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

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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 κB 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 κB, and those of composite elements of CCAAT/enhancer-binding protein/Stat6 or of Stat6/nuclear factor κB, 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 κB. J Invest Dermatol 119:590–599, 2002

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t 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, nonrelated 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 unarranged IgE ε-heavy chain constant region (Ce). This results in the production of untranslatable transcripts containing IgE ε-heavy chain germline exon (It) and Ce sequences and also makes the switch region accessible to the putative recombinase. In the process of switch recombination, It is deleted and the Ce 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
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 adenylyl 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 preswurf surface IgE+ B cells from patients with AD. We further analyzed if KC may inhibit the IL-4 plus anti-CD40-induced germine 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 ± SEM), diagnosed according to the criteria by Hanifin and Rajka (1980). The patients’ serum total IgE value was 2638 ± 501 U per ml (mean ± SEM). Seven out of the 10 patients were treated with topical corticosteroids of mild to strong rank and no patients from patients with mild to severe AD [five men and five women, age 3.7 y (mean ± SEM)]. Seven out of the 10 patients were treated with topical corticosteroids of mild to strong rank and no patients were receiving systemic corticosteroids, antihistergic or antihistamine drugs, or desensitization immunotherapy. Blood was also taken from nonatopic healthy volunteers [five men and four women, age 27.1 ± 4.0 y (mean ± 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), Dibutyryl cAMP (Bt2CAMP), dibutyryl 3’,5’-cyclic guanosine monophosphate (Bt2GMP), N-[2-[(p-bromo- cinnamyl)amino]ethyl-(N-cinnamyl)glycine (Bt2Gly), 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, CD19+ 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 cytomter (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 μg per ml streptomycin, and 2 mM of L-glutamine.

Measurement of IgE or total Ig secretion One × 10⁶ 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 μg 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 supernatant was measured by a sandwich enzymelinked immunosorbent assay (ELISA) previously described (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 described previously (Boyum et al., 1985). From the nonrosette-forming cells, 10–5 Mo of 2 × 10⁶ of sorted surface IgE+ B cells from patients with AD. We further analyzed if KC may inhibit the IL-4 plus anti-CD40-induced germine or mature ε transcripts and ε germline promoter activation. The involvement of AC and cAMP signal in the effects of KC was also investigated.

Northern blotting Sorted surface IgE+ B cells were incubated with the conditions indicated, and total cellular RNA was extracted previously (Albrecht et al., the guanidium isothiocyanate method (Ultraspec, Houston, TX). Aliquots of RNA 2 μg 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 Ce 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 scanning laser 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 promoteron firefly luciferase vector, pGL3 basic (Promega, Madison, WI). Constructs −155/+36 and −109/+36 were obtained by cutting the above vector by TaqI and Smal, 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 (Ichiki et al., 1994). The deletion was confirmed by sequence analysis. p4 × C/EBP-SV-luc, p4 × Stat6-SV-luc, p4 × NF-KB-SV-luc, p3 × C/EBP/Stat6-SV-luc, and p3 × Stat6/NF-KB-SV-luc were constructed by inserting four copies of the CCAAT enhancer binding protein (C/EBP) sequences (5’-GCGTGCTGCTCAATGCAC-3’), Stat6 sequences (5’-AATCGACT- TCCCAAGAACAAGC-3’), or nuclear factor κB (NF-κB) sequences (5’-AAGGAATCTCCCAA-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-κB 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 × 10⁴ of B lymphoma Ramos cells were transiently transfected with 10 μg fiterly luciferase construct and 6 μg 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 μg 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 Galecto-Light kit (Tropix, Bedford, MA). All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbad, 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 lyzed 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 10⁶ cells.

Measurement of cyclic nucleotide phosphodiesterase (PDE) activity B cells were cultured under the indicated conditions, and were lyzed in buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM
ethylenediamine tetraacetic acid, 1 µg per ml aprotinin, 1 µg per ml pepstatin, 1 µg per ml leupeptin, 15 mM benzamidine, and 3.75 mM β-mercaptoethanol. PDE activity of the cell lysate was assayed as described previously (Robicek 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). 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 [2,8-3H] cAMP (30 Ci per mmol) (Amersham), 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 106 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 Dunnet’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 ± SEM 85.5 ± 9.8 ng per ml, n = 10) than that in normal donors (30.6 ± 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 μM) for 30 min, and then incubated with medium alone, 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 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 ± SEM of three separate experiments.

The effects of KC on 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 transfet peripheral blood B cells was unsuccessful (data not shown). As shown in the uppermost five columns of Figure 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 through 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 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.
KC suppresses anti-CD3/CD28-induced IL-4 and IL-5 production. We have recently found that B cell isotype switching to IgE induced inhibition. Promoter activation and in the inhibition by KC of the induction. Be involved in the IL-4 or IL-4 plus anti-CD40-induced germline elements C/EBP, Stat6, and NF-kB. The results also indicate that all the three of Stat6 and NF-kB enhanced enhancer activities C/EBP, Stat6, and NF-kB increased the enhancer activity of C/EBP, Stat6, and NF-kB. It is reported that C/EBP and Stat6 cooperate to activate germline e promoter 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-kB (Berberich et al., 1994; Yanagihara et al., 1996). The transcriptional cooperation of Stat6 and NF-kB is also reported (Strutz and Wistetschlag, 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-kB, or composite elements C/EBP/Stat6, Stat6/NF-kB. IL-4 alone or together with anti-CD40 increased the enhancer activity of C/EBP, Stat6, and NF-kB, and composite elements of C/EBP/Stat6 and Stat6/NF-kB, 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-kB, and also the cooperativity of C/EBP and Stat6 or of Stat6 and NF-kB, 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-kB in the region -167/-115 may be involved in the IL-4 or IL-4 plus anti-CD40-induced germline e 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 countered the KC-induced inhibition of IgE secretion (Fig 5a) and germline and mature e transcripts (Fig 5c) 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 e transcript by IL-4 alone (Fig 5e). Bt2cAMP also counteracted the KC-induced inhibition of germline e promoter activity (Fig 5b) and of C/EBP and NF-kB 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 e transcripts, germline e promoter activity, and C/EBP and NF-kB 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 e 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 e promoter activity by 75% (Fig 5b), of C/EBP enhancer activity by 60% (Fig 5c), and of NF-kB enhancer activity by 67% (Fig 5d) in IL-4 plus anti-CD40-stimulated surface IgE± B cells 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 e transcripts, and germline e promoter activity, and C/EBP and NF-kB 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 e transcripts, and germline e promoter and C/EBP and NF-kB 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...
cAMP level in AD patients’ 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
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 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 μM for 30 min, and then incubated with 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 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 ± SEM (n = 10) in AD patients is shown in the upper half of each figure and the mean ± 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.

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 Cγ1 (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-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
transcription in U266 myeloma cells, however, indicating that cAMP may not be necessary for mature e 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 CREB-responsive element (CRE) on various genes (Gonzalez and Montminy, 1989). IL-4 plus anti-CD40 induced the CRE-dependent OCA-B expression in murine B cells (Stevens et al, 2000). cAMP also promotes the transcriptional activity of C/EBPB and NF-kB (Shirakawa et al, 1989; Bomsztyk et al, 1990; Motomura et al, 1998; Park et al, 1999; Pohinke et al, 1999); PKA induces the synthesis of C/EBPB via CRE on C/EBPB promoter (Berrier et al, 1998; Niehof et al, 2001), and also promotes the nuclear translocation of C/EBPB (Metz and Ziff, 1991; Chinery et al, 1997). PKA phosphorylates cytosolic I-kB associated with NF-kB, which induces the dissociation and nuclear translocation of the free, activated form of NF-kB (Shirakawa and Mizel, 1989), and thus induces NF-kB-dependent gene expression in B cells (Lapointe et al, 1996). Though the IL-4 plus anti-CD40-responsive region on the germline e promoter does not contain consensus CRE, this region contains the binding sites for C/EBP, Stat6, and NF-kB (Delphin and Stavnezer, 1995; Thirenes et al, 1997). IL-4 or IL-4 plus anti-CD40-induced transcription through C/EBPB and NF-kB 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(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 e transcription through the element upstream of Stat6 site, possibly due to the different DNA-binding specificities. On the other hand, the sequence of NF-kB site (-90/-81) downstream of Stat6 in mouse germline e promoter is identical with that in human promoter. KC inhibited the IL-4 plus anti-CD40-induced enhancer activities of NF-kB (-92/-79) and composite Stat6/ NF-kB (-112/-76) elements from murine germline e promoter, as examined by reporter assays using Ramos cells (our unpublished observation). Thus cAMP may be involved in NF-kB-dependent transcription on murine promoter, similarly to that on human promoter.

There may possibly be another mechanism for cAMP-induced germline e transcription; PKA may inhibit the activity of transcriptional repressor. PKA-mediated phosphorylation may suppress the binding of putative repressor(s) to the undefined regulatory element on the IL-4 plus anti-CD40-inducible region, and this may promote the germline e 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 to V(D)J genes, following germline e transcription. Previous studies reported that cAMP-elevating agents promoted the expression of recombination-activating genes RAG1 and RAG2 (Menetski and Gellert, 1991; 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, Ca<sup>2+</sup>, 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 Ca<sup>2+</sup>/calmodulin stimulates AC types 1 and 3, whereas Ca<sup>2+</sup> inhibits AC types 5 and 6 (Hyengar, 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 Ca<sup>2+</sup> 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- IgE- 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.

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