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Purification and partial biochemical characterization of IgE-binding factors secreted by a human B lymphoblastoid cell line

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SUMMARY

IgE-binding factors (IgE-BFs) were purified from the culture supernatant of RPMI-8866 cells, a human lymphoblastoid B-cell line expressing IgE receptors. The material, purified by affinity-chromatography on immunoadsorbents coupled to IgE or to monoclonal antibody against IgE receptor, was comprised of two major components with apparent molecular weight (MW) of 25,000–27,000 and 12,000, as determined by SDS–PAGE and silver staining. Only the 25,000–27,000 MW molecules were identified as IgE-BFs, as demonstrated by (i) their reactivity with MabER in the Western blot and the immunoprecipitation assays, and (ii) their ability to inhibit rosette formation of U937 cells with IgE- but not with IgG-coated erythrocytes. IgE-BFs were purified to homogeneity by combining affinity-chromatography and either DEAE–ion exchange or reverse-phase chromatography on an HPLC system. Chromatofocusing analysis demonstrated the microheterogeneity of IgE-BFs that were comprised of molecules with isoelectric points ranging from 5·0 to 4·4. IgE-BFs were sensitive to treatment with O-glycosidase but not with *N*-glycanase. These molecules were resistant to heat and to pH ranging from 2 to 9; their immunoreactivity was lost after treatment with trypsin and pepsin. Papain digestion of purified IgE-BFs generated 14,000–16,000 MW molecules that were still binding to IgE and to MabER.

INTRODUCTION

The existence of T-cell factors that have affinity for IgE (i.e. IgE-binding factors) and that selectively regulate IgE responses, has been extensively documented in animal models (Ishizaka, 1985; Katz, 1985). Martens *et al.* (1985) have cloned the cDNA coding for rodent IgE-BFs. The recombinant IgE-BFs derived from transfection of Cos monkey cells with these cDNA clones were capable of selectively potentiating IgE responses. They consisted of two species with apparent MW of 60,000 and 11,000. The same authors were able to switch the biological activity of these factors from IgE potentiation to IgE suppression by interfering with their glycosylation, demonstrating that IgE-potentiating and IgE-suppressing IgE-BFs were encoded by a single gene (Ishizaka, 1985), and confirming that the difference between the two factors resided in their carbohydrate moieties (Iwata, Huff & Ishizaka, 1984).

Recently, human T-cell factors that either enhance or suppress the *in vitro* synthesis of IgE by human lymphocytes have been described. Young, Leung & Geha (1984) demonstrated that FcER$^+$ T-cell lines derived from patients with the hyper-IgE syndrome produced IgE-binding factors with IgE-potentiating activity. Deguchi *et al.* (1983) showed that T cells from tuberculosis patients released IgE-suppressive factors when stimulated with tuberculin in the presence of IgE.

More recently, Huff *et al.* (1984) constructed a human T-cell hybridoma producing IgE-binding factors with IgE-potentiating activity. These IgE-BFs were comprised of three species with a MW of 60,000, 30,000 and 15,000; the 30,000 form was biologically inactive. More recently, the same authors described a human T-cell hybridoma capable of secreting IgE-BFs with suppressive activity (Huff, Jardieu & Ishizaka, 1986). In this case, IgE-BFs were made of two species with MWs of 60,000 and

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Abbreviations: BSA, bovine serum albumin; CSN, cell-free culture supernatant; EACA, E-amino caproic acid; EBV, Epstein-Barr virus; EDTA, disodium ethylene diamine tetraacetic acid; E-IgE, bovine erythrocytes coated with IgE; E-IgG, bovine erythrocytes coated with rabbit IgG anti-ox erythrocytes; FcER, lymphocyte receptor for IgE; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; IgE-BFs, IgE-binding factors; IgE-PS, IgE myeloma from patient PS; MabER, monoclonal antibody to FcER; 2-ME, 2-mercaptoethanol; NP-40, nonidet P-40; PBS, phosphate-buffered saline; pI, isoelectric point; PMSF, phenylmethyl sulphonyl fluoride; RIA, radioimmunoassay; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

25,000–30,000, and only the 25,000–30,000 molecules were biologically active.

Previous studies from our laboratory have documented the existence of biologically active human IgE-BFs of B-cell origin (Sarfati *et al.*, 1984b). It was shown that RPMI-8866 cells, a lymphoblastoid cell line expressing IgE receptors, secreted IgE-BFs capable of potentiating the spontaneous *in vitro* synthesis of IgE by B cells from atopic patients. IgE-BFs could also be detected in the culture supernatant of several other EBV-transformed B-cell lines expressing FcER (Sarfati *et al.*, 1984a). We next reported the production of monoclonal antibodies specific to FcER (MabER) (Rector *et al.*, 1985); these antibodies or their $F(ab')_2$ fragments bound the same molecules as IgE, on the surface of FcER-bearing B cells (Nakajima & Delespesse, 1986). It was subsequently found that the same MabER also reacted with FcER on the surface of T cells and of macrophages, and more importantly that they bound to IgE-BFs derived from the same cell lines (Sarfati *et al.*, 1986a). The IgE-binding activity of the culture supernatant from FcER-bearing cell lines could be specifically adsorbed on MabER solid-phase from which it was recovered by acid elution. On the grounds of these observations, two MabER were employed to develop a solid-phase radioimmunoassay for the measurement of IgE-BFs. The molecules detected by this assay appeared to be IgE-BFs since they were retained on IgE- but not on IgG-immunoadsorbents. In the present report, these MabER were used to purify to homogeneity IgE-BFs from the culture supernatant of RPMI-8866 cells and to determine their physicochemical characteristics.

MATERIALS AND METHODS

Reagents and cell line

The RPMI-8866 cell line was obtained from Dr Ralph (Sloan Kettering, New York, NY); U937 cells, a macrophage cell line co-expressing FcER and FcγR, were received from Dr Spiegelberg (Scripp Clinics, La Jolla, CA); MabER were produced as described previously (Rector *et al.*, 1985); IgE myeloma PS was a gift from Drs T. and K. Ishizaka (Johns Hopkins University, Baltimore, MD); trypsin–agarose, neuraminidase–agarose, piperazine–HCl, acetonitrile and trifluoro-acetic acid were purchased from Sigma Chemical (St Louis, MO); pepsin–agarose and papain–agarose were obtained from Pierce Chemical Company (Rockford, IL); *N*-glycanase and O-glycosidase were from Genzyme (Genzyme Corp., Boston, MA); RPMI-1640, FCS, penicillin and streptomycin, were purchased from Flow Labs (Rockville, MD); Affi-gel was coupled to either MabER or IgE at 10 mg/ml according to Bio-Rad manual instructions; polybuffer 74 was obtained from Pharmacia (Uppsala, Sweden).

Preparation of cell-free culture supernatant (CSN) from RPMI-8866 cell line

RPMI-8866 cells were mass-cultured in complete RPMI-1640 culture medium containing 10% FCS for 48 hr at a density of 5×10^5/ml in order to obtain 5×10^9 cells. Cells were then washed twice with HBSS and resuspended in RPMI-1640 without FCS at 5×10^6 cells/ml. After overnight incubation, the supernatant was harvested and supplemented with 0·2% sodium azide, 5 mM EACA and 1·7 mM EDTA. It was passed through a 0·22 μm Millipore filter and concentrated 100-fold with an Amicon ultrafiltration unit equipped with a YM5 membrane (Amicon Corp., Danvers, MA). In some experiments, ^{75}Se-methionine internally labelled proteins were prepared as described elsewhere (Caraux *et al.*, 1983). Briefly, cells were washed with HBSS and incubated for 2 hr with methionine-free medium containing 5% dialysed FCS. They were then incubated with ^{75}Se-methionine (Amersham, 10 Ci/mmol, 0·1 mCi/ml) in RPMI-1640 medium containing 5% FCS. After 18 hr the cells were washed, resuspended in RPMI-1640 without FCS for another 24 hr, and then their CSN was collected.

Radioimmunoassay for the detection of IgE-BFs

IgE-BFs were measured by a solid-phase radioimmunoassay as described previously (Sarfati *et al.*, 1986a). Briefly, 96-well microtitre plates were coated with 150 μl of MabER 176 at a concentration of 15 μg/ml. After overnight incubation, plates were blocked for 2 hr with HBSS–10% FCS, and after washing, 100 μl of the sample were allowed to incubate for 4 hr. The plates were then washed and 100 μl of ^{125}I-MabER 135 ($2–3 \times 10^4$ c.p.m./ng) were added to the wells. After overnight incubation, the wells were washed and counted in a gamma counter. The total radioactivity was ±350,000 c.p.m./well, and the blank was determined by HBSS–10% FCS (±150 c.p.m.)

Affinity chromatography

Twenty-five ml of 100-fold concentrated CSN of RPMI-8866 cells were passed through a 0·22 μm Millipore filter and adsorbed on 2 ml Affi-gel coupled to MabER 30 (clone 20.7.25.A.4.4/30) using a flow rate of 2 ml/hr. The gel was then washed with 20 volumes of PBS containing 0·5 M NaCl and 0·05% Triton X-100, followed by 40 volumes of PBS and three volumes of 0·9% NaCl; it was eluated with 0·1 M glycine–HCl, pH 2·6. The eluate was collected in tubes containing 1 M Tris–HCl, pH 9, and 0·1% SDS (w/v). The same procedure was employed to adsorb IgE-BFs on Affi-gel coupled to IgE-PS or to normal human IgG. In some experiments the glycine eluate was collected in tubes containing 1 M Tris–HCl, pH 9, and 2% Tween 20 (v/v).

DEAE ion exchange chromatography

Affinity-purified IgE-BFs were dialysed against 0·05 M Tris-HCl, pH 8, and layered on a TSK 5PW DEAE column (LKB) equilibrated with the same buffer. The bound material was eluted with a gradient of increasing NaCl concentrations up to 1 M final concentration using a high pressure liquid chromatography (HPLC) system (LKB). Fractions were collected every minute at a flow rate of 1 ml/min; they were tested by RIA for the presence of IgE-BFs.

Reverse-phase HPLC chromatography

IgE-BFs eluted from MabER affinity columns (in PBS containing 2% Tween 20) were applied on a HI-Pore RP 304 column (BioRad, Richmond, CA) equilibrated with 5% acetonitrile (CH_3CN) and 0·1% trifluoro-acetic acid (TFA). The bound material was eluted with a linear gradient of increasing CH_3CN concentrations up to 75% final concentration using a HPLC system. Fractions were collected every 0·5 min at a flow rate of 1 ml/min.

Chromatofocusing

Affinity-purified IgE-BFs (in PBS containing 2% Tween 20) were dialysed against 0·025 M piperazine–HCl buffer, pH 6·3, and applied to a Mono-P column (Pharmacia) equilibrated with

the same buffer. The column was eluted at a constant flow rate of 1 ml/min with 45 ml of polybuffer 74 diluted 1/10, pH 4·5. Fractions were collected every minute and analysed in parallel by RIA and SDS–PAGE.

SDS–PAGE and Western blot analysis

SDS–PAGE was performed according to Laemmli (1970) using a 12% polyacrylamide gel with or without 5% 2-mercaptoethanol. Proteins were detected by silver staining or by autoradiography. For the Western blot assay, they were electrically transferred to a nitrocellulose membrane at 70 V for 4 hr as described elsewhere (Nakajima & Delespesse, 1986). Briefly, the membranes were saturated for 2 hr with Tris-buffered saline (TBS) containing 10% FCS and incubated with MabER coupled to biotin (15 μg/ml). After three washes with TBS, the membranes were reacted with horseradish peroxidase (HRP)–avidin for 4 hr and developed by the enzyme substrate (HRP colour development). Unrelated Mab coupled to biotin was used as a negative control.

Enzymatic and pH treatment

One ml of unconcentrated CSN of RPMI-8866 cells, prepared in serum-free culture medium, was mixed with either 1 unit of insolubilized trypsin or 0·75 U neuraminidase coupled to agarose and treated as described elsewhere (Sarfati *et al.*, 1984b). For pepsin treatment, 1 ml of the same CSN was dialysed against 20 mM acetate buffer, pH 4·5, and incubated at 37° with 50 μl of insoluble pepsin. After 2 hr, the reaction was stopped by centrifugation; the CSN was harvested, dialysed against HBSS and tested by RIA. A similar procedure was employed for papain digestion, using a 20 mM NaH_2PO_4 20 mM cysteine–HCl, 10 mM EDTA Na buffer, pH 6·2, for equilibrating the solid phase and the sample. The reaction was stopped after 5 hr incubation at 37° with rocking. O-glycosidase and *N*-glycanase treatment were performed exactly as described in Genzyme's instruction manual. Removal of sialic acid by neuraminidase was an essential step prior to O-glycosidase digestion. In some experiments, aliquots of unconcentrated 8866 CSN were dialysed for 5 hr against buffer solutions at pH 2 (KCl–HCl) or pH 9 (glycine–NaOH); the samples were subsequently neutralysed to pH 7·4, centrifuged and assayed by RIA.

Immunoprecitation methods

An aliquot of ^{125}I-labelled affinity-purified IgE-BFs (5×10^4 c.p.m./50 μl) was mixed with 2 μg of MabER and incubated for 4 hr at room temperature. The antigen–antibody complexes were precipitated by adding affinity-purified rabbit anti-mouse IgG at the adequate concentration. After overnight shaking at 4°, the precipitate was successively washed three times (with 0·8 ml PBS containing 0·5% NP-40 and 0·05% BSA), dissolved in a 2% SDS solution and counted. Samples were analysed by SDS–PAGE and autoradiography, employing ZAR-5 X-ray film (Eastman Kodak, Rochester, NY) and Dupont lightening and intensifying screen (Dupon de Neumours Co, Wilmington, DE).

Inhibition of rosette formation

The assay was performed exactly as described elsewhere (Sarfati *et al.*, 1984b). Briefly, 30 μl of a preparation containing IgE-BFs were first preincubated with 15 μl of a 2% suspension of IgE-coated erythrocytes (E-IgE) before being rosetted with 15 μl of RPMI-8866 cells (10^7 cells/ml). The preparation was centrifuged at 90 *g* and incubated for 2 hr at 4°. After the addition of 10 μl acridine orange, the pellet was resuspended in HBSS containing 3% BSA, and rosette-forming cells (RFC) were counted under a Leitz fluorescence microscope equipped with a Ploemak 0·63 objective. A lymphocyte tightly surrounded by at least three red cells was considered as a RFC. Samples were coded and counted in duplicate; the examiner was not informed of the code. In some experiments, U937 cells, a macrophage cell line co-expressing FcER and FcγR, were employed. In this case, the same cell preparation was used to measure, in parallel experiments, the inhibition of IgE and IgG rosette formation. In the latter case, bovine erthrocytes were coated with a subagglutinating dose of rabbit IgG anti-ox erythrocytes as described elsewhere (Moretta *et al.*, 1979).

RESULTS

Affinity chromatography

The material isolated from RPMI-8866 CSN by affinity chromatography on Affi-gel columns coupled to either MabER, IgG or IgE was first analysed by SDS–PAGE and silver staining. As shown in Fig. 1a, two major components with apparent MW 25,000–27,000 and 12,000, were identified in the eluate from MabER (Lane 1) and from IgE-Affi-gel (Lane 2). Since the 25,000–27,000 component was comprised of at least three distinct bands, it will be referred to as the 27,000 complex. The latter could not be detected in the eluate from IgG-immunoadsorbent which contained molecules centred at 43,000 and 12,000 (data not shown). These results suggested that the 27,000 complex might be the IgE-BFs as it was the only component

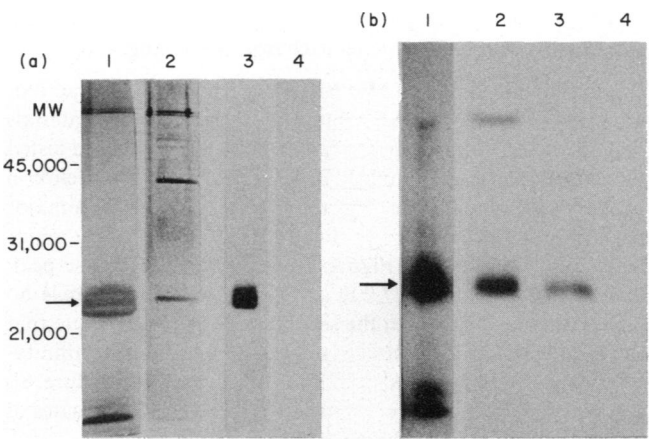

Figure 1. Affinity-chromatography and Western blot: 8866 CSN was adsorbed on Affi-gel coupled to MabER or to IgE (a); the glycine–HCl eluate was analysed on a 12% SDS–PAGE (in the presence of 5% 2-ME) and the proteins were identified by silver staining (Lanes 1 and 2) or by Western blot (Lanes 3 and 4). Lane 1 shows the eluate of MabER 30-Affi-gel, and Lane 2 shows the eluate of IgE–Affi-gel. The eluate of MabER 30-Affi-gel was electrically transferred to a nitrocellulose membrane which was cut into two strips, one was incubated with MabER 30 (Lane 3) and the other with unrelated Mab (Lane 4). In (b), Lane 1 represents ^{75}Se internally-labelled 8866 IgE-BFs purified on MabER 30–Affi-gel; ^{125}I-labelled 8866 IgE-BFs purified on MabER 30–Affi-gel are represented in Lane 2. The same material was immunoprecipitated by MabER 30 (Lane 3) but not by unrelated Mab (Lane 4).

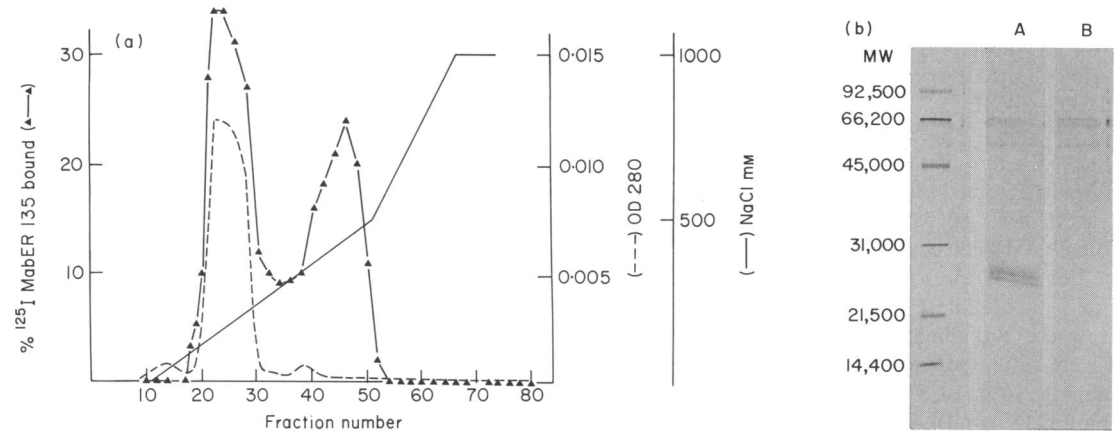

Figure 2. Purification of IgE-BFs by ion exchange chromatography: 1 ml of affinity-purified material was fractionated on a TSK 5PW DEAE column using a flow rate of 1 ml/min. One-ml fractions were collected, and each fraction was diluted 1/20 and tested by RIA (a). One-hundred and fifty microlitres of Fractions 24 and 44 were subjected to 12% SDS–PAGE in the presence of 5% 2-ME and analysed by silver staining (b, Lanes A and B).

binding to both MabER and IgE. To test this hypothesis further, the eluate from MabER-Affi-gel was fractionated by SDS–PAGE and the proteins binding specifically to MabER were detected by Western blot analysis (Fig. 1a); the same eluate was also analysed by immunoprecipitation (Fig. 1b). The 27,000 complex reacted with MabER (Fig. 1a and b, Lane 3) but not with an unrelated monoclonal antibody of the same subclass (Fig. 1a and b, Lane 4). In order to exclude the possibility that the 27,000 complex might be a contaminant of the complete culture medium, we demonstrated that it could be internally labelled (Fig. 1b, Lane 1) by culturing RPMI-8866 cells in methionine-free medium supplemented with ^{75}Se-methionine.

Purification of IgE-BFs by ion exchange chromatography

The material eluted from MabER-Affi-gel column was fractionated on a TSK 5PW DEAE column as described in the Materials and Methods. Fractions were collected every minute and tested in RIA for their content in IgE-BFs. Figure 2a shows the elution profile of the preparation depicted in Fig. 1a (Lane 1). A major peak of IgE-BFs activity was eluted at 0·15 M NaCl and a minor peak at 0·40 M NaCl. Figure 2b shows that the first peak contained the purified 27,000 complex (Lane A), whereas no band could be detected in the second peak (Lane B). Note that the 12,000 molecules contaminating the preparation of affinity-purified IgE-BFs were lost during the purification procedure. So far, we were not able to recover these molecules in the eluates of the ion exchange or of the reverse-phase columns.

Purification of IgE-BFs by reverse-phase chromatography

The same preparation of affinity-purified IgE-BFs was applied on a HI-Pore RP304 column equilibrated with 5% CH_3CN and 0·1% TFA. The material bound was eluted with a linear gradient of acetonitrile. Figure 3 shows that IgE-BFs activity could be separated as a sharp peak at 35% CH_3CN, final concentration. When analysed by SDS–PAGE, this peak contained exclusively the 27,000 complex; this is illustrated in Fig. 5 (Lane 5) where radiolabelled IgE-BFs were purified by this procdure.

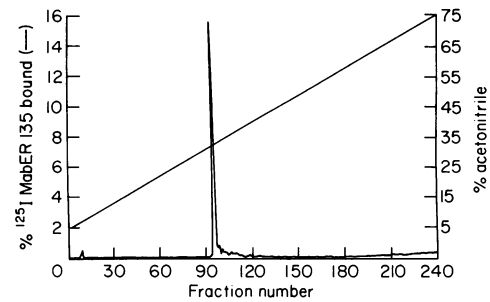

Figure 3. Purification of IgE-BFs by reverse-phase HPLC: affinity-purified IgE-BFs were fractionated on a HI-Pore RP 304 column. Fractions were collected every 0·5 min, diluted 1/10, and tested by RIA.

Isoelectric point

Affinity-purified IgE-BFs was assayed on a chromatofocusing (Mono P) column with a pH gradient from 4·1 to 5·5 (Fig. 4a). The IgE-BFs activity, as measured by RIA, migrated with three major peaks corresponding to pH 5·0, 4·75 and 4·40, respectively, and three minor peaks with pH 5·2, 4·70 and 4·55. This heterogeneous pattern was reproducibly obtained in several experiments. Samples corresponding to pI 5·0–4·3 (as indicated by arrows 1–15) were further analysed by SDS–PAGE and silver staining. Figure 4b shows that the 27,000 complex present in the original preparation of IgE-BFs could be resolved into single bands (Lanes 3, 5–7 and 14) corresponding to the three major peaks of IgE-BFs activity. Note that Fractions 1, 9 and 13 with a low or negative reactivity in RIA had no proteins in the 25,000–27,000 region as detected by silver staining.

Enzymatic treatment of IgE-BFs

In these experiments (Fig. 5), the eluate from MabER-Affi-gel was first radiolabelled and then further purified by either a second cycle of affinity chromatography on MabER-Affi-gel (Lanes 1 and 3) or by reverse-phase chromatography (Lane 5). As seen, IgE-BFs were sensitive to O-glycodidase (Lane 4) but

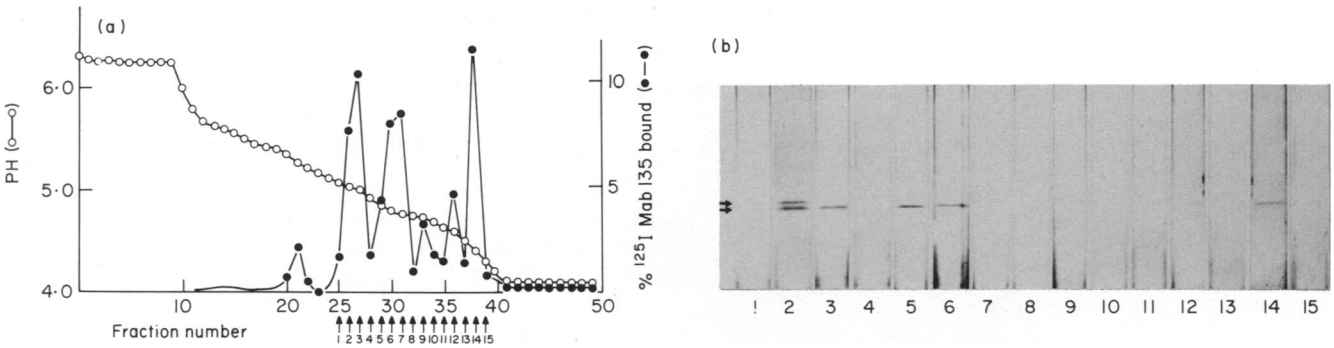

Figure 4. Chromatofocusing: affinity-purified IgE-BFs was fractionated on a Mono-P column at a flow rate of 1 ml/min. Fractions were collected every 1 min and tested in RIA at a dilution of 1/20 (a). In (b), the chromatofocusing fractions were analysed by SDS–PAGE (arrows correspond to the molecular weights of 25,000 and 27,000). Lanes 1–15 correspond to Fractions 1–15 [as indicated by the arrows in (a)].

apparently resistant to *N*-glycanase (Lane 2). Treatment of the 27,000 complex with insoluble papain generated a 16,000 molecule (Lane 6), still binding to MabER 30 (Lane 7) but not to unrelated Mab (Lane 8). Furthermore, the data in Table 1 indicated that papain digestion of crude 8866 CSN did not affect its reactivity in RIA. By contrast, this reactivity was almost completely lost after treatment with trypsin or pepsin. Note that removal of sialic acid from IgE-BFs by neuraminidase treatment did not interfere with their binding to MabER. Finally, IgE-BFs were resistant to pH 2·6 and 9, and their binding to MabERs was not altered by 2 hr of incubation at 56°, nor by 2 min of boiling.

Biological activity of purified IgE-BFs

The data in Table 2 indicated that the material purified from RPMI-8866 CSN by affinity-chromatography on MabER 30-Affi-gel, and thus containing the 27,000 complex and a 12,000 molecule, inhibited the binding of IgE-coated erythrocytes (E-IgE) to FcER on RPMI-8866 cells. Furthermore, the highly purified 27,000 complex, isolated by DEAE ion exchange chromatography, blocked the binding of E-IgE to FcER on both RPMI-8866 cells and U937 cells, whereas it did not interfere with the binding of IgG-coated erythrocytes (E-IgG) to FcγR on U937 cells. Finally, the papain digested 27,000 complex was still capable of inhibiting rosette formation of U937 cells with E-IgE but not with E-IgG.

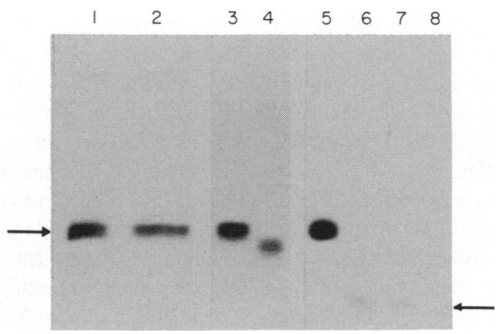

Figure 5. Enzymatic treatment of IgE-BFs: Lanes 1 and 3: radiolabelled IgE-BFs purified on MabER–Affi-gel; Lane 2: *N*-glycanase-treated ^{125}I IgE-BFs; Lane 4: O-glycosidase-treated ^{125}I IgE-BFs; Lane 5: ^{125}I IgE-BFs purified on reverse-phase HPLC; Lanes 6–8: papain-digested ^{125}I IgE-BFs; before precipitation (Lane 6), after immunoprecipitation by MabER 30 (Lane 7) and by unrelated monoclonal antibody (Lane 8). Arrows indicate the molecular weights of 27,000 and 16,000.

Table 1. Sensitivity of IgE-BFs to proteases and extremes of temperature and pH

Treatment*	% ^{125}I MabER 135 bound†	% inhibition
—	9·3	—
Trypsin	2.6	72
—	10·0	—
Pepsin	0·9	91
—	9·4	—
Papain	8·9	4
—	4·2	—
Neuraminidase	4·0	4
—	13·5	—
56°	14·4	0
—	9·1	—
100°	8·7	5
—	8·4	—
pH 2·6	8·6	0
—	10·9	—
pH 9	10·2	7

* Experimental approaches are detailed in the Materials and Methods.

† One representative experiment out of six; this experiment was obtained with unfractionated CSN from RPMI-8866 cell line (total radioactivity = 334,634 c.p.m.; blank = 588 c.p.m.).

DISCUSSION

The present study documents the purification and the physicochemical characterization of human IgE-BFs. These were isolated from the CSN of RPMI-8866 cells, a lymphoblastoid B-cell line known to express FcER (Gonzalez-Molina & Spiegelberg, 1976) and to secrete IgE-BFs capable of modulating the *in vitro* synthesis of human IgE (Sarfati *et al.*, 1984b). Both the purification and the identification of IgE-BFs were based on the

Table 2. IgE and IgG rosette inhibition assays

Inhibitors	RPMI-8866 cells	U937 cells	
	% IgE-RFC*	% IgE-RFC†	% IgG-RFC†
Exp. 1			
—	36±6·2	67±17	87±5
25,000–27,000 and 12,000‡	20±5·5¶	ND	ND
25,000–27,000§	16±3·5¶	42±11¶	88±6
Exp. 2			
—		70±12	87±5
25,000–27,000§		30±10¶	89±6
Papain-digested 25,000–27,000		36±14¶	91±3

* Mean ± 1 SD of three experiments.
† Mean ± 1 SD of four experiments.
‡ Eluate of MabER 30-Affi-gel.
§ Highly purified 25,000–27,000 molecules.
¶ Significantly different from controls ($P < 0{\cdot}01$; paired *t*-test, after angular transformation of the data).

utilization of monoclonal antibodies specific to FcER (Rector *et al.*, 1985) and cross-reacting with IgE-BFs present in the CSN of human B, T and monocyte cell lines expressing FcER (Sarfati *et al.*, 1986a). The existence of antigenic determinants common to IgE-binding factors of T-cell origin and to FcER on B cells has also been reported by others in both the human and the rodent model (Huff *et al.*, 1984; Suemura & Kishimoto, 1985). MabER 30 was selected for affinity purification on the basis of preliminary assays showing its superiority over 12 other MabER for the removal of IgE-BFs from the CSN of RPMI-8866 cells. In keeping with earlier studies (Sarfati *et al.*, 1986a), we found that the eluate of MabER30-Affi-gel displayed an IgE-binding activity, as shown by the IgE rosette inhibition assay. SDS–PAGE analysis of this eluate identified two major components centred at 25,000–27,000 and 12,000, whereas minor components with apparent MW of 43,000 and 60,000 were detected occasionally. The Western blot and the immunoprecipitation assays clearly indicated that the 25,000–27,000 molecules, also named 27,000 complex, were the only molecules reacting with MabER. Note that the 27,000 complex bound not only to MabER30 but also to MabER 135 and 176, employed in the solid-phase RIA. These findings, together with the observation that the eluate from the IgE column, known to contain IgE-BFs and to react strongly with MabER 135 and 176 (Sarfati *et al.*, 1986a), also contained a 27,000 molecule, strongly suggested that the 27,000 complex was IgE-BFs. Indeed, after purification to homogeneity, these molecules were shown to inhibit the binding of IgE to its receptors on both RPMI-8866 and on U937 cells, without interfering with the binding of IgG to its receptors on U937 cells. Since after SDS–PAGE fractionation and silver staining, the 27,000 complex appeared to contain three components or more, it was important to demonstrate in the chromatofocusing experiment, that each of these were reacting with two MabER. These data illustrated the microheterogeneity of IgE-BFs which appeared to contain molecules with pI ranging from 4·4 to 5·0. Because the purification of the 25,000–27,000 IgE-BFs was based on the utilization of Mab against IgE receptors, and since the CSN was prepared by culturing RPMI-8866 cells at a high density in serum-free medium, it might be argued that the 25,000 complex was released in the CSN as a result of cell death and lysis. At least two observations seem to rule out this possibility. First, the ^{75}Se-methionine internally labelled IgE-BFs had the same molecular weight of 25,000–27,000. Second, the 27,000 complex was not detectable in the immunoprecipitate of Nonident-P40-solubilized and surface ^{125}I-labelled RPMI-8866 cells by MabER (Nakajima & Delespesse 1986). These precipitates were made of three components with MW of 65,000–95,000, 45,000 and 37,000, as determined by SDS–PAGE under reducing and non-reducing conditions. Finally, it may be mentioned that, according to ongoing studies, at least some of the *in vivo* produced IgE-BFs (isolated from human serum) are identical to the 27,000 complex described above (M. Sarfati, personal observation). In earlier studies, IgE-BFs were described as molecules with MW of 30,000–40,000 and 10,000–15,000 (Sarfati *et al.*, 1984b). These molecular weights were estimated by filtration through a calibrated Sephadex G 75 column, whereas SDS–PAGE was used in the present study. This may account for the difference in the evaluation of the high MW IgE-BFs, but not for our inability to detect 10,000–15,000 IgE-BFs in the present work. This is best explained by the immediate addition of proteolytic enzyme inhibitors to the CSN of RPMI-8866 cells. Hence, we suggest that the 10,000–15,000 IgE-BFs described earlier are proteolytic cleavage products of the 27,000 complex. Similar observations were made with regard to murine IgG-BFs (Blank *et al.*, 1986) and to rat IgE-BFs (Huff, Uede & Ishizaka, 1982). In support of this view are the present observations that papain digestion of the 27,000 IgE-BFs generated 14,000–16,000 molecules reacting with MabER (Fig. 5) and binding to IgE (Table 2). Morever, Peterson & Conrad (1985) recently reported that a 16,000 molecule binding to both polyclonal anti-FcER and to IgE could be obtained by papain digestion of FcER on RPMI-8866 cell line. Finally, our laboratory has documented the presence in human colostrum of 16,000 IgE-BFs that are capable of suppressing the IgE response of B cells from atopic individuals (Sarfati *et al.*, 1986b). The structure and the antigenic composi-

tion of the colostrum IgE-BFs are presently being compared to those of the 27,000 complex and of its papain fragments. It should also be noted that, in contrast to rodent IgE-BFs (Jardieu *et al.*, 1985), the human 27,000 IgE-BFs could not be reduced into 10,000 and 15,000 molecules in the presence of 2-ME and did not react with Mab to HLA DR and DQ determinants (clones S3/4 and L243, kindly provided by Dr F. Bach, Minneapolis, MN; M. Sarfati, unpublished observation).

IgE-BFs were sensitive to pepsin, trypsin, papain and O-glycosidase (Fig. 5), confirming that they are indeed glycoproteins (Ishizaka, 1985; Young *et al.*, 1984; Sarfati *et al.*, 1984b). *N*-Glycanase treatment did not modify the MW of IgE-BFs. There are at least three possibilities to explain these results: (i) by removing *N*-linked oligosaccharides, *N*-glycanase modifies mainly the charge of IgE-BFs without interfering significantly with their MW; (ii) IgE-BFs are resistant to *N*-glycanase due to steric hindrance (Chu, 1986), and (iii) IgE-BFs do not contain *N*-linked oligosaccharides attached to asparagine residues. Adsorption experiments on lentil lectin–Sepharose aiming to separate IgE-BFs containing *N*-linked mannose-rich oligosaccharides from other species were not conclusive. Indeed, inhibition of IgE rosette formation was evenly found in the eluate and the filtrate of lentil lectin–Sepharose 4B (unpublished observations). These results are in agreement with those of Huff *et al.* (1986). These authors could not demonstrate a clear-cut binding of human IgE-potentiating factors to lentil lectin–Sepharose, unless the hybridoma T cells secreting the IgE-BFs had been treated by bradykinin, or other pharmacological agents. Therefore, in contrast to the rodent system, additional studies are required to determine the precise role of oligosaccharides on the biological function of human IgE-BFs.

In summary, the present study describes two methods to purify to homogeneity human IgE-BFs of B-cell origin. This was achieved by affinity chromatography on MabER immunoadsorbent followed by either HPLC and ion exchange of HPLC reverse-phase chromatography. Because these MabERs were shown to cross-react with IgE-BFs derived from T cells and from macrophages, similarities between the physicochemical characteristics and the biological activities of these molecules are presently under investigation.

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REFERENCES

Blank O., Fridman W.H., Daeron M., Galinha A., Moncuit J. & Neauport-Sautes C. (1986) Size and charge heterogeneity of murine IgG-binding factors (IgG-BF). *J. Immunol.* **136,** 2975.

Caraux J., Chichehian B., Serrow B. & Weigle W.O. (1983) Human soluble Fcγ binding material. I. Immunochemical properties of the material produced by the T cell line KE 37. *J. Immunol.* **130,** 2295.

Chu, F.K. (1986) Requirements of cleavage of high mannose oligosaccharides in glycoproteins by peptide *N*-glycosidase F. *J. biol. Chem.* **261,** 172.

Deguchi H., Suemura M., Ishizaka K., Ozaki Y., Kishimoto S., Yamamura Y. & Kishimoto T. (1983) IgE-class specific suppressor T cells and factors in humans. *J. Immunol.* **131,** 2571.

Gonzalez-Molina A. & Spiegelberg H.L. (1976) Binding of IgE myeloma proteins to human cultured lymphoblastoid cells. *J. Immunol.* **117,** 1838.

Huff T.H. & Ishizaka K. (1984) Formation of IgE-binding factors by human T cell hybridomas. *Proc. natl. Acad. Sci. U.S.A.* **81,** 1514.

Huff T.H., Jardieu P. & Ishizaka K. (1986) Regulatory effects of human IgE-binding factors on the IgE response of rat lymphocytes. *J. Immunol.* **136,** 955.

Huff T.F., Uede T. & Ishizaka K. (1982) Formation of rat IgE-binding factors by rat–mouse T cell hybridomas. *J. Immunol.* **129,** 509.

Huff T.F., Yodoi J., Uede T. & Ishizaka K. (1984) Presence of an antigenic determinant common to rat IgE-potentiating factor, IgE-suppressive factor and Fc receptors on T and B lymphocytes. *J. Immunol.* **132,** 406.

Ishizaka K. (1985) Twenty years with IgE: from the identification of IgE to regulatory factors for the IgE response. *J. Immunol.* **135,** 1.

Iwata M., Huff T.F. & Ishizaka K. (1984) Modulation of the biologic activity of IgG-binding factors. III. Switching of a T cell hybrid clone from the formation of IgE-suppressive factor to the formation of IgE-potentiating factor. *J. Immunol.* **132,** 1286.

Jardieu P., Moore K., Martens C. & Ishizaka K. (1985) Relationship among IgE-binding factors with various molecular weights. *J. Immunol.* **135,** 2727.

Katz D.H. (1985) The IgE antibody system is coordinately regulated by Fc epsilon-positive lymphoid cells and IgE-selective soluble factors. *Int. Archs. Allergy appl. Immun.* **77,** 21.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **227,** 680.

Martens C.L., Huff T.F., Jardieu P., Troustine M.L., Coffman R.F., Ishizaka K. & Moore K.W. (1985) cDNA clones encoding IgE-binding factors from a rat–mouse T cell hybridoma. *Proc. natl. Acad. Sci. U.S.A.* **82,** 2460.

Moretta L., Mingari M.C., Moretta A. & Cooper M.D. (1979) Human T lymphocyte subpopulations: studies of the mechanisms by which T cells bearing Fc receptors for IgG suppress T-dependent B cell differentiation induced by Pokeweed mitogen. *J. Immunol.* **122,** 984.

Nakajima T. & Delespesse G. (1986) IgE receptors on human lymphocytes. I. Identification of the molecules binding to monoclonal anti-Fcε receptor antibodies. *Eur. J. Immunol.* **16,** 809.

Peterson L.H. & Conrad D.H. (1985) Fine specificity, structure and proteolytic susceptibility of the human lymphocyte receptor for IgE. *J. Immunol.* **135,** 2654.

Rector E., Nakajima T., Rocha C., Duncan D., Lestourgeon D., Mitchell R.S., Fisher J., Sehon A.H. & Delespesse G. (1985) Detection and characterization of monoclonal antibodies specific to IgE receptors on human lymphocytes by flow cytometry. *Immunology*, **55,** 481.

Sarfati M., Nutman T., Fonteyn C. & Delespesse G. (1986a) Presence of antigenic determinants common to Fc IgE receptors on human macrophages, T and B lymphocytes and IgE-binding factors. *Immunology*, **59,** 569.

Sarfati M., Rector E., Rubio-Trujillo M., Wong K., Sehon A.H. & Delespesse G. (1984a) *In vitro* synthesis of IgE by human lymphocytes. III. IgE-potentiating activity of culture supernatants from Epstein–Barr virus (EBV) transformed B cells. *Immunology*, **53,** 207.

Sarfati M., Rector E., Wong K., Rubio-Trujillo M., Sehon A.H. & Delespesse G. (1984b) *In vitro* synthesis of IgE by human lymphocytes. II. Enhancement of the spontaneous IgE synthesis by IgE-binding factors secreted by RPMI 8866 lymphoblastoid B cells. *Immunology*, **53,** 197.

Sarfati M., Vanderbeeken Y., Rubio-Trujillo M., Duncan D. & Delespesse G. (1986b) Demonstration of IgE suppressor factors in human colostrum. *Eur. J. Immunol.* **16,** 1005.

Suemura M. & Kishimoto T. (1985) Regulation of human IgE response by T cells and their products. *Int. Archs. Allergy appl. Immun.* **77,** 26.

Young M.C., Leung D.Y.M. & Geha R.S. (1984) Production of IgE potentiating factors in man by T cell lines by bearing Fc receptors for IgE. *Eur. J. Immunol.* **14,** 871.