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ISOLATION AND QUANTITATION OF SERUM IgE LEVELS BY HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

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SUMMARY

High-performance immunoaffinity chromatography on monoclonal antibodies coupled to protein A-coated glass beads is a method for the rapid isolation and quantitation of immunoglobulin E (IgE) from the serum of both adult and pediatric patients. The technique is as sensitive as most immunoassays but takes less than an hour to perform. In addition to measuring total IgE in both plasma and serum, the technique provides biologically active, affinity-isolated IgE, which can be used for other clinical and research studies.

INTRODUCTION

Isolation and measurement of serum or plasma immunoglobulin E (IgE) is often time-consuming and difficult, because the amounts of the immunoglobulin present in human serum or plasma are minute[1]. Chromatographic isolation of IgE has often been hampered by the sheer lack of material present, even in allergic patients. This has been overcome to a certain extent by the use of affinity chromatography, using epsilon heavy chain specific antibodies, immobilized on a matrix. To date, all of these techniques have utilized low-pressure systems and are time-consuming and often of little use in clinical science.

Immunoaffinity chromatography utilizes the specificity of an immobilized antibody to capture and hold its specific antigen[2]. This reaction takes place during the initial phase of the chromatographic procedure and allows all of the unreactive material in the test sample to pass through the column, leaving only the material of interest bound to the immobilized antibody. Recovery of the antigen is achieved by introducing a second buffering system, which will interfere with the binding of the antibody to the antigen. Such buffers can be either acids or chaotropic ions[3]. In either case, the introduction of excessive ions interferes with the charge-bonding between the immobilized antibody and the antigen, thus releasing the antigen which is washed through the column and measured by the column detector.

Glass beads, coated with the bacterial protein, protein A, make a good universal column material for immunoaffinity chromatography[4] and are strong enough to be used in high-performance liquid chromatography (HPLC). Protein A has the

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receptors for IgG antibody tails or Fc portions[5] and, once bound to protein A, the IgG molecule becomes oriented with its antigen receptors facing the mobile phase of the column. This gives the IgG molecule maximum efficiency for trapping antigens, which are passing through the column in the mobile phase.

We have devised a method of high-performance immunoaffinity chromatography (HPIC) for the rapid isolation and quantitation of IgE in both adult and pediatric serum samples using protein A-coated controlled-pore glass beads and a monoclonal antibody, directed against human IgE. In this paper we will describe our system for the rapid isolation and quantitation of human serum or plasma IgE levels by HPIC.

EXPERIMENTAL

Materials

The glass beads used as the column packing material were obtained as controlled-pore glass beads (Pierce Chemicals, Rockford, IL, U.S.A.), which had carbonyl diimidazole-reactive side chains, derived on their glycophase surface. Lyophilized protein A was purchased as a pure product from Pharmacia (Piscataaway, NJ, U.S.A.) and reconstituted to a concentration of 500 μg/ml in 10 ml of 50 m*M* carbonate buffer, pH 9. The anti-IgE monoclonal antibody was laboratory-produced and will be described below.

The protein A–antibody cross linking was achieved with 1-cyclohexyl-3-(2-morpholinethyl) metho-*p*-toluene sulfate carbodiimide, which was obtained through Pierce Chemicals. All columns and fittings were obtained from BioRad Labs. (Rockville Centre, NY, U.S.A.). Comparison studies were performed with IgE immunodiffusion plates (Calbiochem-Behring, San Diego, CA, U.S.A.) and with the total IgE enzyme-linked allergosorbent assay (EAST), obtained from Kalsted Labs. (Austin, TX, U.S.A.).

Monoclonal antibody production

Monoclonal antibodies were produced by the standard technique of Kohler and Milstein[6]. Briefly, BALB/c albino mice (Cumberland View Farm, Clinton, TN, U.S.A.) were injected with human IgE, which had been isolated by affinity chromatography, from a patient with allergy to Aspergillus species. Each animal was then screened, weekly, for the presence of serum antibodies to human IgE. Following the detection of such antibodies, the animals were sacrified and single-cell suspensions were prepared from their spleens. The isolated spleen cells were then polyethylene glycol (PEG)-fused with mouse myeloma cells, and the hydridomas were cloned. Once the clones had become established, they were screened for the presence of mouse anti-human IgE by a solid-phase IgE enzyme-linked immunosorbent assay. Clones which demonstrated production of the desired antibody were isolated and grown.

Column construction

The derivatized glass beads were acid-washed five times in 0.1 *M* hydrochloric acid, followed by extensive washing in doubly distilled water. A volume of 10 ml (5 mg) of the reconstituted protein A was added to 10 g of the washed beads, placed in a 15 ml capped tube and mixed for 18 h at 4°C in an overhead mixer. Following

this incubation, the beads were washed five times in PBS (phosphate buffered saline, 0.01 *M* PO_4^{3-}, pH 7.2) by sedimentation and then added to 10 ml of a solution of PBS, containing 100 ng/ml of mouse monoclonal antibody. The protein A-coated beads were incubated with the monoclonal antibody for 1 h at 4°C in an overhead mixer. Following this incubation, the antibody supernatant was checked by a solid-phase IgE enzyme-linked immunosorbent assay for the efficiency of the IgE antibody binding to the protein A-coated glass beads. The beads were washed five times in PBS by sedimentation and all unreacted protein A sites blocked by reincubating the beads for 1 h at 4°C with a 1 mg/ml solution of normal human IgG Fc fragment in PBS. The beads were washed a further five times in PBS by sedimentation, and the protein A-bound antibody was cross linked into place by incubating the coated beads with 10 m*M* carbodiimide, dissolved in carbonate buffer (pH 9), for a further hour at 4°C in the overhead mixer. Finally, the beads were washed ten times in PBS and then slurry-packed into a 150 × 4.6 mm I.D. column at 1000 p.s.i.

Chromatography

The packed column was installed in a Beckman (Palo Alto, CA, U.S.A.) 340 isocratic chromatography system equiped with an Autochrom (Milford, MA, U.S.A.) Model III OPG/S solvent selection controller, a Beckman Model 112 pump, a Beckman Model 160 UV detector, set at 280 nm, and a Shimadzu (Columbia, MD, U.S.A.) C-RIB peak integrator.

Samples were processed by first passing the plasma or serum through a Millipore (Bedford, MA, U.S.A.) 0.45-μm HV filter and then injecting 100 μl of the sample into the chromatography system. The chromatogram was developed isocratically, in 0.9% sodium chloride–0.1 *M* sodium acetate buffer (pH 6.5) for 20 min at 0.5 ml/min. During this phase, the immobilized antibody captured the IgE and allowed the unreactive material to pass through the column. Following this, the solvent selector was programmed to select the second buffer, 2.5 *M* sodium thiocyanate, and to pass this buffer through the column for the next 30 min. During this phase, the retained IgE was released, passed through the column, and was recorded by the detector and quantitated by the peak integrator. In addition, 200-μl fractions were collected in an ISCO (Lincoln, NB, U.S.A.) Cygnet fraction collector, modified to take 500-μl autoanalyzer cups. The collected fractions were measured by a solid-phase IgE enzyme-linked immunosorbent assay and compared to the integrated peaks, produced in the elution chromatogram. The IgE-containing fractions were pooled and equilibrated with PBS by dialysis. At the end of each elution, the solvent selector was programmed to recycle the column, ready for the next sample.

Other immunological assays for IgE quantitation

Patient samples and IgE standards were also tested by other immunological techniques used in current laboratory practice. HPIC results were compared with those obtained by the old, less sensitive radial immunodiffusion assay (RID), in which levels of serum IgE are quantitated by allowing the antibody to radially diffuse through agarose containing an antibody directed against the heavy chain of the antibody under investigation. This diffusion is allowed to proceed until the diffusing antibody reaches equilibrium with the reactive antibody, in the agarose and a precipitation ring forms. The diameter of this ring is measured and compared to a ref-

erence curve constructed by comparing the precipitation ring diameters formed by standards with their known concentrations. In this way, the results are expressed in ng/ml. This technique is still used by some laboratories to quantitate serum IgE. In addition, comparisons were also made with the EAST, a non-isotopic version of the total IgE radioimmunoassay. Both the RID and East were obtained as commercial kits and performed according to the instructions supplied by the manufacturer.

RESULTS

The production of protein A-coated glass beads is reasonably easy and it is possible to produce both large preparative scale and custom-designed batches. In our experience, the beads remain stable for up to one year provided a suitable preservative is added. Experiments have shown that the column life varies between 1–3 weeks at room temperature but this can be extended to 10–12 weeks, if the column is maintained at 4°C. Under the running conditions, described above, the column life is usually 15–20 cycles but this can be lengthened to 30 cycles by slowing the running conditions to 0.3 ml/min. Antibody binding efficiency was excellent, and in our experience of making 20 columns with this monoclonal antibody, the average binding was 70% of the initial antibody present.

Typical chromatograms obtained from human serum or plasma by HPIC are shown in Fig. 1. Fig. 1A is the chromatogram obtained from a 100-μl sample of a 200-ng/ml standard of human IgE. Two distinct peaks are seen, which correspond to the non-IgE and IgE components of the standard. The IgE peak is clearly separated from the non-IgE material and it eluted at 19.2 min. The chromatogram shown in Fig. 1B demonstrates the pattern from a normal or hypo-allergic person. Again two distinct peaks are produced, but in this example, the first peak is large, due to the fact that the injected sample was human serum. The second, smaller peak corresponds to an IgE level of 12–15 ng/ml. In the third example, the plasma of a patient with allergy to house dust gave the chromatogram shown in Fig. 1C. As in Fig. 1B, the first peak, which represents the non-IgE component of the plasma, is enormous, while the elevated IgE is eluted as a well-defined second peak. This peak which is eluted at 20.9 min, represents an IgE level of 983–990 ng/ml. The apparent peak shift of the eluted IgE, seen in the figures is an artifact produced by the integrator. The larger IgE load, in the hyper-allergic sample, taking longer to elute and pass through the column.

Comparison studies between the HPIC system, RID, and EAST on IgE standards demonstrated that the HPIC system had a good correlation with the EAST and was much more sensitive than RID. This was also evident when two standards from the EAST Kit and both the hypo- and hyper-allergic patient samples were tested by all three techniques. The efficiency of the column was shown to be good when comparing the 280 nm peak integration values, for the eluted IgE peaks, with the values obtained by examining the eluted peak fractions by the solid-phase enzyme-linked assay for IgE. The integration value was shown to represent all of the IgE present plus a 5 ng additional non-IgE fraction in the hypo-allergic sample and a 30 ng non-IgE fraction in the hyper-allergic sample. Only 1–5 ng non-IgE fractions were detected in the reference standards. In the twenty-five samples that have been analyzed to-date, this trend has remained unchanged. Table I summarizes the results

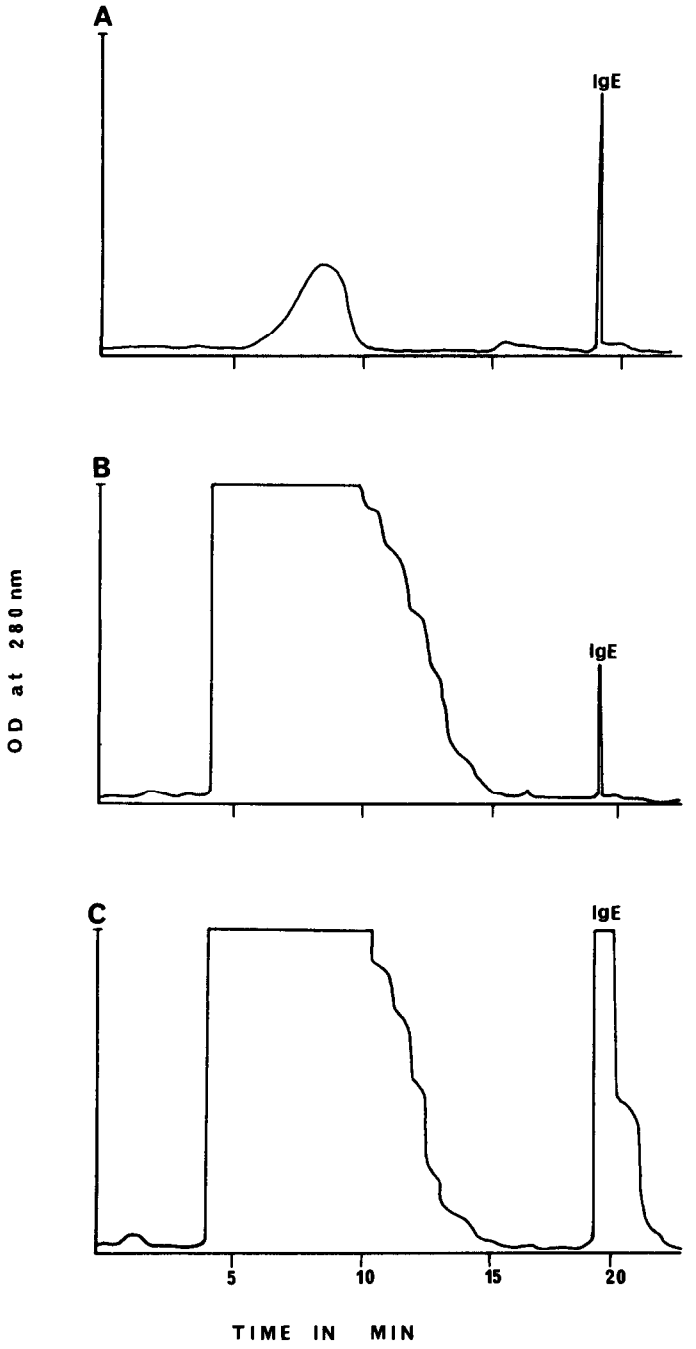

Fig. 1. Typical chromatograms produced by the HPIC technique. A demonstrates the isolation of an IgE standard. B shows the pattern produced by a normal or hypo-allergic patient serum sample and C illustrates the chromatogram produced by a plasma sample from an hyper-allergic patient with an allergy to house dust.

TABLE I

COMPARISON OF HPIC WITH RID AND EAST

All values (ng/ml) are given as the mean of triplicate samples ± S.E.M.

	HPIC	*RID*	*EAST*
Test sample			
IgE Standard (330 ng/ml)	320 ± 1.5	283 ± 3.2	323 ± 1.5
IgE Standard (25 ng/ml)	19 ± 1.0	50	22 ± 1.5
Hypo-allergic patient	9 ± 2.0	50	14 ± 2.2
Hyper-allergic patient	979 ± 5.8	584 ± 12.7	990 ± 5.0
IgE Calibration curve			
20 ng/ml Standard	18 ± 1.8	50	19 ± 1.0
100 ng/ml Standard	101 ± 2.6	74 ± 6.1	103 ± 3.7
200 ng/ml Standard	194 ± 3.1	174 ± 6.7	197 ± 2.6
400 ng/ml Standard	395 ± 4.5	350	394 ± 2.8

obtained by HPIC peak integration readings at 280 nm with RID and EAST results, on the same samples. The values given are the mean of triplicate samples ± the standard error of the mean (S.E.M.). Pearson's correlation coefficient was calculated for the means of the HPIC and EAST results and was shown to greater than 97% correlation.

DISCUSSION

The introduction of affinity chromatography techniques into HPLC is not new and many different workers have explored this area[7,8]. Many different types of packing material have been investigated but the greater success appears to come from the application of porous and activated silica and controlled-pore glass beads[8–10,4]. The use of carbonyl diimidazole as an active coupling side chain has also been explored[7,11,12], although applied to low-pressure soft gels rather than to high-pressure packings. The advantage of using a high-pressure system is that minute samples can be analyzed quickly with sophisticated optical instrument and direct readings can be obtained. The application of protein A-coated glass beads provides an ideal packing material for immobilizing antibodies for affinity chromatography of human immunoglobulins in a wide variety of different body fluids.

IgE represents a minute fraction of the human immunoglobulin repertoire, except in hypersensitive states, such as asthma and allergies[13,14]. In the latter, the normal levels of circulating IgE may increasse by up to ten-fold and the total IgE levels may become clinically important. Food, drug, and environmental allergies are common in both adult and pediatric patients, and activation of the IgE system produces the release of a series of chemical mediators, which increase vascular permeability, bronchial smooth muscle contraction, and skin eruptions, all common to hypersensitivity shock[15,16]. Measurement of serum IgE is important in pediatric patients where persistently elevated IgE indicates the subsequent development of asthma and other allergic disorders[17].

Although there was little difference between the results obtained by HPIC and

EAST, HPIC of a 100-μl sample was accomplished in 40 min and the IgE was isolated for further testing. This enables rapid assessment of potentially allergic patients within the hour, facilitating the clinical management of drug or food allergy patients. In addition, the isolated IgE, collected in an active form, is available for further testing.

CONCLUSIONS

HPIC is a fast method for the isolation and quantitation of biologically active IgE from serum or plasma of both adult and pediatric patients. The use of the protein A-coated glass beads provides a suitable antibody column packing, which can be used for other HPLC systems. In this way. HPIC technology combines the speed and precision of HPLC with the power of immunological specificity to biological materials.

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