower titres of antigen

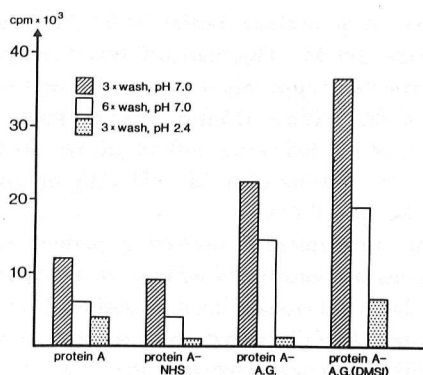


Fig. 1. Batch-wise utilization of immunosorbents. Four immunosorbents were prepared from protein A-sepharose and a) buffer b) NHS c) patient serum (affinity coupled) and d) patient serum (affinity and covalently coupled). The sorbents were incubated with radioactive allergen extract and washed 3 times with phosphate buffer, additionally 3 times with this buffer and finally 3 times with glycine buffer. The amount of remaining radioactivity was determined after each of the 3 washing procedures.

specific IgG₁ and IgG₄, was used for preparation of an immunosorbent, the radioactivity bound to immunosorbents with or without patient serum became almost indistinguishable. Washing the sorbents with low pH reduced the radioactivity, although it seems that protein A-Sepharose itself adsorbs some radiolabelled antigens rather strongly. The finding that some antigen could not be eluted from the covalently bound IgG-sorbent made from serum A.G. correlates with the finding that although it is possible to break the binding between protein A and IgG with low pH, the same does not apply to the IgG-antigen binding (see below).

Due to great difficulties in washing out unspecifically bound radiolabelled allergen extract from immunosorbents, as illustrated by the high amount of radioactivity bound to protein A- and NHS-immunosorbents in the experiment depicted in Fig. 1, we chose a chromatographic procedure. Two columns were prepared: one utilizing the affinity between IgG and protein A-Sepharose, the other reinforcing the binding by means of the bifunctional coupling agent dimethylsuberimidate. Fig. 2 shows

the results of application of equal amounts of labelled allergen extract on the two columns. After elution with PBS (pH 7.4) the pH was lowered by addition of acetate buffer pH 4.2 and later glycine buffer pH 2.4 to the column. In the figure the radioactivity has been normalized, i.e. the activity of each fraction has been expressed relatively to the total activity eluted from the column.

When the column is washed with buffer of neutral pH, the major part of the radioactivity is eluted. It appears from Fig. 2, that the initial peaks have "tails" due to interaction of the radioactive material with the chromatographic column. This tailing was mainly caused by interaction between allergen extract and protein A-sepharose, as it was also present when extract was applied to protein A-sepharose with omission of IgG. It can be seen that even when a peak is eluted at pH 4.2, it is possible to elute a substantial part of the activity at pH 2.4. Compared with the batch procedure, the affinity-bound IgG column produces a higher yield (Table 1) when the same amount of serum is used for the preparation. By means of this procedure it is possible to minimize the non-specifically bound allergen extract.

When neat radiolabelled allergen extract was applied to the covalently coupled IgG column, radioactivity but not light absorbing material was detected in the acid eluate due to the low amount of protein. This indicated that less than 0.1% of the covalently coupled IgG was coeluted at this pH, i.e. that no substantial hydrolysis of the protein A-DMSI-IgG covalent bindings had occurred. This estimate is based on a comparison with the absorbance observed when affinity-bound IgG was eluted from protein A-sepharose.

Characterization of patient-related antigen

To identify the antigens produced by the covalently coupled IgG-column, the eluted antigens were chromatographed on a gel permeation column (Fig. 3). From the figure it appears that a large amount of low-molecular weight radioactivity (see Material and Methods) contained in the labelled extract, has disappeared

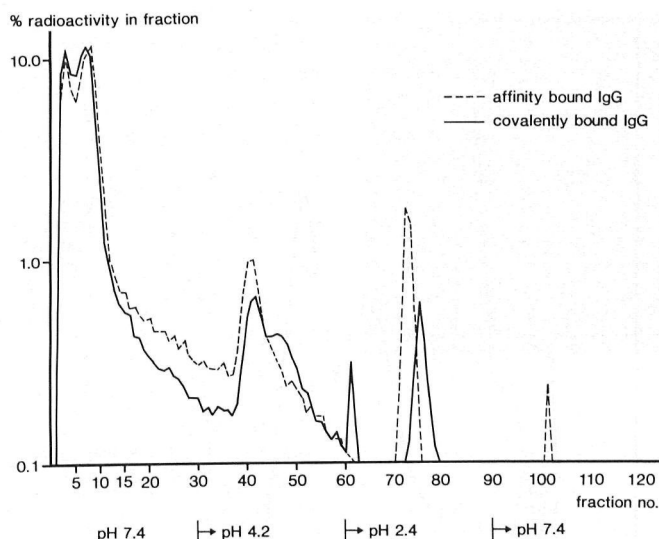


Fig. 2. Isolation of patient-related antigens by means of affinity chromatography. To an affinity-IgG and a covalent-IgG column (see text for explanation) was added radioactive allergen extract (5×10^5 cpm). The columns were eluted with neutral and low pH's and the radioactivity in the collected fractions was determined in a gamma-counter. The results have been normalized, i.e. the percentage of the totally eluted radioactivity appearing in one fraction.

in the patient-IgG immunosorbed antigen preparation. The high molecular weight fractions (> 10 kdalton) involving three partly separated peaks are now dominating.

Fig. 4 shows autoradiographs of crossed immunoelectrophoretic patterns of radiolabelled, native allergen extract (4.a) and purified patient-related antigen (4.c) both included in a CIE with native allergen extract. A Coomassie

staining of the CIE plate from which 4.c is made, is shown in Fig. 4.b. On the autoradiograph of the purified extract one precipitate only is visible, as opposed to 11 precipitates on the autoradiograph of radiolabelled native allergen extract.

Fig. 5 shows the IgE- and IgG₄-CRIE autoradiographs of the test serum. The CIE-plates for the CRIE were made with the native allergen extract. The IgE-response is directed against several precipitates, as opposed to IgG₄ which binds to one precipitate only. This precipitate appears to be identical to that of Fig. 4 c, i.e. the antigen purified with the patient serum. The two autoradiographs are, however, not identical, because a larger volume of antigen had to be applied for the CIE plate of Fig. 4.c. In both cases, the IgG₄-CRIE and CIE of labelled patient-related antigen, the radioactivity was associated with the largest of the precipitates. These radioactive precipitates had equal horizontal displacement from the well,

Table 1

Percentage of radioactive allergen extract eluted from columns of protein A-sepharose with affinity-bound IgG, covalently bound IgG and protein A-sepharose with no serum applied. See Legend to Fig. 2 for details

	Peak at pH 4.2 %	Peak at pH 2.4 %	Column total %
Affinity-bound IgG	6.4	4.2	10.6
Covalently bound IgG	5.6	1.9	7.5
Protein A-Sepharose	0.4	0.2	0.6

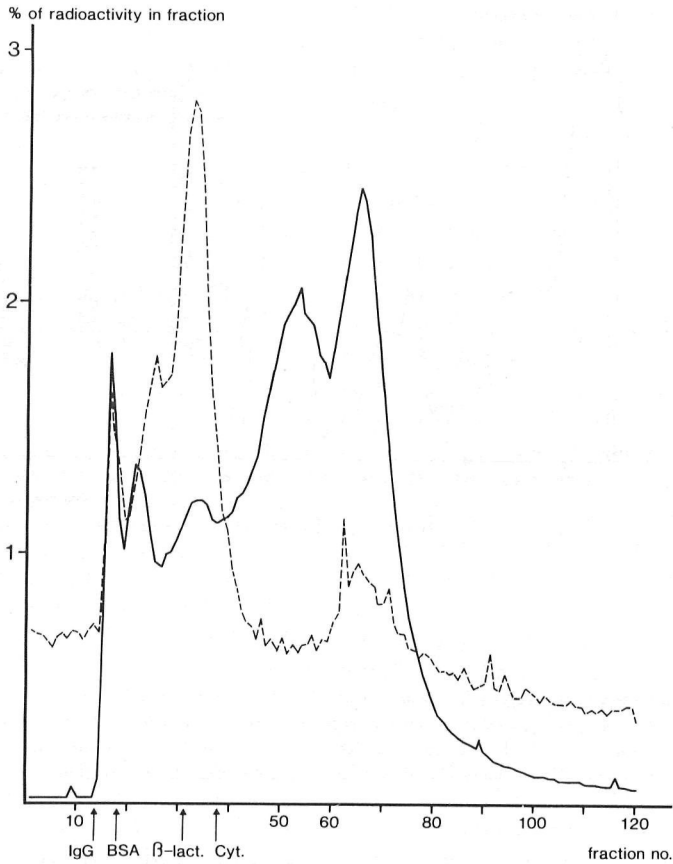


Fig. 3. Gel permeation chromatography (Sephadex G-75) of radiolabelled, native allergen extract (—) and patient-related antigen purified on a covalent-IgG column (---). The positions of molecular weight markers used for calibration of the column are indicated. The results have been normalized.

indicating that the proteins were of the same electrophoretic mobility. This suggests that the purified antigen is identical to the IgG₄-binding antigen in the IgG₄-CRIE.

Affinity-purified IgG

If immune complexes consisting of patient IgG and patient-related allergen/antigen should express a modifying effect on the immune system, the IgG-molecules should be functionally intact after the affinity chromatography. The antigen-binding capability of patient IgG after elution from protein A-Sepharose was investigated.

Patient serum was applied to the column, and the elution scheme was the same as described above. Fractions eluted from the column were collected, neutralized and analysed for antigen-binding activity in IgG-subclass-RAST for IgG₁ and IgG₄ (the serum did not contain antigen-specific IgG₂ and IgG₃). The absorbance at 280 nm shows that most serum components are eluted at neutral pH. However, measurements of RAST activity indicate that more than 90 % of the IgG₁ and 70 % of the IgG₄ RAST activity is recovered in fractions eluted at pH 2.4. Less than 10 and 25 % of the two subclasses are eluted at pH 4.2. No detectable RAST activity

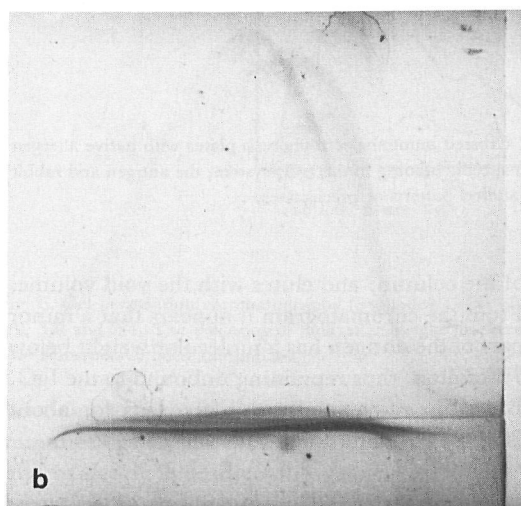
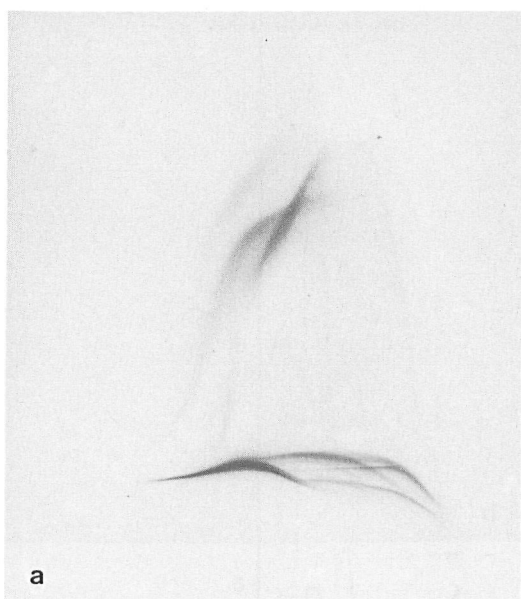


Fig. 4. Crossed immunoelectrophoresis of a) autoradiography of a CIE plate in which radiolabelled, native allergen extract was added to native extract; b) Coomassie Brilliant Blue staining of a CIE plate in which patient-related antigen purified on a covalent-IgG column was added to native extract; c) autoradiography of the CIE plate from b.

is eluted at neutral pH. When reappplied to the protein A-sepharose column, the eluted IgG bound quantitatively to the protein A (results not shown).

Immune complexes of IgG and antigen

When non-covalently bound IgG is eluted together with patient-related antigen, immune

complexes are formed. As most of the antigens have molecular weights below 80 kdalton, these immune complexes would be separated in IgG (150 kdalton) and antigens, if the complexes could be dissociated and chromatographed according to size.

Fig. 6 shows the results of experiments, in which immune complexes eluted from protein A-sepharose at pH 2.4 were rechromatographed

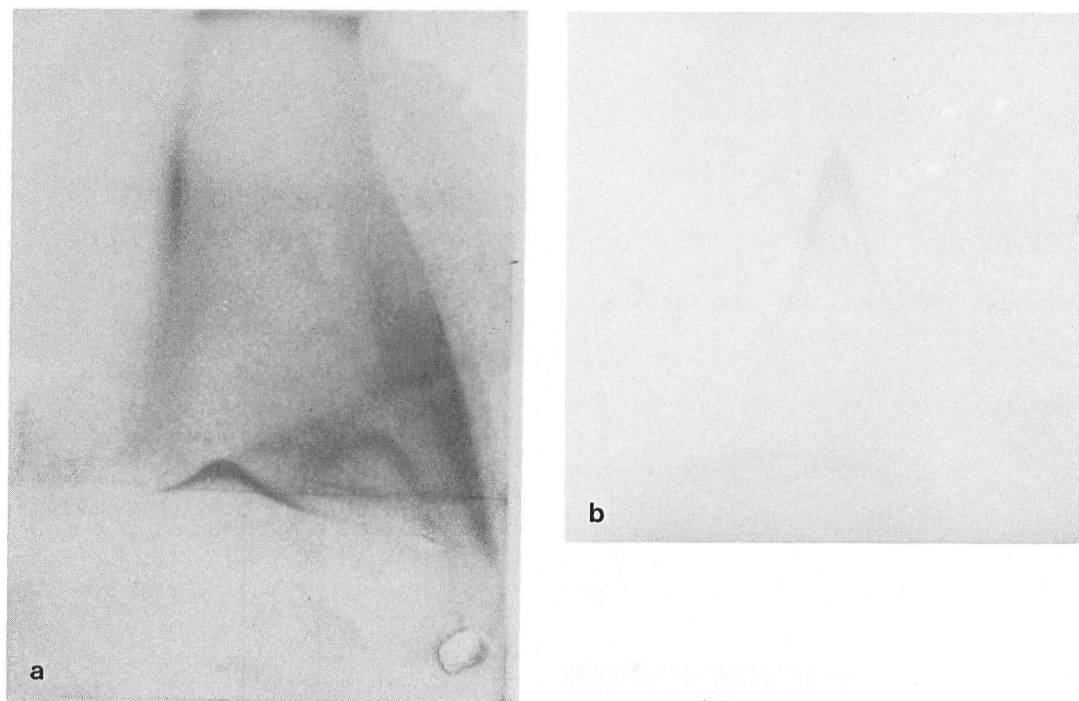


Fig. 5. IgE (a) and IgG₄ (b) crossed radioimmuno-electrophoresis. Crossed immunoelectrophoresis plates with native allergen extract were incubated with patient serum. In order to reduce non-specific binding in the IgG₄-system, the antigen and rabbit antibody for the CIE-plate were both diluted, thus offering an altered pattern of precipitates.

on a gel filtration column by means of pH varying between 4.2 and 2.4. From the figure it is seen that lowering of the pH causes an increasing dissociation of the immune complexes, but although the binding between IgG and protein A is completely broken at pH 2.4, a considerable amount of antigen is still bound to IgG.

Fig. 7 shows the results of gel filtration on a Sephacryl-400 column separating very large molecules. The components of the native, radio-labelled allergen extract have molecular weights below 150 kdalton, whereas the individual antigen preparation, i.e. the immune complexes, ranges broadly from 150 kdalton to more than 1,000 kdalton. This indicates that the antigens are bound in immune complexes with a varying number of IgG molecules. The peak early in the chromatogram probably reflects that some immune complexes are beyond the exclusion limit

of the column, and elutes with the void volume. From the chromatogram it appears that a minor part of the antigen has a molecular weight below 70 kdalton, thus remaining unbound to the IgG. Based on radioactivity this accounts for about 20 % of the antigen.

DISCUSSION

Previously, it has been demonstrated that it is possible to produce immune complexes containing patient-related allergen/antigen and patient IgE (20) or IgG_{1,2,4} (21). However, separation of the individual antigens from the immunoglobulin has not been possible. To establish the identity between the purified antigens and the ones causing the patient to react, the antigen molecules themselves should be purified.

In the present study we have prepared an

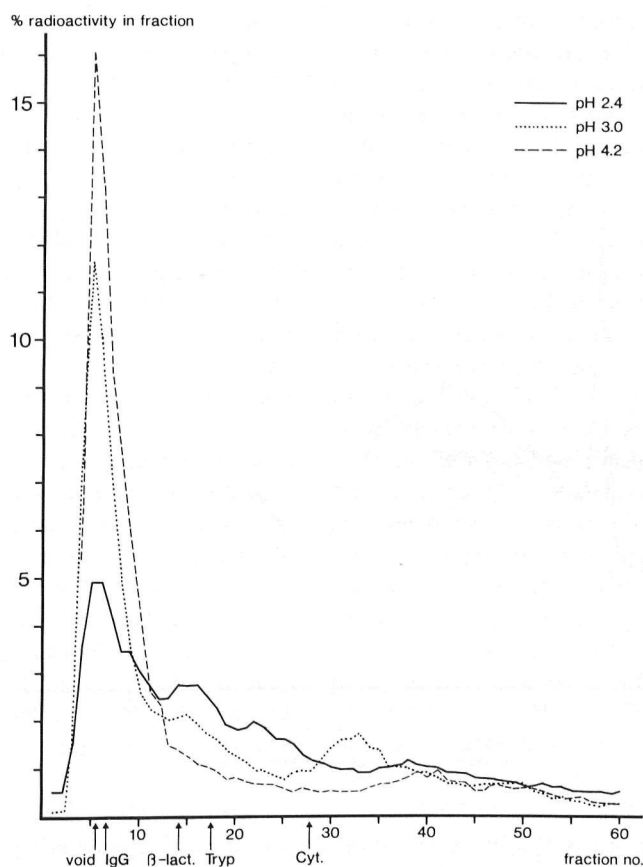


Fig. 6. Gel permeation chromatography (Sephadex G-75) of complexes between patient-IgG and radioactive antigens at pH 4.2, 3.0 and 2.4. The positions of molecular weight markers used for calibration (neutral pH) of the column are indicated. The results have been normalized.

immunosorbent, in which the IgG-molecules are covalently bound to the gel matrix. With the purpose of achieving the right orientation with $F(ab)_2$ pointing into the solution, we used protein A-sepharose as basis instead of cyanogen-bromide activated sepharose (16), which will orientate the immunoglobulins randomly. Protein A will bind IgG in the Fc part, thus leaving the antigen-binding site sterically free to bind ligands from the solution. IgG was "anchored" by means of the bifunctional agent dimethylsuberimide (5), which has been shown to crosslink protein A and IgG in batch procedures (9) and in column techniques (18). This was done in order to prevent IgG from

being eluted from the sorbent under dissociating conditions.

The results (Figs. 1 and 2) demonstrated that it was practicable to prepare an immunosorbent as described, but the batch-technique was discouraging due to a high non-specific binding of radiolabelled allergen extract to immunosorbents without patient serum. A residual amount of about 20% of radioactivity adhered to the patient-IgG-sorbent even after washing with low pH. This was also noted several times when patient-IgG was covalently coupled to a chromatographic column, although the residual antigen reached only 5–10% of the total radioactivity as estimated by means of a portable

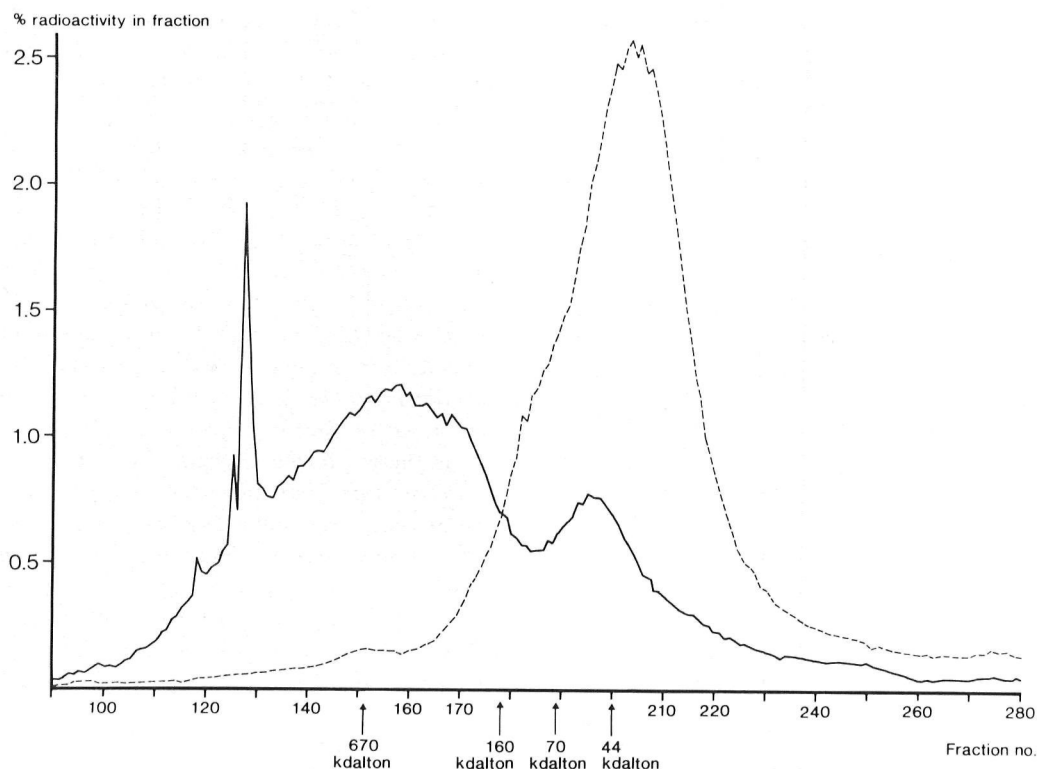


Fig. 7. Gel permeation chromatography (Sephacryl-400, pH 7.4) of native, radiolabelled allergen extract (----) and complexes between patient-IgG and radioactive antigens (—). The positions of molecular weight markers used for calibration of the column are indicated. The results have been normalized.

monitor. The radioactivity could be only slowly eluted even by buffers of low pH, and the reproducibility of the phenomenon suggests that the strong binding is not caused by reactive groups of DMSI. More likely, a minor part of the specific IgG expresses very high affinity towards the antigen, and more vigorous conditions will be needed to dissociate the IgG-antigen complex. This hypothesis is supported by the fact that when immune complexes are chromatographed at low pH, a substantial part of the antigen is bound to IgG (Fig. 6).

The batch procedure showed a considerable non-specific binding (Fig. 1). It is remarkable that protein A-sepharose itself shows higher non-specific binding than when coated with human IgG. This may reflect the blockage of binding sites on the gel matrix rather than any

affinity of protein A to allergen extract, as protein A without IgG is present in both experiments (calculated from the IgG-binding capacity of the protein A-sepharose gel as reported by the manufacturer). It was found that when conducting purification on a column, only a minor fraction of the radiolabelled extract was eluted from protein A-sepharose without serum (Table 1). This is contrasted by the batch-experiment, in which non-specifically bound extract could be removed from the protein A-Sepharose with buffers of both neutral and low pH.

When the column is eluted for a longer period, "bleeding" occurs. In the batch procedure this phenomenon is paralleled by the fall in activity when the immunosorbents containing specific antibodies are washed additionally three times. The polyclonal antibody

response towards the antigens must be considered to represent a broad spectrum of affinities, low affinity antibodies possibly causing the bleeding.

Fig. 3 shows that radiolabelled allergen extract includes a large part of the low molecular weight range (cf. Material and Methods). Apparently this part of the extract is not antigenic as the patient-related antigen shows little radioactivity at molecular weights below 10 kdalton. This is in accordance with Brandt & Yman (2) who found most of the allergenic activity of a dog dander extract in the molecular weight range of 10–70 kdalton.

The largest peak of the purified patient-related antigen appears at about 10 kdalton, and coincides with a smaller peak of the crude extract. As at least 10 different proteins are contained in the crude extract (Fig. 4 a, b), it is, however, not possible to further establish the identity between the peaks. From the chromatogram it appears that 3 peaks are detectable in the patient-related extract. The immunoelectrophoresis (Fig. 4 c), however, shows one single precipitate only, which corresponds to one precipitate in the IgG₄ crossed radioimmunoelectrophoresis autoradiograph (Fig. 5). We do not know the reason for this, but an explanation might be that the inclusion of radioactive antigen in a crossed immunoelectrophoresis and the IgG₄-CRIE have a sensitivity which is too low to detect labelled antigen and human antibody, respectively.

A comparison of the IgE- and IgG₄-antibody-response to the individual components of the crude allergen extract (Fig. 5) shows that the IgE-response covers more precipitates. This might be explained by a lower sensitivity of the IgG₄-CRIE as suggested above. However, the IgG-response during immunotherapy with grass pollen, moulds and insect venoms tends to be broader than the IgE-response, and can easily be determined by means of the IgG-subclass-CRIE (to be published).

The RAST activity of the IgG has been checked after elution from the protein A-column at low pH. As the RAST activity does not imply the IgG-molecule to be functionally intact, it tells us at least that the antigen-bind-

ing capacity of the molecule is undiminished. Furthermore, from Fig. 7 it can be seen that a major part of the radioactivity appears at molecular weights beyond that of IgG, which indicates that the antigen is bound in immune complexes. This is in conformity with the results presented in Fig. 6, which shows that even at very low pH a considerable part of the antigen is unable to dissociate from IgG. Both the monoclonal antibody in the IgG-RAST and protein A are able to bind to the patient-IgG after elution from the column, thus offering two indications that the Fc-part of the IgG molecule is unaltered by the treatment.

From the results we conclude that it is possible to produce patient-related antigens and immune complexes containing both the patient-related antigen and the immunoglobulin G of the patient. Recent studies suggest that polymerized allergens with high molecular weight and reduced ability of diffusion from the injection site have improved efficacy and reduced side effects compared with native allergen extracts (10). Immune complexes between IgG and allergen would be suspected to have the same properties and, further, the Fc-portions of the IgG-molecules might enhance opsonization by macrophages, thus improving presentation of allergen to immunocompetent cells in lymph-nodes.

In spite of our use of sera from hyposensitized patients, the yield of the preparations was very low when expressed in terms of per cent of total activity. This does not imply an equally low yield expressed in biological units, i.e. the amount of allergen relevant to the particular patient. Studies are in progress to elucidate this aspect.

The above procedure utilizing covalent coupling of immunoglobulin to the sorbent should be applicable to an anti-IgE column binding patient-IgE. So it would be possible to produce small amounts of patient-related allergen for initiating immunotherapy. During the first months of therapy the patient will develop an IgG-immune response restricted to the antigens used for hyposensitization, i.e. the actual allergens recognized by the patient's IgE. This IgG could subsequently be utilized for produc-

tion of either free patient-related allergen or immune complexes between IgG and allergen as discussed above. The method also offers the possibility to purify panels of individual allergens by means of sera from patients with monocomponent allergies, or different columns containing patient sera with only one allergen component in common.

ACKNOWLEDGEMENTS

The excellent technical and secretarial assistance of Ms. Lisbeth Abrahamsen, Ulla Minuva and Anne Larsen is greatly appreciated. L. K. Poulsen is a Research Fellow at the University of Copenhagen. This study was supported by the Dagmar Marshall Foundation and Merchant E. Willumsens Grant.

REFERENCES

- Aalberse, R. C., Dieges, P. H., Knul-Bretlova, V., Vooren P., Aalbers, M. & van Leuwen, J.: IgG4 as a blocking antibody. *Clin. Rev. Allergy* 1, 289-302, 1983.
- Brandt, R. & Yman, L.: Dog dander allergens. Specificity studies based on the radioallergosorbent technique. *Int. Arch. Allergy Appl. Immunol.* 61, 361-370, 1980.
- Cooke, R. A., Barnard, J. H., Hebal, S. & Stull, A.: Serological evidence of immunity with coexisting sensitization in a type of human allergy (hay fever). *J. Exp. Med.* 62, 733-751, 1935.
- Cooke, R., Loveless, M. & Stull, A.: Studies on immunity in a type of human allergy (hay fever): serological response of non-sensitive individuals to pollen injections. *J. Exp. Med.* 66, 689-696, 1937.
- Davies, G. E. & Stark, G. R.: Use of dimethyl suberimidate, a crosslinking agent, in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci.* 66, 651-656, 1970.
- Djurup, R.: The subclass nature and clinical significance of the IgG antibody response in patients undergoing allergen-specific immunotherapy. *Allergy* 40, 469-486, 1985.
- Djurup, R., Malling, H.-J., Søndergaard, I. & Weeke, B.: The IgE and IgG subclass antibody response in patients allergic to yellow jacket venom undergoing different regimens of venom immunotherapy. *J. Allergy Clin. Immunol.* 76, 46-55, 1985.
- Djurup, R., Søndergaard, I., Magnusson, C. G. M. & Weeke, B.: A three-layer immunoradiometric assay for determination of IgG subclass antibodies in human sera ("IgG subclass RAST"). *Allergy* 39, 51-63, 1984.
- Geersten, D. M. & Marchalonis, J. J.: A rapid, novel method for the solid-phase derivatization of IgG antibodies for immune-affinity chromatography. *J. Immunol. Methods* 24, 305-309, 1978.
- Grammer, L. C., Shaughnessy, M. A. & Patterson, R.: Modified forms of allergen immunotherapy. *J. Allergy Clin. Immunol.* 76, 397-401, 1985.
- Greenwood, F. C., Hunter, W. M. & Glover, J. S.: The preparation of ¹³¹I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114-123, 1963.
- Harboe, N. M. G. & Ingild, A.: Immunization, isolation of immunoglobulins and antibody titre determination. *Scand. J. Immunol.* 17, 345-351, 1983.
- Højby, N. & Axelsen, N. H.: Crossed immunoelectrophoresis as modified for quantitative purposes. *Scand. J. Immunol.* 17, 125-134, 1983.
- Lee, W. Y. & Schon, A. H.: Suppression of reaginic antibody formation. III. Relationship between immunogenicity and suppression of tolerogenicity of hapten-carrier conjugates. *J. Immunol.* 116, 1711-1718, 1976.
- Lichtenstein, L. M., Holtzman, N. A. & Burnett, L. S.: A quantitative in vitro study of the chromatographic distribution and immunoglobulin characteristics of human blocking antibody. *J. Immunol.* 101, 317-324, 1968.
- Porath, J.: General methods and coupling procedures. In Jacoby, W. B. & Wilchek, M. (eds.): *Methods in enzymology*, vol. XXXIV: Affinity techniques. Enzyme purification, Part B. Academic Press, New York, 1974.
- Poulsen, L. K. & Weeke, B.: Aluminium hydroxide adsorbed allergens used in modified RAST. *Allergy* 40, 405-416, 1985.
- Reeves, H. C., Heeren, R. & Malloy, P.: Enzyme purification using antibody crosslinked to protein A agarose: Application to Escherichia coli NADP-isocitrate dehydrogenase. *Anal. Biochem.* 115, 194-196, 1981.
- Søndergaard, I., Djurup, R. & Weeke, B.: Characterization of IgG-subclass antibodies to individual allergens by crossed radioimmuno-electrophoresis. *Allergy* 39, 622-629, 1984.
- Søndergaard, I. & Weeke, B.: Purification of patient-related allergens by means of bio-affinity chromatography on a sepharose-anti IgE-patient IgE immunosorbent. *Allergy* 39, 473-479, 1984.
- Søndergaard, I. & Weeke, B.: Isolation of patient-related antigens from allergen extract by means of protein A-sepharose-patient IgG_{1,2,4} bio-affinity chromatography. *Allergy* 39, 551-559, 1984.
- Weeke, B., Søndergaard, I., Lind, P., Aukrust, L. & Löwenstein, H.: Crossed radioimmuno-electrophoresis (CRIE) for identification of allergens and determination of the antigenic specificities of patients' IgE. *Scand. J. Immunol.* 17, 265-272, 1983.

Address

Lars K. Poulsen, M.Sc.

Laboratory of Medical Allergology

Medical Dept. TTA 7542

Rigshospitalet

Tagensvej 20

DK-2200 Copenhagen N

Denmark

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.