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Detection and characterization of IgE-binding factors (IgE-BF) within supernatants of the cell line RPMI-8866, normal human sera and sera from atopic patients

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SUMMARY

IgE-binding factors (IgE-BF) have been shown to be important regulatory factors for IgE induction and suppression. The analysis of IgE-binding factor activity by a modified inhibition radioimmunoassay (RIA), as well as by monoclonal antibodies (mAb) was carried out in the supernatant of the $Fc_\varepsilon RII^+$ cell line RPMI-8866 as well as in human sera. Kinetics of IgE-BF showed optimal release from RPMI-8866 cells after 3–4 days. Gel filtration of the supernatant indicated binding activity at < 100,000, 45,000 and 25,000 MW. Within normal human sera two peaks of IgE-BF activity were obtained at 45,000 and 25,000 MW. In sera with high IgE levels (atopic dermatitis) a peak at < 100,000 was MW detected. Within this peak endogenous IgE was present. Addition of sodium dodecylsulphate induced a release of IgE-BF with a MW of 60,000.

INTRODUCTION

Recent interest has focused on molecules controlling the growth of B lymphocytes. In this regard the *de novo* appearance of antigens, such as CD23, at a distinct point during the B-cell programme suggests that these molecules are involved as foci for external signals in guiding the cells most likely to the next phase of response (Gordon & Guy, 1987). The low-affinity receptor for IgE ($Fc_\varepsilon RII$), which is identical to CD23 (Kikutani *et al.*, 1986), has been implicated recently in a variety of functions (Gordon & Guy, 1987; Kikutani *et al.*, 1986; König *et al.*, 1985; Katz, 1984; Geha, 1984). It appears as an early stage-specific marker in the ontogeny of the IgM-bearing B cell in humans. In humans the CD23 is also exposed on T cells in patients with elevated IgE (Prinz *et al.*, 1987; Thompson *et al.*, 1983), while in rodents $Fc_\varepsilon RII$ seems to be present mainly on T lymphocytes. $Fc_\varepsilon RII$ can also be detected on T lymphocytes (Kanowith-Klein & Saxon, 1986; Deguchi *et al.*, 1983; Prinz *et al.*, 1987; Thompson *et al.*, 1983), macrophages (Sarfati *et al.*, 1986a), eosinophils (Capron *et al.*, 1981) and platelets (Joseph *et al.*, 1986). Activation of these cells induces an amplification of the inflammatory response with the release of preformed and newly generated mediators. The expression of CD23 is enhanced in the presence of IL-4 (Gordon *et al.*, 1986) as well as IgE- and EBV-transformed cells (Thorley-Lawson, 1985). This could lead to more available receptors on which B-cell growth factors (BCGF), such as the cleavage products of the CD23 molecule, may act and provide greater autocrine-enhancing activities.

Soluble isotype-specific binding factors (BF) play an important role in the mechanism of immunoglobulin E antibody regulation, as has been shown in rodents (Ishizaka, 1985) as well as in the human system (König *et al.*, 1985; Katz, 1984; Geha, 1984). Several groups described IgE-BF from T cells that either potentiated or suppressed the IgE synthesis (Kanowith-Klein & Saxon, 1986; Deguchi *et al.*, 1983; Huff & Ishizaka, 1984; Young, Leung & Geha, 1984; Ishizaka & Sandberg, 1981). Those binding factors were induced after stimulation of the cells with IgE; IgE-BF are also secreted from hybridoma T-cell lines (Huff & Ishizaka, 1984), $Fc_\varepsilon RII$-bearing peripheral B cells (Gonzales-Molina & Spiegelberg, 1977) and from the lymphoblastoid B-cell line RPMI-8866 (Jensen, Sand & Spiegelberg, 1984; Sarfati *et al.*, 1984 a, c, d). It has been shown that the biological functions of the IgE-BFs depend on the glycosylation of the factors. In this regard glycosylated BFs enhance and unglycosylated factors suppress the IgE production (Sarfati *et al.*, 1984c; Yodoi, Hirashima & Ishizaka, 1982; Sarfati *et al.*, 1984a; Leung *et al.*, 1986). The detection of IgE-BF has been shown previously by the rosette assay. This test, however, is not suitable for a multitude of samples under study. An alternative method was established as an inhibition-RIA (Chen *et al.*, 1983). This technique allows the semi-quantitative detection of

Abbreviations: BCGF, B-cell growth factor; EBV, Ebstein–Barr virus; $Fc_\varepsilon RII$, low affinity receptor for IgE; FCS, fetal calf serum; IgE-BF, IgE-binding factor; IL-4, interleukin-4; mAb, monoclonal antibody; MW, molecular weight; RIA, radioimmunoassay; SD, standard deviation; SDS, sodium dodecylsulphate.

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IgE-BF by inhibiting the binding of IgE to anti-IgE in the presence of IgE-BF. A more specific determination of IgE-BF is based on the cross-reaction of monoclonal antibodies (mAb) specific for the low-affinity IgE-receptor ($Fc_\varepsilon RII$) with IgE-BF (Sarfati *et al.*, 1986a; Nakajima & Delespesse, 1986; Sarfati, Rubio-Trujillo & Wolowiec, 1986b; Delespesse *et al.*, 1986; Rector *et al.*, 1985). The monoclonal antibodies recognize three components of the receptor with molecular weights of 65–90,000, 45,000 and 37,000 (Nakajima & Delespesse, 1986). Ikuta *et al.* (1987) generated monoclonal antibodies that reacted with a 20–25,000 MW and a 45,000 MW component of $Fc_\varepsilon RII$ from human B cells.

It has been shown that the supernatants of hybridoma T-cell lines contain IgE-binding factors of 25–30,000 and 30,000 MW, as well as 15,000 MW (Huff & Ishizaka, 1984). Sarfati *et al.* (1984c) described two IgE-BFs with molecular sizes in the range of 15,000 and 30–40,000 MW within RPMI-8866 supernatant. Furthermore, IgE-BF with 60,000 and 15,000 MW were detected within the plasma of patients with the hyper-IgE syndrome (Leung *et al.*, 1986; Sarfati *et al.*, 1986a). All these data support the idea that IgE-BFs are essential components in the regulation of IgE production.

It was the purpose of our study to compare the detection of IgE-BF as obtained by the inhibition-RIA with the data raised with the mAb-RIA. As a source of IgE-BF, were studied the cell supernatants of the cell line RPMI-8866, the sera of healthy volunteers and the sera of patients with atopic dermatitis.

MATERIALS AND METHODS

Preparation of cell-free RPMI-8866 culture supernatant

RPMI-8866 cells (generous gift of Dr G. Delespesse, Hôpital Notre-Dame, Montreal, Canada) were cultured in RPMI-1640 medium (Gibco, Eggenstein) supplemented with 10% FCS and in serum-free HB 101 medium, NEN, Dreieich. The supernatant was collected from cultures grown up to a density of $1–2 \times 10^6$ cells per ml by centrifugation. Subsequently, the supernatant was concentrated (10–20-fold) by ultrafiltration (Amicon, Witten; YM5), dialysed and finally passed through a 0·22 μm Millipore filter. Aliquots of the supernatant were stored at $-20°$.

Gel filtration

Gel filtrations were performed on Fractogel TSK HW 55 S colums (Merck, Darmstadt) (2·4 × 90 cm; 2·4 × 70 cm) at 4° and on AcA 44 columns (LKB, Gräfelfing) (2·4 × 90 cm). Phosphate-buffered saline was used as standard elution buffer. The flow rate using Fractogel was 20 cm/hr. AcA columns were performed with a flow rate of 5 cm/hr. Fractions of 5 ml were collected and stored at $-20°$. Beriglobin (Behringwerke AG, Marburg) (150,000 MW), ovalbumin (48,000 MW) and vitamin B_{12} (13,500 MW) were used as marker proteins for the determination of the molecular weights.

RIA for IgE measurement

Removal U-bottomed wells were coated with 0·1 ml of goat anti-human IgE (Medac, Hamburg; 1·5–2 μg/ml) for 4 hr at 37°. After washing and saturation of the free protein-binding sites with PBS supplemented with 0·05% Tween 20 and sodiumazide (0·05%), appropriate dilutions of the test samples were incubated overnight. The bound amount of IgE was determined by radiolabelled anti-IgE (0·1 ml; 1·5–2 μg/ml; 100,000 c.p.m.). The maximum of bound radioactivity reached 15% of the total activity. The standard deviation of duplicates amounted under 5%.

Detection of the IgE-BF with the inhibition RIA

A modified binding RIA was used for the detection of inhibition by IgE-BF (Chen *et al.*, 1983). As described above for the IgE-RIA, U-bottomed wells were coated with anti-IgE (0·2 ml; Medac) and saturated with PBS/0·05% Tween 20/0·05% sodiumazide. Subsequently 0·1 ml of the sample under study and 0·1 ml of human IgE (10–193 ng/ml; Behring, Behringwerke) were added to the wells and incubated over night at room temperature. After thorough washing ^{125}J-labelled anti-IgE was added for 4 hr at 37°. The inhibition of the binding of labelled anti-IgE in the presence of the samples was calculated in reference to the control. The value in the absence of the BF sample served as a control; it represented 100% binding. Inhibition was calculated by indicating the c.p.m. in the presence of the BF sample to the 100% value (absence of the BF sample). Each sample was determined in quadruplicates.

RIA for IgE-BF with monoclonal antibodies

The RIA for IgE-BF with mAb specific for $Fc_\varepsilon RII$ (generous gift by Dr G. Delespesse) cross-reacting with IgE-BF was performed as has been previously described by Dr G. Delespesse: removal wells were coated with mAb 176 (generous gift of Dr G. Delespesse) (2–10 μg/ml; diluted in bicarbonate buffer, pH 9·0) overnight at room temperature. After washing and saturation with PBS supplemented with 10% FCS, the samples were added for 4 hr at room temperature. Subsequently, the wells were washed again and incubated with the ^{125}J-labelled mAb 135 overnight. The c.p.m. of 100 μl of the labelled sample amounted to 100,000 up to 300,000 c.p.m. After a last wash the bound radioactivity was determined. As 100% served the total radioactivity, results for the IgE-BF were expressed in relation to the 100% value. The standard deviation ranged under 10%.

RESULTS

Detection of IgE-BF within the crude supernatant of the cell line RPMI-8866

The presence of IgE-BF activity within the unseparated cell-free supernatant of the cell line RPMI-8866 was analysed by the inhibition-RIA. For this purpose microtitre plates were coated with anti-IgE. Aliquots (100 μl) of the concentrated (10-fold) RPMI supernatant were added followed by a subsequent addition of IgE diluted from 200 ng/ml up to 3 ng/ml. The percentage of inhibition was calculated for IgE concentrations ranging from 25 ng/ml to 200 ng/ml. As is apparent from Fig. 1, the concentrated RPMI cell supernatant led to an inhibition by 80%. No cross-reaction was obtained in this assay with either the medium, its supplements (FCS/Pen/Strep) or human IgG (200 ng/ml).

Since IgE-BF is structurally related to the low-affinity receptor for IgE ($Fc_\varepsilon RII$), monoclonal antibodies were applied to detect the presence of IgE-BF in the cell supernatant of RPMI-8866 cells which were cultured either in RPMI-1640 medium (10% FCS; Fig. 2a) or in serum-free HB 101 medium

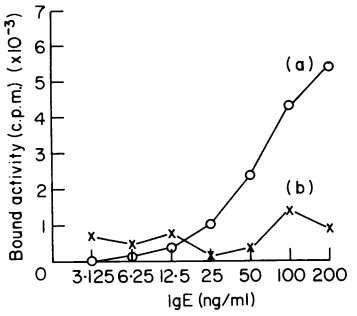

Figure 1. Inhibition of the IgE–anti-IgE binding by RPMI-8866 supernatant. This figure demonstrates the data obtained by the inhibition-RIA: removal wells were coated with anti-human IgE. Subsequently 100 μl of concentrated (10-fold) RPMI-8866 supernatant were added. IgE standard dilution (100 μl) from 3 to 100 ng/ml was added. The amount of bound IgE was detected by radiolabelled anti-IgE. Curve (a) IgE standard curve (100% control); (b) shows the results of the inhibition experiment. Each sample was determined in quadruplicates. The SD of each point was below 10%.

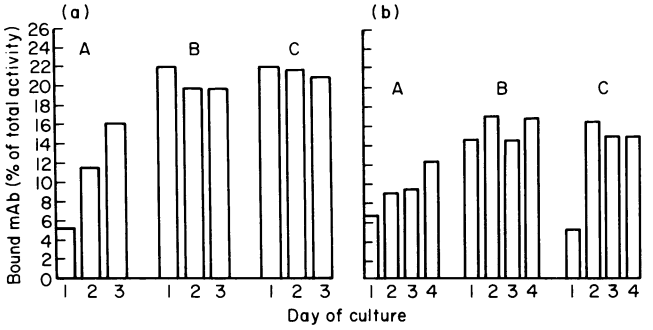

Figure 2. Kinetics of IgE-BF secretion from the cell line RPMI-8866 detected by monoclonal antibodies against $Fc_\varepsilon RII$ (mAb). RPMI-8866 cells (A, 1×10^5; B, 1×10^6; C, 2×10^6 cells/ml) were incubated in RPMI-1640 medium supplemented with 10% FCS (a) as well as in serum-free HB 101 medium (b). During the culture aliquots of the supernatant were obtained and the BF content was determined by the mAb-RIA. Each sample was analysed in quadruplicates. The radioactivity of the monoclonal antibody per well amounted to 200,000–300,000 c.p.m. The results are indicated as percentage of binding of the total activity.

(Fig. 2b). Several concentrations of the cells (1×10^5, 1×10^6, 2×10^6 cells/ml) were incubated for 1–4 days. The 10-fold concentrated samples were analysed for IgE-BF activity. It is shown that after 1 day of culture the concentrated supernatant bound up to 40% of the radiolabelled monoclonal antibody. Highest BF-activity was obtained after Day 3 or 4 of culture at a cell concentration of 2×10^6 cells/ml. With either culture method a similar percentage of binding was achieved. The monoclonal antibody was able to detect IgE-BF at a 1:64 dilution of the native serum-free RPMI-8866 supernatant. Repeated thawing and freezing reduced nearly all of the IgE-BF activity. Storage of the supernatant over 1 year reduced the BF activity by 50% (data not shown).

Detection of IgE-BF within fractionated supernatants

Further characterization of IgE-BF within the RPMI-8866 cell supernatant was carried out after subjecting appropriate fractions to gel filtration. Samples of concentrated supernatants (5 ml) were applied to a Fractogel TSK HW 50 S column. The resulting fractions were collected and IgE-BF was determined by the inhibition-RIA. Figure 3a represents the mean values after five gel filtrations. It is shown that three maxima of inhibition at molecular weights of < 100,000, 45,000 and 25,000 were obtained. Similar results were observed by autoradiographic studies (data not shown).

In order to achieve a better separation of the 100,000 MW and 45,000 MW peaks, the supernatants were also subjected to AcA 44 columns. IgE-BF was detected with either the inhibition-RIA or by the monoclonal antibody against IgE-BF. Two peaks of BF activity were obtained at 25,000 MW and 15,000 MW, respectively. It may be suggested that the 15,000 MW component represents a breakdown product of the high MW IgE-BFs. In 50% of the experiments ($n = 6$) an additional 45,000 MW component was observed.

Detection of IgE-BF within human sera

The detection of IgE-BF in human sera is disturbed by the presence of endogenous IgE when the inhibition-RIA is applied. In order to analyse the MW distribution of IgE-BFs within

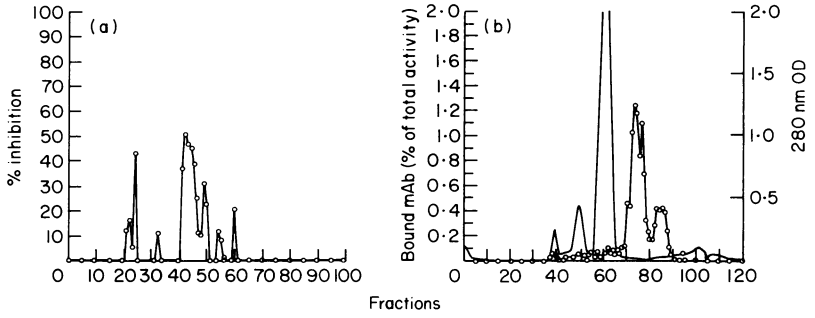

Figure 3. (a) Detection of IgE-BF activity within the cell supernatants of RPMI-8866 after gelfiltration. Aliquots from the 10-fold concentrated RPMI-8866 supernatant were applied on gelfiltration columns (Fractogel HW 50 S); fractions of 5 ml were collected. Each fraction was analysed by inhibition-RIA and the percentage of inhibition was calculated as described elsewhere. The figure indicates the mean values of four experiments. The maximal values of inhibition (fraction 40–45) reached up to 50%. (b) Comparison of the inhibition-RIA and the mAb-RIA with regard to IgE-BF content. RPMI-8866 cell supernatant was fractionated on AcA 44 gelmaterial. Plots without symbols represent the protein profile at OD 280 nm; (o—o) the BF activity detected by mAb-RIA (c.p.m.) Two maxima of BF activity corresponding to 25,000 (fractions 70–75) and 15,000 MW (fractions 75–80) were obtained. The high molecular weight IgE-BFs (100,000, 45,000 MW) were not detected. The parallel analysis of the same fractions by inhibition-RIA (a) clearly shows the presence of the high molecular BFs.

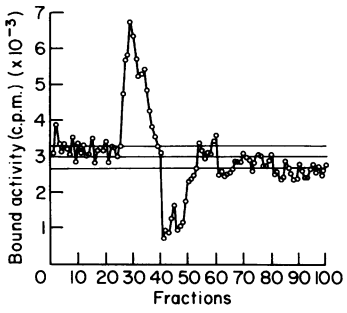

Figure 4. Detection of IgE-BF within normal human serum. The gelfiltration was performed on AcA 44 gelmaterial. The BF content was determined by the inhibition-RIA. Figure 4 indicates the data as c.p.m. and not as percentage inhibition in order to quantify endogenous IgE. The mean value of the positive control (100% binding) as well as its standard deviation are marked as horizontal lines. By this procedure the IgE content (maximum) and the IgE-BF activity (minima) are demonstrated by one plot.

serum, human sera (5 ml, $n=5$) were fractionated on AcA 44 columns. The endogenous IgE activity was analysed, as well as the IgE-BF activity by the inhibition-RIA (Fig. 4). The peak at fraction 31 represents the maximum of endogenous IgE activity, the two minima within fractions 41 (45,000 MW) and 49 (25,000 MW) correspond to the IgE-BF activities. The position and the shape of these peaks correlate to those obtained for the fractionated RPMI-8866 supernatant. These experiments clearly demonstrate that no overlapping of the IgE peak with the IgE-BFs at 45,000 MW and 25,000 MW are obtained. Similar results were obtained after fractionation on TSK 50 S columns.

IgE-BF in patients with atopic dermatitis

It has been described that IgE-binding factors are responsible for the enhanced IgE levels in patients with atopic dermatitis. In order to study the molecular weight of IgE-BF, the sera from patients with atopic dermatitis ($n=11$) were subjected to gel filtrations on AcA columns. A representative experiment is shown (Fig. 5a). As is apparent, unlike normal serum the binding factor activity was not present within the molecular weight range of 45,000–25,000. The endogenous IgE level eluted within the fractions 31–38. Thus, it appeared that IgE-BF might be complexed to IgE. Representative sera ($n=4$) were also chromatographed on AcA columns in the presence of 0·2% SDS. An inhibitory peak was obtained with a maximum at 60,000 MW. This component was also apparent when a serum with a low IgE level (600 ng/ml) was analysed.

When monoclonal antibody was applied to analyse IgE-BF activity within the fractions, maximal binding activity was obtained at a MW of < 100,000 (Fig. 5b, fraction 25). This peak coincides with the peak for IgE. These data suggested the presence of a complex consisting of IgE and BF.

Comparison of IgE-BF activity within various sera

The monoclonal antibody allows the detection of IgE-BF within the sera even in the presence of IgE. Therefore the BF content of crude normal sera ($n=9$), sera of patients with atopic dermatitis ($n=12$) and patients with wheat flour allergy ($n=69$) was determined. Each serum was tested at least three times in triplicate. Normal donors show IgE-BF activity which comprises 0·1% of the total radioactivity (200,000 c.p.m.) of the monoclonal antibody. These results were used to classify the sera in three groups, which correspond to a normal, high and low IgE-BF content. In addition serum IgE levels were also determined and correlated to the IgE-BF. No linear correlation of IgE-BF with serum IgE was obtained.

DISCUSSION

The low-affinity receptor for IgE ($Fc_\varepsilon RII = CD23$) has been implicated recently in a variety of functions. It appears as an early stage-specific marker in the ontogeny of the IgM-bearing B cells in humans.

The expression of CD23 is enhanced in the presence of IL-4 as well as by IgE and in EBV-transformed cells. One may suggest that this leads to more available receptors on which the B cell-derived B-cell growth factor (BCGF), which is the cleavage product of the $Fc_\varepsilon RII$, itself may act and provide greater autocrine-enhancing activities (Gordon *et al.*, 1986; Swendeman & Thorley-Lawson, 1987).

Indeed $Fc_\varepsilon RII$-bearing cells have been described as secreting IgE-binding factors with regulatory functions (enhancing, suppressive) as to IgE antibody synthesis. Our results demonstrate the detection of IgE-binding factors by the inhibition-RIA as

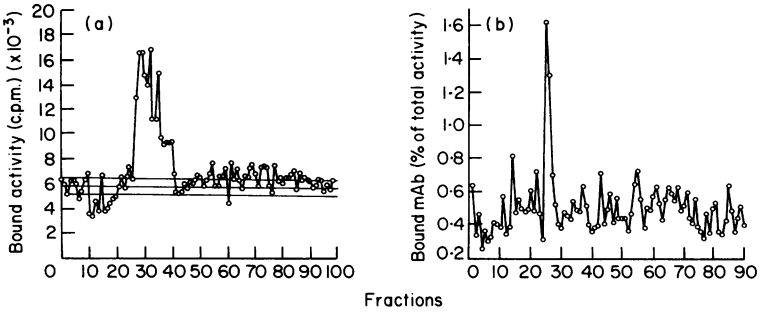

Figure 5. (a) Detection of IgE-BF within sera of patients suffering from atopic dermatitis by inhibition-RIA. Samples of the sera (5 ml) were separated by gelfiltration (AcA 44 column); the fractions were analysed by inhibition-RIA. A representative experiment of $n=11$ experiments is shown. Values are plotted in c.p.m. (See Fig. 4.) to demonstrate the endogenous IgE. (b) Detection of IgE-BF within sera of patients with atopic dermatitis by mAb-RIA. The figure indicates the data as percentage of total activity (Fig. 2). Maximal binding is observed within the high molecular weight range.

well as by monoclonal antibodies against the $Fc_{\varepsilon}RII$. With the inhibition-RIA three main components which reveal IgE-binding activity within the RPMI-8866 supernatant were demonstrated with molecular weights of < 100,000, 45,000 and 25,000 MW. In several cases an additional component—most likely a breakdown product—with 15,000 MW was detected. The data agree with the previously obtained results by Sarfati *et al.* (1987) and Ikuta *et al.* (1987). In their studies with the rosette inhibition assay, IgE-BF with three MW were detected (90,000, 45,000, 25,000). These data apparently suggest a similarity of the rosette inhibition assay and the inhibition-RIA according to Chen *et al.* (1983).

Human sera of healthy volunteers showed IgE-BFs with molecular weights of 45,000 and 25,000. Sera of patients with atopic dermatitis, however, demonstrated, after chromatography under physiological conditions, no IgE-binding activities ($n = 11$). Under denaturing conditions IgE-binding activities at a molecular weight of 60,000 were obtained. It appears that this component can also be detected in cases with lower or intermediate IgE levels (600 ng/ml).

The inhibition-RIA does not exclude the existence of a high molecular weight IgE-BF within sera containing endogenous IgE, while the IgE peak would interfere with the detection system. Thus the data indicate that IgE-BFs exist under various conditions and raises the question as to their pathophysiological role.

In this regard, Leung *et al.* (1986) analysed IgE-BF from patients with hyper-IgE syndrome. A 15,000 and a 60,000 MW component were analysed which, according to their data, could be responsible for the elevated IgE levels. They interpreted this component as an IgE-enhancing factor. IgE-binding factors from three of four patients expressed 15,000 and 60,000 MW components and showed enhancing activity on IgE synthesis from atopic B cells and cells with recent exposure to allergens.

The monoclonal antibody assay, which is described against $Fc_{\varepsilon}RII$, is not disturbed by endogenous IgE levels. Three components at 45,000, 25,000 and 15,000 MW within fractionated RPMI-8866 supernatant are apparent. In atopic sera the monoclonal antibody also detects IgE-BF directly complexed with IgE; the inhibition assay, however, recognizes this component under conditions when the complexed IgE is exposed towards denaturing conditions and BF dissolved from IgE. It is not clear whether IgE complexed with BF exert regulatory functions.

IgE-BFs have been suggested to exert regulatory functions. It has been shown that CD23 ($Fc_{\varepsilon}RII$) expression is confined to IgM-bearing cells. After the isotype switch the cells not only stop the expression of CD23 but also loose their capacity to do so. There is evidence that the B-cell derived BCGF and the cleaved CD23 molecule are in fact the same. One may also imply that a major effect of T-cell derived BCGF is to induce an autocrine growth factor cascade to process cell-bound CD23 to its autostimulatory or regulatory (suppressive) extracellular form (Gordon & Guy, 1987). In this regard it is interesting to correlate IgE-BF with the IgE levels. It has been described that a positive correlation existed in patients with rhinitis and asthma (Delespesse, Sarfati & Rubio-Trujillo, 1987).

In our studies, which compared patients with atopic dermatitis ($n = 22$) and wheat flour allergy ($n = 90$), a direct correlation between IgE-BF and IgE levels was not obtained. It is unclear up to now whether a high IgE level is the result of excessive or the consequence of a decreased IgE suppressive factor. This would imply that not the absolute quantities but only the ratio of the functionally different IgE-BFs is decisive for IgE regulation. Our results thus show that IgE-BFs as soluble factors of the CD23 occur in different molecular weight species. Their relevance for the expression of the various disease processes is unclear and under study.

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