

Isolation of Patient-Related Antigens from Allergen Extracts by Means of Protein A-Sepharose-Patient IgG_{1,2,4} Bio-Affinity Chromatography

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A method is described for bio-affinity purification of patient-specific IgG reactive antigens by using protein A-sepharose allergic patient serum and the corresponding antigen/allergen extract. The optimal conditions for each step have been determined and it is shown that the non-specific binding of both serum proteins and antigens can be kept at very low levels by a high ionic strength. The specificity of the reaction has been shown by biospecific elution of ¹²⁵I-labelled antigens by non-labelled antigens and also by comparison of the amount of antigen bound by sera from hyposensitized patients with that from non-atopic individuals. It is suggested that the isolated antigen-IgG-immune complexes could be used for a specific and active immunotherapy.

Key words: affinity chromatography; immunotherapy; protein A-sepharose; specific IgG.

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CLINICAL ASPECTS

Evidence from different sources indicates that IgG plays an immunoregulatory role on the cellular level and does not merely function as a blocking antibody. Immune complexes of IgG and antigen could function in immunotherapy by eliciting tolerogenic signals to B and/or T cells and thus abrogate IgE synthesis without interfering with ongoing IgG synthesis. The present paper describes a method of producing patient-specific IgG_{1,2,4}-antigen immune complexes by affinity chromatography for use in more effective immunotherapy of allergic diseases in the future.

Patients with allergic reactions to grass pollen, animal hair and dander, house dust mite and other allergen sources will react by formation of specific IgE and IgG antibodies against a few of the several antigens which can be detected by crossed immunoelectrophoresis (CIE) and crossed radio immunoelectrophoresis (CRIE) (1, 14, 20, 21, 27, 28).

Different patients react to different antigen combinations in a particular allergen extract, but in a way that reactions are seen more often to some allergens (major allergens) than to others (minor allergens). For both diagnostic

and therapeutic reasons it would be of value to isolate these patient-specific allergens. It has been shown (18, 19) that patient IgE can be used in an anti-IgE-patient-IgE-allergen affinity chromatographic system to isolate patient specific allergens from a complex extract, but the yield in terms of µg allergen/ml serum is low. However, as there is a greater quantity of IgG in serum than IgE it seemed tempting to try to use an affinity chromatographic system based on IgG instead of IgE. Firstly, this could solve the problem about similarities and/or differences in the reactivity of IgG and IgE to the

different antigens in an allergen extract and secondly, if similarities in the reactivity were found, the system could be used for diagnostic and therapeutic purposes.

The use of protein A-sepharose as immunosorbent for isolation of human IgG_{1,2,4} is well known (7, 8). Furthermore, protein A-sepharose has been used both for radioimmunoassay of blocking antibodies (12) and for the detection of anti-IgE antibodies in human serum (11). Recently, a method has been devised for immobilizing antibodies on protein A-sepharose by a cross-linking agent. The immobilized antibody has then been used to isolate the corresponding antigen (16) instead of cross-linking the IgG molecules to cyanogen bromide activated sepharose or other activated material. The advantage of protein A-sepharose as an immunosorbent is that the binding of antibodies occurs through the F_c part of the antibody, thus leaving this in proper orientation for combination with antigen (13).

The purpose of the present study was to show that bio-affinity chromatography on a protein A-sepharose immunosorbent can be used to isolate allergens to which a certain patient reacts with IgG_{1,2,4}, and that undesirable non-specific binding of both serum proteins and allergens could be kept at very low levels. The applications of the method will be discussed.

MATERIAL AND METHODS

Analytical methods

Crossed immunoelectrophoresis (CIE), rocket immunoelectrophoresis (RIE), and fused rocket immunoelectrophoresis (FURIE) were performed as described previously (17, 24, 25). Total IgE was determined by the paper radio immunosorbent test (PRIST) from Pharmacia (Uppsala, Sweden). Al-RAST was performed according to Weeke (26).

Antigens

Standardized aqueous extracts of timothy pollen (*Phleum pratense*), 3 mg/ml, and of the house dust mite (*Dermatophagoides pteronyssinus*), 4 mg/ml,

were obtained from Allergologisk Laboratorium (ALK) A/S (Copenhagen, Denmark). Both extracts were without added preservative and kept frozen until use. The extracts contained a number of antigens including all the major allergens as measured by CIE and crossed radio immunoelectrophoresis (CRIE).

Antibodies

Rabbit antibodies against the allergen extracts were prepared by immunizing rabbits for more than 1 year with the standardized extracts according to Harboe & Ingild (5). Monospecific antibodies raised in rabbits against different human serum proteins were obtained from DAKO A/S (Copenhagen, Denmark).

Labelled antigens and antibodies

¹²⁵I-labelled anti-human IgE was obtained from Pharmacia (Uppsala, Sweden) and the timothy pollen extract was labelled by the chloramine T method (9). The labelling of the pollen extract was controlled by CIE followed by autoradiography. An amount of the labelled extract corresponding to 10⁵ cpm was mixed with the amount of non-labelled extract normally used for CIE as a carrier for the labelled antigens, and CIE was performed in the usual manner using rabbit antibodies against timothy pollen antigens. The washed and dried immunoplate was then placed on an X-ray film for 14 days for autoradiography.

Immunosorbent

Protein A-sepharose C1 4 B was obtained from Pharmacia (Uppsala, Sweden).

Test sera

Sera from 1,000 donors were pooled and used as a normal reference serum together with sera from non-atopic individuals. The serum from the 1,000 donor pool contains 80 U/ml IgE and 10 SU/ml (class 2) specific IgE to grass pollen and 5 SU/ml (class 2) specific IgE to house dust mite as measured by the Al-RAST method (26).

Serum from a patient (H.D.) allergic to timothy pollen was used in the experiments. The patient is a 26-year-old female allergic to grass pollen and for 6 years preseasonally treated with Allpyral® 5-grass-mix. Her serum contains 945 U/ml IgE as determined by the PRIST technique and 214 SU/ml (class 4) as determined by the Al-RAST method. This serum has been kept frozen for several years and is used as an allergic control serum.

Apparatus

The chromatographic experiments were performed in 5 ml plastic syringes and the flow through the column was controlled by a multi-flow roller pump from Ole Dich Instrument-makers (Hvidovre, Denmark). The fractions were collected in a fraction collector from LKB (Bromma, Sweden). Radioactivity was measured in a gamma-counter from Palle Medicoteknik (Copenhagen, Denmark).

Chemicals

All chemicals were of analytical grade from E. Merck (Darmstadt, G.F.R.) and used as received. Solutions of sodium thiocyanate should be freshly prepared. Phosphate buffer pH 7.0, 0.07 M contained 1.5 mM sodium azide to prevent microbial growth.

Principle

Protein A-sepharose packed in a column was used to immobilize human IgG_{1,2,4} by reaction with the F_c part of the IgG. Other serum proteins were washed away and the immuno-

sorbent was incubated by recycling with an antigen extract. Surplus of antigen was then washed away and the IgG-antigen complexes were eluted from the column by lowering the pH to 2.5, or by the use of chaotropic ions (see also Fig. 1). The eluate was collected in a small amount of concentrated buffer solution to bring the pH to neutral immediately after elution.

Procedure

All the experiments were conducted at room temperature. Five ml of protein A-sepharose were packed in a 5 ml plastic syringe with a piece of filterpaper and some glass beads on the bottom of the column. The column was then equilibrated with phosphate buffer pH 7.0, 0.07 M containing 0.5 M NaCl, and 3 ml of serum was recycled for 1 h at 25 ml/h. The column was washed with 6 column volumes of buffer and the allergen extract, usually 1 ml, containing 4×10^5 cpm labelled antigen or 3 mg/ml non-labelled antigen, was recycled for 2 h. After washing with 6 column volumes of buffer the IgG-allergen complexes were eluted with 6 column volumes of acetate buffer pH 2.5, 0.05 M containing 0.5 M NaCl. Finally, the column was washed with 6 column volumes of phosphate buffer pH 7.0, 0.07 M containing 0.5 M NaCl and 1.5 mM NaN₃. The eluate was analysed by RIE for content of IgG and other serum proteins, and FURIE was used for screening of the allergen content. Finally, the allergen-containing fractions were analysed by CIE with intermediate gel containing anti-human IgG in order to split the immune complexes.

RESULTS

Binding of IgG and other serum proteins to the immunosorbent

The binding of IgG was shown to be maximum after 1 h of recycling at 25 ml/h as measured by RIE using rabbit antibodies against human IgG. The washing procedure was evaluated by measuring both IgG as well as albumin in the effluent from the column as a marker for non-

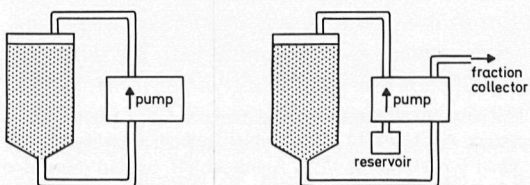


Fig. 1. Schematic drawing of the affinity chromatographic experiments.

specific binding. This showed that both IgG and albumin reached undetectable levels after washing with 6 column volumes. The non-specific binding of other serum proteins could be kept below 1% when 0.5 M NaCl was added to the incubation buffer. Albumin was shown to be a good marker of non-specific binding, thus RIE, using rabbit antibodies against human albumin, could be used as control of the non-specific binding in the experiments. The omission of NaCl in the buffer considerably increased the non-specific binding, indicating that the non-specific binding was due mainly to ionic interactions, probably to the bound IgG.

Binding of antigen to the sepharose-protein A-IgG matrix

a) *Labelled grass pollen antigens.* Control of labelling by the use of CIE showed that all the antigens detected by CIE were also labelled (Fig. 2). The time needed for maximum binding of antigens as well as the length of the washing procedure were determined by using the labelled antigens. It was shown that maximum binding was achieved in 2 h and that 6 column volumes of buffer were needed to remove non-bound antigen.

The results from typical experiments using serum from an allergic hyposensitized patient (H.D.) and normal control serum are shown in Fig. 3, and were shown to be highly repro-

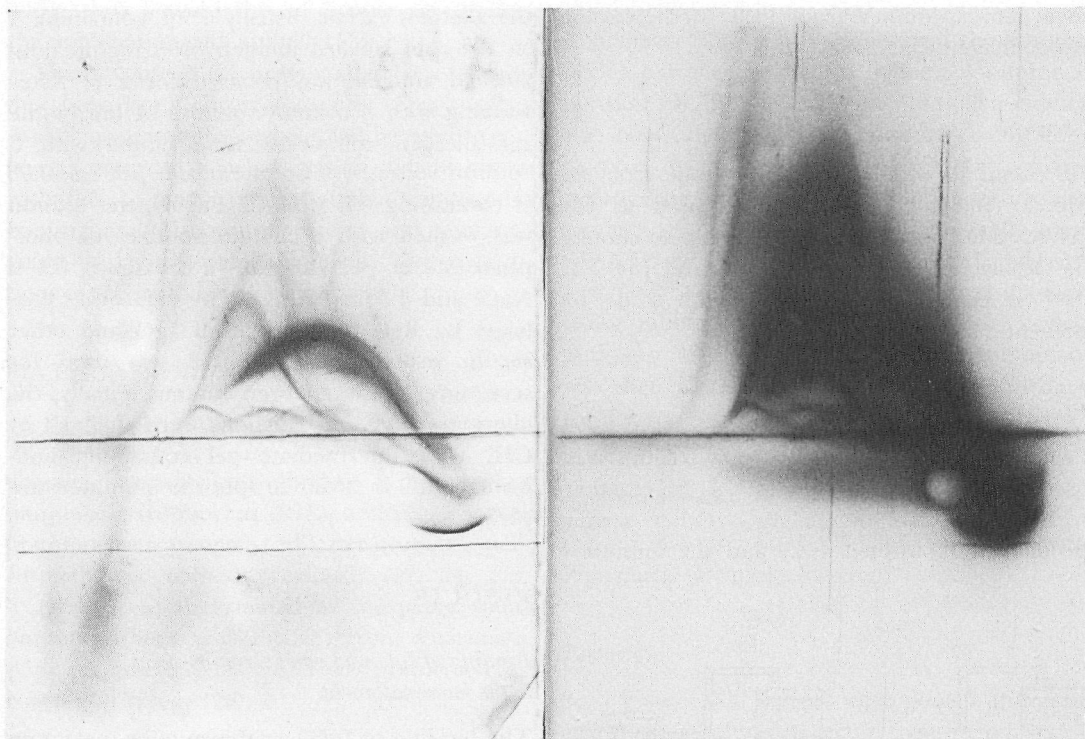


Fig. 2. Crossed immunoelectrophoresis of non-labelled and ^{125}I -labelled timothy pollen antigens. Left: CIE of non-labelled timothy pollen antigens. Antigen: 10 μl 3 mg/ml timothy pollen extract. Antibody: 12 $\mu\text{l}/\text{cm}^2$ rabbit antibodies against timothy pollen antigens. Conditions: First dimension electrophoresis 10 V/cm for 30 min at 15°C. Anode at left. Second dimension electrophoresis 2 V/cm for 18 h at 15°C. Anode at top. Right: CIE of labelled timothy pollen antigens. Antigen, antibodies and conditions as above except that labelled timothy pollen antigens 10^5 cpm were added to the non-labelled antigens and that after electrophoresis the immunoplate was placed on an X-ray film for autoradiography for 14 days.

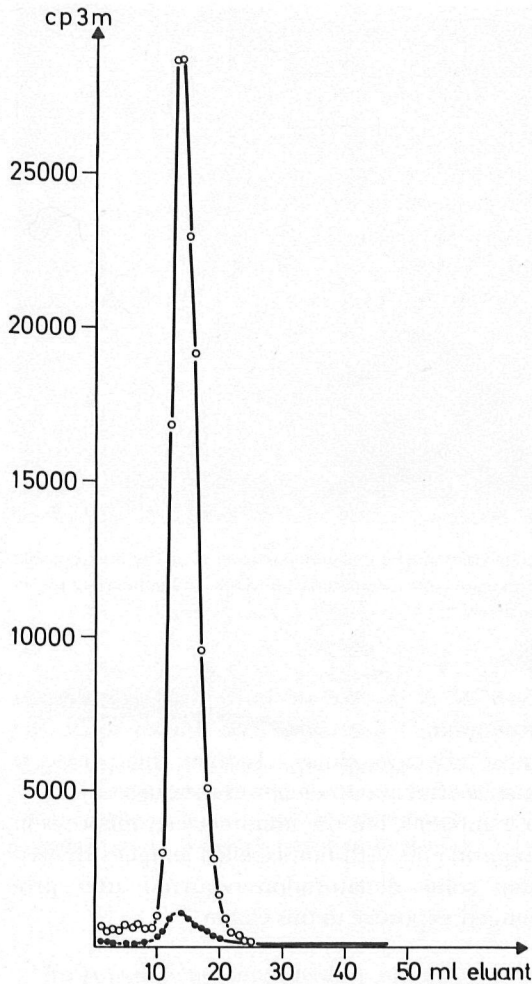


Fig. 3. Elution profile from a protein A-sepharose column incubated with serum, washed, incubated with labelled timothy pollen antigens, washed and finally eluted at pH 2.5. Column: 5 ml protein A-sepharose. Sera: 3 ml serum from an allergic hyposensitized patient (O) or 3 ml serum from a non-atopic individual (●). Antigens: ^{125}I -labelled timothy pollen antigens 4×10^5 cpm. Eluant: Acetate buffer pH 2.5, 0.05 M containing 0.5 M NaCl.

ducible. The amount of bound and eluted antigen when using normal serum was shown to be 5% that of the amount when using patient serum. In order to test further the specificity of the binding an attempt was made to elute, biospecifically, the labelled antigen by non-labelled antigen, followed by elution at low pH. The results are shown in Fig. 4. This showed that non-labelled antigens slowly elute labelled anti-

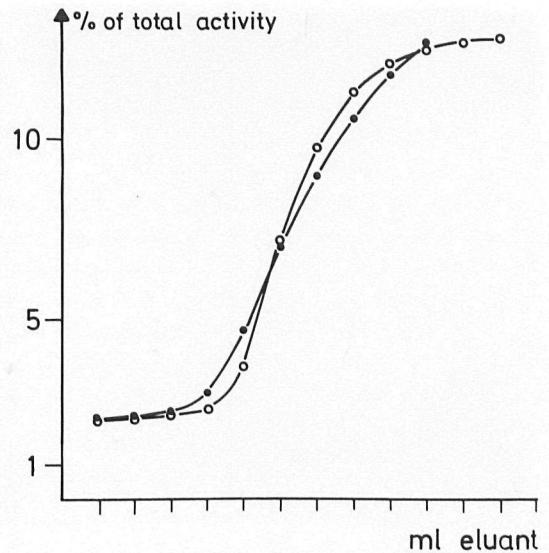


Fig. 4. Biospecific elution (zone sharpening effect). ^{125}I -labelled antigens eluted with non-labelled antigens from the same source followed by elution at pH 2.5. Conditions same as in Fig. 3 except that before elution at pH 2.5 1 ml 0.15 M NaCl (●) or 1 ml timothy pollen extract (O) was added to the column.

gens, and when the pH was subsequently lowered the eluted peak was sharper than the peak eluted without preceding biospecific elution.

As a final test of the specificity, the same experiments were repeated using an extract of the house dust mite instead of the grass pollen extract. These experiments showed that in these cases there was no binding of house dust mite antigens to the immunosorbent.

Experiments with sera from patients reacting to house dust mite and horse hair and dander showed that the same experimental conditions could be used in these cases.

b) *Non-labelled antigens.* The fused rocket immunoelectrophoresis elution profiles from column experiments using both normal and patient serum and extract from timothy pollen are shown in Fig. 5. Similar experiments with extract from house dust mite and the same patient serum showed no binding. Thus, it was shown that the non-specific binding of antigen was undetectable, even when, as in contrast to the

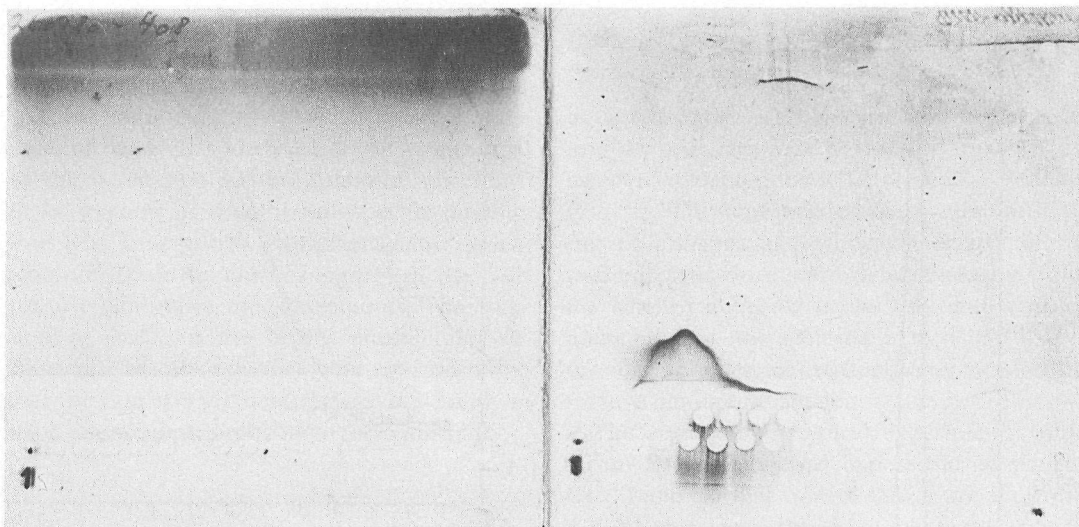


Fig. 5. Fused rocket immunoelectrophoretic elution profile of non-labelled antigens. Conditions same as in Fig. 3 except that the antigens were 1 ml 3 mg/ml timothy pollen antigens. To the left serum from a non-atopic individual and to the right serum from an allergic hyposensitized patient was used in the column experiment.

experiments with labelled antigens, larger amounts of antigen were used.

The eluted fractions consisting of antigen-IgG complexes were further analysed by CIE using rabbit antibodies against timothy pollen antigens. In ordinary CIE a diffuse precipitate was formed due to the presence of human IgG directed against the antigens. However, when anti-human IgG was placed in an intermediate gel the immune complexes were split up and several precipitates were formed (Fig. 6). Similar experiments with antigens from house dust and horse hair and dander and serum from patients allergic to these antigens gave comparable results, indicating that the system can be used without modifications with different antigens. The results were shown to be highly reproducible both in terms of IgG and antigen binding. The column could be used at least 10 times without showing any deterioration when stored in phosphate buffer pH 7.0, 0.07 M containing 0.5 M NaCl and 1.5 mM NaN_3 .

Evaluation of different elution buffers

Buffers with different pH all containing 0.5 M NaCl were tried as well as the chaotrope

NaSCN, 3 M. Acetate buffer pH 2.5, 0.05 M containing 0.5 M NaCl was shown to be the most effective eluant. Sodium thiocyanate is just as effective an eluant as measured by labelled antigens, but the immunoelectrophoresis in experiments with non-labelled antigens showed that some denaturation occurred after prolonged exposure to this eluant.

DISCUSSION

The need for purification of allergens for use in analysis and hyposensitization leads to the proposal by Søndergaard & Weeke (18) of using immobilized anti-human IgE for the purification of IgE reactive allergens. As a natural consequence, an IgG system was further investigated because it is well suited as a model system for investigations on specificity and kinetics. Furthermore, if IgE and IgG can be shown to be directed against the same antigenic determinants, or at least the same allergen molecules, it can be used directly for allergen isolation. The affinity chromatography on protein A-sepharose could be optimized so that non-specific binding is reduced to a very low level without impairing the specific reaction.

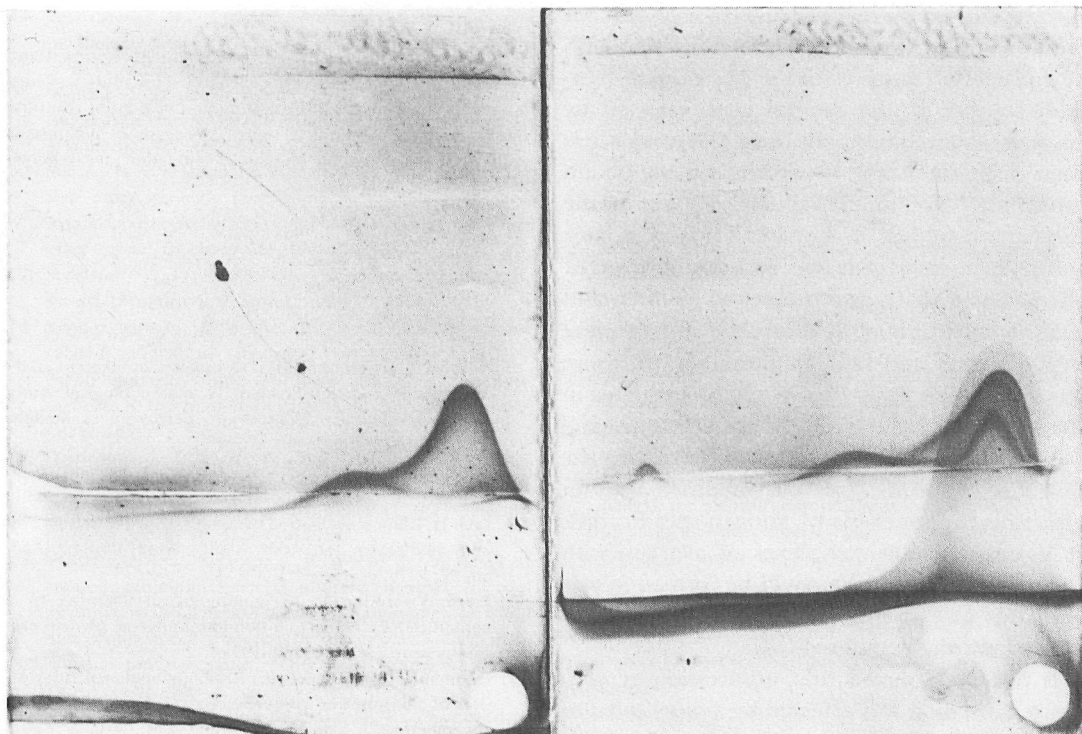


Fig. 6. CIE analysis of the eluted antigen/IgG immune complexes. Antigens: 50 μ l of the eluate from the experiment shown in Fig. 5. Intermediate gel to the left: empty. Intermediate gel to the right: 25 μ l/cm² anti-human IgG. Upper gel: Contains 12 μ l/cm² rabbit antibodies to timothy pollen antigens. Conditions: First dimension electrophoresis 10 V/cm for 30 min at 15°C. Anode to the left. Second dimension electrophoresis 2 V/cm for 18 h at 15°C. Anode at top.

This is mainly accomplished by inclusion of 0.5 M NaCl in the buffers as was shown in the IgE system (19), indicating that non-specific binding is mainly due to ionic forces. The specificity of the reaction was demonstrated both by comparing the binding of antigens to patient and normal IgG and, very convincingly, by the peak sharpening effect of a biospecific eluant. Furthermore, no binding was detected to either normal or grass pollen-allergic patients' IgG when an extract of house dust mite was used. The eluted antigen IgG complexes were analysed by CIE and it was shown that inclusion of anti-human IgG in the intermediate gel enabled visualization of the different antigens included in the complex. Thus, it was not necessary to separate IgG and antigen by gel filtration before analysis.

The eluant was shown to be very important, as the often used chaotrophe NaSCN, in contrast to 0.05 M acetate buffer at pH 2.5 containing 0.5 M NaCl, when used with timothy pollen antigens leads to partial denaturation of the antigens. However, when an eluant of low pH is used the eluate must be brought to a neutral pH immediately after elution.

The method can be used as an analytical tool together with crossed radio immunoelectrophoresis to identify the components in complex allergen/antigen extracts to which the patient reacts with both IgG and IgE. The low degree of non-specific binding and the shown biospecificity make the system very reliable, and further work on the similarity of IgG and IgE specificity and affinity is in progress in our laboratory.

As discussed below, the use of isolated immune complexes for immunotherapy may also be considered. Suppression of the primary synthesis of IgE in the rat has been effected by passively administered antibody (22), and it has been suggested that hyperimmune γ -globulin could play an immunoregulatory role in the production of IgE in humans (15). In a later study (2) it was shown that in a combination of allergen-specific (ragweed-specific) globulin and rush desensitization, as compared to treatment with albumin and rush immunotherapy, there was significant reduction in symptom scores in the group of patients receiving both active and passive immunotherapy. Furthermore, the rise of allergen-specific IgE was modified without affecting the synthesis of antigen-specific IgG. It is possible that complexes of allergen with homologous γ -globulin could be formed *in vivo* and thus give rise to the observed suppression of the immune response (3, 4).

It has been shown that suppression of antitoxin formation was affected by a much smaller number of antitoxin molecules when they were administered as antigen-antibody complexes (23). In addition it has been shown that formation was suppressed, not only of antibodies directed against the antibody-bound determinants, but also of those directed to unrelated determinants on the same antigen molecule (6).

A possible mechanism on the cellular level whereby the immune complexes could elicit a tolerogenic signal could be through antigen-reactive cell opsonization (ARCO) (10). According to this mechanism for antibody-mediated immune response the antigen reactive cells (B and/or T cells) are opsonized by antigen-antibody complexes (or by anti-idiotypic antibody) and are subsequently destroyed by macrophages.

We now propose that instead of combining active and passive immunotherapy it might be useful to use the method described in the present paper to produce patient-specific immune complexes and use these for immunotherapy.

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