dilutions of PCR mimics (competitors) mixed with a constant amount of the cDNA sample (targets). The PCR was cycled under suitable conditions, as described previously [10]. PCR products were analyzed by electropheresis on 2% agarose gels and visualized by ethidium bromide staining. The bands of targets and competitors were scanned and quantified using the Multianalyst hardware and software system (Bio-Rad Laboratories, Hercules, CA). The results were expressed as attomol/μg RNA for germline Cε and β-actin.

In vitro ERK assay

DG75 cells (1×10^7 cells/ml) were stimulated with or without anti-CD40 mAb (1 μg/ml) for the indicated times, lysed with lysis buffer, immunoprecipitated with anti-phospho-ERK antibody, and washed with kinase reaction buffer containing 25 mM Tris–HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. The immunocomplexes were resuspended in 50 μl kinase reaction buffer containing 2 μg GST-Elk1, 200 μM ATP, and 20 μCi [α-^{32}P]ATP. After 30-min incubation at 30 °C, the products were separated by SDS–PAGE, followed by autoradiography.

Results

Analysis of association of TRAF family proteins with CD40

To determine the association of TRAF family proteins with CD40 in DG75 cells, the lysates from unstimulated or anti-CD40-stimulated cells were immunoprecipitated with respective antibodies to TRAF1, 2, 3, 4, 5, and 6 and were blotted wih anti-CD40 antibody. As shown in Fig. 1, association of TRAF2, 3, 5, and 6 with CD40 was detected in an unstimulated state, and the levels of association between these four proteins and CD40 did not change even after CD40 ligation. In contrast, no association of TRAF1 and TRAF4 with CD40 was observed in both unstimulated and stimulated cells, although these two proteins were detected by reprobing the same blot with anti-TRAF1 and anti-TRAF4 antibodies, respectively (data not shown).

AS ODN for TRAF3 inhibits CD40-mediated upregulation of IL-4-driven germline Cε transcription

To downregulate the activity of TRAF2, 3, 5, and 6, DG75 cells were pretreated with or without AS or S ODNs for respective TRAFs. Forty eight-hour pretreatment with AS ODNs (1 and 5 μM) decreased each level of the corresponding TRAF proteins in a concentration-dependent manner, whereas all the S ODNs had no effects (Fig. 2). Based on these observations, we examined which

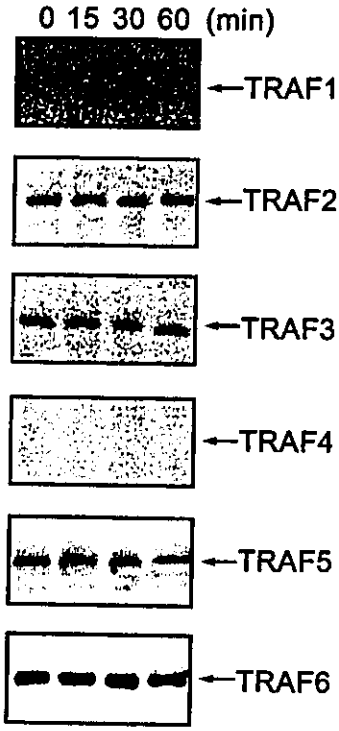

Fig. 1. Analysis of CD40-associated members of the TRAF family. Lysates from DG75 cells stimulated with anti-CD40 (1 μg/ml) for the indicated times were immunoprecipitated with respective anti-TRAF antibodies and subjected to immunoblot analysis with anti-CD40. Results shown are representative of three experiments.

TRAF protein is involved in the enhancement of germline Cε transcription. Competitive RT-PCR was performed for the quantitative analysis of its mRNA level. Stimulation of DG75 cells with IL-4 for 48 h induced expression of germline Cε transcripts (458 ± 37 attomol/μg RNA, mean ± SE of triplicate cultures), whereas anti-CD40 stimulation did not. Upon IL-4 plus anti-CD40 stimulation, germline Cε transcript levels were significantly increased approximately threefold (1378 ± 166 attomol/μg RNA) (Fig. 3A). Using such a quantative assay system, we investigated the effects of AS and S ODNs for TRAF2, 3, 5, and 6 on CD40-mediated upregulation of IL-4-driven germline Cε transcription. As summarized in Fig. 3B, pretreatment with only AS ODN (5 μM) for TRAF3 inhibited enhanced germline Cε transcription, and the inhibition was significant ($p < 0.05$, paired t test). These ODNs had no effect on the transcription in response to IL-4 alone (data not shown). In this experiment, the effects of MAPK inhibitors were also examined. PD98059 (10 μM) was found to inhibit the CD40-mediated augmentation of germline Cε transcription, whereas SB203580 (10 μM) was not.

TRAF3 is involved in CD40-mediated ERK activation

Because not only AS ODN for TRAF3 but also a MEK1 inhibitor, PD98059, showed an inhibitory effect

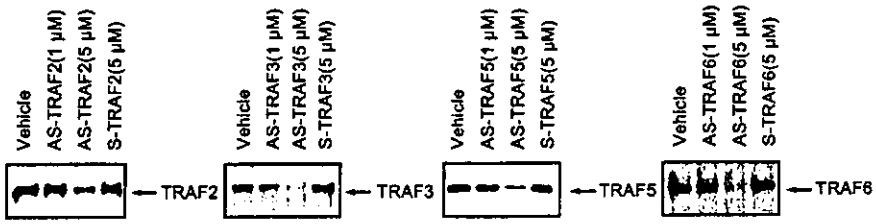

Fig. 2. Effects of antisense (AS) and sense (S) ODNs for TRAF2, 3, 5, and 6 on their protein expression. Lysates from DG75 cells treated for 48 h with each AS or S ODN at a concentration of 1 or 5 μM were analyzed by immunoblotting with respective anti-TRAF antibodies. Results shown are representative of three experiments.

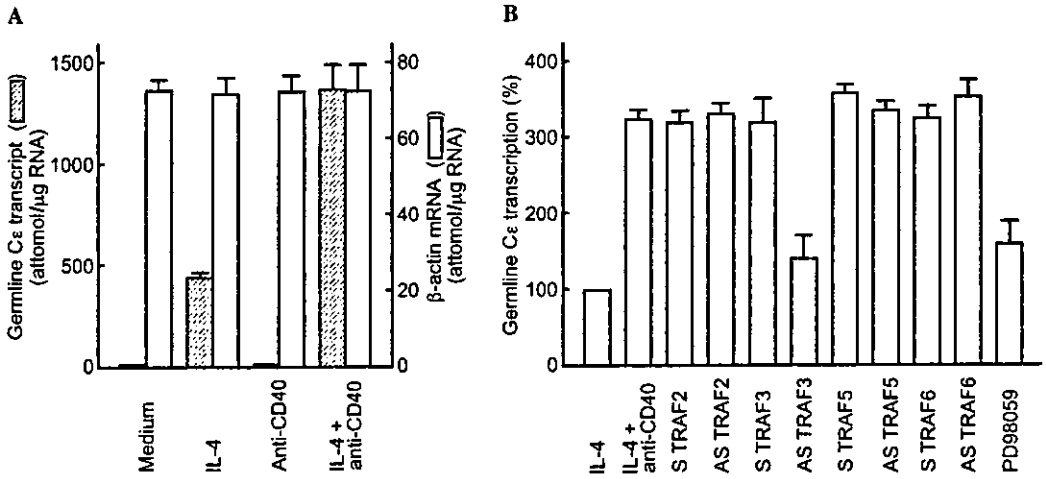

Fig. 3. Quantitative analysis of germline Cε transcription. (A) DG75 cells were cultured for 48 h with medium alone, IL-4 (2 ng/ml), anti-CD40 (1 μg/ml), or a combination of them. (B) Cells were pretreated either with antisense (AS) or sense (S) ODN (5 μM) for TRAF2, 3, 5, or 6 for 48 h or with PD98059 or SB203580 (each 10 μM) for 30 min, followed by IL-4 plus anti-CD40 stimualtion for 48 h. In each experiment, total cellular RNA was extracted and subjected to competitive RT-PCR analysis of germline Cε and β-actin mRNA. The bands of competitors and amplified targets were scanned and quantified, as described under Materials and methods. Values represent the mean ± SE of three different experiments, each performed in duplicate.

on the CD40-dependent enhanced germline Cε transcription, we next examined whether CD40 ligation could induce the threonine and tyrosine phosphorylation of ERK. DG75 cells were stimulated with anti-CD40 mAb, and the phosphorylation of ERK was analyzed by immunoblotting. Fig. 4A shows that anti-CD40 stimulation induced increased phosphorylation of ERK. ERK activity was further evaluated by in vitro kinase assay using GST–Elk-1 fusion protein as a substrate. Increased phosphorylation of the fusion protein, which reached a maximum between 5 and 10 min, was detectable in the lysates from the stimulated cells (Fig. 4B). Then, we examined whether TRAF proteins could be involved in ERK activation following CD40 engagement. Pretreatment with AS ODN (5 μM) for TRAF3, but not with that for TRAF2, 5, or 6, significantly inhibited ERK activity following CD40 ligation, as determined by in vitro kinase assay using GST–Elk-1 (Fig. 4B). Similarly, 30-min pretreatment with PD98059 (10 μM) also decreased CD40-induced ERK activity. These results demonstrated that TRAF3 was involved in the activation of ERK in anti-CD40-stimulated DG75 cells.

Discussion

Members of TRAF family have been shown to associate with the cytosolic domain of CD40 molecule and are involved in the signal transduction pathway of CD40 [3–5]. In this report, we demonstrated that TRAF3 constitutively associates with CD40 and mediates upregulation of IL-4-driven germline Cε transcription, possibly via MEK1/ERK pathway, in a human Burkitt's lymphoma B cell line, DG75.

It has been reported that CD40-mediated ERK activation is detected in some but not all the B cell type, whereas JNK activation is reproducibly observed in these cells. For example, CD40-mediated ERK activation is detected in normal murine B cells but not in WEHI-231 B lymphoma, whereas JNK activation is induced in both cells [12]. There is little information on whether TRAF is required for CD40-dependent ERK activation and, if so, which TRAF member is involved in such an activation. Our results indicate that TRAF3 and MEK1 are involved in the activation of ERK in CD40-dependent signaling, because CD40 engagement induced the enhancement of ERK activity in DG75 cells

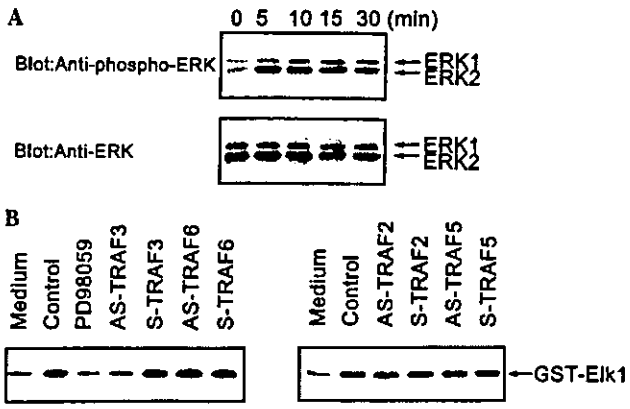

Fig. 4. TRAF3-dependent activation of ERK following CD40 ligation. (A) Lysates from DG75 cells stimulated with anti-CD40 (1 μg/ml) for the indicated times were immunoprecipitated with anti-ERK antibody and blotted with a specific antibody to phosphorylated threonine and tyrosine of ERK (phospho-ERK). Expression of ERK was verified by reprobing the same membrane with anti-ERK antibody. (B) Effects of PD98059 and antisense (AS) and sense (S) ODNs for TRAF 2, 3, 5, and 6 on CD40-mediated induction of ERK activity. DG75 cells were pretreated either with PD98059 (10 μM) for 30 min or with each AS or S ODN for 48 h and then stimulated with anti-CD40 (1 μg/ml) for 10 min. The lysates from unstimulated or stimulated cells were immunoprecipitated with an anti-phospho-ERK antibody and subjected to in vitro ERK assay using GST-Elk-1 as a substrate. Results shown are representative of three or four experiments.

and this enhancement was abrogated by downregulation of TRAF3 and by PD98059, a specific inhibitor of MEK1. In contrast, TRAF2, 5, and 6 are not involved in the ERK activation. Several lines of evidences suggest that these TRAFs can activate JNK, whereas TRAF3 cannot [5,13]. It is therefore likely that the activation of ERK and JNK is dependent on different TRAFs in CD40-dependent signaling.

Activation of NF-κB is another important event downstream of CD40 signaling. It is an important transcription factor for germline Cε transcription and IL-4 alone can induce NF-κB activation [14,15]. The IL-4-induced activation is independent of TRAFs because the IL-4 receptor does not contain the interaction domain with this family. Two distinct NF-κB binding sites are located in the human germline Cε promoter and appear to bind with different types of NF-κB complexes [14,16]. Thus, IL-4 and CD40 engagement might induce different NF-κB complexes for the respective sites, and occupation of both sites may result in an augmentation of germline Cε transcription. Previous reports suggest that TRAF2, 5, and 6 are capable of mediating CD40-induced NF-κB activation through their ability to bind activators of the IκB kinase complex such as NIK [5,13]. However, the role of each TRAF appears to be cell-type specific. TRAF2 is considered very important for NF-κB activation in non-B cells [17], but not in B cells [18,19]. Furthermore, two groups demonstrated that TRAF6 plays an important role in germline Cε transcription through NF-κB activation [7,8], whereas another group reported the TRAF6-independent NF-κB activation [9]. In this study, downmodulation of TRAF2, 5 and 6 by AS ODN had no effect on the germline Cε transcription, suggesting the presence of TRAF-independent mechanism for the NF-κB activation. Indeed, we have already reported that the activation of phosphatidylinositol 3-kinase following CD40 ligation mediates the NF-κB activation in DND39 and DG75 B lymphomas [20,21]. Such a redundancy in the mechanism of NF-κB activation might be associated with the level of each TRAF protein and with the number of CD40 molecules on the cell surface. In contrast, both AS ODN for TRAF3 and PD98059 showed inhibitory effect on CD40-mediated up-regulation of IL-4-driven germline Cε transcription. Because PD98059 did not affect the germline Cε transcription induced by IL-4 alone, only the CD40-mediated upregulation is susceptible to the inhibitory effect. Moreover, Leo et al. [8] reported that TRAF3 is involved in the germline Cε transcription induced by IL-4 plus anti-CD40 in a manner that is independent of NF-κB activation. Taken together, TRAF3-dependent MEK1 activation is involved in CD40-mediated augmentation of germline Cε transcription. Although ERK is a likely component downstream of MEK1 in this response, the involvement of other components cannot be excluded.

Because CD40 engagement, in addition to IL-4, is required to induce IgE protein synthesis, CD40-mediated signaling that is not shared by IL-4 is indispensable for DNA recombination in IgE class switching. Whether CD40 signaling for the augmentation of germline Cε transcription and that for the DNA recombination is overlapping or totally different is currently unclear. Further studies will be required to identify the downsteam of TRAF3-MEK1 pathway contributing to these events.

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Original Article

Recombinant soluble form of the high-affinity IgE receptor α subunit and anti-IgE antibody inhibit IgE synthesis by IgE-expressing B cells through distinct pathways

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Abstract

Background: Both a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and anti-IgE antibody have been shown to be involved in the regulation of IgE synthesis. However, the mechanisms of IgE regulation by two such IgE-binding agents remain unclear. In the present study, we investigated whether rsFcεRIα and anti-IgE antibody modulated IgE synthesis in an identical or different manner.

Methods: Normal human B cells stimulated with interleukin (IL)-4 plus anti-CD40 antibody were analyzed for the regulatory effects of rsFcεRIα and anti-IgE antibody on the expression of Cε transcipts, the autocrine production of IL-6 and the induction of apoptosis.

Results: Both rsFcεRIα and anti-IgE antibody inhibited mature Cε transcription, without affecting germline Cε transcription. In addition, rsFcεRIα was effective in decreasing IL-6 production at a later stage when IgE-expressing B cells were generated, whereas $F(ab')_2$, but not the Fab fragment, of anti-IgE antibody induced apoptosis in the cells. Although these three agents almost equally recognized IgE expressed on B cells, rsFcεRIα was unable to induce apoptotic cell death and the Fab fragment was similarly ineffective in the regulation of IL-6 production. The addition of IL-6 to cultures containing rsFcεRIα significantly restored its suppressive effect on IgE synthesis.

Conclusions: These results indicate that regulation of IgE synthesis by rsFcεRIα differs from that by anti-IgE antibody.

Key words: apoptosis, divalent recognition, IgE-binding agents, IgE-expressing B cells, interleukin-6, monovalent recognition.

Introduction

It is well known that IgE-binding factors derived from T cells and anti-IgE antibodies, which target IgE-expressing B cells, exert selective effects on the IgE response.[1–4] We have previously shown that, in addition to anti-IgE antibodies, a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα), which binds free IgE with an affinity as high as that of native FcεRI, can modulate IgE synthesis by binding to the surface IgE on B cells.[5] Although the membrane-bound form of IgE is a common target for such potent IgE-binding agents, there are some differences in efficacy between monovalent and divalent recognition. For example, anti-IgE antibodies, regardless of anaphylactogenic or non-anaphylactogenic antibodies, are involved in surface IgE cross-linking-mediated inhibition of IgE synthesis in human B cell cultures stimulated with interleukin (IL)-4 plus anti-CD40 monoclonal antibody (mAb).[4] Supportive evidence for this divalent recognition is that $F(ab')_2$, but not Fab fragments, of anti-IgE antibodies have an inhibitory effect. In contrast with the Fab fragment, rsFcεRIα effectively decreases IgE synthesis, possibly via monovalent recognition, because the stoichiometry for

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the IgE binding of the rsFcεRIα has been shown to be 1 : 1.[6,7] However, the mechanisms by which rsFcεRIα and anti-IgE antibodies inhibit IgE synthesis remain unclear.

Previous investigations have suggested that the differentiation of IgE-expressing B cells into IgE-secreting cells may be subject to positive or negative regulation by several different factors.[2-4,8-10] Of these factors, one positive regulating factor is the autocrine production of IL-6, which provides a late amplification signal for IgE synthesis, whereas a negative regulating factor is the induction of apoptosis, which may eliminate IgE-expressing B cells. However, it is presently unknown whether IgE-expressing B cells respond to a signal delivered through their surface IgE with decreased IL-6 production or with apoptotic cell death.

In the present study, we have examined the relative contributions of rsFcεRIα and Fab and $F(ab')_2$ fragments of anti-IgE mAb to endogeneous IL-6 production and to apoptosis induction in IgE-expressing B cells generated by IL-4 plus anti-CD40 mAb stimulation of normal human B cells.

METHODS

The rsFcεRIα and mouse anti-rsFcεRIα mAb (CRA-1, IgG2b) were the same as described previously.[5] The CRA-1 had no ability to compete with IgE in binding to rsFcεRIα. Mouse antihuman IgE mAb (G7-18, IgG1) was obtained from PharMingen (San Diego, CA, USA). Although the epitope specificity of G7-18 was not described in the attached data sheet, our preliminary observation that G7-18 induced anaphylactic histamine release from atopic human peripheral leukocytes revealed that G7-18 recognizes cytophilic IgE on basophils. The Fab and $F(ab')_2$ fragments of G7-18 were prepared using the IgG1 Fab and $F(ab')_2$ preparation kit (Pierce Chemical, Rockford, IL, USA) according to the manufacturer's instructions. The fragments had undetectable contaminants of the intact antibody, as determined by a 10% non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The ability of G7-18 and its fragments to bind to IgE was determined by flow cytometry using the FcεRIα-expressing transfectant (a T cell hybridoma, MA5.8)-fixed IgE. No significant differences in binding activity were observed among G7-18 and Fab and $F(ab')_2$ fragments when each of them was added to the transfectant.

Cell preparation and cultures

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood obtained from normal healthy adults who had given informed consent to participate in the study and cytophilic IgE was removed by treatment with acid buffer, as described previously.[5] Depletion of $CD2^+$, $CD14^+$ and $CD56^+$ cells from PBMC was performed using magnetic beads coated with appropriate mAbs or streptavidin (Dynal, Oslo, Norway), as described elsewhere.[11] The purity of $CD19^+$ B cells obtained was always > 98% as determined by flow cytometry. Highly purified B cells were suspended at a concentration of 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 2 mmol/L l-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Aliquots of the cell suspension in a total volume of 0.2 mL were cultured in 96-well round-bottomed plates (Costar, Cambridge, MA, USA) with or without 2 ng/mL recombinant human IL-4 (Genzyme, Cambridge, MA, USA) plus 1 μg/mL of an agonistic mouse antihuman CD40 mAb (EA-5, IgG1; Ancell, Bayport, MI, USA) for the indicated time periods. In some experiments, recombinant human IL-6 (Genzyme) at appropriate concentrations was added to the culture.

IgE measurement

IgE levels in culture supernatants were measured by an isotype-specific radioimmunoassay using two different anti-IgE mAbs, as described previously.[5] Although rsFcεRIα and G7-18 at 1–1000 ng/mL, regardless of their concentrations, showed 25–30% inhibition of the assay, no significant differences were observed within these ranges when the standard curve was constructed by calculating least-square lines. For example, these were $\log y = 1.00 \log x - 0.43$ ($r = 0.999$; vehicle) versus $\log y = 0.99 \log x - 0.58$ ($r = 0.999$; 1 ng rsFcεRIα), $\log y = 1.02 \log x - 0.57$ ($r = 0.998$; 1000 ng rsFcεRIα), $\log y = 1.01 \log x - 0.54$ ($r = 0.998$; 1 ng G7-18) and $\log y = 0.98 \log x - 0.59$ ($r = 0.997$; 1000 ng G7-18). The Fab and $F(ab')_2$ fragments of G7-18 gave similar results. Thus, either rsFcεRIα or G7-18 at the highest concentration used in the present study was usually added to the corresponding control supernatant before the assay. This procedure enabled the exact determination of IgE levels in the supernatants containing rsFcεRIα or G7-18, despite entirely different recognition of Fcε

epitopes. The net synthesis of IgE was calculated by subtracting the value of preformed IgE released in the presence of both cycloheximide and puromycin.[5,11]

Competitive reverse transcription–polymerase chain reaction for germline and mature Cε transcription

Extraction of total cellular RNA and cDNA synthesis by reverse transcription (RT) was performed as described previously.[11] Levels of cDNA were quantified by means of a competitive polymerase chain reaction (PCR) method.[12] Germline Cε, mature Cε and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mimics were constructed by using a commercial construction kit (Clontech Laboratories, Palo Alto, CA, USA). Briefly, two rounds of PCR amplification were conducted to construct the mimics. The first PCR was cycled using two composite primers according to the manufacturer's protocol. Each composite primer had the target germline Cε, mature Cε or GAPDH gene primer sequence attached to a short nucleotide stretch of sequence designed to hybridize to opposite strands of a *v-erbB* gene fragment. The sequences of composite primers were as follows: germline Cε, 5'-ATCCACAGGCACCAAATGGACGACCCGCAAGTGAAATCTCCTCCG-3' and 5'-GCCAGGTCCACCACCAGACAGGTGATCTGTCAATGCAGTTTGTAG-3'; mature Cε, 5'-TCGACTTCTGGGGCCAAGGGCGCAAGTGAAATCTCCTCCG-3' and 5'-GCCAGGTCCACCACCAGACAGGTGATCTTCGGAGTCAACGGATTTGGTCGCAAGTGAAATCTCCTCCG-3' and 5'-CATGTGGGCCATGAGGTCCACCACTCTGTCAATGCAGTTTGTAG-3'. The first PCR products were amplified in a second round of PCR with primers specific for germline Cε, mature Cε or GAPDH to confirm that the PCR mimics obtained had the gene-specific primer sequences.[11] Competitive PCR was performed using serial 10-fold dilutions of PCR mimics (competitors) mixed with PCR amplification reactions containing a constant amount of the cDNA sample (targets). The PCR was cycled under suitable conditions, as described previously.[11] The PCR products were analyzed by electropheresis on 2% agarose gels and visualized by ethidium bromide staining. The bands of targets and competitors were scanned and quantified using the Multianalyst hardware and software system (Bio-Rad Laboratories, Hercules, CA, USA). The results obtained were expressed as zepto mol/μg RNA for germline or mature Cε and amol/μg RNA for GAPDH.

Detection of membrane-bound IgE

Cells trapping rsFcεRIα via their surface IgE were reacted with biotinylated CRA-1 and phycoerythrin (PE)-conjugated streptavidin, as described previously.[5] With regard to anti-IgE mAb binding to surface IgE, cells were incubated with biotinylated Fab or $F(ab')_2$ fragment of G7-18 (1 μg) for 2 h at 4°C, followed by incubation with PE-conjugated streptavidin for 30 min. In each experiment, an isotype-matched control mAb or its fragment was used for negative staining. Stained cells were analyzed by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA, USA) using a gate for lymphocytes. Fluorescence data were collected by using logarithmic amplification.

Apoptosis measurements

Detection of apoptotic cells was performed by using a commercially available apoptosis detection kit (Medical & Biological Laboratories, Nagoya, Japan) based on the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5'-triphosphate (dUTP)-biotin nick end-labeling method (TUNEL).[13] Briefly, cells were fixed with 4% paraformaldehyde, followed by permeabilization with 70% ethanol. After washing, reaction buffer containing TdT and fluorescein isothiocyanate (FITC)-labeled dUTP was added to the cell pellet in order to enable *in situ* visualization of DNA fragmentation at the single cell level. Fluorescein isothiocyanate-labeled dUTP alone was used for negative staining. Stained cells were analyzed by flow cytometry. Cells in the low forward scatter/high side scatter subpopulation were apoptotic, as described in detail elsewhere.[14]

IL-6 measurement

Quantification of IL-6 present in culture supernatants was determined using a commercial ELISA kit (BioSource International, Camarillo, CA, USA). This assay was not affected by the addition of rsFcεRIα or G7-18 at any concentration.

Statistical analysis

Paired *t*-test was used to determine the significance of the results.

RESULTS

Inhibitory effect of rsFcεRIα and G7-18 on IgE synthesis

B cells from five different donors were stimulated in the absence or presence of rsFcεRIα or G7-18 at 1–1000 ng/mL for 14 days with IL-4 plus anti-CD40 mAb. As shown in Fig. 1, both rsFcεRIα and G7-18 induced a concentration-dependent inhibition of IgE synthesis and the maximal inhibition was approximately the same for 100 ng/mL rsFcεRIα and 1000 ng/mL G7-18. Their effects on germline and mature Cε transcription were further investigated using RNA from B cells that had been stimulated for 12 days. A representative result of competitive RT-PCR is depicted in Fig. 2a. The results obtained from three different donors are summarized in Fig. 2b. Both rsFcεRIα (100 ng/mL) and G7-18 (1000 ng/mL) induced marked inhibition of mature Cε transcription, without affecting germline Cε transcription. These data are in keeping with previous reports.[4,5]

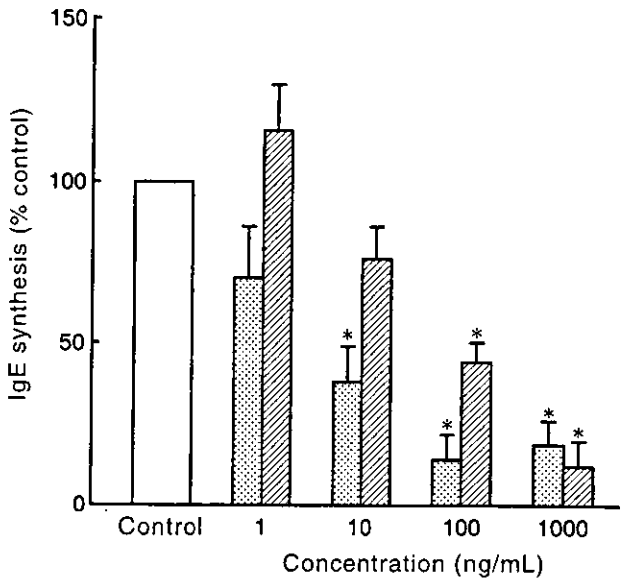

Fig. 1 Inhibitory effect of a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and G7-18 on IgE synthesis. B cells were stimulated in the absence (□) or presence of rsFcεRIα (▨) or G7-18 (▨) at 1–1000 ng/mL with interleukin-4 plus anti-CD40 antibody for 14 days. Control culture supernatants were supplemented with rsFcεRIα or G7-18 before the assay. Net IgE synthesis was assessed by subtracting the value of preformed IgE. The mean (± SEM) IgE levels in control cultures were 27.1 ± 8.9 ng/mL. Results are expressed in terms of percent change (mean ± SEM of five different experiments, each performed in triplicate). *$P < 0.05$ compared with control. Unlike G7-18, an isotype-matched control antibody had no inhibitory effect on IgE synthesis (data not shown).

Binding of rsFcεRIα and G7-18 to IgE-expressing B cells

Both rsFcεRIα and G7-18 were examined for their capacity to bind to IgE expressed on B cells. For this purpose, B cells were stimulated for 5 days with IL-4 plus anti-CD40 mAb and were then analyzed by flow cytometry. As shown in Fig. 3, surface IgE expression was detected on 12.7 ± 1.5% (mean ± SEM; $n = 3$) stimulated cells when rsFcεRIα was used. Also, G7-18 and its Fab and $F(ab')_2$ fragments almost equally recognized surface IgE. In each assay, surface IgE started to be expressed between 3 and 5 days after stimulation, reached a peak level at 5 days and rapidly declined thereafter (data not shown). Nevertheless, the Fab fragment (330 ng/mL) adjusted to the equal molar concentration of the intact antibody (1000 ng/mL), unlike the $F(ab')_2$ fragment (670 ng/mL) or rsFcεRIα (100 ng/mL), was ineffective in the inhibition of IgE synthesis (Fig. 4). The results obtained with G7-18 confirmed the previous finding[4] that inhibition of IgE synthesis by anti-IgE antibodies, irrespective of their epitope specificity, requires divalent recognition of surface IgE and demonstrate that surface IgE binding does not necessarily lead to decreased IgE synthesis.

Effect of rsFcεRIα and G7-18 on apoptosis

To elucidate the cellular mechanisms by which rsFcεRIα and G7-18 inhibit IgE synthesis, we first investigated whether surface IgE-mediated apoptosis of B cells was inducible. For this purpose, B cells were cultured in the absence or presence of rsFcεRIα (100 ng/mL), G7-18 (1000 ng/mL), the Fab fragment (330 ng/mL) or the $F(ab')_2$ fragment (670 ng/mL) with IL-4 plus anti-CD40 mAb. After 6 days, apoptotic cells were analyzed by flow cytometry using TdT and FITC-labeled dUTP. A representative result of three experiments is shown in Fig. 5. Although there were no significant differences in the percentage of apoptotic cells among medium (27.3 ± 1.5%), rsFcεRIα (28.6 ± 2.3%) and the Fab fragment (26.9 ± 1.9%), the $F(ab')_2$ fragment (34.7 ± 1.6%) and G7-18 (35.2 ± 1.8%) induced significant cell death by apoptosis ($P < 0.05$). This result implies that, despite the net induction of apoptosis in no more than 7% of stimulated cells, approximately 50% of the IgE-expressing B cells generated at a rate of approximately 13%

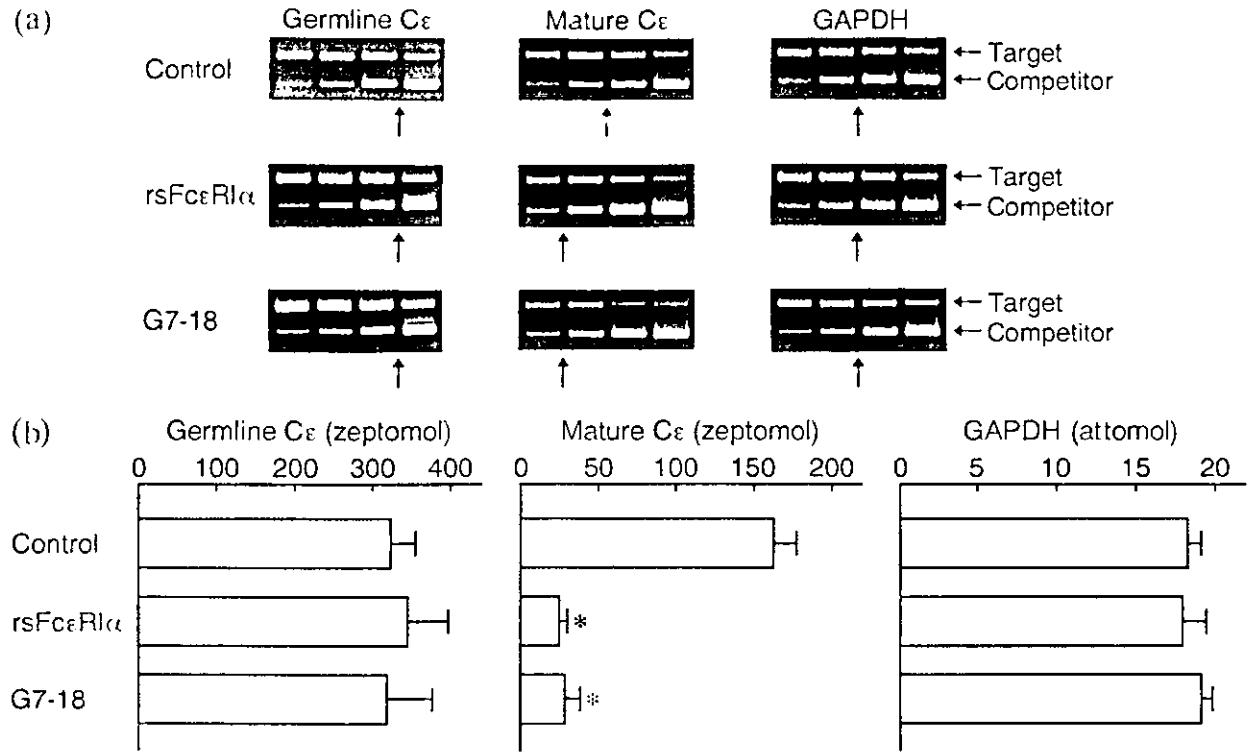

Fig. 2 Effect of a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and G7-18 on germline and mature Cε transcription. B cells were stimulated in the absence or presence of rsFcεRIα (100 ng/mL) or G7-18 (1000 ng/mL) with interleukin-4 plus anti-CD40 antibody for 12 days. After extraction of total cellular RNA, competitive reverse transcription–polymerase chain reaction (PCR) analysis was performed as described in Methods. (a) cDNA levels for germline Cε, mature Cε and GAPDH in a donor. Each lane shows progressive 10-fold increments of competitor DNA spiked into the PCR tube with a constant amount of target cDNA. Arrows indicate a target/competitor ratio of 1.0. (b) Image data were converted to digital values (zepto mol or amol/μg total RNA) by using computer software. Results represent the mean ± SEM of three different experiments, each performed in triplicate. *$P < 0.01$ compared with control.

(see Fig. 3) fell into apoptosis following surface IgE cross-linking.

Effect of rsFcεRIα and G7-18 on IL-6 production

Next, we addressed whether rsFcεRIα and G7-18 could regulate the autocrine production of IL-6. Culture supernatants from B cells stimulated in the absence or presence of rsFcεRIα(100 ng/mL) or Fab (330 ng/mL) and $F(ab')_2$ (670 ng/mL) of G7-18 with IL-4 plus anti-CD40 mAb were harvested 1–10 days post-stimulation and were analyzed for secreted IL-6. Figure 6 shows the results obtained with three different donors who were high responders for both IgE and IL-6 production. In control cultures, IL-6 started to be produced during 1 and 3 days after stimulation and gradually increased afterwards. It was found that rsFcεRIα was effective in decreasing IL-6 levels produced at 5–10 days after stimulation, but not at 1–3 days. A similar result was obtained with $F(ab')_2$ but not Fab fragment of G7-18 capable of inducing apoptosis in IgE-expressing B cells. This was most likely due to apoptotic cell induction. In contrast, the suppressive effect of rsFcεRIα on IgE synthesis, when assessed at 14 days after stimulation, was significantly restored by adding IL-6 (300–1000 pg/mL) at the onset of cell culture, although this restoration was incomplete (Fig. 7). In contrast, inhibition of IgE synthesis by the $F(ab')_2$ fragment was not restored, even by the addition of IL-6. These results showed that, unlike G7-18, rsFcεRIα was, to some extent, involved in regulating the IL-6-dependent differentiation of IgE-expressing B cells into IgE-secreting cells.

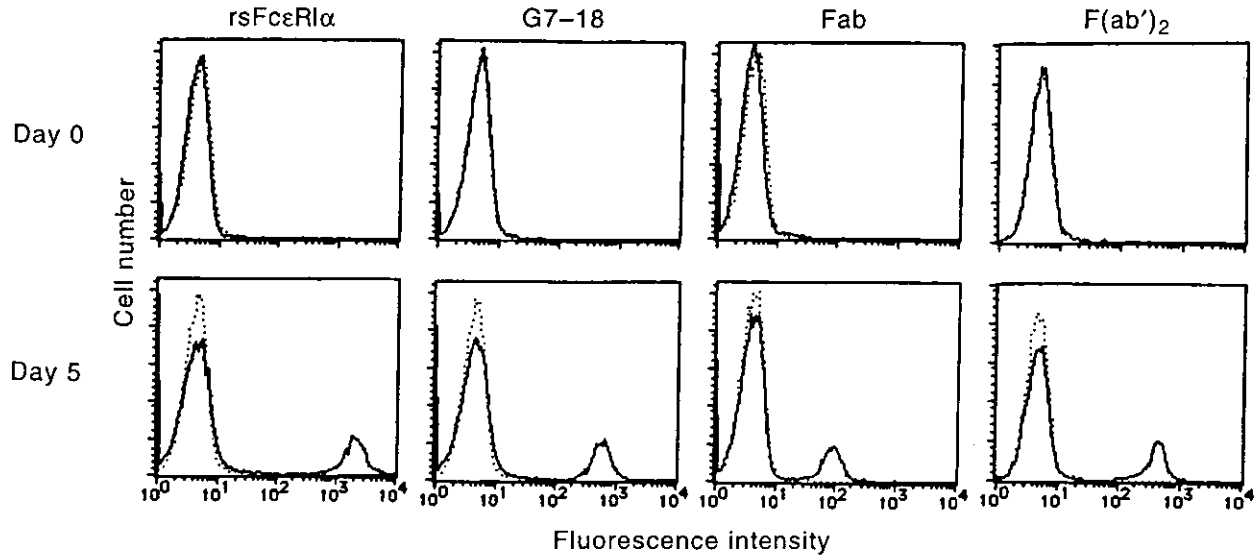

Fig. 3 Binding of a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and G7-18 to surface IgE. B cells were stimulated with interleukin-4 plus anti-CD40 antibody for 5 days. Cells were incubated either with rsFcεRIα or with biotinylated G7-18 or its Fab or $F(ab')_2$ fragment. Cells trapping rsFcεRIα were reacted with biotinylated CRA-1, followed by incubation with phycoerythrin (PE)-conjugated streptavidin. Cells trapping the biotinylated antibody were incubated with PE-conjugated streptavidin. In each experiment, background staining was obtained by incubating cells with an isotype-matched control antibody or its fragment. Stained cells were analyzed by flow cytometry. Results are representative of three experiments.

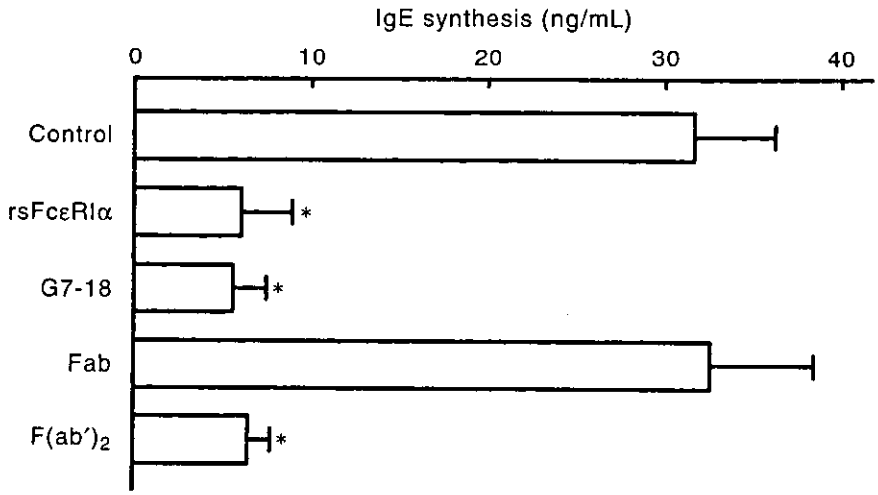

Fig. 4 Effect of a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and G7-18 on IgE synthesis. B cells were stimulated in the absence or presence of rsFcεRIα (100 ng/mL), G7-18 (1000 ng/mL), the Fab fragment (330 ng/mL) or the $F(ab')_2$ fragment (670 ng/mL) with interleukin-4 plus anti-CD40 antibody for 14 days. Control culture supernatants were supplemented with rsFcεRIα or G7-18 before the assay. Net IgE values represent the mean ± SEM of four different experiments, each performed in triplicate. *$P < 0.05$ compared with control.

Discussion

The present study shows that, in normal human B cell cultures stimulated with IL-4 plus anti-CD40 mAb, rsFcεRIα decreases IL-6 production by IgE-expressing B cells, possibly via monovalent recognition, whereas G7-18, an anti-IgE mAb, induces apoptosis in the cells through surface IgE cross-linking. The decrease in IL-6 secretion is of significance because the addition of IL-6 significantly restored the suppressive effect of rsFcεRIα on IgE

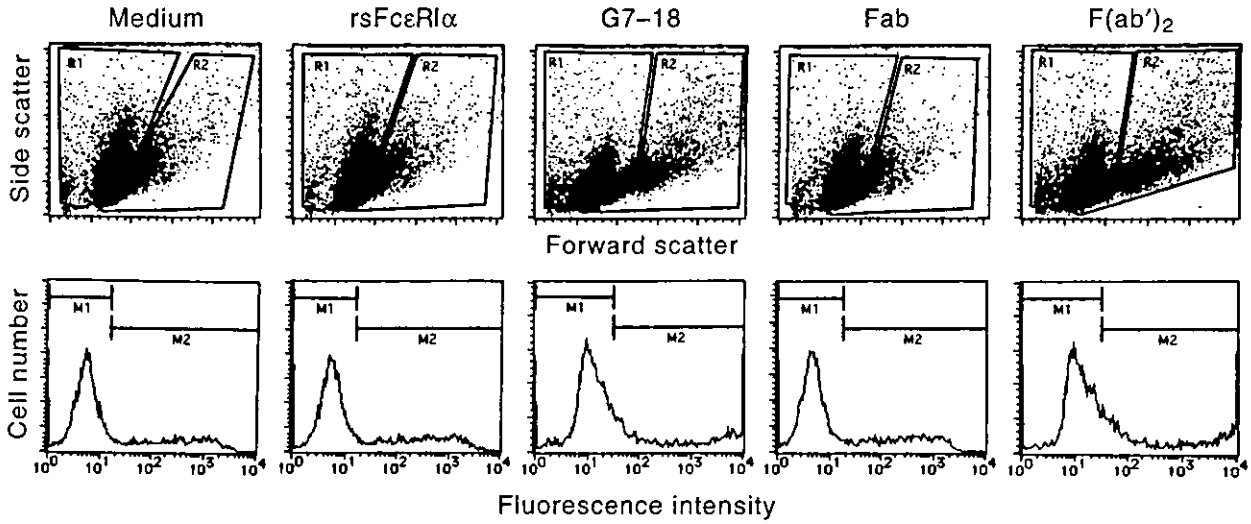

Fig. 5 Effect of a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and G7-18 on apoptosis. B cells were cultured in the absence or presence of rsFcεRIα (100 ng/mL), G7-18 (1000 ng/mL), the Fab fragment (330 ng/mL) or the $F(ab')_2$ fragment (670 ng/mL) with interleukin-4 plus anti-CD40 antibody for 6 days. Apoptotic cells, which were identified as the low forward scatter/high side scatter subpopulation (R1), were stained with terminal deoxynucleotidyl transferase plus fluorescein isothiocyanate (FITC)-labeled deoxyuridine 5'-triphosphate (dUTP). Background staining was obtained by incubating cells with FITC-labeled dUTP (M1). Apoptotic cells (M2) in total B cells were analyzed by flow cytometry. Results are representative of three experiments.

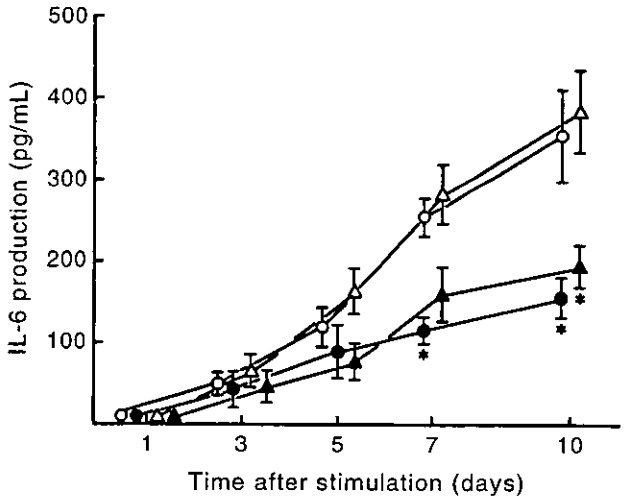

Fig. 6 Effect of a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcεRIα) and G7-18 on the autocrine production of interleukin (IL)-6. B cells were cultured in the absence (○) or presence of rsFcεRIα (100 ng/mL; ●), Fab (330 ng/mL; △) or $F(ab')_2$ (670 ng/mL; ▲) of G7-18 with IL-4 plus anti-CD40 antibody for up to 10 days. Culture supernatants were harvested at the indicated time points and analyzed for IL-6 levels. Results represent the mean ± SEM of three different experiments, each performed in triplicate. $^*P < 0.05$ compared with control.

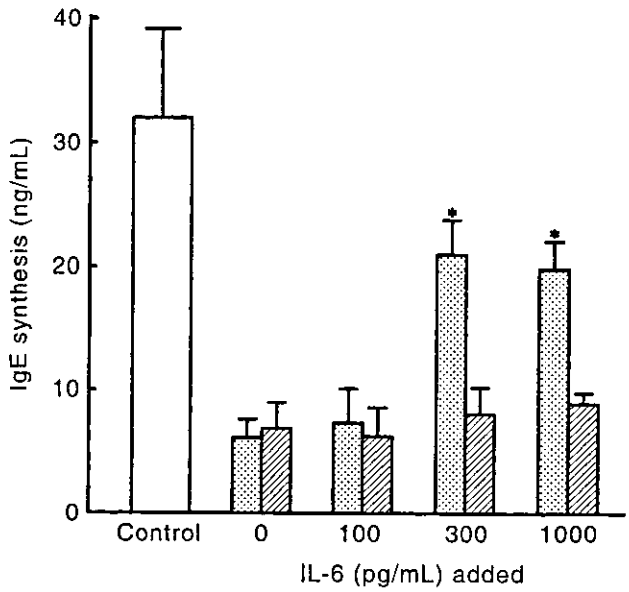

Fig. 7 Effect of addition of interleukin (IL)-6 on a recombinant soluble form of the high-affinity IgE receptor α subunit (rsFcε-RIα)- or G7-18-induced inhibition of IgE synthesis. B cells were stimulated with IL-4 plus anti-CD40 antibody for 14 days. Either rsFcεRIα (100 ng/mL; ⊠) or $F(ab')_2$ fragment (670 ng/mL; ▨) of G7-18 mixed with or without IL-6 was added at the onset of the cell culture. Control culture supernatants were supplemented with rsFcεRIα or the $F(ab')_2$ fragment before the assay. Net IgE values represent the mean ± SEM of three different experiments, each performed in triplicate. $^*P < 0.05$ compared with the culture containing rsFcεRIα alone.

synthesis. Evidence that the induction of apoptosis is specific for divalent recognition is that $F(ab')_2$, but not the Fab fragment of G7-18, was effective. These findings provide the explanation for the difference between mechanisms for rsFcεRIα- or anti-IgE mAb-induced inhibition of IgE synthesis.

Recombinant soluble FcεRIα is involved in the surface IgE-mediated attenuation of IL-6 production. Indeed, rsFcεRIα was able to decrease IL-6 levels produced at a later stage when IgE-expressing B cells started to be generated without affecting IL-6 production at an early stage. The involvement of the autocrine production of IL-6 is supported by previous reports that showed that a neutralizing anti-IL-6 antibody inhibited the T cell-independent induction of IgE synthesis by IL-4 plus anti-CD40 mAb[8] and that these two stimuli synergized for the activation of nuclear factor-κB, which regulates IL-6 transcription and production.[9] However, IL-6 transcription was not affected by rsFcεRIα because its induction reached a peak level at an early stage of B cell activation (data not shown). In contrast, unlike rsFcεRIα, neither IL-6 production nor IgE synthesis was inhibited by the Fab fragment of anti-IgE mAb. This result indicates that simple monovalent recognition of surface IgE does not necessarily contribute to decreased IL-6 production and suggests that the signal provided exclusively via surface IgE trapping rsFcεRIα may be critical in regulating functional IL-6 secretion. Furthermore, the observation that the suppressive effect of rsFcεRIα on IgE synthesis could be significantly, but incompletely, restored by the addition of IL-6 suggests that IL-6-independent yet unidentified events also may be involved in the regulation of IgE synthesis. Although the FcεRI binding site has been shown to be located more to the beginning of the Cε3 domain of IgE,[15,16] no amino acid replacements within this fragment have been obtained.[17,18] It therefore seems unlikely that functional polymorphisms exist within the *Cε3* gene that may alter the rsFcεRIα–surface IgE interaction. Although it is not known whether the binding of rsFcεRIα to IgE-expressing B cells leads to conformational changes in surface IgE for signal transduction that are functionally associated with the selective modulation of accumulation of mature Cε transcripts, the mechanism of rsFcεRIα-induced inhibition of IgE synthesis can be at least partly explained by the decrease in IL-6 production by IgE-expressing B cells.

Our observation that $F(ab')_2$ but not Fab fragment of anti-IgE mAb inihibited mature Cε transcription and IgE synthesis is in accordance with a previous report describing that a cross-linking of surface IgE was required for the inhibitory activity.[4] An interesting finding was that the $F(ab')_2$ fragment induced apoptosis in IgE-expressing B cells through surface IgE cross-linking. This finding is in line with several reports[19–21] showing that mature B cell subsets, including germinal center and memory B cells, undergo apoptosis upon subsequent ligation of surface Ig. Because surface IgM ligation has been shown to induce caspase-dependent and -independent apoptosis, the apoptotic pathways coupled to the surface IgE may involve activation of pro-apoptotic signals, including effector caspases.[21,22] The apoptotic cell induction we found was seen in approximately 50% of IgE-expressing cells, but the remainder were resistant to surface IgE-mediated apoptosis. Nevertheless, mature Cε transcription was markedly inhibited by the $F(ab')_2$ fragment. Therefore, it is likely that additional events, which are independent of cell death by apoptosis, are also involved in the regulation of IgE synthesis. One possible event is that apoptosis-resistant IgE-expressing B cells may have fallen into anergy after divalent recognition. Similar phenomena have been obtained with IgE memory B cells, which can be specifically inactivated by treatment with anti-IgE antibodies, resulting in abrogation of secondary IgE responses.[23,24] Collectively, inhibition of IgE synthesis by anti-IgE mAb is most likely due to the combination of surface IgE-mediated apoptosis and anergy in IgE-expressing B cells.

Although the anti-IgE mAb used in the present study is anaphylactogenic, non-anaphylactogenic humanized or chimeric anti-IgE mAbs have been produced that bind to free IgE and surface IgE on B cells but not to IgE bound to FcεRI or FcεRII/CD23 on various cell types.[25–29] These properties are similar to those of rsFcεRIα, which traps IgE via its Cε3 domain, responsible for receptor binding. The non-anaphylactogenic anti-IgE mAbs reduced serum levels of free IgE in atopic patients to low or almost undetectable levels. This reduction is rapid, which is due predominantly to the formation of complexes with IgE. Anti-IgE therapy has also been shown to inhibit allergic inflammation and to improve symptoms.[26,28,29] Similar results have been obtained with rsFcεRIα in experimental animal models.[6,7,30] In addition to such pharmacologic modulation of IgE-mediated responses, anti-IgE mAb and rsFcεRIα selectively inhibit IgE synthesis by binding to surface IgE on B cells, although their immunoregulatory mechanisms are entirely different. Thus, it is also expected from previous and present studies that anti-IgE mAb or rsFcεRIα treatment of atopic patients may eliminate the terminal differentiation of IgE-expressing B cells, including

memory cells, into IgE-secreting cells, with ameliorated effects on allergic symptoms.

In summary, we have shown that monovalent recognition of surface IgE by rsFcεRIα, but not by Fab fragment of anti-IgE mAb, leads to a decrease in IL-6 production by IgE-expressing B cells and that divalent recognition by the $F(ab')_2$ fragment induces apoptosis in the cells. It is clear from these findings that regulation of IgE synthesis by rsFcεRIα differs from that by anti-IgE mAb. Further studies are needed to define the nature of the putative factors conferring inhibition of IL-6 secretion by rsFcεRIα or induction of apoptosis by anti-IgE mAb in IgE-expressing B cells.

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Regulation of IgE Synthesis: From Past To Present

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Summary

Allergen-specific IgE synthesis contributes to induction and maintenance of allergic symptoms. Recent advances in elucidating the signaling pathways to produce the terminal differentiation of B cells into IgE-secreting plasma cells have led to new insights into the molecular mechanisms involved in IgE synthesis. This differentiation is regulated at the level of transcriptional activation by multiple pathways of B cell triggering. Despite great effort, the whole picture of the switch recombination machinery responsible for IgE isotype switching remains unclear. Identification and characterization of the switch recombinase are the next challenge for the future studies.

Introduction

More than 35 years have passed since IgE was identified in 1966 as a new immunoglobulin isotype that plays a key role in the pathogenesis of allergic disease (1). Active attention has been paid in the past three decades to elucidate the regulatory mechanisms of the IgE response. The earliest reports in the 1970s and 1980s described that T cells could produce several soluble factors responsible for the regulation of IgE synthesis. This implied that B cell differentiation into IgE-secreting plasma cells was subject to regulation by different factors. It was shown in the 1980s and 1990s that the immune system was controlled by a balance between Th1 and Th2 cells, allergic individuals had Th2 polarization, and Th2 cells served to induce IgE synthesis by producing IL-4/IL-13 and expressing CD40 ligand (CD40L, CD154). It was also found that IL-4 and IL-13 induced germline Ce transcription by utilizing a common receptor component (IL-4Rα, CD124) and subsequent engagement of CD40 by CD40L activated DNA switch recom-

bination leading to IgE isotype switching. The increasing understanding of the cellular and molecular events underlying IgE synthesis has allowed the identification of new targets for IgE regulation. The present article provides a current overview of the regulatory mechanisms of human IgE synthesis.

Cell Types Supporting IgE synthesis by B Cells

Induction of allergen-specific IgE synthesis requires the cognate interactions between B and Th2 cells. In the presence of the Th2 cell-derived IL-4 or IL-13, the B cell undergoes germline Ce transcription that is a critical initiating step for IgE isotype switching. The IL-4Ra, which forms a complex either with the common γ chain (γc, CD132) or with the IL-13Rα1 (CD213a1), plays a key role in IL-4- or IL-13-dependent germline Ce transcription. Actually, B cells of X-linked severe combined immunodeficiency patients with mutations in the γc gene can express germline Cε transcripts in response to IL-4 or IL-13 stimulation (2). CD40L, a non-covalent trimer expressed on the activated Th2 cell, in conjunction with IL-4 or IL-13, induces IgE isotype switching through cross-linking of CD40 constitutively expressed on the B cell. Ligation of CD40 also mediates rescure from apoptosis, proliferation and terminal differentiation into IgE antibody-secreting plasma cells. The central role of the interaction of CD40 with CD40L in Ig class switching has been shown in patients with X-linked hyper-IgM syndrome, who have mutations in the CD40L gene that result in defective isotype switching (3).

In addition to Th2 cells, Tc2 cells, γδ T cells, mast cells, basophils, and eosinophils secrete IL-4 and/or IL-13 and express CD40L after immunologic or non-immunologic stimulation. Such cellular responses allow adjacent B cells to induce IgE isotype switching and differentiation into IgE-secreting plasma cells. More recently, glucocorticoids have been shown to up-regulate CD40L expression both in T and B cells (4). Thus, several cell types are involved in the induction of IgE synthesis. Although the production of specific IgE antibody by a given B cell is critically dependent on the interaction with an allergen-specific Th2 cell, other lineage cells with a Th2-like phenotype contribute to polyclonal IgE production by different B cells. In this respect, such cells also may be important targets for achieving the down-regulation of IgE synthesis.

Molecular Events in IgE Isotype Switching

The human CH gene family consists of nine functional genes and two pseudogenes. The organization of the CH locus located at the 3′ side of a given VH segment, a D segment, and a JH segment that complete a VH region sequence is as follows: 5′–Cμ–Cδ–Cγ3–Cγ1–Cψε–Cα1–Cωγ– Cγ2–Cγ4–Cε–Cα2–3′. A DNA recombination involved in isotype switching takes place between two switch (S) regions located at the 5′ side of each CH gene,

except Cδ and Cψγ. The S regions include Sμ, Sγ, Sα, and Sε, each of which is composed of trandem repetition of short unit sequences. Although the Se region is also present before the Cψε gene, this region is not involved in recombination because of the defect in a part of the exon. Furthermore, the germline IH exons (Iμ, Iγ, Iα, and Iε) are located 5′ to each functional S region. With the exception of constitutive activation of the Im promoter, the other IH promoter is activated in response to cytokine stimulation, leading to transcription of the IH exon, the S region, and the CH exons. Because transcripts of the S region are spliced out by splicing factors, the resultant transcripts are germline CH transcripts, expression of which directs isotype switching by regulating the accessibility of a particular S region to a putative common recombinase system. This type of recombination involves the Sμ region as one of the pair, and the Sγ, Sα, or Sε region is involved as the other partner. Engagement of CD40 by CD40L in the presence of a particular cytokine plays a crucial role in a given switch recombination. During recombination, the DNA segment between the expressed VH and CH genes is looped out as a circle and deleted from the chromosome. Although the switch recombinase has not as yet been identified, activation-induced cytidine deaminase (AID), a B cell-specific RNA editing enzyme, has recently been reported to be expressed after cytokine and CD40L stimulation and to be involved in regulation or catalysis of the DNA modification step of isotype switching (5, 6). Indeed, AID deficiency causes the autosomal recessive form of the hyper-IgM syndrome characterized by defective switch recombination.

In the course of switching to IgE, activation of the Ie promoter induced by IL-4 or IL-13 initiates transcription of the Iε exon, the Sε region, and the Cε1-4 exons. The Iε promoter contains binding elements for STAT6, C/EBP, PU.1, NF-κB, and Pax-5. A genetic variant of IL-4Rα, Ile50Val, which has been identified in relation to atopic asthma, associates with IL-4 or IL-13 activity and upregulates STAT6 activation (7). Although the essential role of JAK-dependent STAT6 in the activation of the Ie promoter is well established, PKC-dependent PU.1 cooparates with STAT6 for the synergistic activation of the Ie promotor. Then, splicing cuts the transcript of the Se region, thereby allowing expression of germline Ce transcripts. Several studies have suggested that spliced switch transcripts bind the DNA of the corresponding S region and induce stable RNA/DNA hybrids that are a target for both the ribonuclease and the putative switch recombinase (8, 9). It seems therefore that processing of germline Cε transcripts may be of importance in directing Sμ–Sε recombination. Furthermore, CD40 ligation not only up-regulates IL-4- or IL-13-driven germline Ce transcription due to full activation of the Ie promoter that results from at least NF-κB activation, but also induces expression of AID that plays a role downstream of the germline Cε transcription. AID expression is followed by activation of the recombination machinery that allows the deletion of the intervening DNA between the Sμ region and the targeted Sε region. Furthermore, nuclear factors, such as

Ku70/80 and DNA-PK, are required to perform Sμ–Sε recombination. This switch recombination results in the juxtaposition of the Cε gene to the expressed gene of the variable region and in the subsequent induction of mature Ce transcription and IgE synthesis. Thus, CD40 signaling activates multiple pathways that are important for AID expression and isotype switching. However, the nature of the key second messengers involved in the activation of the switch recombination machinery is still unknown.

Conclusions

The expotential increase in the understanding of the cellular mechanisms of IgE synthesis has led to the development of agents capable of regulating B cell differentiation into IgE-secreting plasma cells. However, the molecular mechanisms involved in IgE isotype switching are incompletely understood. Elucidation of the merging point of IL-4Rα and CD40 signaling pathways that are required for AID expression and IgE switching will provide potential new strategies for the isotype-specific regulation of IgE synthesis.

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(14) Predominant expression of *950delCAG* of IL-18R α chain cDNA is associated with reduced IFN-γ production and high serum IgE levels in atopic Japanese children

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Background: We previously reported that serum IgE levels were negatively correlated with the amount of IFN-γ produced by phytohemagglutinin-stimulated or IL-12–stimulated PBMCs and that one of the mechanisms of the pathogenesis of atopy was the reduced IFN-γ production, which led to upregulated IgE production.
Objective: IL-18 is also known to be a strong inducer of IFN-γ production. However, it has not yet been determined whether IL-18 is associated with atopic disease.
Methods: We investigated the response to IL-18 or IL-12 stimulation and the sequence of IL-18 receptor (IL-18R) α chain cDNA in 41 nonatopic controls and 39 atopic patients.
Results: Serum IgE level was negatively correlated with IFN-γ production by PBMCs stimulated with IL-18. The IL-18R α chain cDNA of atopic patients was sequenced. We identified a 3-base deletion of the IL-18R α chain cDNA (*950delCAG*), which was generated by alternative splicing, as determined on the basis of genomic sequence data for the IL-18R α chain gene. PBMCs with the predominant expression of *950delCAG* significantly showed the reduced IFN-γ production after IL-18 stimulation. There was a significant difference in the expression pattern of the IL-18R α chain transcript between the atopic patients and the nonatopic controls.
Conclusion: According to these results, the dominant expression of the *950delCAG* transcript of IL-18R α chain cDNA, which was associated with reduced IFN-γ production by IL-18 stimulation and high serum IgE levels, is predisposition to some atopic diseases. (J Allergy Clin Immunol 2002;109:669-75.)

Key words: Atopy, IL-18, IFN-γ, IgE, IL-18 receptor

Abbreviations used
950delCAG: Three-base deletion of the IL-18 receptor α chain cDNA
NK: Natural killer
PHA: Phytohemagglutinin
IL-18R: IL-18 receptor

A variety of biological functions have been associated with human IL-18, including induction of proliferation of activated T cells, enhancement of natural killer (NK) cytotoxity, induction of IFN-γ and GM-CSF production, and promotion of a T_H1-type T-cell response.[1-4] The activity of IL-18 is via an IL-18 receptor (IL-18R) complex. This IL-18R complex is composed of a binding chain termed *IL-18R* α, a member of the IL-1R family previously identified as the IL-1R–related proteins, and a signaling chain, also a member of the IL-1R family. The IL-18R complex recruits the IL-1R–activating kinase and TNF–associated factor 6, which phosphorylates nuclear factor κB–inducing kinase, with subsequent activation of nuclear factor κB.[5-7]

With respect to IL-18 and atopic diseases, the data of Kodama et al[8] support a role for IL-18 in the complex pathogenesis of allergic inflammation in which IL-18 limits the development of the local inflammatory response to antigen. In vivo, the synergistic action of IL-12 and IL-18 is necessary to prevent T_H2-like cell differentiation, and this consequently inhibits the development of airway symptoms in a mouse model of allergic asthma.[9] A contrary result has also been reported. IL-18 has the potential to stimulate basophils, but when coadministered with IL-12, it exhibits an antiallergic action in vivo.[10] IL-18 promotes expression of the T_H2 phenotype in vivo, potently induces allergic sensitization,[11] induces T_H2 cytokine and CD154 expression, and can contribute to $CD4^+$ T-cell–dependent, IL-4–independent IgE production.[12]

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Abbreviations used
HDM: House dust mite
TCR: T-cell receptor

We previously reported that serum IgE level was negatively correlated with the amount of IFN γ secreted by PBMCs and that IFN-γ production by IL-12–stimulated PBMCs in an atopic group was lower than that in a control group.[13,14] Furthermore, serum IgE level was negatively correlated with IFN-γ production by PBMCs stimulated with IL-12, one of the reasons being that reduced IFN-γ production after IL-12 stimulation was associated with heterozygous IL-12R β2 mutations in atopic subjects.[15]

IL-18, a novel inflammatory cytokine that shares biological functions with IL-12, is known as a strong IFN-γ–inducing factor. In this study, we examined IFN-γ production in PBMCs after stimulation with IL-18 and the IL-18R α chain cDNA sequence. We show that predominant expression of *950delCAG* of IL-18R α chain cDNA is associated with reduced IFN-γ production after stimulation with IL-18 and high serum IgE levels in some atopic patients.

MATERIALS AND METHODS

Patients and control subjects

All of the atopic and control patients were outpatients at our hospitals. All were free of acute infection at the time of testing. The atopic group consisted of 39 children who had IgE levels higher than 150 IU/mL, specific IgE levels higher than 3.5 IU/mL, and clinical symptoms such as bronchial asthma or atopic dermatitis. The mean age ± 1 SD of the atopic patients was 6.9 ± 4.4 years, and their mean IgE ± 1 SD level was 2062.4 ± 3240.2 IU/mL. The diagnosis of bronchial asthma was made according to the criteria of the American Thoracic Society, and that of atopic dermatitis was made according to the criteria of Hanifin. Specific IgE antibodies for house dust, *Dermatophagoides*, hen egg, and cow milk were measured with a fluoroenzyme immunoassay by means of a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden). A specific IgE level higher than 3.5 IU/mL was considered positive. No patients had been receiving systemic steroids.

Each of the 41 nonatopic controls had a negative history of atopic disease. Their serum IgE levels were lower than 150 IU/mL, and the specific IgE levels for the 4 allergens were lower than 3.5 IU/mL. The mean age ± 1 SD of the nonatopic controls was 4.8 ± 6.7 years, and their mean IgE ± 1 SD level was 42.2 ± 35.1 IU/mL.

Informed consent was obtained from all subjects or their parents.

Cell preparation

PBMCs were isolated from 5 mL heparinized blood of control donors and patients by gradient centrifugation in Ficoll-Paque (Pharmacia). PBMCs were suspended at a density of 10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Cell culture

PBMCs (10^6/mL) were cultured in the presence or absence of 10 μg/mL phytohemagglutinin (PHA; Gibco BRL, Grand Island, NY), 5 IU/mL recombinant human IL-12 (R & D systems, Inc, Wiesbaden, Germany), or 400 ng/mL recombinant human IL-18 for 24 hours in a final volume of 1 mL in a round-bottom tube (Falcon 2059, Becton Dickinson Labware, Lincoln Park, NJ) at 37°C in a humidified atmosphere containing 5% CO_2.[14] The recombinant human IL-18 used had been prepared in our laboratory through use of an *Escherichia coli* expression system (according to an unpublished method developed by Kato et al). The endotoxin level of the recombinant human IL-18 was less than 0.1 pg per 1 μg of the protein.

Assays of cytokines

Culture supernatants in the test tubes incubated for 24 hours were spun to remove cells after the cultures. IFN-γ concentrations were measured through use of a human IFN-γ ELISA kit (Ohtsuka, Tokyo, Japan); the detection limit was 15.6 pg/mL.[14] Results of some samples were confirmed by duplicate measurement ($P < .0001$; $r = 0.994$).

IgE assay

Plasma samples obtained from heparinized blood were stored at −30°C. Plasma IgE concentrations were determined with a chemiluminescent enzyme immunoassay. The values were regarded as the serum IgE concentrations.

Sequencing of IL-18R α chain cDNA

RNA was extracted from PBMCs cultured in the presence of PHA for 24 hours through use of an Isogen kit (Nippon Gene, Tokyo, Japan). Fragments of IL-18R α chain cDNA were amplified, ligated to a T-vector (Novagen, Madison, Wis), and sequenced through use of an ABI 377 DNA sequencer (Applied Biosystems, Indianapolis, Ind). The conditions for the PCRs were 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes.

For concise detection of *950delCAG*, 40 cycles of PCR were performed through use of the sense primer 5′-^{891}ATTGCACTGTGGCCAGCACG–3′ and the antisense primer 5′-^{991}TCCTCTTGTGAAGACGTGGC–3′. The nucleotide was numbered according to the Gene Bank Database U43672. The fragments were electrophoresed on 15% polyacrylamide gel to detect the size difference between normal and mutant fragments. The fragments were simultaneously ligated to the T-vector and sequenced through use of the autosequencer.

Amplification of the genomic region of *950delCAG* of IL-18R α chain

Genomic DNA was purified from a polynuclear cell fraction with a Sepa Gene (Sanko Jyunyaku, Tokyo, Japan), used according to the manufacturer's instructions. The conditions for detection of *950delCAG* from genomic DNA amplified by PCR were 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes for 40 cycles. PCR was performed through use of the sense primer 5′-^{909}CGGGAGGCACAGACACCAAA–3′ and the antisense primer 5′-^{982}GAAGACGTGGCCTGGGATAT–3′.

Statistical analyses

The significance of the difference between the 2 groups was analyzed by means of the Student *t* test. A multiple regression model was used to simultaneously evaluate the effects of the log-transformed independent variables on IFN-γ production and incorporate the effect of IL-12, IL-18, or PHA as a categorical grouping variable.

Association of the differences in the expression pattern of *950delCAG* of IL-18R α chain transcript and IgE or IFN-γ production stimulated by IL-18 was confirmed by ANOVA and then analyzed by Fisher's protected least significant difference test.

Differences in the expression pattern of *950delCAG* of IL-18R α chain transcript between the atopic group and the control group were compared through the use of χ^2 statistics.