

Purification of immunoglobulin E (IgE) antibodies from sera with high IgE titers^a

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Abstract

A three stage method for the ultrapurification of polyclonal IgE from human serum is reported using anion exchange chromatography followed by monoclonal antibody based positive and negative affinity chromatography. Following dialysis of 25–100 ml of serum (2.3–14 μ g IgE/ml, $n = 4$) against 0.05 M Tris pH 8, each specimen was subjected to diethylaminoethyl (DEAE)-cellulose chromatography (serum/matrix = 1/4). IgE was eluted with 0.05 M Tris, 0.05 M NaCl pH 8, yielding an IgE recovery of 61–93%, with removal of $\sim 90\%$ of other serum proteins and an IgE purity ([IgE]/[Igs]) of 0.1–1.1%. After adjusting to 0.1 M NaCl and concentrating ~ 30 -fold, the eluted IgE was further purified by affinity chromatography using a panel of IUIS/WHO-documented mouse monoclonal anti-human immunoglobulin antibodies (α hIg-MAbs). First, the IgE-enriched DEAE-cellulose chromatography fraction was incubated in a batch mode with two α hIgE-Fc MAbs (HP6029, HP6061) coupled to CNBr-Sepharose, CL-4B. IgE was eluted with 0.05 M glycine pH 2.8 and immediately neutralized. The IgE recovery was 32–52% and IgE purity was 72–97%. Silver-stained SDS-PAGE and noncompetitive solid-phase two-site immunoenzymetric assays for total human IgA, IgE, IgG and IgM indicated that IgA, IgG and IgM were the only contaminants. Next, the IgE was concentrated 10–30-fold in the presence of 0.1% HSA. One IgE specimen was ultrapurified in a batch mode by negative selection chromatography using three pairs of α hIg-MAbs (α hIgA: HP6111 + HP6123; α hIgG: HP6017 + HP6046; α hIgM: HP6081 + HP6083) coupled to CNBr-Sepharose, CL-4B. IgE purity increased from 91% to $> 99.9\%$ with $\sim 70\%$ recovery of IgE for this step. The ultrapurified IgE antibody was shown to be functionally reactive for allergen and Fc ϵ RI receptors on human basophils. We conclude that α hIg-MAbs are powerful tools to

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facilitate the affinity purification of functionally active human IgE from serum; however, when the analyte is present in low concentration, a carrier protein needs to be added to minimize non-specific loss of the material during this process.

Keywords: IgE, human; Purification; Chromatography, ion exchange ; Chromatography, affinity; Monoclonal antibody, murine

1. Introduction

Since its discovery in 1967 (Johansson and Bennich, 1967; Ishizaka and Ishizaka, 1967) IgE and its receptors have been extensively studied to provide a better understanding for their role in host defense against parasites and in the etiology of allergic diseases (for review see Sutton and Gould, 1993). Since IgE constitutes a minuscule fraction of the total human immunoglobulin in serum from non-atopic individuals (50–300 ng/ml versus 7–12 mg/ml of IgG), it has been difficult to isolate IgE (Ishizaka, 1985; Ikeyama et al., 1986). The discovery of two patients each exhibiting a myeloma protein of the IgE immunoglobulin (Ig) class (Johansson and Bennich (1967), IgE(ND); Ishizaka et al. (1970), IgE(PS)) at a serum concentration up to 10 mg/ml enabled their purification and subsequent biochemical and biological characterization. More recently, IgE has been purified from the culture fluid of the human myeloma cell line SK007/U266 (Ikeyama et al., 1986), which secretes human IgE(ND) (Nilsson et al., 1970). Early attempts to isolate human IgE from serum with high IgE titers were hampered by difficulties in assessing the purity of the final IgE preparations as a result of a lack of standardized quantitative assays for IgE and problems associated with contaminating serum proteins (Ishizaka and Ishizaka, 1967; Aalberse et al., 1973).

With the availability of human immunoglobulin-specific MAbs, quantitative immunoassays for the clinically indicated Ig classes and subclasses have been developed (Hamilton et al., 1988; Hamilton and Adkinson, 1992). Moreover, these MAbs have been used to fractionate human serum IgG into its four subclasses by immunoaffinity chromatography (Pird et al., 1984; Lambin et al., 1993). In this report we describe a rapid method for the purification of IgE from human serum.

DEAE-cellulose chromatography initially removes all non-immunoglobulin proteins and affinity chromatography (AC) with a panel of IUIS/WHO-documented α hIg-MAbs allows selective isolation of IgE (positive AC) and removal of residual contaminating IgA, IgG and IgM (negative AC).

2. Materials and methods

2.1. Buffers and general methods

All chemicals unless otherwise specified were obtained from Sigma Chemical Co. (St. Louis, MO). Tris buffer (TB) was 0.05 M Tris buffer pH 8.0; 10 $\times$ TB was 0.5 M Tris buffer pH 8.0. Tris buffered saline (TBS) contained 0.05 M Tris buffer pH 8.0 supplemented with 0.1 M NaCl and 10 $\times$ TBS was 0.5 M Tris buffer pH 8.0 supplemented with 1 M NaCl. Glycine buffer was 0.05 M glycine pH 2.8. Phosphate-buffered saline (PBS) was 0.8 w/v% NaCl, 0.02 w/v% KH_2PO_4 , 0.22 w/v% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 w/v% KCl at pH 7.4 ± 0.1 without further titration. PBS-Tween (PBS-T) was PBS with 0.01 w/v% thimerosal and 0.055 v/v% Tween 20. PBS with bovine serum albumin (PBS-BSA) was PBS with 0.01 w/v% thimerosal and 1 w/v% BSA. Citrate-phosphate buffer (70 mM) was 0.564 w/v% citric acid (anhydrous) and 1.116 w/v% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$. ABTS substrate was 0.055 w/v% of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in 70 mM citrate-phosphate buffer (store at 4°C for 1 week). Pipes (P) contains 25 mM Pipes, 110 mM NaCl, and 5 mM KCl adjusted to pH 7.4. Unless otherwise stated, all experimental procedures were performed at room temperature. The protein content of chromatography fractions were estimated from their adsorbance at 280 nm (A_{280}) using a flow spectrophotometer (Milton Roy,

Fisher Scientific, Fair Lawn, NJ). To minimize losses of purified IgE on surfaces by non-specific interactions, fractions were collected in polypropylene tubes.

2.2. IgE antibody-containing sera

EDTA-plasma was collected from atopic donors (total serum IgE: 2.5–13 $\mu\text{g/ml}$) using standard plasmapheresis procedures (RH Typing Laboratory, Baltimore, MD). Conversion of plasma to serum was performed by adding 2 ml of 1 M CaCl_2 and 500 U of bovine thrombin per 100 ml of plasma while stirring in a beaker. After 1 h, the serum was collected by extensively mincing the clot, aliquoted in 25 ml volumes and frozen.

2.3. Ion exchange chromatography

Prior to each ion exchange chromatography experiment, 25 ml of serum were dialysed extensively against TB at 4°C using Spectra/Por membranes (Spectrum, Houston, TX) with a molecular weight cut off of 50 000. During a 24 h period, 4 liters of dialysis fluid were changed three times (the first two were performed at 4°C and the final dialysis at room temperature). Initial conditions using small aliquots of serum were designed and are described in protocols 1a and 1b.

2.3.1. Protocol 1a

Diethylaminoethyl (DEAE) cellulose (DE52, Whatman, Maidstone, England) (10 g) was equili-

brated in 100 ml 0.01 M Tris buffer pH 8. 1 ml of serum (GG, 13 μg IgE/ml) was dialyzed against the same buffer as described above. After removing cellulose fines and transferring 5 ml of the wet gel to a small column, the dialyzed serum was applied to the gel bed (serum/matrix $\approx 1/4$). Fractions were stepwise eluted with Tris buffer pH 8 of increasing molarity (0.01, 0.02, 0.03, 0.05, 0.1 M). A_{280} values were monitored and IgE and IgG levels in each fraction were detected by IEMA (see below).

2.3.2. Protocol 1b

This procedure was identical to protocol 1a except NaCl was added stepwise (0.05, 0.1 M NaCl in 0.05 M Tris buffer pH 8) to increase the molarity of the buffer for elution beyond 0.05 M. After determining the correct conditions we designed the final protocol, termed protocol 2.

2.3.3. Protocol 2

DEAE-cellulose was equilibrated by stirring 250 g of pre-swollen DEAE in 1.5 liters of 10 $\times$ TB (DEAE/buffer = 1/6) at pH 8.0. The fines were removed by sedimenting the gel for 20 min in a 2 liters beaker, decanting the supernatant and washing with 2 liters of TB. This was repeated three times, approximately 100 ml of swollen DEAE-cellulose were packed into a 5 $\times$ 30 cm chromatography column (Bio-Rad cat. #737-5031, Bio-Rad Laboratories, Richmond, CA) to form a 5 $\times$ 7 cm column bed. The column was washed with five bed volumes of TB and then 25 ml of

Table 1
Characteristics of human immunoglobulin-specific murine antibodies^a

Assay specificity	Form of antibodies	Clone	Mouse isotype	Human Ig specificity
IgA	Purified (capture)	HP6111	IgG1	IgA PAN Fc
	Biotinylated (detection)	HP6123B	IgG1	IgA PAN Fd
IgG	Purified (capture)	HP6046	IgG1	IgG PAN Fd
	Biotinylated (detection)	HP6017B	IgG2a	IgG PAN Fc
IgE	Purified (capture)	HP6061	IgM	IgE Fc
	Biotinylated (detection)	HP6029B	IgG1	IgE Fc
IgM	Purified (capture)	HP6083	IgG1	IgM Fc
	Biotinylated (detection)	HP6081B	IgG1	IgM Fc

^a These MAbs were used in pairs for IEMA detection of IgA, IgG, IgE and IgM and in affinity purification of IgE (Hamilton and Morrison, 1993).

dialyzed serum (serum/matrix = 1/4) were applied to the gel. The gel was eluted with 400 ml of TB, collecting 50 ml fractions while monitoring at A_{280} . After the flow through of the first peak, 10 ml fractions were eluted with TB supplemented with 0.05 M NaCl and analysed at A_{280} until the second peak had been eluted (Fig. 2a). A final eluate was collected in 50 ml fractions after applying 200 ml of $10 \times$ TB to the column. The IgE-containing fractions with $A_{280} > 0.1$ were combined, adjusted to a final molarity of 0.05 M Tris, 0.1 M NaCl and concentrated to 10 ml using a diaflow PC 30 ultrafiltration membrane (Amicon, W.R. Grace & Co, Beverly, MA), molecular weight cut off 30 kDa.

2.4. Preparation of anti-human IgA, IgG, IgM and IgE affinity matrices

The α Ig-MAbs (Table 1) were coupled in pairs (two different α Ig-MAbs per Ig isotype) to CNBr-activated agarose (Sephacrose 4B-CL, Pharmacia, Sweden) as described (Hamilton and Adkinson, 1986). In brief, 0.5 mg of each antibody is added in coupling buffer (0.1 M Na_2CO_3 and 0.1 M NaCl, pH 8.6) for each ml of packed agarose previously activated with CNBr in dimethylformamide (March et al., 1974). Following an overnight orbital mixing at 45° , uncoupled active sites were saturated with ethanolamine (1 h at 25°C) and washed with high (8.5) and low (4.3) pH buffers. The binding capacity and non-specific binding of the sorbent was quality controlled using a double reciprocal plot analysis (Aalberse, 1974).

2.5. Affinity purification

Affinity purification was performed in a batch mode using 14 ml polypropylene round bottom tubes (Falcon #2059, Becton Dickinson Labware, Lincoln Park, NY). One ml of packed α IgE beads was washed four times by repeated resuspension in 10 ml of TBS and centrifugation ($750 \times g$, 3 min). The concentrated IgE-containing DEAE-cellulose chromatography fraction (10 ml) was then added to the beads and the mixture was rotated for 12 h. The beads were sedimented by

centrifugation ($750 \times g$, 3 min) and the supernatant was collected. The beads were washed ten times with 10 ml of TBS and recentrifuged to remove unbound material until the A_{280} was ≤ 0.01 . Bound material was eluted three times with 10 ml of glycine buffer with 1 min rotations, followed each time by centrifugation ($750 \times g$ for 3 min). The supernatant was immediately neutralized after each elution with 1 ml of $10 \times$ TBS and stored at 4°C until analyzed.

2.6. SDS-PAGE and silver staining

Samples (50 μl) of the first glycine eluate and the IgE-containing fraction after DEAE-cellulose chromatography were diluted in Tris-glycine SDS sample buffer (Novex, San Diego, CA), heated for 5 min at 95°C and applied under nonreducing conditions to precasted Tris-glycine 4–12% gradient acrylamide minigels (Novex). Wide-range protein standards (Mark 12, Novex) and affinity purified human myeloma IgE(PS) (Calbiochem, La Jolla, CA) as a positive control were run in parallel with eluted proteins. Electrophoresis was performed in Tris-glycine SDS running buffer in a minigel chamber (Novex) according to the instructions of the manufacturer at constant voltage of 125 V for 1.5–2 h. The gels were developed with a sensitive silver stain as previously described (Blum et al., 1987) using micropure H_2O .

2.7. Concentration of IgE after affinity purification

To minimize losses of purified IgE on equipment surface areas, 100 μl of a 10% w/v HSA (Sigma cat. #A8763) were added to 10 ml of the glycine eluate (final HSA concentration: 0.1% w/v) prior to concentration with a centricon 30 (Amicon). Moreover, the sample chamber was also preincubated with 2.7 ml of 1% w/v HSA 1/2 h prior to use to further minimize non-specific loss. Following removal of this solution, IgE-containing glycine eluate was applied to the centricon chamber and centrifuged (15 min, $5000 \times g$, 4°C) in a precooled ultracentrifuge with a 45° angel rotor (SM-24, Sorvall). The filtrate was concentrated to 250 μl and diaflowed two times (15 min, $5000 \times g$, 4°C) with 2.5 ml of $1 \times$ Pipes

(Fisher Scientific) to avoid any further dialysis. After concentration to 100 μ l (30–45 min, 5000 $\times$ g, 4°C), the device was inverted, an additional 100–200 μ l of 1 $\times$ Pipes were added and the eluate was centrifuged (5 min, 5000 $\times$ g, 4°C). The concentrated material was aliquoted and stored at –20°C.

2.8. IgA, IgE, IgG and IgM immunoenzymetric assays (IEMAs)

Two site monoclonal antibody based solid phase IEMAs for human IgA, IgG, IgE and IgM were performed as previously described for IgE (Hamilton and Adkinson, 1992) and IgG (Hamilton et al., 1988). In these assays flat-bottom Immulon IV 96-well microtiter plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with the appropriate capture antibody (Table 1) diluted in PBS to 10 μ g/ml just prior to use. Each plate was washed with PBS-T, using a Corning washing system and 0.2 ml of PBS-BSA were added to each well to block unreacted sites (1 h, 23°C). After four washes with PBS-T, 0.1 ml of buffer, reference (9–11 two-fold dilutions in duplicate), control and test samples were added to the respective wells. The plates were then incubated for 2 h at 23°C. Following a PBS-T buffer wash, the appropriate biotin-conjugated antibody was added (0.1 ml per well, 1 μ g/ml in PBS-BSA, see Table 1) and the plates were incubated for 1 h at 23°C. Following four further PBS-T buffer washes, streptavidin-horseradish peroxidase (avidin-HRP; Sigma S5512) was added (0.1 ml per well, 1 μ g/ml in PBS-BSA) and the plates were incubated for 1 h at 23°C. Plates were finally washed four times, and 0.1 ml/well of 1 mM ABTS substrate in 70 mM citrate-phosphate buffer containing 1 μ l of 30% hydrogen peroxide per ml of ABTS was added. The HRP enzyme reaction was stopped with 0.1 ml of 2 mM sodium azide as the top point on the standard curve reaches an A_{410} of 1.5–2.0 U. Absorbance was read on a microtiter plate reader at 410 nm (Dynatech, MR 4000), and optical densities of unknown samples were interpolated from the standard curve by using an IBM-PC-based cubic spline-fitting program (Johns Hopkins University, Baltimore, MD).

2.9. Ultrapurification of IgE by negative selection chromatography

The final purification step was performed in a batch mode using 1.5 ml Eppendorf tubes. α hIgA, α hIgG and α hIgM-Sepharose (200 μ l, 50% v/v, Table 1) were washed three times by repeated suspension in 1 ml of PBS supplemented with 1 w/v% HSA and centrifugation (750 $\times$ g, 3 min). The supernatant was removed after centrifugation leaving 100 μ l of packed beads (ratio 2:2:1 between α hIgA-, α hIgG- and α hIgM-Sepharose). Washed uncoupled Sepharose (100 μ l) was used as a control. After affinity purification of one IgE preparation (F1491) the resulting concentrate (700 μ l) was added in equal volumes to the MAb-coupled and uncoupled beads. The mixture was slowly rotated for 12 h at 23°C to permit maximal binding of the contaminating Igs to the appropriate beads. After centrifugation, the supernatant representing the flow through was collected and analyzed for IgA, IgG, IgM and IgE levels.

2.10. Definition of recovery and purity

Since IgA, IgG and IgM were the main contaminants after IgE purification, routine protein assays were insufficiently sensitive to detect these small levels. Therefore, our working definitions of IgE recovery and purity were as follows: Total recovery was defined as the IgE recovered in the eluate as a percent of the IgE quantity in the initial serum. Purity of IgE was defined as the quantity of IgE in the eluate in percent as a function of the total amount of immunoglobulins (IgA, IgG, IgM and IgE) in the same specimen.

2.11. Demonstration of functionality

The functional properties of affinity purified IgE were studied in terms of its ability to bind to Fc ϵ RI receptors by passive sensitization using washed lactic acid stripped human basophil leukocytes (Schleimer et al., 1982; Pruzansky et al., 1983). IgE from the same donor (GG) before and after purification was normalized to 2 μ g/ml. Cells were incubated with concanavalin A (Sigma)

or polyclonal anti-IgE at different concentrations in the presence of 5 mM Ca^{2+} at 37°C for 40 min. Aliquots of the cell-free supernatant were finally assessed for IgE-mediated stimulation by monitoring the histamine content by an automated fluorometric assay (Siraganian, 1975). The results were computed as the mean of duplicate determinations and were expressed as percent HR, by dividing total cellular histamine after subtracting the level of spontaneous histamine release from unstimulated basophils. Total cellular histamine content was obtained by lysing cells in 2% perchloric acid.

Immunoreactivity of the affinity purified IgE antibody was studied by its ability to directly bind allergen in the Pharmacia CAP-system (Kleine-Tebbe et al., 1992). After normalization of the total IgE level to 2 $\mu\text{g}/\text{ml}$ specific IgE was detected to cat (e1), house dust mite (d1) and ragweed (w1) allergens and expressed as $k_A\text{U}/\text{ml}$, according to the manufacturer's instructions.

3. Results

Four human sera with elevated IgE levels were used to optimize the IgE purification method. After extensive dialysis, serum sample GG (Table 2) was applied to the DEAE-cellulose column. Fractions eluted by stepwise increases in the elution buffer molarity were analyzed for their optical density at 280 nm and for IgE and IgG levels

by IEMAs. Using DEAE-cellulose chromatography protocol 1a, the major protein and IgG peak were detected in the unbound fraction (Fig. 1a, fraction 1–4). Increasing to 0.05 M eluted additional non-IgE protein. IgE was eluted at an ionic strength of 0.1 M. Smaller stepwise increases in the salt concentration were used in protocol 1b to improve the separation of IgE from other serum proteins. The major protein and IgG peaks (~90% of the total IgG) were eluted in the unbound fraction (Fig. 1b, fraction 1–6). In contrast, IgE was eluted as a sharp peak with 0.05 M Tris, containing 0.05 M NaCl. Further increases in the ionic strength eluted a broad peak of non-IgE proteins, together with trace quantities of IgE and IgG (Fig. 1b).

Based on these results, DEAE-cellulose chromatography (protocol 1b) was scaled up to process 25 ml of serum (protocol 2). The chromatogram in Fig. 2a demonstrates a good separation of IgE from the majority of other serum proteins using 0.05 M Tris, 0.05 M NaCl, pH 8. Two broad protein peaks with very low IgE levels were seen in the flow through eluted with the first buffer (0.05 M Tris) and at 0.5 M Tris respectively. The three other serum samples subjected to this procedure produced almost identical chromatographic profiles (data not shown). Levels of IgA, IgE, IgG, IgM and percentage recovery and purity for IgE are presented in Table 2. This column purification step increased the IgE purity 10 fold while recovering 61–93%

Table 2

Immunoglobulin levels before and after purification of IgE from four sera using DEAE-based ion exchange chromatography and consecutive affinity purification

	Volume (ml)	IgA ($\mu\text{g}/\text{ml}$)	IgG ($\mu\text{g}/\text{ml}$)	IgM ($\mu\text{g}/\text{ml}$)	IgE ($\mu\text{g}/\text{ml}$)	IgE recovery (%)	IgE purity (%)
Serum (GG)	25	3 740	19 480	2 610	14	(100)	0.05
DEAE eluate	310	6.5	165	0.45	0.83	74	0.48
αIgE eluate #1	11	0.86	1.2	0.006	16.4	52	89
Serum (F1491)	25	2 080	7 110	550	12	(100)	0.12
DEAE eluate	260	9.6	66	0.2	0.87	75	1.13
αIgE eluate #1	10.7	0.1	0.3	0.006	12	43	97
Serum (BG)	99	2 310	9 570	1 670	2.9	(100)	0.02
DEAE eluate	700	11.1	105	0.3	0.38	93	0.33
αIgE eluate #1	10.5	0.01	2.4	0.01	9.6	35	80
Serum (PC)	99	1 710	9 570	2 410	2.3	(100)	0.02
DEAE eluate	700	15	154	0.3	0.2	61	0.12
αIgE eluate #1	11	0.18	2.4	0.01	6.7	32	72

of the IgE protein. Following a 25–35-fold concentration, the combined IgE eluate (GG) was subjected to affinity purification using CNBr-activated agarose beads coupled with two α hIgE-MAbs (HP6061 and HP6029) that bind to non-overlapping determinants on the IgE Fc. Slow rotation was performed for 12 h to ensure maximal binding of the IgE to the solid phase MAb. A broad peak of non-IgE serum proteins was washed

out in the first unbound fractions. Washing was continued until no further decrease of the OD reading was detected (Fig. 2b). The majority of the IgE was collected in the first 0.05 M glycine, pH 2.8 fraction. When the other sera were affinity purified using the same protocol, their chromatography profiles were almost identical to the pattern depicted in Fig. 2b (data not shown). IgE purity following this procedure was 72–97% while the recovery ranged from 32–52% (Table 2).

SDS-PAGE analysis of the DEAE-chromatography eluates obtained with the 4 IgE specimens revealed several bands (Fig. 3, lane C1, C2 and C3). The most prominent band at 200 kDa may represent IgG, which was detected in high levels by the IEMA (Table 2). After affinity purification, a broad, dense band was seen at 230 kDa (Fig. 3, lane B1 and B2) representing purified IgE. A band at the same location was produced by the purified myeloma-IgE control (lane A1 and A2). However, a weakly silver-stained band was detected at 200 kDa even in the affinity purified material of both IgE samples (Fig. 3, lane B1 and 2). This band most likely represents contaminating immunoglobulins (IgA and IgG), that were also detected by the IEMA in the same samples (Table 2).

To obtain ultrapure (> 99%) IgE for biochemical studies, the remaining IgG and IgA contaminating proteins were removed by 'negative' affinity chromatography. This was accomplished by incubating the affinity purified IgE from a representative atopic patient (F1491) with α hIgA-, α hIgG- and α hIgM-Sepharose beads. The remaining non-IgE immunoglobulins were removed without significant losses in purified IgE (Table 3). The overall procedure achieved a final IgE purity of > 99.9%.

Concanavalin A and anti-IgE induced histamine release from human basophils showed almost identical dose response curves after passive sensitization with unpurified or the affinity purified IgE (specimen GG, Fig. 4). This indicates that the binding capacity of the Fc $_{\epsilon}$ portion of the IgE to its receptor and to Fc-specific stimuli is unaltered. Allergen-specific IgE levels as measured before and after purification were comparable indicating that antibody immunoreactivity

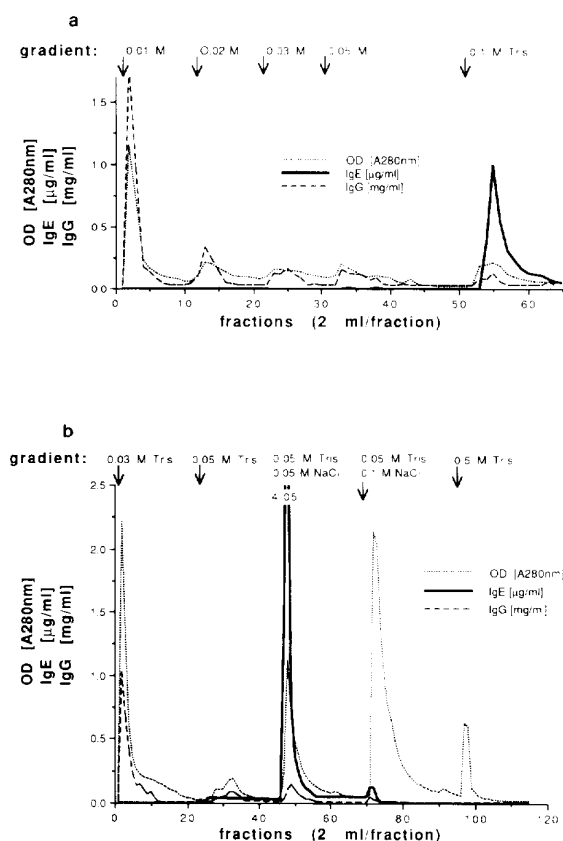


Fig. 1. Chromatograms are presented based on two different protocols for anion exchange purification of IgE from serum, using DEAE-cellulose. The top arrows in each figure indicate the stepwise increasing buffer gradients. The x axis indicates the fraction numbers; the y axis shows OD readings at 280 nm and IgE (μ g/ml) and IgG levels (mg/ml), detected with the IEMA. a: molarity of Tris (pH 8) was stepwise increased to separate IgG and other serum proteins from the IgE fraction. b: to enhance the separation of IgE from other serum proteins, NaCl was added in a stepwise manner to the Tris buffer.

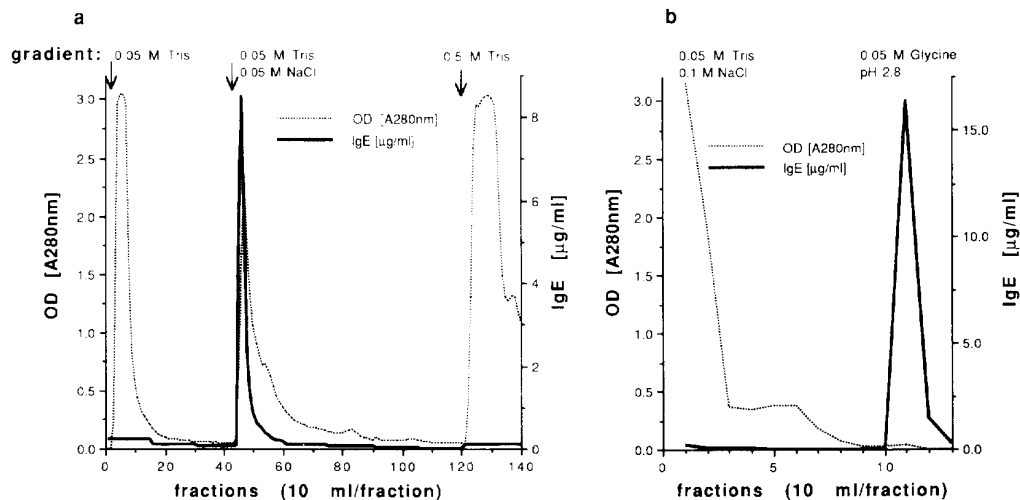


Fig. 2. Chromatograms obtained using the scaled up anion exchange purification (a) and consecutive affinity purification of IgE (b) protocols. The serum processed in the illustration contained high IgE levels (14 $\mu\text{g/ml}$). Fraction numbers are given on the x axis; adsorbance readings at 280 nm are shown on the left y axis and IgE levels ($\mu\text{g/ml}$) are shown on the right y axis of each figure.

was maintained after the purification process (Table 4). These data demonstrate that the IgE samples produced in the overall affinity purification procedure were functionally active and highly immunoreactive.

4. Discussion

Investigators have attempted to isolate polyclonal IgE antibodies from guinea pig (Karol et al., 1991), canine (Peng et al., 1993), equine (Suter and Fey, 1988) and bovine serum (Thatcher and Gershwin, 1988). Moreover a number of monoclonal IgE molecules have been isolated from rat myelomas (Bazin et al., 1974), mouse hybridomas (Böttcher and Hämmerling, 1978) and mouse α -nitrophenyl human IgE-secreting transfectomas (Neuberger et al., 1985). While some of the principles used in these studies may be applicable, the species-specificity of most immunological reagents have not been of particular help in any purification of polyclonal human IgE from serum.

Previous studies isolated human IgE from the myeloma cell line U266, using a tedious procedure involving ammonium sulfate precipitation,

affinity chromatography on lysine-Sepharose 4B, ion exchange chromatography on DEAE-Sepharcel, gel filtration, recycling chromatography on Sephacryl S-300 and extraction of bovine proteins on an anti-bovine serum rabbit IgG-Sepharose 4B column (Ikeyama et al., 1986). A more concise combination of ammonium sulfate precipitation, immunoaffinity chromatography and gel filtration produced better yields and a comparable purity of the isolated myeloma-IgE. However, it has always been difficult to remove all contaminating serum proteins without the use of purified α hIg-antibodies (Ishizaka, 1985; Aalberse et al., 1973). In the present study, we have combined the high loading capacity of anion exchange resin (microgranular DEAE) chromatography to remove non immunoglobulin proteins (Rossomando, 1990) with the specificity of monoclonal antibody based affinity chromatography.

The DEAE microgranular cellulose chromatography of the dialyzed serum samples provided a good recovery of IgE (mean: 76%, range: 61–93%) and resulted in the removal of approx. 90% of irrelevant protein which represented a ten-fold enhancement in IgE purity. The simple technique of stepwise salt elution from micro-

granular DEAE minimized the inactivation of IgE that has been observed in the case of salt precipitation of IgG (Phillips et al., 1984). The

remaining contaminating IgG (~ 10%) probably had very similar charge properties to IgE (Gorevic et al., 1985). The availability of IUIS/WHO-

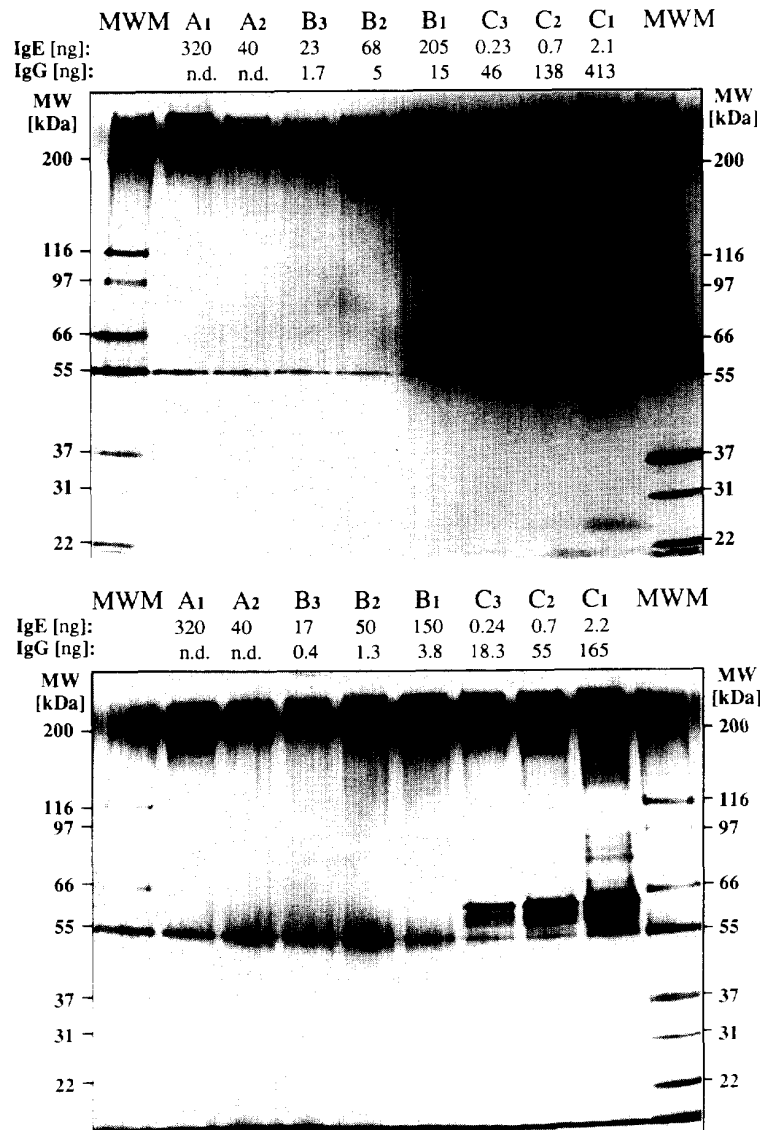


Fig. 3. Protein samples at different stages in the purification of IgE from two sera (upper figure: GG; lower figure: F1491) were separated under nonreducing conditions by SDS-PAGE on 4–12% gradient gels and developed using a sensitive silver stain. The outer right and left lane (MWM) show marker proteins; their molecular weight is given on the left and right lane beside the gel (MW). Lanes A1 and A2 represent two dilutions of a purified myeloma IgE used as reference protein. Lanes B1–B3 show bands obtained with dilutions of the eluted IgE after affinity purification, lanes C1–C3 show protein bands obtained with dilutions of the eluted material after ion exchange purification of IgE from serum. The amounts of IgE and IgG in each sample that were detected by IEMA and corrected for dilution are presented on the top of each gel.

Table 3

Immunoglobulin levels after ultrapurification of affinity-purified polyclonal IgE

Affinity adsorbent	Immunoglobulin levels (ng/ml)			
	IgA	IgG	IgM	IgE
Uncoupled Sepharose (sham control)	1 400	1 000	< 10	23 900
α hIgA + α hIgG + α hIgM-Sepharose	< 10	< 2	< 2	20 600

Contaminating immunoglobulins were selectively removed by incubation with a mixture of six anti-human Ig monoclonal antibodies (Table 1) coupled to CNBr-activated agarose beads.

documented MAbs to the four immunoglobulin classes enabled the positive and negative affinity chromatography steps. MAb based affinity adsorbents may be preferable to polyclonal adsorbents since milder, less denaturing conditions can be used for antigen elution (Pird et al., 1984). The binding capacity was enhanced by the use of MAbs of two different clones with the same Ig class specificity for each antibody. Optimal bind-

ing of IgE had been determined to occur after a 12 h incubation period; 50% of the IgE from the DEAE fraction was recovered and the purity was increased 164 fold (range: 80–625-fold). When purified rabbit antibodies were used for the affinity purification of polyclonal human IgE (Aalberse, 1974), their batch procedure produced comparable IgE yields, but more contamination of non-IgE proteins were present.

SDS-PAGE analysis using 4–12% acrylamide gels with a broad protein separation range (22 to > 200 kDa) showed a uniform band at 55 kDa running through the lanes of all gels that most likely reflects an artifact, while it has not been reported in the original description of the silver stain protocol (Blum et al., 1987) and may be related to non-specific background staining of the precasted gels used. We analyzed several dilutions of the same purified IgE-preparations to semiquantitatively evaluate the levels of impuri-

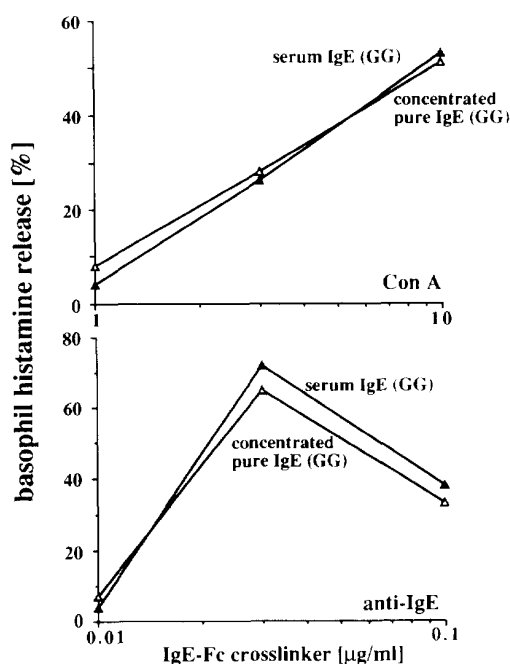


Fig. 4. Concanavalin A and anti-IgE induce the same amount of histamine release from human basophils after passive sensitization with unpurified and purified IgE samples of donor GG. Lactic acid treated basophils were passively sensitized with 2 μ g/ml of IgE from starting serum or affinity purified IgE. The cells were then stimulated with different concentrations of ConA or anti-IgE as shown.

Table 4

Allergen-specific IgE levels before and after purification of IgE from four sera using DEAE-based ion exchange chromatography and consecutive affinity purification

	Cat (e1) (kAU/ml)	<i>D. pteronys</i> . (d1) (kAU/ml)	Ragweed (w1) (kAU/ml)
Serum (GG)	6.1	12.7	8.9
α IgE eluate #1	15.1	25.4	22.7
Serum (F1491)	0.5	0.6	41.5
α IgE eluate #1	14.3	25.7	42.8
Serum (BG)	13.2	78.5	16.4
α IgE eluate #1	21.1	59.6	31.5
Serum (PC)	0.47	20.8	40.9
α IgE eluate #1	14.7	39.0	54.6

Total IgE levels were normalized to 2 μ g/ml by appropriate sample dilutions before allergen-specific IgE antibodies to cat, housedust mite and ragweed allergens were detected by the CAP system.

ties. The faint bands at 200 kDa in the affinity purified samples (Fig. 3, B1–B3) and the detection of IgA and IgG in the IEMA indicate that these two non-IgE isotypes represent the primary contaminants. Purified IgE and the IgE myeloma control both exhibited a 230 kDa MW band in nonreducing gels. This size estimate is consistent with previous molecular weight estimates with purified IgE(ND) myeloma from U266 (Ikeyama et al., 1986) or dog serum (Peng et al., 1993). Interestingly, the purified myeloma IgE sample also showed a sharp band between stacking and running gel (Fig. 3, A1 and A2) that was probably oligomers or aggregates of the protein that could not enter the gel.

The greatest challenge was preventing the loss of the purified IgE on equipment surfaces as a result of non-specific absorption. For this reason, we minimized the need for extensive dialysis. The same buffer system for ion-exchange and affinity purification was used with minor adjustments in the molarity of the elution buffer. Diaflowing the affinity purified IgE after its final concentration with $1 \times$ Pipes avoided an additional dialysis step. However, we needed to introduce a carrier protein (99.9% purified HSA) to minimize the loss of purified IgE during its final concentration. All subsequent procedures including ultrapurification with selective removal of remaining Ig-like contaminants were performed in the presence of a carrier protein. Although this could reintroduce protein contaminants, it was a necessity to prevent > 90% loss. To insure that the purification of IgE is as complete as possible, any residual IgD should have been removed using negative affinity chromatography with monoclonal antibodies to IgD. However, the major contaminant was IgG, as shown by the Ig amounts detected after positive affinity chromatography with α hIgE-MAbs, whereas IgA, IgM and most likely IgD represented only a minor portion of the contaminants. We therefore chose not to attempt any removal of residual IgD in this study.

It has been reported, that ligands might leach off the matrix during affinity purification (Ostrove, 1990). We did not control for this during the positive and negative affinity purification. However, the latter one did not expose the matrix

to acidic conditions and leakage of ligands from the matrix might therefore be of minor importance. Major leakage of ligands during positive affinity purification by α hIgE beads is unlikely since separation of the eluate by SDS-PAGE revealed only minor contaminants at 200 kDa. These were most likely due to other serum Igs which were detected by quantitative immunoassays.

The purpose of this study was to purify IgE for enzymatic digestion of its oligosaccharides with the ultimate goal of determining whether glycosylation is necessary for interaction with a serum factor, that causes IgE-dependent histamine release from human basophils (MacDonald et al., 1987). In summary, we have established a rapid purification method with acceptable yields of polyclonal IgE from serum of allergic donors with elevated IgE levels. We conclude that α hIg-MAbs are powerful tools to facilitate the purification of human IgE from serum; however, depending on the starting concentration of IgE, a carrier protein needs to be added to avoid excessive loss of isolate during its final purification steps.

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