

Purification of Patient-Related Allergens by Means of Bioaffinity Chromatography on a Sepharose Anti-IgE Patient IgE Immunosorbent

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A method is described for bioaffinity chromatographic purification of patient-specific IgE reactive allergens by using sepharose coupled rabbit anti-human IgE antibodies, patient serum and an allergen extract. The conditions for this bioaffinity chromatographic procedure have been optimised, and it was shown that the nonspecific binding of serum proteins and antigens can be kept at very low levels by a high ionic strength. The specificity of the method has been determined by showing that allergens to which the patient did not react were not bound to the affinity matrix. We suggest that the isolated allergens could be used for more specific immunotherapy in the future.

Key words: allergens; IgE; bioaffinity chromatography.

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CLINICAL ASPECTS

Individual allergic patients react to different allergens in a given allergen extract as determined by crossed radioimmuno-electrophoresis (CRIE). Therefore we suggest that the extracts used for immunotherapy should be tailored to the individual need. The use of highly purified extracts might reduce the amount of allergen needed to produce the desired effect.

The allergen extracts used today for immunotherapy contain a number of antigens to which the actual patient has no IgE response as measured with the radioallergen sorbent test (RAST) and crossed radioimmuno-electrophoresis (CRIE). From the point of view of both the patient and the immunologist it would be appropriate with extracts containing only those components to which the patient's IgE-system is directed. Purification of allergens by chemical and physicochemical methods for use in diagnosis and treatment of allergy has in recent years reached great sophistication (5, 9).

Studies on hyposensitization using major allergens, or a single major allergen instead of a mixture of allergens, are in progress (19), but it is not practicable with present day methodology to produce allergen extracts tailored to the

individual patient. The development of techniques for covalent coupling of proteins to sepharose by cyanogen bromide (8) has made available new methods for purification of allergens. The parallel development of the RAST and CRIE techniques (17, 18) together with the development in bioaffinity chromatography tempted us to try to isolate patient-related allergens by means of an immobilized anti-human IgE and using the IgE of the patient as a second antibody. It was the increasing use of the CRIE technique as a sensitive method for use in allergen standardization (1, 5, 16, 17) that led to the idea that this system could be reversed and used for allergen isolation (12). The possible use of such allergens would in the utmost consequence be highly specific immunotherapy. It could also be used for isolation of

small quantities of radiolabelled allergens using serum from patients reacting to one or more allergens and later use the isolated allergens in immunoassays of different kinds i.e. blocking antibody assay, determination of affinity constants. It is the aim of the present study to investigate further, different aspects of a bio-affinity chromatographic system for use in allergen isolation. In a system used for isolation of individual allergens it is very important to bring the nonspecific binding down to very low levels. Therefore the problem of nonspecific binding of serum proteins and allergens is also investigated in this study and some generally applicable methods are devised.

MATERIAL AND METHODS

Analytical methods

The determination of total IgE was performed using the paper radioimmunosorbent test (PRIST) from Pharmacia. Crossed immunoelectrophoresis (CIE) and rocket immunoelectrophoresis (RIE) were carried out according to Weeke (13, 14) and fused rocket immunoelectrophoresis (FURIE) according to Svendsen (11). Crossed radioimmuno-electrophoresis (CRIE) was performed according to Weeke et al. (17).

Antigen

A standardized aqueous extract of timothy pollen (*Phleum pratense*) 3 mg/ml was obtained from Allergologisk Laboratorium ALK A/S (Copenhagen, Denmark). The extract contained at least 20 antigens including all the major allergens as measured by CIE and CRIE.

Antibodies

Anti-human IgE antibodies were raised in rabbits and used as the isolated immunoglobulin fraction or purified by immunoabsorption on sepharose coupled IgE (6). The antibodies were shown by CIE not to react with human IgG. Rabbit antibodies against timothy pollen antigens were prepared by immunizing rabbits for

more than 1 year with the standardized timothy pollen extract as described by Harboe & Ingild (3). Monospecific rabbit antibodies against different human serum proteins were obtained from DAKO A/S (Copenhagen, Denmark).

Coupling of anti-human IgE to CNBr activated sepharose

Sephacrose C1 4 B activated with CNBr was obtained from Pharmacia (Uppsala, Sweden). The coupling of anti-human IgE to CNBr activated sepharose followed the procedure recommended by the manufacturer. The amount of antibody coupled was approximately 2.5 mg/ml gel. Normally 5–10 mg protein per ml gel is recommended, but in order to minimize the nonspecific binding of other serum proteins as well as antigens the concentration was kept lower.

Chemicals

Chemicals used for preparation of buffers were analytical grade from E. Merck (Darmstadt, West Germany) and were used as received.

Test sera

A pool of sera from 1000 donors was used as control. It contained 100 IU/ml of IgE. Serum from a patient (H.D.) allergic to timothy pollen was used as test serum. The patient was a 26-year-old female allergic to grass pollen, who has been preseasonally treated for 6 years with Allpyral® 5-grass mix. Her serum contained 945 IU/ml of IgE as determined by PRIST and 214 SU/ml (allergy class 4) as determined by the A1-RAST (15). This serum has been kept frozen for several years and is frequently used as an allergic control serum.

¹²⁵I-labelled reagents

¹²⁵I-labelled anti-human IgE was obtained from Pharmacia (Uppsala, Sweden) and the timothy pollen extract was labelled according to Hunter (4). The labelling of the timothy pollen extract was controlled by CIE. An amount of the label-

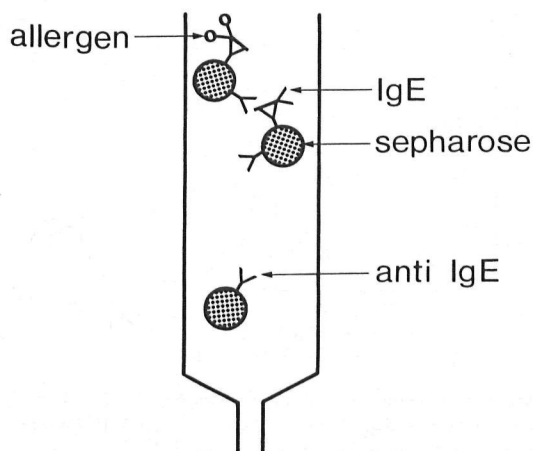


Fig. 1. Schematic drawing showing a column packed with sepharose particles to which rabbit anti-human IgE antibodies are bound. The other steps in the procedure, where IgE and, finally, allergen is bound to the immunosorbent are also shown.

led extract corresponding to 10^5 cpm was mixed with the amount of unlabelled 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. The washed and dried immunoplate was then placed on an X-ray film for 14 days for autoradiography.

Apparatus

Columns for the chromatographic experiments were either 5 ml plastic syringes or a K 16/20 column from Pharmacia (Uppsala, Sweden). The flow through the column was controlled by a multiflow roller pump from Ole Dich Instrument makers (Hvidovre, Denmark). The fractions were collected on a fraction collector from LKB (Bromma, Sweden).

Principle

The chromatographic system consists of a solid phase bound rabbit antibody against human IgE as the primary antibody. This immunosorbent is then reacted with human IgE from an allergic individual. After elution of the other serum proteins, an allergen extract is allowed to react

with this secondary antibody. Finally, the allergen and the secondary antibody are eluted by changing the pH and the ionic strength, or by use of chaotropic ions (Fig. 1).

Binding of IgE to anti-IgE sepharose

In order to optimize the conditions used for binding of IgE to the anti-IgE sepharose a series of batch experiments were performed. Fifty μ l of the immunosorbent was incubated with IgE containing serum, and the influence of pH, ionic strength and time on the binding was studied. The amount of bound IgE was measured by using ^{125} I-anti IgE as marker.

Binding of allergen to the sepharose anti-IgE-IgE

The conditions for optimal binding of allergen to the sepharose anti-IgE-IgE immunosorbent in terms of pH, ionic strength and time was evaluated by both column and batch experiments using labelled allergens. All experiments were carried out at 20°C.

Experiments with ^{125} I-labelled allergens

The experiments were run in 5 ml columns packed with sepharose coupled anti-human IgE equilibrated with 0.05 M citrate buffer pH 6.2. Five ml serum was recycled for 5 h. After washing of the column with 10 column volumes of 0.05 M citrate buffer pH 6.2, 0.5 ml of ^{125} I-labelled timothy pollen extract corresponding to 1.2×10^5 cpm was applied to the column and recycled for 1 h. The column was then washed with 6 column volumes of 0.05 M citrate buffer pH 6.2 and the bound allergens were eluted by stepwise lowering of the pH and increasing the ionic strength using 0.1 M acetate buffer containing 1.0 M NaCl pH 4.0 and 0.05 M citrate buffer containing 1.0 M NaCl pH 2.6, or by using 3 M NaSCN.

Experiments with non-labelled allergens

A column containing 30 ml of sepharose coupled anti-human IgE was incubated for 3 h with 12.5 ml of serum and washed as described.

One ml of allergen extract was then recycled for 5 h, the column washed and eluted with 3 M NaSCN. The eluate was collected in 1 ml fractions and analyzed by fused rocket immuno-electrophoresis.

Nonspecific binding of serum proteins

Determination of the nonspecific binding of 11 human serum proteins to the sepharose anti-human IgE were made in a column containing 30 ml of sepharose coupled anti-human IgE equilibrated with 0.07 M phosphate buffer containing 0.5 M NaCl. The column was incubated with 45 ml serum by recycling for 1 h. The column was washed with 15 column volumes of phosphate buffer 0.07 M containing 0.5 M NaCl. After washing, the column was eluted with 5 column volumes of 3 M NaSCN and the protein content of the collected 5 ml fractions was determined by RIE. The IgE content was measured before and after recycling by the PRIST technique.

RESULTS

Binding of IgE to sepharose anti-IgE

The batch experiment showed that pH could be varied between 5.5 and 8.0 with only small

Table 1
Nonspecific binding of 11 serum proteins to sepharose anti-IgE-IgE in 0.07 M phosphate buffer pH 7.0 containing 0.5 M NaCl column 30 ml and amount of serum 45 ml

Protein	bound in % of total amount
IgA	0.23
IgG	0.91
IgM	0.91
Albumin	0.03
C ₃ -complement	not detectable
α ₂ -macroglobulin	0.03
Transferrin	not detectable
Orosomucoid	not detectable
Ceruloplasmin	not detectable
α ₁ -antitrypsin	not detectable
Haptoglobin	not detectable

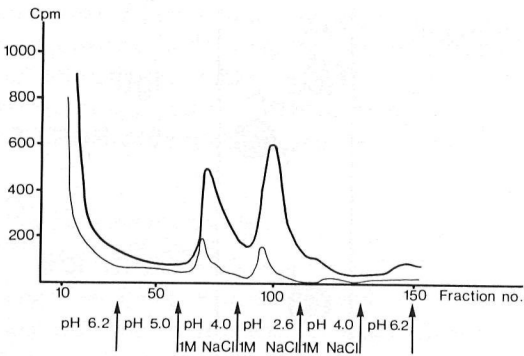


Fig. 2. The elution profile of ¹²⁵I-labelled allergens from a column containing 5 ml sepharose coupled rabbit anti-human IgE preincubated with 5 ml serum for 3 h and stepwise eluted by changing the pH and ionic strength. The broad line indicates what has been bound by a patient serum and the thin line shows the same experiment, but with normal human serum.

differences in the binding of IgE. The concentration of NaCl in the incubation buffer could be as high as 2 M with a loss of bound IgE of only 8%. When evaluating the time needed for binding of IgE to the immunosorbent one has to take into consideration that prolonged incubation leads to increased specific binding as well as increased nonspecific binding. Batch experiments showed that half maximal binding was obtained in 30 min and that maximal binding was obtained in 72 h. Thus the incubation times chosen were a compromise.

The capacity of the immunosorbent for binding of IgE was shown to be large enough to bind 1.2×10^4 IU IgE because the amount of IgE in the effluent from the column after incubation with 12.5 ml test serum containing 945 IU/ml IgE was not detectable with the PRIST technique.

Nonspecific binding of serum proteins

In a series of column experiments it was shown that the nonspecific binding of serum proteins to the sepharose anti-IgE matrix could be considerably reduced by including 0.5 M NaCl in the incubation buffer and by prolonging the washing steps. The addition of 0.5 M NaCl lowers the nonspecific binding of IgA, IgM,

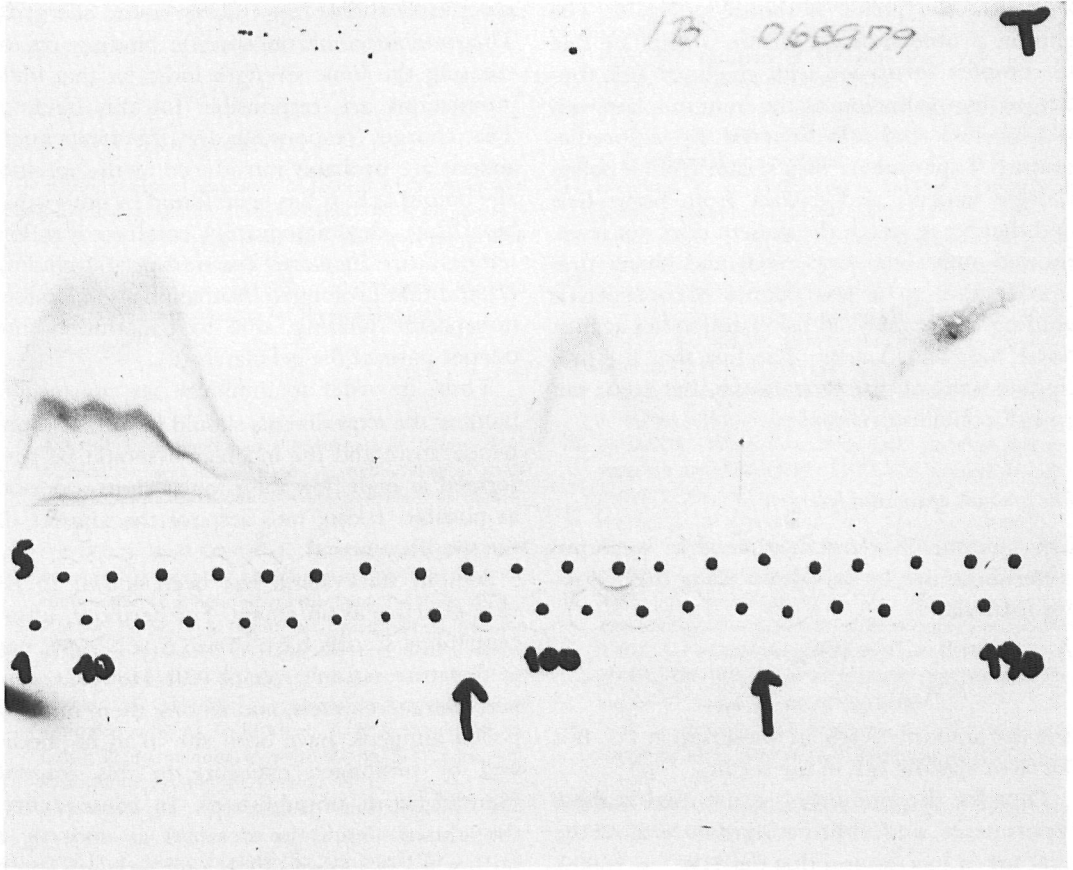


Fig. 3. Fused rocket immunoelectrophoretic elution profile from a column experiment using non-labelled allergens. Thirty ml of sepharose coupled anti-human IgE incubated for 3 h with 12.5 ml of serum and washed. Then 1 ml of allergen extract was recycled for 5 h, the column washed and eluted with 3 M NaSCN. The electrophoresis was run in 1% agarose with the upper part containing rabbit antibodies to timothy pollen antigens. Conditions: 18 h with 2 V/cm at 15°C. Anode at top.

IgG, C₃-complement and albumin 5 to 10 times, with a decrease in the specific binding of only 6%. The effect on the other tested serum proteins was not measurable as the nonspecific binding of these proteins could not be measured under any of the elution conditions. The nonspecific binding of 11 serum proteins to the sepharose anti-IgE matrix as measured by RIE is outlined in Table 1.

Binding of allergens to the bound specific IgE

The result of a typical experiment using ¹²⁵I-labelled timothy pollen allergens and 5 ml serum in a 5 ml column is shown in Fig. 2. It is

seen that the first elution peak appears after changing to pH 4.0 and 1 M NaCl and that further decrease in pH to 2.6 and 1 M NaCl elutes a comparable amount of allergens. As can be seen from these experiments it is necessary to decrease the pH to 2.6 in order to elute all the bound allergens. It was shown in similar experiments that elution with 3 M NaSCN is an effective alternative, but other experiments (Søndergaard, unpublished) show that grass pollen allergens, and probably also other allergens, may be denatured by prolonged contact with this eluant.

The fused rocket immunoelectrophoretic elution profile from an experiment with extract

from timothy pollen is shown in Fig. 3. The elution profile shows a diffuse precipitate due to complex formation with co-eluted IgE confirmed by incubation of the immunoplate with ^{125}I -labelled anti-IgE followed by autoradiography. Experiments with serum from a pollen allergic patient, and extract from horse hair and dander to which the patient does not react, showed only one very small and sharp precipitate due to a low degree of nonspecific binding when analyzed using antibodies against horse hair and dander. The fact that the precipitate was not diffuse indicates that it was not an IgE-containing complex.

The amount of isolated allergen

The amount of allergens isolated in a column experiment can be calculated using the following formula:

$$\frac{A \times 2.5 \times 2 \times B \times \text{MW}_{\text{ag}}}{\text{MW}_{\text{IgE}}} = \text{amount in ng.}$$

A is the amount of IgE in the serum in IU; B is the % of specific IgE in the serum.

Thus for the amount of serum used in these experiments, and anticipating that 30% of the total IgE is specific and that the $\text{MW}_{\text{ag}} = 15000$, an amount of 1.5 μg of specific allergens can be isolated. This figure was confirmed both by the immunoelectrophoretic analysis and in the experiments using labelled allergens.

DISCUSSION

The experiments performed with ^{125}I -labelled timothy pollen antigens showed that it is possible to use the proposed bioaffinity chromatography system to isolate allergens. It was shown that the system is specific, as the amount of eluted allergen reflects the amount of specific IgE. The experiment with non-labelled antigens showed that the system can be scaled up, thus allowing isolation of μg quantities of allergen. The measurement of the nonspecific binding showed that by including 0.5 M NaCl in the incubation and washing buffer the nonspecifically bound human proteins were kept at a very low level, thus increasing the ratio between

specifically and nonspecifically bound allergens. The inhibition of nonspecific binding by increasing the ionic strength indicates that ionic interactions are responsible for this binding. The charges responsible for the ionic interactions are probably introduced by the specifically bound IgE. It has been found by others that bioaffinity chromatography conducted at low temperature increases the nonspecific binding (7) and that prolonged incubation also increases nonspecific binding, due to penetration into deeper parts of the gel matrix (2).

Thus, in order to minimize the nonspecific binding the experiments should be run at room temperature and the incubation should be performed at high flow rates for as short a period as possible, taking into account the kinetics of the specific reaction.

Sodium thiocyanate has been shown to be very effective as an eluant. Furthermore, ultracentrifugal studies have shown 3 M NaSCN not to denature serum proteins (10). However, certain allergen extracts, and among them timothy pollen antigens, have been shown to be modified by prolonged exposure to this reagent (Søndergaard, unpublished). In consequence, the eluate should be desalted at once or a buffer at low pH and high ionic strength could be used and neutralized just after elution has been performed. The immunosorbent shows no deterioration, even after many elutions using both of these elution reagents, if it is washed just after elution.

The method could be modified to include a gel filtration step immediately after the elution in order to separate the eluted IgE from the allergens, assuming that the IgE-allergen complexes are dissociated at the elution conditions. In this way it would be possible to obtain pure allergens to which a particular patient reacts. Furthermore, in cases where a patient only reacts to one allergen as measured with the CRIE technique it would be possible in a simple way to obtain a sample of this allergen and use it for further characterization of the IgE-allergen binding by measuring affinity constants. Studies along these lines are in progress in our laboratory. Other possibilities may be immunotherapy with the IgE-allergen complexes in

order to induce tolerance or hyposensitize with small amounts of the patient-specific allergens.

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