

Ketoconazole Suppresses Interleukin-4 plus Anti-CD40-Induced IgE Class Switching in Surface IgE Negative B Cells from Patients with Atopic Dermatitis

Naoko Kanda and Shinichi Watanabe

Department of Dermatology, Teikyo University, School of Medicine, Tokyo, Japan

We previously reported that antimycotic agent ketoconazole suppressed interleukin-4 production in T cells from patients with atopic dermatitis. We herein studied if ketoconazole may suppress B cell IgE class switching. Interleukin-4 plus anti-CD40-induced IgE secretion was enhanced in peripheral blood surface IgE⁻ B cells from atopic dermatitis patients compared to those from normal donors, and the secretion was inhibited by ketoconazole. Ketoconazole suppressed interleukin-4 plus anti-CD40-induced germline and mature ϵ transcripts in surface IgE⁻ B cells. Ketoconazole also inhibited interleukin-4 plus anti-CD40-induced activation of germline ϵ promoter in human Burkitt lymphoma Ramos cells. The regions -171/-155 bp containing CCAAT/enhancer-binding protein element and -155/-109 bp containing Stat6 and nuclear factor κ B elements were required for the ketoconazole-induced inhibition of the germline ϵ promoter activity. Ketoconazole inhibited interleukin-4 plus anti-CD40-induced enhancer activities of CCAAT/enhancer-

binding protein and nuclear factor κ B, and those of composite elements of CCAAT/enhancer-binding protein/Stat6 or of Stat6/nuclear factor κ B, but did not alter that of Stat6 in Ramos cells. cAMP analog reversed the inhibitory effects of ketoconazole on interleukin-4 plus anti-CD40-induced IgE secretion, germline and mature ϵ transcripts, and ϵ germline promoter activation. Interleukin-4 plus anti-CD40 increased intracellular cAMP by activating cAMP-synthesizing adenylate cyclase in surface IgE⁻ B cells, and the increase was greater in the cells from atopic dermatitis patients than in those from normal donors. Ketoconazole suppressed interleukin-4 plus anti-CD40-induced activation of adenylate cyclase in surface IgE⁻ B cells. These results suggest that ketoconazole may suppress interleukin-4 plus anti-CD40-induced B cell IgE class switching by inhibiting cAMP signal, and stress its prophylactic effects on allergic diseases. **Key words:** cAMP/C/EBP/nuclear factor κ B. *J Invest Dermatol* 119:590-599, 2002

It has recently been reported that systemic and/or topical treatment with an antimycotic drug, ketoconazole (KC), is effective for patients with atopic dermatitis (AD) (Clemmensen and Hjorth, 1983; Back *et al*, 1995; Broberg and Faergemann, 1995). This is partly caused by the fungicidal or fungistatic effects of KC (Agut *et al*, 1992; Elewski, 1993) as skin-localized fungi such as *Malassezia furfur* may act as allergens for IgE-mediated allergic responses in AD (Tengvall Linder *et al*, 1996). Direct immunomodulatory effects, however, unrelated to fungi, appeared to mediate the therapeutic efficacy of KC on AD, such as the inhibition of the leukotriene synthesis in

polymorphonuclear leukocytes (Bettens *et al*, 1986), or the inhibition of reactive radical nitric oxide production in macrophages (Baroni *et al*, 1999).

AD is characterized by abnormally increased IgE production (Leung, 1995). The allergen-induced ligation of IgE on the surface of mast cells induces the release of chemical mediators, such as leukotrienes, histamine, or prostaglandins, and causes immediate hypersensitivity reaction, which is followed by a late phase inflammatory reaction involving T cells or eosinophils, leading to the development of atopic eczema (Leung, 1995; Werfel *et al*, 1995). It is thus a key therapeutic target for AD to prevent B cell isotype switching to IgE. The B cell IgE switch requires soluble factors such as interleukin-4 (IL-4) and a T-cell-contact-dependent signal such as the stimulation of CD40 molecules (Worm and Henz, 1997). IL-4 firstly induces transcription from the germline promoter located upstream of the gene segments encoding unrearranged IgE ϵ -heavy chain constant region (C ϵ). This results in the production of untranslatable transcripts containing IgE ϵ -heavy chain germline exon (I ϵ) and C ϵ sequences and also makes the switch region accessible to the putative recombinase. In the process of switch recombination, I ϵ is deleted and the C ϵ gene is positioned directly downstream of the variable region genes (V(D)J genes) encoding antigen specificity (Worm and Henz, 1997). The switch recombination induced by IL-4 is potentiated by the

Manuscript received October 29, 2001; revised April 23, 2002; accepted for publication May 3, 2002.

Reprint requests to: Dr. Naoko Kanda, Department of Dermatology, Teikyo University, School of Medicine, 11-1, Kaga-2, Itabashi-Ku, Tokyo 173-8605, Japan. Email: nmk@med.teikyo-u.ac.jp

Abbreviations: AC, adenylate cyclase; AD, atopic dermatitis; AP-1, activator protein 1; Bt₂, dibutyl; C ϵ , IgE ϵ -heavy chain constant region; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, cAMP response element binding protein; H-89, N-[2-((p-bromocinnamyl)aminoethyl)-5-isoquinolinesulfonamide]; I ϵ , IgE ϵ -heavy chain germline exon; KC, ketoconazole; NF- κ B, nuclear factor κ B; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PDE, cyclic nucleotide phosphodiesterase.

interaction of CD40 on B cells with CD40 ligand on activated T cells (Shapira *et al*, 1992).

T cells from AD patients predominantly produce T helper 2 type cytokines enhancing IgE synthesis, such as IL-4, IL-5, or IL-13, in response to inhalant allergens like house dust mite (Wierenga *et al*, 1991). The overexpression of T helper 2 type cytokines may induce B cell IgE class switch in AD patients (Grewe *et al*, 1998). We recently found that KC inhibited anti-CD3 plus anti-CD28-induced IL-4 and IL-5 production in human T cells by inhibiting the activity of adenylate cyclase (AC), which synthesizes cAMP (Kanda *et al*, 2001). This indicates that KC may indirectly suppress B cell IgE class switch by inhibiting the production of cytokines inducing this switch. In this study, we examined if KC may directly suppress IL-4 plus anti-CD40-induced IgE class switching in preswitch surface IgE- B cells from patients with AD. We further analyzed if KC may inhibit the IL-4 plus anti-CD40-induced germline or mature ϵ transcripts and ϵ germline promoter activation. The involvement of AC and cAMP signal in the effects of KC was also investigated.

MATERIALS AND METHODS

Patients with AD and normal donors Peripheral blood was taken from patients with mild to severe AD [five men and five women, age 26.4 ± 3.7 y (mean $\pm$ SEM)], diagnosed according to the criteria by Hanifin and Rajka (1980). The patients' serum total IgE value was 2638 ± 501 U per ml (mean $\pm$ SEM). Seven out of the 10 patients were treated with topical corticosteroids of mild to strong rank and no patients were receiving systemic corticosteroids, antiallergic or antihistaminic drugs, or desensitization immunotherapy. Blood was also taken from nonatopic healthy volunteers [five men and four women, age 27.1 ± 4.0 y (mean $\pm$ SEM)]. The serum total IgE value in the normal donors was 78 ± 12 U per ml. At the time of the investigation, the AD patients and normal donors did not have active infection by fungi, viruses, or bacteria, and were not receiving topical or systemic antimycotic medication. All the patients and controls were informed of the objectives and methods of this study, and consented to participate.

Reagents Recombinant human IL-4 was purchased from Genzyme (Cambridge, MA). Anti-CD40 monoclonal antibody was from Pharmingen (San Diego, CA). Dibutyl cAMP (Bt_2cAMP), dibutyl 3',5'-cyclic guanosine monophosphate (Bt_2cGMP), N-[2-(p-bromocinnamyl)aminoethyl]-5-isoquinolinesulfonamide (H-89), 1-oleoyl-2-acetyl-sn-glycerol (OAG), and 3-isobutyl-1-methylxanthine were obtained from Calbiochem (La Jolla, CA). KC was from Janssen Pharmaceutica (Beerse, Belgium). These agents were dissolved in dimethylsulfoxide as 100 mM stock solution and were kept in the dark until used.

Human surface IgE- B cells and B cell lines Peripheral blood mononuclear cells from AD patients and normal donors were isolated by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden), as described previously (Boyum, 1968), and were allowed to adhere to plastic dishes for 1 h at 37°C. From the nonadherent cells, CD56- cells were isolated by negative selection using immunomagnetic beads (Dynal, Great Neck, NY), as described previously (Gee *et al*, 1987), and were incubated with neuraminidase-treated sheep erythrocytes as described previously (Farrant *et al*, 1985). From the nonrosette-forming cells, CD14 and CD3 cells were isolated by immunomagnetic negative selection and were used as B cells. This B cell population was > 97% CD19+, and the contamination of CD14+, CD3+, or CD56+ cells was < 1%. The purified B cells were incubated for 30–45 min on ice with fluorescein-isothiocyanate-labeled goat antihuman IgE antibody (Cappel, West Chester, PA). Surface IgE- B cells were sorted on a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA) with single-color fluorescence as described previously (Jabara *et al*, 1990).

Epstein-Barr-virus-negative, surface IgM+ human Burkitt lymphoma Ramos cells and IgE-producing human myeloma U266 cells were maintained in RPMI 1640 (Gibco/BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 5×10^{-5} M of 2-mercaptoethanol, 100 U per ml penicillin, 100 μ g per ml streptomycin, and 2 mM of L-glutamine.

Measurement of IgE or total Ig secretion One $\times 10^6$ of sorted surface IgE- B cells were preincubated with or without KC at indicated concentrations for 30 min, and then incubated with 200 U per ml IL-4

and 1 μ g per ml anti-CD40 in the presence or absence of KC for another 14 d in 1 ml per well of RPMI 1640 supplemented with 10% fetal bovine serum in 24-well plates at 37°C in an atmosphere of 5% CO₂. The activity of IgE or total Ig in the culture supernatants was measured by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (Punt *et al*, 1994; Coqueret *et al*, 1997). The sensitivity of the assay for IgE or total Ig was 0.3 ng per ml or 1 ng per ml, respectively.

Proliferation assays Surface IgE- B cells were cultured as above in triplicate in 96-well plates at a density of 5×10^4 cells per well in a final volume of 200 μ l for 5 d. 1 μ Ci of ³H-thymidine (Amersham, Arlington Heights, IL) was added to each well during the last 16 h of culture, and the cells were harvested and ³H-thymidine uptake was measured by liquid scintillation.

Northern blotting Sorted surface IgE- B cells were incubated under the conditions indicated, and total cellular RNA was extracted by the guanidium isothiocyanate method (Ultraspec, Houston, TX). Aliquots of RNA 2 μ g were electrophoresed in a 1% agarose gel and transferred to nitrocellulose membranes. Blots were hybridized with ³²P-labeled probe that was a BamHI fragment of genomic DNA containing whole C ϵ exons (Gauchat *et al*, 1990). The membranes were exposed to X-ray films (Hyperfilm MP; Amersham) for 17 h at -80°C. Autoradiograms were scanned using a Molecular Dynamics computing densitometer (model 300 A; Molecular Devices, Menlo Park, CA). For standardizing the amount of applied RNA, the hybridized filter was stripped and rehybridized to ³²P-labeled probe that was a HindIII fragment of genomic DNA encoding human β -actin.

Plasmids, transfection, and luciferase assays A fragment of the human ϵ germline promoter (-214/+36 relative to the transcriptional start site) was amplified by polymerase chain reaction as described previously (Ichiki *et al*, 1993; Ikizawa and Yanagihara, 2000). The amplified products were digested with BamHI and HindIII and inserted into the promoterless firefly luciferase vector, pGL3 basic (Promega, Madison, WI). Constructs -155/+36 and -109/+36 were obtained by cutting the above vector by TaqI and SmaI, respectively, and re-ligating. Construct -171/+36 was obtained by polymerase chain reaction using upstream primer carrying a XhoI site and was inserted into the basic vector as XhoI/HindIII fragment as described previously (Albrecht *et al*, 1994). The deletion was confirmed by sequence analysis. p4 $\times$ C/EBP-SV-luc, p4 $\times$ Stat6-SV-luc, p4 $\times$ NF- κ B-SV-luc, p3 $\times$ C/EBP/Stat6-SV-luc, and p3 $\times$ Stat6/NF- κ B-SV-luc were constructed by inserting four copies of the CCAAT enhancer binding protein (C/EBP) sequences (5'-GCTGTTGCTCAATCGAC-3'), Stat6 sequences (5'-AATCGACTTCCCAAGAACAG-3'), or nuclear factor κ B (NF- κ B) sequences (5'-AAGGGAACCTTCCAA-3'), or three copies of the sequences containing C/EBP and Stat6 elements on germline ϵ promoter (-167/-138) or of the sequences containing Stat6 and NF- κ B elements on germline ϵ promoter (-155/-115), respectively, in front of minimal SV40 promoter upstream of firefly luciferase reporter as described previously (Delphin and Stavnezer, 1995; Pesu *et al*, 2000). 1×10^7 of B lymphoma Ramos cells were transiently transfected with 10 μ g firefly luciferase construct and 6 μ g control plasmid Rous sarcoma virus- β -galactosidase by treatment with 0.5 mg per ml DEAE-dextran (Pharmacia, Uppsala, Sweden) for 30 min at 37°C. Each transfection was performed in triplicate. After transfection, the cells were split into aliquots and cultured in the presence or absence of IL-4 (200 U per ml) and anti-CD40 (1 μ g per ml). After 48 h, cell extracts were prepared, and luciferase activities were quantified using the luciferase assay system (Promega) according to the manufacturer's instruction. The same cell extracts were assayed for β -galactosidase activity using chemiluminescent Galacto-Light kit (Tropix, Bedford, MA). All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbach, Germany). The results obtained in each transfection were normalized for β -galactosidase activity and expressed as relative luciferase activity.

Measurement of cAMP amount B cells were cultured under the indicated conditions, and were harvested and lysed with ethanol. The cell lysates were centrifuged and the supernatants were dried under vacuum. The dried samples were dissolved in acetate buffer (pH 5.8), and cellular cAMP contents were measured with an ELISA kit (Amersham) according to the manufacturer's instruction. The sensitivity of the assay was 12 fmol per assay well. The cellular cAMP level was presented as pmol per 10^6 cells.

Measurement of cyclic nucleotide phosphodiesterase (PDE) activity B cells were cultured under the indicated conditions, and were lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM

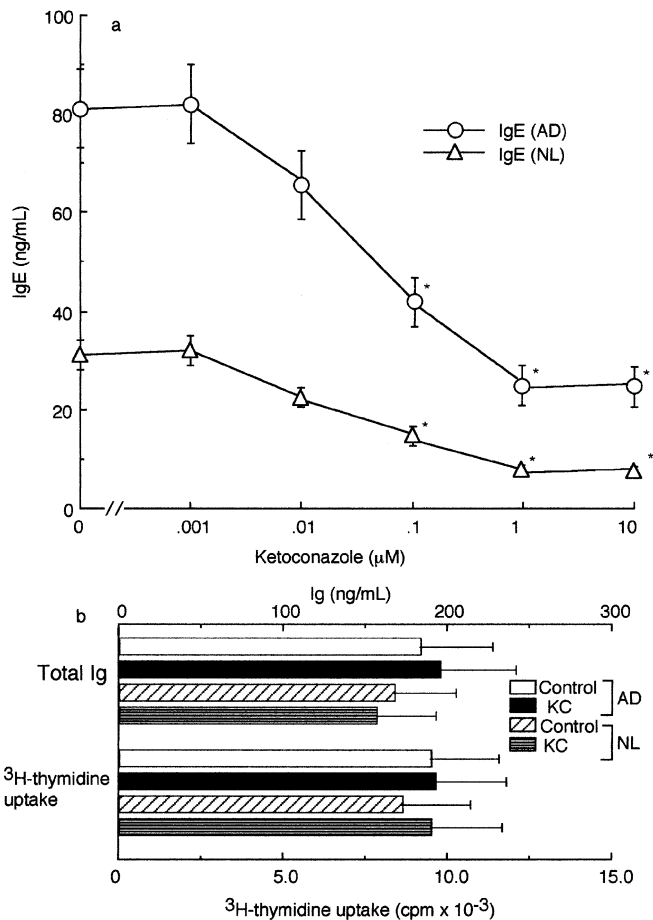


Figure 1. The effects of KC on IL-4 plus anti-CD40-induced IgE or total Ig secretion and proliferation in surface IgE- B cells from AD patients and normal donors. (a) The surface IgE- B cells from an AD patient or a normal donor were preincubated for 30 min with medium alone or with medium containing KC at indicated doses, and then incubated with 200 U per ml IL-4 plus 1 μg per ml anti-CD40 in the presence or absence of KC for 14 d. The culture supernatants were assayed for IgE by ELISA. Values are the mean $\pm$ SD of triplicate cultures. * p < 0.05 versus control cultures with IL-4 plus anti-CD40 without KC, by one-way analysis of variance with Dunnett's multiple comparison test. The data shown in the figure are representative of five separate experiments using surface IgE- B cells from five different AD patients and five different normal donors. (b) The surface IgE- B cells were cultured as described above with IL-4 plus anti-CD40 in the presence or absence of KC 1 μM, and total Ig secretion was assessed after 14 d whereas ³H-thymidine uptake was analyzed after 5 d. The data are mean $\pm$ SEM (n = 10 for AD patients; n = 9 for normal donors). The mean $\pm$ SEM of background values with medium alone in AD patients versus normal donors was as follows: total Ig, 16.2 $\pm$ 4.8 ng per ml versus 15.8 $\pm$ 4.9 ng per ml; 1023 $\pm$ 302 cpm versus 1131 $\pm$ 345 cpm, respectively. There were no significant differences between AD patients and normal donors in total Ig secretion or ³H-thymidine uptake of background and of culture with IL-4 plus anti-CD40 alone (p > 0.1 by Student's t test).

ethylenediamine tetraacetic acid, 1 μg per ml aprotinin, 1 μg per ml pepstatin, 1 μg per ml leupeptin, 15 mM benzamidin, and 3.75 mM β-mercaptoethanol. PDE activity of the cell lysate was assayed as described previously (Robicsek *et al*, 1991) using 1 mM [2,8-³H] cAMP (30 Ci per mmol) (Amersham) as a substrate. The assays were performed in 40 mM Tris-HCl (final pH 8.0), 10 mM MgCl₂ at 37°C for 10 min, and PDE activity was presented as pmol cAMP hydrolyzed per min per mg protein.

Measurement of AC activity The B cell lysate was centrifuged at 23,600g for 10 min. The pellet was used as a particulate fraction for AC assays as described previously (Salomon *et al*, 1974; Choi *et al*, 1992).

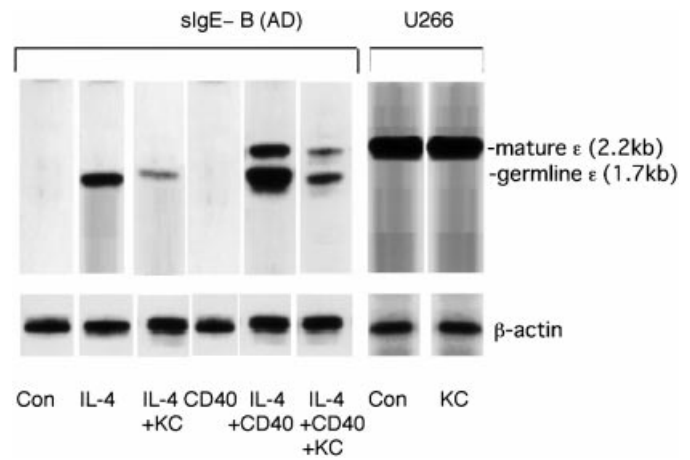


Figure 2. The effects of KC on IL-4 and/or anti-CD40-induced germline and mature ε transcripts in surface IgE- B cells from an AD patient. The surface IgE- B cells from an AD patient were preincubated for 30 min with medium alone or with medium containing KC at 1 μM, and then incubated with 200 U per ml IL-4 and/or 1 μg per ml anti-CD40 in the presence or absence of KC for 8 d. RNA was isolated and hybridized with a probe complementary to Cε or β-actin. The data shown in the figure are representative of five separate experiments using B cells from five different AD patients. RNA was also isolated from IgE-producing myeloma U266 cells after 8 d of culture in the presence or absence of KC 1 μM and was hybridized with the same probes as a control.

The AC activity of the particulate fraction was measured at 37°C for 10 min in 20 mM Tris-HCl (pH 7.4), 1 mM [α-³²P] ATP (30 Ci per mmol) (Amersham), 1 mM [γ-³²P] cAMP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM MgCl₂, 0.2 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 20 mM creatine phosphate, and 100 units per ml creatine phosphokinase. AC activity was presented as pmol cAMP formed per min per mg protein.

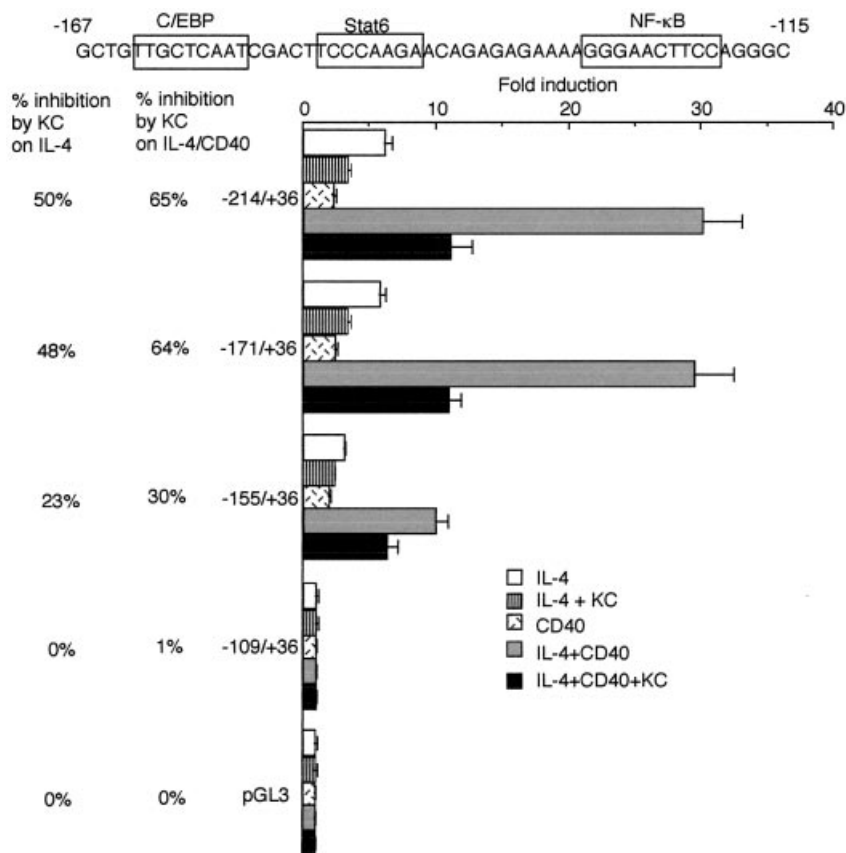
Assays of cAMP-dependent protein kinase (protein kinase A, PKA) The B cell lysate was assayed for the activity of PKA using an ELISA kit (Medical and Biological Laboratories, Nagoya, Japan) by examining the phosphorylation of plate-bound peptide substrate in the presence or absence of 2 μM cAMP for 10 min at room temperature. The plates were sequentially incubated with biotinylated antibody against the phosphorylated substrate, peroxidase-conjugated streptavidin, and o-phenylenediamine, and then the reaction was stopped, and optical density at 492 nm was read. The PKA activity was expressed as an activity ratio, which is defined as the optical density in the absence of exogenous cAMP divided by that in the presence of cAMP.

Statistical analyses Student's t test was used for the comparison of mean amounts in AD and normal groups. One-way analysis of variance with Dunnett's multiple comparison test was used for the data in **Fig 1(a)**. Paired t test was used for the data in **Fig 1(b)**. One-way analysis of variance with Scheffe's multiple comparison test was used for the data in **Figs 5 and 6**. Spearman's correlation coefficient was determined for the data in **Fig 7**. A value of p < 0.05 was considered significant.

RESULTS

The effects of KC on IL-4 plus anti-CD40-induced IgE secretion in surface IgE- B cells from AD patients and normal donors First, the effects of KC were examined on IL-4 plus anti-CD40-induced secretion of IgE by surface IgE- B cells from AD patients and normal donors. The IL-4 plus anti-CD40-induced IgE secretion was significantly higher in AD patients (mean $\pm$ SEM 85.5 $\pm$ 9.8 ng per ml, n = 10) than that in normal donors (30.6 $\pm$ 4.6 ng per ml, n = 9; p < 0.01 by Student's t test). The enhanced IgE secretion in AD patients did not appear to be due to contamination of surface IgE+ B cells as no surface IgE+ cells were detectable in the sorted surface IgE- population upon

Figure 3. IL-4 and/or anti-CD40-induced activation and KC-induced inhibition of germline ϵ promoter in Ramos cells. Ramos cells were transiently transfected with luciferase reporter plasmids driven by serially 5'-deleted ϵ germline promoters together with β -galactosidase vector. The cells were split into aliquots and preincubated with KC (1 μ M) for 30 min, and then incubated with medium alone, IL-4 (200 U per ml), anti-CD40 (1 μ g per ml), or IL-4 plus anti-CD40, in the presence or absence of KC for 48 h. The cells were harvested, and luciferase activities of the cell lysates were assayed and standardized for β -galactosidase activities. The results are shown as fold induction above the relative luciferase activity in unstimulated cells incubated with medium alone. The data represent the mean $\pm$ SEM of three separate experiments. Percent inhibition by KC on IL-4- or IL-4 plus anti-CD40-induced promoter activation is depicted to the far left. The putative transcription factor binding sites in the critical region of the ϵ germline promoter are shown at the top.



reanalysis by flow cytometry (data not shown). Furthermore, medium alone, IL-4 alone, or anti-CD40 alone did not induce detectable IgE secretion in surface IgE⁺ B cells from either AD patients or normal donors. KC dose-dependently reduced the IL-4 plus anti-CD40-induced IgE secretion: 65.1% inhibition *versus* controls in AD patients and 75.2% inhibition in normal donors at 1 μ M (Fig 1a). Thus KC inhibited IL-4 plus anti-CD40-induced IgE secretion in AD patients' and normal donors' surface IgE⁺ B cells. As 1 μ M appeared to be the optimal concentration for the inhibitory effects of KC, this concentration was used in the further experiments. In parallel, we examined the effects of KC on total Ig secretion and proliferation. KC did not alter IL-4 plus anti-CD40-induced total Ig secretion or ³H-thymidine uptake in surface IgE⁺ B cells from either AD patients or normal donors (Fig 1b). Thus the inhibitory effect of KC on these cells was specific to IgE secretion. We next examined if KC may inhibit germline or mature ϵ transcripts.

The effects of KC on IL-4 plus anti-CD40-induced germline and mature ϵ transcripts in surface IgE⁺ B cells Northern blots using C ϵ probe revealed two ϵ mRNA species, 2.2 kb and 1.7 kb (Fig 2). The 2.2 kb ϵ mRNA was the same size as that from IgE-secreting myeloma U266, suggesting that the 2.2 kb mRNA may be mature productive ϵ transcript and the 1.7 kb mRNA may be the germline ϵ transcript, which was consistent with the previous studies (Gauchat *et al*, 1990). IL-4 alone induced germline C ϵ transcript whereas anti-CD40 alone did not, and IL-4 plus anti-CD40 resulted in further increased germline C ϵ transcript and also induced mature C ϵ transcript in surface IgE⁺ B cells from AD patients. KC reduced IL-4 or IL-4 plus anti-CD40-induced germline C ϵ mRNA level by 62% or by 67% *versus* controls, respectively, as examined by the intensity normalized for that of β -actin. KC also reduced IL-4 plus anti-CD40-induced mature C ϵ mRNA level by 66%. Similar results were obtained in surface IgE⁺ B cells from normal donors (data not shown). In contrast, KC did

not reduce mature ϵ mRNA level in U266 cells. Thus KC reduced the germline and mature ϵ mRNA levels in surface IgE⁺ B cells, but did not inhibit mature ϵ mRNA expression in myeloma cells, which have completed IgE class switch.

The effects of KC on IL-4 or IL-4 plus anti-CD40-induced activation of germline ϵ promoter We then assessed if KC may also inhibit germline ϵ promoter activity in Epstein-Barr-virus-negative Burkitt lymphoma Ramos cells secreting IgM but not IgE. Ramos cells were transiently transfected with the luciferase reporter construct driven by germline ϵ promoter (-214/+36), and subsequently stimulated with IL-4 and/or anti-CD40. The attempt to transfect peripheral blood B cells was unsuccessful (data not shown). As shown in the uppermost five columns of Fig 3, IL-4 alone increased ϵ germline promoter (-214/+36) activity, as examined by relative luciferase activity, and anti-CD40 further potentiated the IL-4-induced promoter activation though anti-CD40 alone only modestly increased the promoter activity. KC suppressed the germline ϵ promoter activation induced by IL-4 alone or together with anti-CD40. According to the percentage inhibition, the promoter activation by IL-4 alone may be the main target of KC-induced inhibition whereas additional inhibition of the combined response to IL-4 plus anti-CD40 was negligible. Thus KC appeared to inhibit germline or mature ϵ transcripts by mainly inhibiting IL-4-induced germline ϵ promoter activation, though it is also possible that KC may inhibit the switch recombination to IgE following germline ϵ transcription.

To determine the sequences on germline ϵ promoter involved in the KC-induced inhibition, we transfected Ramos cells with serially 5'-deleted promoters linked to luciferase reporter, and compared the effects of KC on promoter induction by IL-4 alone or together with anti-CD40. The activity of -171/+36 promoter was increased by IL-4, anti-CD40, or their combination to extents comparable to those in -214/+36 promoter, and the magnitude of the inhibition by KC on the inducibility was similar to that in

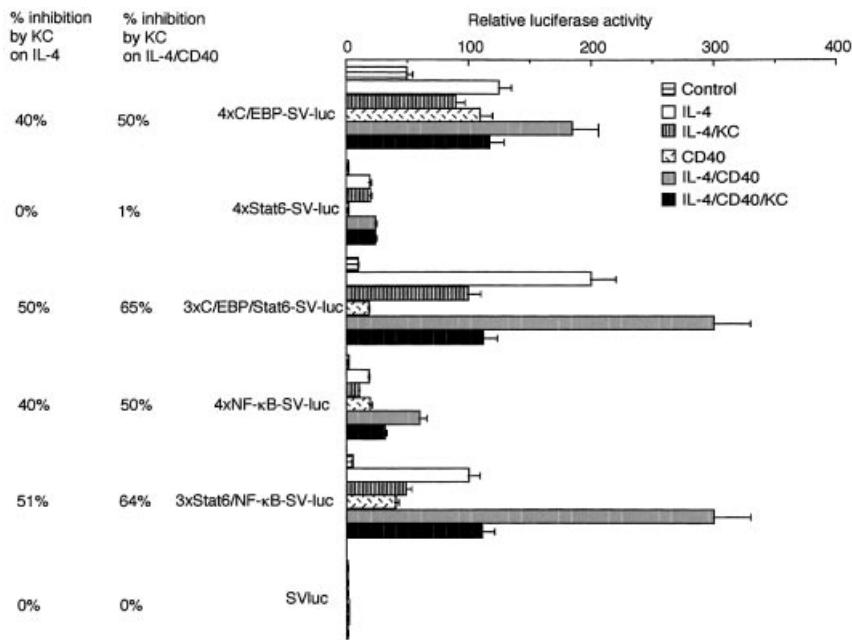


Figure 4. IL-4 and/or anti-CD40-induced activation and KC-induced inhibition on the enhancer activities of C/EBP, Stat6, NF-κB, or their composite elements. Ramos cells were transiently transfected with luciferase reporter plasmids driven by C/EBP, Stat6, NF-κB element, or composite elements of C/EBP/Stat6 or of Stat6/NF-κB, or enhancerless reporter plasmid together with β-galactosidase vector. The cells were split into aliquots and preincubated with KC (1 μM) for 30 min, and then incubated with medium alone (control), IL-4 (200 U per ml), anti-CD40 (1 μg per ml), or IL-4 plus anti-CD40 in the presence or absence of KC for 48 h. The cells were harvested, and luciferase activities of the cell lysates were assayed. The results are shown as relative luciferase activities normalized for β-galactosidase activities, and represent mean ± SEM of three separate experiments. Percent inhibition by KC on IL-4 or IL-4 plus anti-CD40-induced increase of enhancer activity is depicted to the far left.

–214/+36 promoter. After deletion of the –174/–155 region, a significant proportion (54% or 62%, respectively) of the inducibility by IL-4 or IL-4 plus anti-CD40 was lost, and KC-induced inhibition on the inducibility greatly decreased. A further deletion of –155/–109 completely abolished the residual inducibility and inhibition. These results suggest that the –174/–155 region may mainly confer the germline ε promoter induction by IL-4 or IL-4 plus anti-CD40 and may be required for KC-mediated inhibition of the induction. The –155/–109 region may also be involved in the inducibility and inhibition.

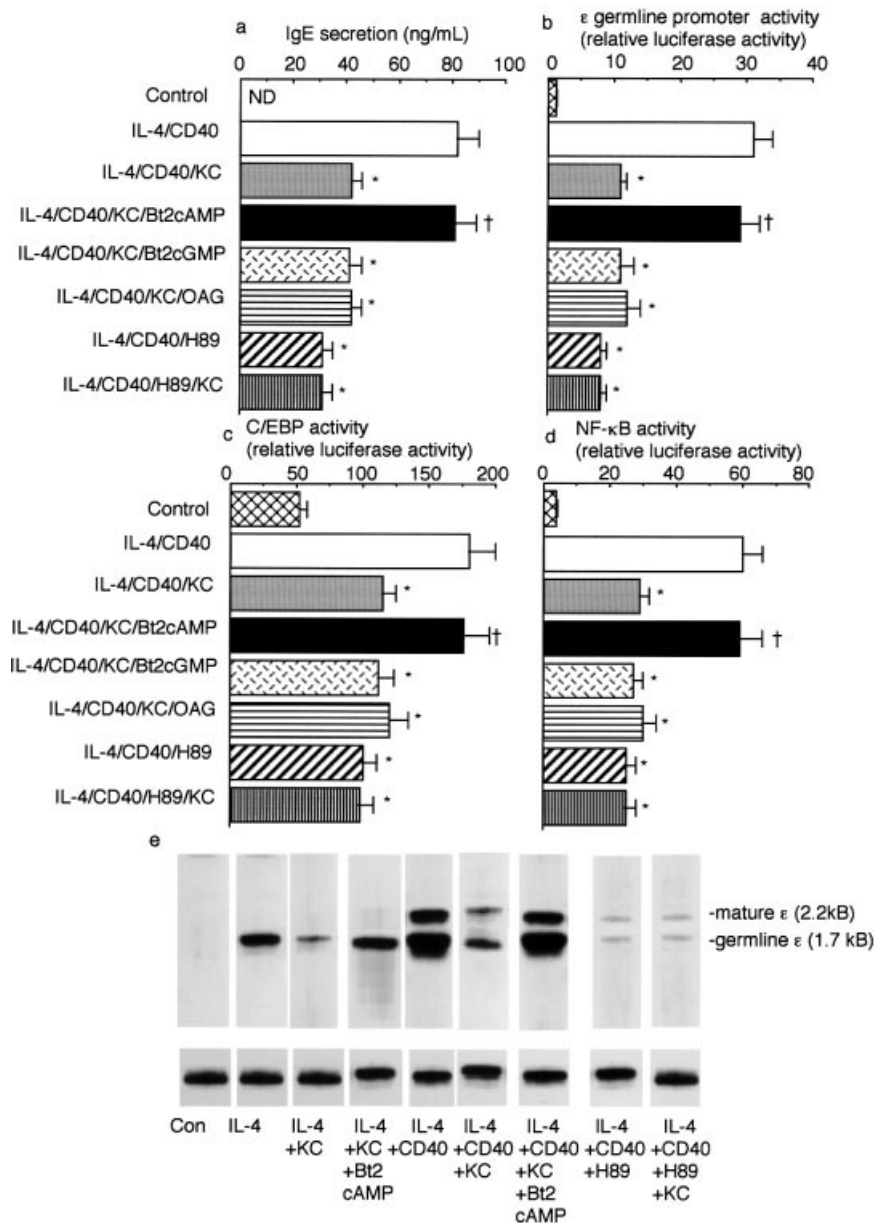
The –174/–155 region contains the binding site for transcription factor C/EBP (–163/–155), and the –155/–109 region contains Stat6 (–149/–142) and NF-κB (–129/–120) sites (Fig 3, top; Thienes *et al*, 1997). Thus these three elements in the region –167/–115 (Fig 3, top) may confer the promoter induction by IL-4 alone or together with anti-CD40, and may be required for the inhibition by KC. It is reported that C/EBP and Stat6 cooperate to activate germline ε transcription in response to IL-4 (Mikita *et al*, 1998; Pesu *et al*, 2000). Anti-CD40 and IL-4 enhance the transcriptional activity of NF-κB (Berberich *et al*, 1994; Yanagihara *et al*, 1996). The transcriptional cooperation of Stat6 and NF-κB is also reported (Stutz and Woistetschlager, 1999; Monticelli and Vercelli, 2001). We thus examined if KC may inhibit the IL-4 or IL-4 plus anti-CD40-induced enhancer activities C/EBP, Stat6, NF-κB, or composite elements C/EBP/Stat6, Stat6/NF-κB. IL-4 alone or together with anti-CD40 increased the enhancer activity of C/EBP, Stat6, and NF-κB, and composite elements of C/EBP/Stat6 and Stat6/NF-κB, and the increases were inhibited by KC except for IL-4-induced Stat6 enhancer activity (Fig 4). These results suggest that KC may inhibit the IL-4 or IL-4 plus anti-CD40-induced enhancer activities of C/EBP and NF-κB, and also the cooperativity of C/EBP and Stat6 or of Stat6 and NF-κB, though KC may not directly inhibit the enhancer activity of Stat6. The results also indicate that all the three elements C/EBP, Stat6, and NF-κB in the region –167/–115 may be involved in the IL-4 or IL-4 plus anti-CD40-induced germline ε promoter activation and in the inhibition by KC of the induction. According to the percentage inhibition, the enhancer activities induced by IL-4 alone appeared to be the main target of KC-induced inhibition.

CAMP-induced reversal from the KC-induced inhibition on B cell isotype switching to IgE We have recently found that KC suppresses anti-CD3/CD28-induced IL-4 and IL-5 production

in human T cells by suppressing cAMP signal (Kanda *et al*, 2001). We thus examined if the KC-induced inhibition of B cell IgE class switching may also involve cAMP, by using cAMP analog Bt₂cAMP, which activates cAMP-dependent PKA. Bt₂cAMP counteracted the KC-induced inhibition of IgE secretion (Fig 5a) and germline and mature ε transcripts (Fig 5e) in IL-4 plus anti-CD40-stimulated surface IgE⁺ B cells from AD patients and normal donors (data not shown). Bt₂cAMP also reversed the KC-induced inhibition of germline ε transcript by IL-4 alone (Fig 5e). Bt₂cAMP also counteracted the KC-induced inhibition of germline ε promoter activity (Fig 5b) and of C/EBP and NF-κB enhancer activities (Fig 5c, d) in IL-4 plus anti-CD40-stimulated Ramos cells. These results indicate that the cAMP/PKA-dependent signaling pathway may be involved in the KC-mediated inhibition of IgE class switching. On the other hand, cGMP analog Bt₂cGMP or PKC activator OAG did not counteract the KC-induced inhibition on IgE secretion, germline and mature ε transcripts, germline ε promoter activity, and C/EBP and NF-κB enhancer activities. The specific PKA inhibitor H-89 reduced IL-4 plus anti-CD40-induced IgE secretion by 71% (Fig 5a) and suppressed germline and mature ε transcripts (Fig 5e) in surface IgE⁺ B cells from AD patients. H-89 also reduced the IL-4 plus anti-CD40-induced increase of germline ε promoter activity by 75% (Fig 5b), of C/EBP enhancer activity by 60% (Fig 5c), and of NF-κB enhancer activity by 67% (Fig 5d) in Ramos cells. In contrast, H-89 did not inhibit Stat6 enhancer activity induced by IL-4 or IL-4 plus anti-CD40 (data not shown). These results suggest that the cAMP/PKA signaling pathway may be required for IL-4 plus anti-CD40-induced IgE secretion, germline and mature ε transcripts, germline ε promoter activity, and C/EBP and NF-κB enhancer activities, but not that of Stat6. In the presence of H-89, KC did not further suppress the IL-4 plus anti-CD40-induced IgE secretion, germline and mature ε transcripts, and germline ε promoter and C/EBP and NF-κB enhancer activities. This confirms that the cAMP/PKA pathway may be the main target for the KC-induced inhibition of IgE class switching. These results suggest that the KC-induced inhibition of IgE class switching may be attributable to inhibition of the cAMP/PKA signaling pathway. We then examined whether KC may alter cAMP level in IL-4 plus anti-CD40-stimulated surface IgE⁺ B cells.

The effects of KC on cAMP signal in IL-4 plus anti-CD40-stimulated surface IgE⁺ B cells At 10 min of incubation, IL-4 alone, anti-CD40 alone, or IL-4 plus anti-CD40 increased the

Figure 5. The reversal by cAMP analog Bt₂cAMP of KC-induced inhibition of IgE secretion, germline ϵ promoter activity, enhancer activities of C/EBP and NF- κ B, and germline and mature ϵ transcripts. (a) Surface IgE⁺ B cells from five different AD patients were preincubated for 30 min with medium alone or with Bt₂cAMP 100 μ M, Bt₂cGMP 100 μ M, OAG 100 ng per ml, or H-89 0.1 μ M in the presence or absence of KC 1 μ M, and then incubated with IL-4 200 U per ml plus anti-CD40 1 μ g per ml for 14 d in the presence or absence of the above-mentioned agents. IgE secretion was analyzed by ELISA. The results are shown as mean $\pm$ SEM ($n = 5$). ND, not detectable. (b–d) Ramos cells were transiently transfected with luciferase reporter plasmids driven by ϵ germline promoter (–171/+36) (b) or C/EBP element (c) or NF- κ B element (d) together with β -galactosidase vector. The cells were split into aliquots and preincubated with the indicated agents as above, and then incubated with IL-4 plus anti-CD40 in the presence or absence of the respective agents. After 48 h, the cells were harvested and luciferase activities of the cell lysates were assayed. The results are shown as relative luciferase activity normalized for β -galactosidase activity and represent the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ versus cultures with IL-4 plus anti-CD40 alone, and † $p < 0.05$ versus cultures with IL-4 plus anti-CD40 plus KC, by one-way analysis of variance with Scheffé's multiple comparison test. (e) Surface IgE⁺ B cells from an AD patient were incubated under the indicated conditions for 8 d. RNA was isolated and hybridized with a probe complementary to C ϵ or β -actin. The data shown in the figure are representative of five separate experiments using B cells from five different AD patients.



cAMP level in AD patients' surface IgE⁺ B cells 3.8-fold, 3.1-fold, and 6.1-fold of controls, respectively (**Fig 6a, upper half**). KC reduced the IL-4 plus anti-CD40-induced increase of cAMP by 80% in the patients' cells. IL-4 alone, anti-CD40 alone, or IL-4 plus anti-CD40 increased the cAMP level in normal donors' surface IgE⁺ B cells 1.8-fold, 1.5-fold, or 2.5-fold of controls, respectively, and KC reduced the IL-4 plus anti-CD40-induced increase of cAMP by 95% (**Fig 6a, lower half**). Though the basal cAMP level was not different between AD patients' (mean $\pm$ SEM 1.80 ± 0.51 pmol per 10^6 cells; $n = 10$) and normal donors' surface IgE⁺ B cells (2.01 ± 0.53 pmol per 10^6 cells; $n = 9$; $p > 0.10$ by Student's *t* test), the cAMP level in IL-4 plus anti-CD40-stimulated cells was higher in AD patients (11.2 ± 2.1 pmol per 10^6 cells) than in normal donors (5.0 ± 1.0 pmol per 10^6 cells, $p < 0.01$, by Student's *t* test). In both AD patients and normal donors, the cAMP level in IL-4 plus anti-CD40-stimulated surface IgE⁺ B cells correlated with the IL-4 plus anti-CD40-induced IgE secretion (**Fig 7**). These results indicate that the IL-4 plus anti-CD40-induced increase of cAMP may be responsible for IgE secretion. The IL-4 plus anti-CD40 enhanced PKA activity in AD patients' and normal donors' surface IgE⁺ B cells (**Fig 6b**). Though

the constitutive PKA activity in surface IgE⁺ B cells was not different between AD patients (0.20 ± 0.03) and normal donors (0.21 ± 0.04 ; $p > 0.1$, by Student's *t* test), PKA activity in IL-4 plus anti-CD40-stimulated cells was higher in AD patients (0.75 ± 0.08) than in normal donors (0.45 ± 0.05 ; $p < 0.01$ by Student's *t* test), which paralleled the results in cAMP level. KC suppressed the IL-4 plus anti-CD40-induced activation of PKA in AD patients' and normal donors' surface IgE⁺ B cells (**Fig 6b**) in parallel with the reduction of cAMP (**Fig 6a**). IL-4 alone, anti-CD40 alone, and IL-4 plus anti-CD40 increased AC activity of AD patients' surface IgE⁺ B cells 4.3-fold, 3.8-fold, and 7.0-fold of controls, respectively, and increased that of normal donors' surface IgE⁺ B cells 2.7-fold, 2.0-fold, and 4.0-fold of controls, respectively (**Fig 6c**). Though the basal AC activity of surface IgE⁺ B cells was not different between AD patients (30.5 ± 6.1 pmol per min per mg protein) and normal donors (31.3 ± 6.2 pmol per min per mg protein; $p > 0.10$ by Student's *t* test), the AC activity in IL-4 plus anti-CD40-stimulated cells was significantly higher in AD patients (210.3 ± 31.2 pmol per min per mg protein) than in normal donors (121.1 ± 19.1 pmol per min per mg protein; $p < 0.05$ by Student's *t* test). KC reduced the IL-4 plus anti-CD40-induced

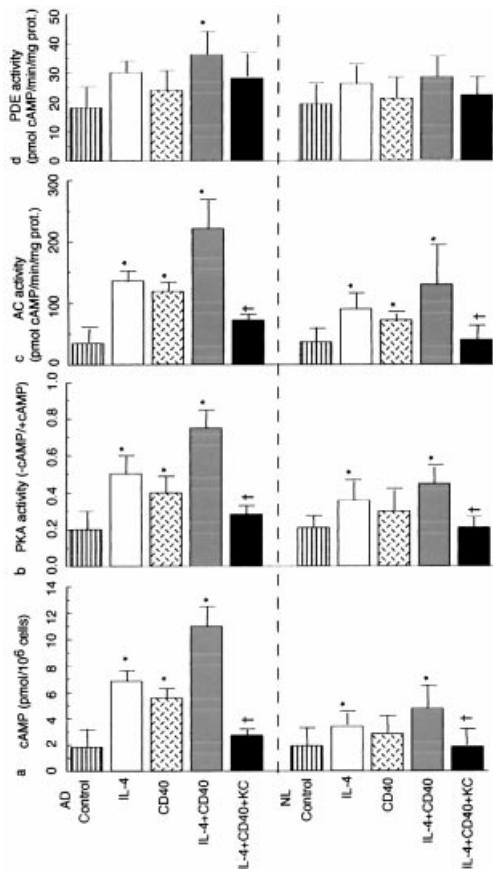


Figure 6. IL-4 and/or anti-CD40-induced increase and KC-induced reduction of the intracellular cAMP level (a), PKA (b), AC (c), and PDE (d) activities in surface IgE⁺ B cells from AD patients and normal donors. Surface IgE⁺ B cells from 10 AD patients and nine normal donors were preincubated with KC 1 μ M for 30 min, and then incubated with IL-4 (200 U per ml), anti-CD40 (1 μ g per ml), or IL-4 plus anti-CD40 in the presence or absence of KC for 10 min. Intracellular cAMP level, the activities of PKA and PDE in the cell lysates, or AC activity in the particulate fraction were analyzed as described in *Materials and Methods*. The mean $\pm$ SEM ($n = 10$) in AD patients is shown in the upper half of each figure and the mean $\pm$ SEM ($n = 9$) in normal donors is shown in the lower half. * $p < 0.05$ versus values with medium alone, and † $p < 0.05$ versus values with IL-4 plus anti-CD40 alone, by one-way analysis of variance with Scheffe's multiple comparison test.

increase of AC activity by 78% and by 94% of controls in AD patients' and normal donors' surface IgE⁺ B cells, respectively. As the upregulation of cAMP-hydrolyzing PDE activity also decreases cAMP level, we analyzed whether KC may enhance the PDE activity of IL-4 plus anti-CD40-stimulated surface IgE⁺ B cells. IL-4 plus anti-CD40 enhanced PDE activity of surface IgE⁺ B cells from AD patients or normal donors moderately, 1.9- or 1.7-fold of controls, respectively, 10 min after the stimuli, and the PDE activity was not enhanced but rather slightly reduced by KC (Fig 6d), indicating that the reduction of cAMP level by KC may not be caused by the enhancement of PDE activity. In Ramos cells, IL-4 plus anti-CD40 increased AC and PKA activities and cAMP level, and the increases were suppressed by KC, but their PDE activity was not enhanced by KC (data not shown). These results suggest that KC may suppress the IL-4 plus anti-CD40-induced activation of AC and thus may suppress the IL-4 plus anti-CD40-induced cAMP signal in AD patients' and normal donors' surface IgE⁺ B cells and Ramos cells.

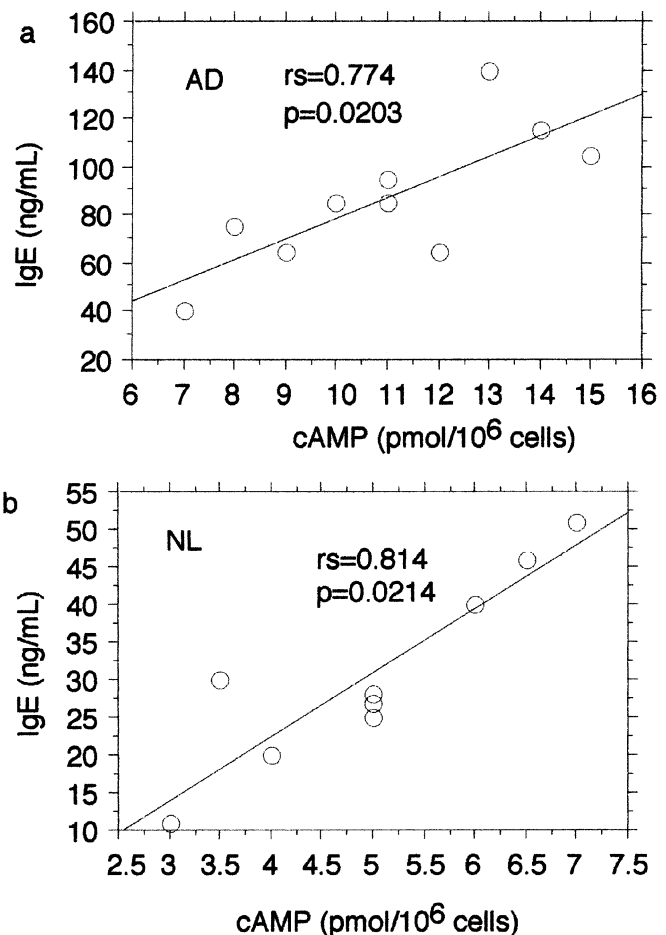


Figure 7. Correlation between cAMP level and IgE secretion in IL-4 plus anti-CD40-stimulated surface IgE⁺ B cells from AD patients (a) and normal donors (b). Surface IgE⁺ B cells from 10 AD patients and nine normal donors were incubated with IL-4 (200 U per ml) plus anti-CD40 (1 μ g per ml). The intracellular cAMP level was analyzed after 10 min whereas IgE secretion was assayed after 14 d. r_s = Spearman's correlation coefficient.

DISCUSSION

In this study, KC suppressed ϵ germline transcription by inhibiting AC and its product cAMP. KC also inhibits AC in human T cells (Kanda *et al*, 2001) and rat anterior pituitary cells (Stalla *et al*, 1988, 1989). It is known that IL-4 increases cAMP level in B cells (Finney *et al*, 1990). The binding of IL-4 to its receptor activates the receptor-associated *src* kinase *fyn*, which phosphorylates and activates phospholipase C γ 1 (Ikizawa *et al*, 1994). The activated phospholipase C generates inositol 1,4,5-triphosphate and diacylglycerol; the former induces intracellular Ca²⁺ signal and the latter activates PKC (Bihoreau *et al*, 1991). Ca²⁺ complexed with cytosolic calmodulin activates AC (Iyengar, 1993), and PKC activates AC by phosphorylation (Bihoreau *et al*, 1991; Iyengar, 1993; Jacobowitz and Iyengar, 1994; Watson *et al*, 1994). PKC-mediated phosphorylation also suppresses the inhibitory guanine-nucleotide-binding protein, which inactivates AC (Chen and Iyengar, 1993), and induces the stimulatory guanine-nucleotide-binding protein to activate AC (Bell *et al*, 1985). The ligation of CD40 on resting B cells also increases cAMP (Knox *et al*, 1993; Kato *et al*, 1994), possibly via phospholipase C (Ren *et al*, 1994). cAMP at least partially conferred the germline ϵ promoter activation by IL-4 and anti-CD40. KC-mediated inhibition of cAMP signal led to the suppression of IL-4 or IL-4 plus anti-CD40-induced ϵ germline transcription. KC did not inhibit mature

ϵ transcript in U266 myeloma cells, however, indicating that cAMP may not be necessary for mature ϵ transcript in cells that have completed IgE class switch. Furthermore KC did not inhibit IL-4 plus anti-CD40-induced total Ig secretion or proliferation even in surface IgE⁺ B cells. This indicates that cAMP may not be responsible for total Ig secretion or proliferation in these cells.

The cAMP-activated PKA phosphorylates cAMP-responsive element binding protein (CREB) and promotes its transcriptional activity through cAMP-responsive element (CRE) on various genes (Gonzalez and Montminy, 1989). IL-4 plus anti-CD40 induced the CRE-dependent OCA-B gene expression in murine B cells (Stevens *et al*, 2000). cAMP also promotes the transcriptional activity of C/EBP β and NF- κ B (Shirakawa *et al*, 1989; Bomsztyk *et al*, 1990; Motomura *et al*, 1998; Park *et al*, 1999; Pohnke *et al*, 1999); PKA induces the synthesis of C/EBP β via CRE on C/EBP β promoter (Berrier *et al*, 1998; Niehof *et al*, 2001), and also promotes the nuclear translocation of C/EBP β (Metz and Ziff, 1991; Chinery *et al*, 1997). PKA phosphorylates cytosolic I- κ B associated with NF- κ B, which induces the dissociation and nuclear translocation of the free, activated form of NF- κ B (Shirakawa and Mizel, 1989), and thus induces NF- κ B-dependent gene expression in B cells (Lapointe *et al*, 1996). Though the IL-4 plus anti-CD40-responsive region on the germline ϵ promoter does not contain consensus CRE, this region contains the binding sites for C/EBP, Stat6, and NF- κ B (Delphin and Stavnezer, 1995; Thienes *et al*, 1997). IL-4- or IL-4 plus anti-CD40-induced transcription through C/EBP and NF- κ B was at least partially mediated by cAMP. On the other hand, IL-4-induced activation of Stat6 may not require cAMP signal. The previous studies reported that the activity of Stat6 may be regulated by serine/threonine kinase different from PKA or PKC (Pesu *et al*, 2000). The transcriptional activators binding to C/EBP, Stat6, and NF- κ B sites interact with one another on ϵ germline promoter and thus synergistically enhance the transcription (Delphin and Stavnezer, 1995; Iciek *et al*, 1997; Mikita *et al*, 1998; Monticelli and Vercelli, 2001). cAMP may indirectly promote the cooperation of Stat6 with C/EBP or with NF- κ B in the transcription by enhancing the activity of C/EBP and NF- κ B. Such transcriptional cooperation may be indirectly inhibited by KC via suppression of cAMP.

It is reported that the overexpression of C/EBP β promotes the transcription through the composite element C/EBP/Stat6 on human germline ϵ promoter (Shen and Stavnezer, 2001), and recombinant C/EBP β can bind to the putative C/EBP element on this promoter (Mikita *et al*, 1998). The binding of endogenous C/EBP family proteins has not been reported, however. Thus the other cAMP-inducible transcription factor(s) may bind to this element and confer the transcription. As activating transcription factor/CREB family proteins can bind to C/EBP element (Fawcett *et al*, 1999; Zhou and Finch, 1999), these may bind to the putative C/EBP site on human germline ϵ promoter. This possibility should be further examined.

In murine germline ϵ promoter, the sequence (5'-TTAGTCAAC-3', -120/-112 relative to the transcriptional start site) upstream of Stat6 site is different from the human sequence, and this element is bound by activator protein 1 (AP-1) family proteins, and is thus redefined as AP-1 site (Shen and Stavnezer, 2001). AP-1 proteins transactivated murine germline ϵ promoter but did not transactivate human promoter (Shen and Stavnezer, 2001). When Ramos cells were transfected with luciferase reporter plasmids containing tandem repeats of AP-1 (5'-TGCCTTAGTCAAC-3', -124/-112) or composite element AP-1/Stat6 (5'-TGCCTTAGTCAACTTCCCAAGAAGACAG-3', -124/-99) from mouse germline ϵ promoter, IL-4 plus anti-CD40-induced enhancer activities of these elements were not inhibited by KC (our unpublished observation). This indicates that cAMP may not be responsible for the AP-1-dependent transcription induced by IL-4 plus anti-CD40. Though cAMP induces the expression of AP-1 family c-Fos (Ahn *et al*, 1998), cAMP-independent protein kinase(s), such as stress-activated protein kinases (Berberich *et al*, 1996), may regulate the AP-1-mediated transcription. Thus cAMP

may differently regulate human *versus* murine germline ϵ transcription through the element upstream of Stat6 site, possibly due to the different DNA-binding specificities. On the other hand, the sequence of NF- κ B site (-90/-81) downstream of Stat6 in mouse germline ϵ promoter is identical with that in human promoter. KC inhibited the IL-4 plus anti-CD40-induced enhancer activities of NF- κ B (-92/-79) and composite Stat6/NF- κ B (-112/-76) elements from murine germline ϵ promoter, as examined by reporter assays using Ramos cells (our unpublished observation). Thus cAMP may be involved in NF- κ B-dependent transcription on murine promoter, similarly to that on human promoter.

There may possibly be another mechanism for cAMP-induced germline ϵ transcription; PKA may inhibit the activity of transcriptional repressor. PKA-mediated phosphorylation may suppress the binding of putative repressor(s) to the undefined negative regulatory element on the IL-4 plus anti-CD40-inducible region, and this may promote the germline ϵ transcription. This mechanism is proposed for IL-4-induced CD25 expression in human B cell lines (Mckay *et al*, 2000). cAMP may also promote the switch recombination that juxtaposes C ϵ to V(D)J genes, following germline ϵ transcription. Previous studies reported that cAMP-elevating agents promoted the expression of recombination-activating genes RAG1 and RAG2 (Menetski and Gellert, 1990; Casilas *et al*, 1995). KC may counteract the cAMP-induced inhibition of transcriptional repressor and/or class switch recombination. These possibilities should be further examined.

IL-4 plus anti-CD40-induced cAMP signal in surface IgE⁺ B cells was greater in AD patients than in normal donors (**Fig 6**), and this may contribute to the enhanced IgE secretion in the former. This indicates that AC in surface IgE⁺ B cells from AD patients may be more sensitive to IL-4 or anti-CD40-induced signals such as phospholipase C, Ca²⁺, and/or PKC, compared to normal donors. This may possibly be due to the difference in AC isoforms between AD patients and normal donors. PKC stimulates AC types 2 and 7, and Ca²⁺/calmodulin stimulates AC types 1 and 3, whereas Ca²⁺ inhibits AC types 5 and 6 (Iyengar, 1993). Thus the amounts of AC types 2 and 7 and/or 1 and 3 may be increased and/or those of AC types 5 and 6 may be reduced in AD patients' surface IgE⁺ B cells. Another possible reason is that IL-4 or anti-CD40 may more potently activate phospholipase C in AD patients' surface IgE⁺ B cells, which may lead to the higher levels of PKC activation and/or Ca²⁺ signal, and may result in the greater level of AC activation. Alternatively, some surface IgE⁺ B cells in AD patients may have already started IgE class switch *in vivo*, and AC of these cells may have higher responsiveness to IL-4 or anti-CD40. These possibilities should be further examined.

The stimulation of CD3 activates AC via phospholipase C in T cells (Bihoreau *et al*, 1991). In our previous study, anti-CD3/CD28-induced cAMP signal was greater in AD patients' T cells compared to normal donors, and this correlated with the enhanced IL-4 production in the former (Kanda *et al*, 2001). Thus in AD patients the responsiveness of AC appears to be enhanced in both surface IgE⁺ B cells and T cells, which may lead to the high potentiality for IgE class switch in the former and to the increased production of IgE-class-switch-inducing IL-4 in the latter. The enhanced responsiveness of AC in both cell types may thus totally induce the abnormally enhanced IgE synthesis in AD patients.

Our results indicate that KC may prevent IgE class switch in surface IgE⁺ B cells and thus prevent the initiation of IgE-mediated allergic reactions. The responsiveness to KC may vary with B cell activation status and/or B cell sources, however. Knox *et al* (1993) reported that anti-CD40 decreased cAMP level in human surface CD39⁺ IgD⁺ B cells from tonsillar germinal centers though it increased cAMP in high density resting B cells. Thus cAMP signal may be distinctly regulated by anti-CD40 or IL-4 in different B cell subsets, and thus its contribution to IgE class switch may differ with the different subsets. We should further examine the involvement of cAMP in IgE class switch and the effects of KC on the switch in different B cell subsets. We should also examine if KC may suppress

B cell IgE class switch induced by stimuli other than IL-4 or anti-CD40. In particular IL-13 increases intracellular cAMP as well as IL-4 (Sozzani *et al*, 1995). We are now studying the effects of KC on IL-13 plus anti-CD40-induced IgE class switching in human surface IgE⁺ B cells.

REFERENCES

- Agut J, Palacin C, Salgado J, Casas E, Sacristan A, Ortiz JA: Direct membrane-damaging effect of sertaconazole on *Candida albicans* as a mechanism of its fungicidal activity. *Arzneimittelforschung* 42:721-724, 1992
- Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C: A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of *c-Fos*. *Mol Cell Biol* 18:967-977, 1998
- Albrecht B, Peiritsch S, Woitschlager M: A bifunctional control element in the human IgE germline promoter involved in repression and IL-4 activation. *Int Immunol* 6:1143-1151, 1994
- Back O, Scheynius A, Hohnsson SG: Ketoconazole in atopic dermatitis: therapeutic response is correlated with decrease in serum IgE. *Arch Dermatol Res* 287:448-451, 1995
- Baroni A, Ruocco V, de Paolis P, Cicatiello L, Esumi H, Tufano MA: Ketoconazole inhibits lipopolysaccharide-induced activation of the nitric oxide synthase gene in the murine macrophage cell line J774. *Arch Dermatol Res* 291:54-58, 1999
- Bell JD, Buxton IL, Brunton LL: Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. *J Biol Chem* 260:2625-2628, 1985
- Berberich I, Shu GL, Clark EA: Cross-linking CD40 on B cells rapidly activates nuclear factor- κ B. *J Immunol* 153:4357-4366, 1994
- Berberich I, Shu G, Siebelt J, Woodgett JR, Kyriakis JN, Clark EA: Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases. *EMBO J* 15:92-101, 1996
- Berrier A, Siu G, Calame K: Transcription of a minimal promoter from the NF-IL-6 gene is regulated by CREB/ATF and SP1 proteins in U937 promonocytic cells. *J Immunol* 161:2267-2275, 1998
- Bettens JR, Loots W, Somers Y, Coene MC, de Clerck F: Ketoconazole inhibits the biosynthesis of leukotrienes *in vitro* and *in vivo*. *Biochem Pharmacol* 35:883-891, 1986
- Bihoreau C, Heurtier A, Enjalbert A, Corvaia N, Bensussan A, Degos L, Kordon C: Activation of the CD3/T cell receptor (TcR) complex or of protein kinase C potentiate adenylyl cyclase stimulation in a tumoral T cell line: involvement of two distinct intracellular pathways. *Eur J Immunol* 21:2877-2882, 1991
- Bomsztyk K, Toivola B, Emery DW, Rooney JW, Dower SK, Rachle NA, Sibley CH: Role of cAMP in interleukin-1-induced κ light chain gene expression in murine B cell line. *J Biol Chem* 265:9413-9417, 1990
- Boyum A: Separation of leukocytes from peripheral blood and bone marrow. *Scand J Clin Laboratory Invest* 21 (Suppl. 97):77-89, 1968
- Broberg A, Faergemann J: Topical antimycotic treatment of atopic dermatitis in the head/neck area. A double-blind randomized study. *Acta Derm Venereol (Stockh)* 75:46-49, 1995
- Casillas AM, Thompson AD, Cheshier S, Hernandez S, Aguilera RJ: RAG-1 and RAG-2 gene expression and V(D)J recombinase activity are enhanced by protein phosphatase 1 and 2A inhibition in lymphocyte cell lines. *Mol Immunol* 32:167-175, 1995
- Chen J, Iyengar R: Inhibition of cloned adenylyl cyclases by mutant-activated G_i - α and specific suppression of type 2 adenylyl cyclase inhibition by phorbol ester treatment. *J Biol Chem* 268:12253-12256, 1993
- Chinery R, Brockman JA, Dransfield DT, Coffey RJ: Antioxidant-induced nuclear translocation of CCAAT/enhancer-binding protein β . *J Biol Chem* 272:30356-30361, 1997
- Choi EJ, Xia Z, Storm DR: Stimulation of the type III olfactory adenylyl cyclase by calcium and calmodulin. *Biochemistry* 31:6492-6498, 1992
- Clemmensen OJ, Hjorth N: Treatment of dermatitis of the head and neck with ketoconazole in patients with type I sensitivity to *Pityrosporum orbiculare*. *Semin Dermatol* 2:26-29, 1983
- Coqueret O, Boichot E, Lagente V: Selective type IV phosphodiesterase inhibitors prevent IL-4-induced IgE production by human peripheral blood mononuclear cells. *Clin Exp Allergy* 27:816-823, 1997
- Delphin S, Stavnezer J: Characterization of an interleukin 4 (IL-4) responsive region in the immunoglobulin heavy chain germline ϵ promoter: regulation by NF-IL-4, a C/EBP family member and NF- κ B/p50. *J Exp Med* 181:181-192, 1995
- Elewski BE: Mechanisms of action of systemic antifungal agents. *J Am Acad Dermatol* 28:S28-S34, 1993
- Farrant J, Bryant AE, Lever AML, Edwards AJ, Knight SC, Webster ADB: Defective low-density cells of dendritic morphology from the blood of patients with common variable hypogammaglobulinaemia: low immunoglobulin production on stimulation of normal B cells. *Clin Exp Immunol* 61:189-194, 1985
- Fawcett TW, Martindale JL, Guyton KZ, Hai T, Holbrook NJ: Complexes containing activating transcription factor (ATF)/cAMP-responsive element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate *Gadd153* expression during the stress response. *Biochem J* 339:135-141, 1999
- Finney M, Guy GR, Michell RH, Gordon J, Dugas B, Rigley KP, Callard RE: Interleukin 4 activates human B lymphocytes via transient inositol lipid hydrolysis and delayed cyclic adenosine monophosphate generation. *Eur J Immunol* 20:151-156, 1990
- Gauchat J-F, Leberman DA, Coffman RL, Gascan H, de Vries JE: Structure and expression of germline transcripts in human B cells induced by interleukin 4 to switch to IgE production. *J Exp Med* 172:463-473, 1990
- Gee AP, Lee C, Sleasman JW, Madden M, Ugelstad J, Barret DJ: T lymphocyte depletion of human peripheral blood and bone marrow using monoclonal antibodies and magnetic microspheres. *Bone Marrow Transplant* 2:155-163, 1987
- Gonzalez GA, Montminy MR: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59:675-680, 1989
- Grewe M, Bruijnzeel-Koomen CAFM, Schopf E, Thepen T, Langeveld-Wiltschut AG, Ruzicka T, Krutmann J: A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 19:359-361, 1998
- Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh) Suppl* 92:44-47, 1980
- Ichiki T, Takahashi W, Watanabe T: Regulation of the expression of human C ϵ germline transcript. *J Immunol* 150:5408-5417, 1993
- Iciek LA, Delphin SA, Stavnezer J: CD40 cross-linking induces Ig ϵ germline transcripts in B cells via activation of NF- κ B. Synergy with IL-4 induction. *J Immunol* 158:4769-4779, 1997
- Ikizawa K, Kajiura K, Koshio T, Yanagihara Y: Possible role of tyrosine kinase activity in interleukin 5-induced expression of germline C ϵ transcripts in a human Burkitt lymphoma B-cell line, DND39. *J Allergy Clin Immunol* 94:620-624, 1994
- Ikizawa K, Yanagihara Y: Possible involvement of Shc in IL-4-induced germline ϵ transcription in a human B cell line. *Biochem Biophys Res Commun* 268:54-59, 2000
- Iyengar R: Molecular and functional diversity of mammalian Gs-stimulated adenylyl cyclases. *FASEB J* 7:768-775, 1993
- Jabara HH, Schneider LC, Shapira SK, *et al*: Induction of germ-line and mature C ϵ transcripts in human B cells stimulated with rIL-4 and EBV. *J Immunol* 145:3468-3473, 1990
- Jacobowitz O, Iyengar R: Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. *Proc Natl Acad Sci USA* 91:10630-10634, 1994
- Kanda N, Enomoto U, Watanabe S: Anti-mycotics suppress interleukin-4 and interleukin-5 production in anti-CD3 plus anti-CD28-stimulated T cells from patients with atopic dermatitis. *J Invest Dermatol* 117:1635-1646, 2001
- Kato T, Kokuho T, Tamura T, Nariuchi H: Mechanisms of T cell contact-dependent B cell activation. *J Immunol* 152:2130-2138, 1994
- Knox KA, Johnson GD, Gordon J: Distribution of cAMP in secondary follicles and its expression in B cell apoptosis and CD40-mediated survival. *Int Immunol* 5:1085-1091, 1993
- Lapointe R, Lemieux R, Olivier M, Darveau A: Tyrosine kinase and cAMP-dependent protein kinase activities in CD40-activated human B lymphocytes. *Eur J Immunol* 26:2376-2382, 1996
- Leung DYM: Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol* 96:302-318, 1995
- Mckay CE, Hewitt EL, Ozanne BW, Cushley W: A functional role for interleukin (IL)-4-driven cyclic AMP accumulation in human B lymphocytes. *Cytokine* 12:731-736, 2000
- Menetski JP, Gellert M: V(D)J recombination activity in lymphoid cell lines is increased by agents that elevate cAMP. *Proc Natl Acad Sci USA* 87:9324-9328, 1990
- Metz R, Ziff E: cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to trans-locate to the nucleus and induce *c-fos* transcription. *Genes Dev* 5:1754-1766, 1991
- Mikita T, Kurama M, Schindler U: Synergistic activation of the germline ϵ promoter mediated by Stat6 and C/EBP. *J Immunol* 161:1822-1828, 1998
- Monticelli S, Vercelli D: Molecular regulation of class switch recombination to IgE through ϵ germline transcription. *Allergy* 56:270-278, 2001
- Motomura T, Kawayama S, Takagi M, *et al*: Increased interleukin-6 production in mouse osteoblastic MC3T3-E1 cells expressing activating mutant of the stimulatory G protein. *J Bone Miner Res* 13:1084-1091, 1998
- Niehof M, Kubicka S, Zender L, Manns MP, Trautwein C: Autoregulation enables different pathways to control CCAAT/enhancer binding protein β (C/EBP β) transcription. *J Mol Biol* 309:855-868, 2001
- Park EA, Song S, Vinson C, Roesler WJ: Role of CCAAT/enhancer-binding protein β in the thyroid hormone and cAMP induction of phosphoenolpyruvate carboxykinase gene transcription. *J Biol Chem* 274:211-217, 1999
- Pesu M, Takaluoma K, Aittomaki S, Lagerstedt A, Saksela K, Koaven PE, Silvennoinen O: Interleukin-4-induced transcriptional activation by Stat6 involves multiple serine/threonine kinase pathways and serine phosphorylation of Stat6. *Blood* 95:494-502, 2000
- Pohnke Y, Kempf R, Gellersen B: CCAAT/enhancer-binding proteins are mediators in the protein kinase A-dependent activation of the decidual prolactin promoter. *J Biol Chem* 274:24808-24818, 1999
- Punt CJA, Barbutto JAM, Zhang H, Grimes WJ, Hatch KD, Hersh EM: Anti-tumor antibody produced by human tumor-infiltrating and peripheral blood B lymphocytes. *Cancer Immunol Immunother* 38:225-232, 1994
- Ren CL, Morio T, Fu SM, Geha RS: Signal transduction via CD40 involves activation of *lyn* kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase C γ 2. *J Exp Med* 179:673-680, 1994
- Robicsek SA, Blanchard DK, Djeu JY, Krzanowski JJ, Szentivany A, Polson JB: Multiple high-affinity cAMP-phosphodiesterase in human T-lymphocytes. *Biochem Pharmacol* 42:869-877, 1991
- Salomon Y, Londons D, Rodbell M: A highly sensitive adenylyl cyclase assay. *Anal Biochem* 58:541-548, 1974
- Shapira SK, Vercelli D, Jabara HH, Fu SN, Geha RS: Molecular analysis of the

- induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. *J Exp Med* 175:289–292, 1992
- Shen C-H, Stavnezer J: Activation of the mouse Ig germline ϵ promoter by IL-4 is dependent on AP-1 transcription factors. *J Immunol* 166:411–423, 2001
- Shirakawa F, Mizel SB: *In vitro* activation and nuclear translocation of NF- κ B catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol Cell Biol* 9:2424–2430, 1989
- Shirakawa F, Chedid M, Suttles J, Pollok BA, Mizel SB: Interleukin 1 and cyclic AMP induce κ immunoglobulin light-chain expression via activation of an NF- κ B like DNA-binding protein. *Mol Cell Biol* 9:959–964, 1989
- Sozzani P, Cambon C, Vita N, Sequelas M-H, Caput D, Ferrara P, Pipy B: Interleukin-13 inhibits protein kinase C-triggered respiratory burst in human monocytes. Role of calcium and cyclic AMP. *J Biol Chem* 270:5084–5088, 1995
- Stalla GK, Stalla J, Huber M, Loeffler JP, Holtt V, von Werder K, Muller OA: Ketoconazole inhibits corticotropic cell function *in vitro*. *Endocrinology* 122:618–623, 1988
- Stalla GK, Stalla J, von Werder K, Muller OA, Gerzer R, Holtt V, Jakobs KH: Nitroimidazole derivatives inhibit anterior pituitary cell function apparently by a direct effect on the catalytic subunit of the adenylate cyclase holoenzyme. *Endocrinology* 125:699–706, 1989
- Stevens S, Wang L, Roeder RG: Functional analysis of the OCA-B promoter. *J Immunol* 164:6372–6379, 2000
- Stutz AM, Woistetschlager M: Functional synergism of STAT6 with either NF- κ B or PU.1 to mediate IL-4-induced activation of IgE germline gene transcription. *J Immunol* 163:4383–4391, 1999
- Tengvall Linder M, Johansson C, Zargari A, *et al*: Detection of *Pityrosporum orbiculare* reactive T cells from skin and blood in atopic dermatitis and characterization of their cytokine profiles. *Clin Exp Allergy* 26:1286–1297, 1996
- Thienes CP, de Monte L, Monticelli S, Busslinger M, Gould HJ, Vercelli D: The transcription factor B cell-specific activator protein (BSAP) enhances both IL-4- and CD40-mediated activation of the human germline promoter. *J Immunol* 158:5874–5882, 1997
- Watson PA, Krupinski J, Kempinski AM, Frankenfield CD: Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. *J Biol Chem* 269:28893–28898, 1994
- Werfel S, Massey W, Lichtenstein LM, Bochner BS: Preferential recruitment of activated, memory T lymphocytes into skin chamber fluids during human cutaneous late-phase allergic reactions. *J Allergy Clin Immunol* 96:57–65, 1995
- Wierenga EA, Snoek M, Jansen HM, Bos JD, van Lier RA, Kapsenberg ML: Human atopen-specific types 1 and 2 helper cell clones. *J Immunol* 147:2942–2949, 1991
- Worm M, Henz BM: Molecular regulation of human IgE synthesis. *J Mol Med* 75:440–447, 1997
- Yanagihara Y, Basaki Y, Ikizawa K, Kajiura K, Koshio T, Akiyama K: Involvement of nuclear factor-B activation in IgE synthesis in human B cells. *J Allergy Clin Immunol* 98:S224–S229, 1996
- Zhou J, Finch PW: Identification of a novel transcriptional regulatory element within the promoter region of the keratinocyte growth factor gene that mediates inducibility to cyclic AMP. *Biochim Biophys Acta* 1446:71–81, 1999