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Spontaneous expression of IL-4 mRNA in lymphocytes from children with atopic dermatitis

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SUMMARY

Normal lymphocytes do not generally produce or secrete lymphokines in the resting or unstimulated state and only express or release cytokines following activation. Recently, the spontaneous production of intracellular interferon-gamma (IFN-γ) and spontaneous secretion of IL-6 has been documented in patients with atopic dermatitis. These findings indicated that lymphocytes had been previously activated *in vivo*. Such *in vivo* activation may also be associated with spontaneous production of IL-4. As measurement of IL-4 secretion by immunoassay is complicated by poor sensitivity, and only provides information on the net amount of cytokine present after secretion, adsorption, consumption and degradation have occurred, IL-4 mRNA expression in peripheral blood lymphocytes from children with atopic dermatitis and controls was examined by polymerase chain reaction (PCR)-assisted mRNA amplification. Spontaneous expression of IL-4 mRNA was detected in four of eight patients with severe atopic dermatitis. Following stimulation *in vitro*, seven of eight atopic patients demonstrated detectable IL-4 mRNA. In comparison, no spontaneous expression of IL-4 mRNA was found in controls, and only six of 10 controls expressed IL-4 mRNA in stimulated cultures. The spontaneous expression of IL-4 mRNA in unstimulated cultures from children with atopic dermatitis supports the possibility that previous *in vivo* activation has occurred, and suggests that IL-4 production is increased *in vivo* in atopic dermatitis. This *in vivo* activation together with the constitutive expression of IL-4 mRNA are likely to contribute to the spontaneous *in vitro* production of IgE in atopic patients.

Keywords IL-4 atopy mRNA childhood

INTRODUCTION

Atopic individuals demonstrate spontaneous B cell production of IgE *in vitro* which is considered to be an indicator of high atopic state [1,2]. Recent studies have elucidated that two signals are required for induction of IgE synthesis [3]. The first signal is delivered by IL-4 which induces isotype switch to IgE in B cells and transcription of germ-line Cε transcripts. A second B cell-activating signal, delivered by activated T cells, Epstein–Barr virus (EBV) or engagement of CD40 on B cells, is then required for transcription of productive ε-mRNA and subsequent IgE synthesis. The finding that anti-IL-4 antibody inhibits this spontaneous IgE production in peripheral blood mononuclear cell (PBMC) cultures from atopic individuals suggests that spontaneous IL-4 production in these cultures may contribute to the IgE synthesis [2,4].

Spontaneous generation of other cytokines has been reported in atopic individuals. We have recently found increased interferon-gamma (IFN-γ)-producing cells [5] and constitutive transcription of IFN-γ mRNA [6] in unstimulated PBMC cultures from children with atopic dermatitis (AD), and spontaneous secretion of IL-6 by T cells from adults with AD was documented by Toshitani *et al.* [7]. These findings suggested that T cells had been activated *in vivo*. Such *in vivo* T cell activation may also be associated with spontaneous production of IL-4. Several studies have reported increased IL-4 secretion in stimulated PBMC cultures from atopic donors [4,8,9]. However, consistent evidence of spontaneous IL-4 secretion in unstimulated cultures from atopic patients has not been presented. Although spontaneous IL-4 secretion was documented in T cell cultures from 15 asthmatic adults [10] and in mononuclear cell cultures from four of 21 adults with elevated IgE [4], no spontaneous IL-4 secretion was noted in mononuclear cell cultures from 18 children [8] and 21 adults [9] with AD. In these studies, IL-4 was measured by immunoassay [4,8, 10] which is currently hampered by poor sensitivity and only provides information on the net amount of IL-4 present after secretion, uptake, adsorption and degradation have occurred, or by biological assay [9] which is associated with poor

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specificity. Using *in-situ* hybridization, Brown *et al.* reported an 'increased percentage' of IL-4 mRNA-positive T cells in unstimulated cultures from AD patients (1%) compared with controls (0%), though it is uncertain whether this difference is truly significant, and the presence of IL-4 mRNA in unstimulated cultures was not confirmed by Northern analysis or reverse transcription/polymerase chain reaction (PCR) [11]. As examination of mRNA expression provides more direct information regarding production of IL-4, we have employed a method of PCR-assisted mRNA amplification which is reported to allow assessment of mRNA expression with increased sensitivity from small numbers of cells [12,13]. The expression of IL-4 mRNA in PBMC from children with severe AD and from non-atopic controls was examined to determine whether atopic patients have spontaneous transcription of IL-4 which could reflect *in vivo* activation and contribute to spontaneous IgE production. IL-4 mRNA expression in stimulated peripheral blood lymphocytes was also examined to investigate whether the increased IL-4 secretion previously reported in atopic subjects is associated with increased IL-4 mRNA.

MATERIALS AND METHODS

Reagents

Reagents were: Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), purified phytohaemagglutinin (PHA-P; Wellcome Diagnostics, Dartford, UK); phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (Sigma Chemical Co., St Louis, MO); cyclosporin A (Sandoz, North Ryde, Australia); RPMI 1640 medium, fetal calf serum (FCS), L-glutamine, penicillin and streptomycin (Flow, UK); Quanticlone IgE immunoradiometric assay kit (Kallestad Diagnostics Inc, Chaska, MN); Taq polymerase (Perkin Elmer Cetus, Norwalk, CT); RNasin, acetylated bovine serum albumin (BSA) and dNTPs (Promega, Madison, WI); and reverse transcriptase (GIBCO BRL, Gaithersburg, MD).

Patients

Heparinized venous blood samples were obtained from:

(i) eight children with severe AD who had elevated levels of serum IgE. These children had an acute exacerbation of AD, with a total clinical severity score $\geqslant 7$ and involvement of at least 20% of their body surface area. The total clinical severity score was defined as the sum of the individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for each of six parameters (pruritus, erythema, oedema/papulation, lichenification, scaling, and erosion/weeping) [14]. The extent of skin involvement was estimated using the rule of nines [14]. Levels of IgE were elevated above the normal limits for age in all cases (normal IgE 0–0·2 years <6 U/ml, 0·2–0·5 years <45 U/ml, 0·5–0·7 years <20 U/ml, 0·7–1 year <25 U/ml, 1–2 years <35 U/ml, 2–4 years <130 U/ml, 4–7 years <150 U/ml, >7 years <200 U/ml). The mean serum IgE level was 10 355 U/ml (range 61–30 000 U/ml) and the mean age was 4·0 years (range 3 months to 12 years). All patients were treated with topical steroids with or without emollient wet dressings. None had received oral steroid therapy for at least 1 month before testing;

(ii) ten age-matched, non-atopic controls. These were children admitted for elective surgery at the Royal Children's Hospital who had a negative history of atopic disease and plasma IgE levels within normal limits for age. The mean serum IgE was 13·6 U/ml (range 5–27 U/ml) and the mean age was 3·7 years (range 3 months to 12 years). They were otherwise well, free of acute infection, and not on any medication at the time of testing. Heparinized venous blood was obtained immediately on induction of anaesthetic.

We have previously examined expression of IFN-γ mRNA in these patients and controls, and the results have been accepted for publication [6].

Cell cultures and IL-4 assay

PBMC were separated from heparinized blood by density gradient centrifugation over Ficoll–Hypaque. For measurement of IL-4 secretion, PBMC were cultured at a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. PHA-P (10 μg/ml) was used as a stimulus. Supernatants were harvested at 24 h, as we have previously found maximal secretion of IL-4 in atopic and control children at this time [8]. IL-4 was measured on an indirect sandwich ELISA as detailed previously [8]. Recombinant human IL-4 was used as a standard. The mean (±s.d.) for 50 replicates of a negative control was 14·2 pg/ml (±5·0).

IgE assay

Plasma IgE levels were determined by immunoradiometric assay using Quanticlone IgE IRMA kits according to the manufacturer's instructions.

RNA extraction and reverse transcription

For RNA extraction, 4×10^5 PBMC (200 μl, 2×10^6/ml) were cultured in sterile Eppendorf tubes. PMA 50 ng/ml in combination with calcium ionophore 2·0 μM (PMA/Ca) was used as a stimulus. Where cyclosporin A (CsA) was added to cultures, this was added at a final concentration of 100 ng/ml before addition of PMA/Ca. Cultures were harvested at 6 h, as maximal IL-4 mRNA expression was demonstrated at this time in cultures from normal children and adults. Cells were isolated by brief centrifugation and then stored in 200 μl 4 M guanidinium isothiocyanate (GuSCN) at −70°C until further processing. RNA extraction was performed using phenol/chloroform extraction and ethanol precipitation as described previously [15]. Briefly, 1 μg tRNA, 200 μl 4 M GuSCN, 40 μl 2 M Na-acetate, 400 μl water-saturated acid phenol and 100 μl chloroform-iso-amyl alcohol (48:2) were added to the lysates with thorough vortexing after each addition. After incubation on ice for 15 min, the mixture was centrifuged at 10 000 *g* for 15 min at 4°C. The aqueous phase was recovered and RNA was precipitated overnight at −20°C in 100% ethanol. Precipitates were pelleted by centrifugation at 10 000 *g* for 20 min at 4°C, washed once with 75% ethanol in diethylpyrocarbonate-treated distilled water (DEPC-dH_2O) and repelletted at 10 000 *g* for 20 min at 4°C. Pellets were then vacuum dried, resuspended in 13·5 μl DEPC-dH_2O, incubated at 65°C for 5 min and cooled on ice. cDNA was synthesized from oligo-dT-primed RNA by reverse transcription with M-MLV reverse transcriptase (GIBCO BRL). The total RNA mixture was incubated with 200 U M-MLV reverse transcriptase, 40 U RNasin, 0·5 mM dNTPs, 20 μg/ml primer dT, 100 μg/ml acetylated BSA, 10 mM dithiothreitol, 50 mM Tris-HCl pH 8·3, 75 mM

KCl and 3 mM $MgCl_2$, in a final volume of 30 μl for 1 h at 37°C. The final cDNA product was made up to 100μl with sterile distilled water and stored at 4°C for subsequent cDNA amplification by PCR.

Polymerase chain reaction

Primer sequences for the internal control, β_2 microglobulin (β_2-M), were 5′ CTC GCG CTA CTC TCT CTT TCT GG 3′ for the upstream primer, and 5′ GC TTA CAT GTC TCG ATC CCA CTT AA 3′ for the downstream primer (Clontech, Palo Alto, CA). Reaction mixtures for β_2-M PCR contained 2 μl sample, 200 μM of each dNTP, 0·5 U Taq polymerase, 40 pM of each primer, 10 mM Tris-HCL, 1·5 mM $MgCl_2$, 50 mM KCl, 0·01% gelatine. Reaction times on a Perkin-Elmer Cetus DNA Thermal Cycler were 94°C 1 min, 50°C 2 min and 72°C 3 min for 30 cycles, followed by 7 min extension at 72°C. Primer sequences for IL-4 were 5′ GTT CTT CCT GCT AGC ATG TG 3′ for the upstream primer, and 5′ ATT TCT CTC TCA TGA TCG TC 3′ for the downstream primer. These primer sequences were confirmed to specifically amplify IL-4 cDNA by Southern blot analysis using a digoxigenin-labelled internal probe. Reaction mixtures for IL-4 PCR contained 5 μl sample, 200 μM of each dNTP, 0·5 U Taq polymerase, 25 pM of each primer, 10 mM Tris-HCL, 1·5 mM $MgCl_2$, 50 mM KCl, 0·01% gelatine. Reaction times for IL-4 PCR were 94°C 1 min, 50°C 2 min, and 72°C 3 min for 35 cycles, followed by 10 min extension at 72°C. A cDNA-positive control, a negative control and a mol. wt ladder were run with all PCR reactions. cDNA products were visualised by gel electrophoresis in 2% agarose after ethidium bromide staining.

Separate cycle course experiments confirmed linearity of amplification for β_2-M and IL-4 cDNA over 20–35 cycles and 25–40 cycles, respectively (Fig. 1). Comparison of the intensity of ethidium bromide staining for β_2-M after 30 cycles and IL-4 after 35 cycles therefore provided an indication of the amount of mRNA amplified in each sample. As total mRNA was extracted from a standard number of cells and PCR amplification was carried out on a standard amount of reverse transcribed cDNA in all cases, equivalent amounts of the internal control β_2-M would be expected in various patient samples allowing comparative assessment of IL-4 mRNA expression in patients and controls.

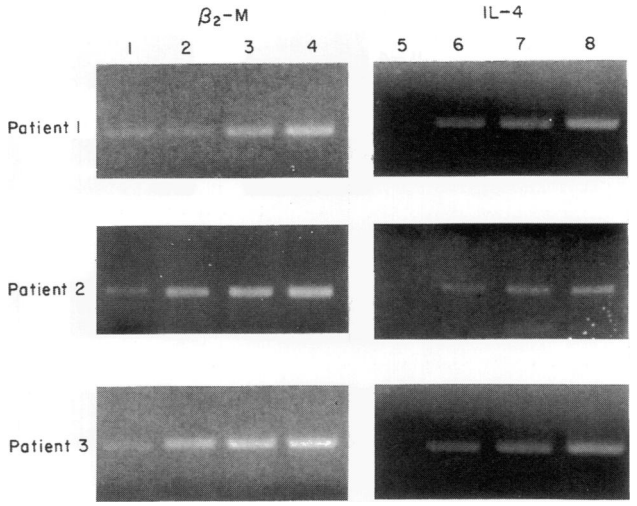

Fig. 1. Polymerase-chain reaction (PCR)-assisted amplification of reversed transcribed β_2 microglobulin (β_2-M) and IL-4 mRNA. Lanes 1–4, 20, 25, 30 and 35 cycles of amplification for β_2-M cDNA, respectively; lanes 5–8, 25, 30, 35 and 40 cycles, respectively, of amplification for IL-4 cDNA. Intensity of ethidium bromide staining increased with increasing cycle number for both β_2-M and IL-4. Subsequent comparisons of mRNA between subjects were therefore carried out using 30 cycles of amplification for β_2-M and 35 cycles for IL-4.

RESULTS

Expression of IL-4 mRNA in PBMC cultures from children with AD and controls

IL-4 mRNA expression in unstimulated and PMA/Ca-stimulated PBMC cultures from eight atopic children and 10 age-matched non-atopic controls was examined by reverse transcription/PCR. Expression of β_2-M mRNA was similar in all samples from patients and controls. No IL-4 mRNA was detected in unstimulated cultures from controls. Stimulation with PMA/Ca induced detectable levels of IL-4 mRNA in six of the 10 controls (60%) (Fig. 2a). The four controls who failed to express IL-4 mRNA in stimulated cultures were aged 3 months, 12 months, 20 months and 3 years, whereas the controls who had detectable IL-4 mRNA in stimulated cultures were aged 8 months, 12 months, 6·5 years, 7 years, 11·5 years and 12 years. In contrast to the controls, spontaneous expression of IL-4 mRNA was noted in unstimulated cultures from four of eight children with AD, and seven of the eight atopic children (88%) expressed IL-4 mRNA in PMA/Ca-stimulated cultures (Fig. 2b). The single AD patient who failed to express IL-4 mRNA in stimulated culture was aged 3 months. Expression of IL-4 mRNA in stimulated cultures appeared to be greater in atopic patients compared with controls based on intensity of ethidium bromide staining (Figs 2a,b).

Kinetics of IL-4 mRNA expression in children with AD and controls

To determine if atopic children demonstrated constitutive expression of IL-4 mRNA in freshly isolated PBMC, expression of IL-4 mRNA was examined at 0 h in PBMC from two atopic children who had detectable IL-4 mRNA in 6-h unstimulated cultures and two non-atopic controls (Fig. 3). No IL-4 mRNA was detected at 0 h in PBMC from controls. In contrast, both children with AD demonstrated constitutive expression of IL-4 mRNA at 0 h. The kinetics of IL-4 mRNA expression was also examined in these two atopic children and two controls following 3 h, 6 h, 12 h, and 24 h stimulation with PMA/Ca (Fig. 3). In the non-atopic subjects, IL-4 mRNA was first detected at 3–6 h after stimulation *in vitro*, and rapidly returned to undetectable levels after 6 h. In comparison, IL-4 mRNA expression in the children with AD, which was first detectable at 0 h, increased further following 3–6 h of stimulation and persisted until 12 h before becoming undetectable.

Effect of cyclosporin A on IL-4 mRNA expression in children with AD and controls

To determine if the spontaneous IL-4 mRNA expression in unstimulated cultures from children with AD could be reduced

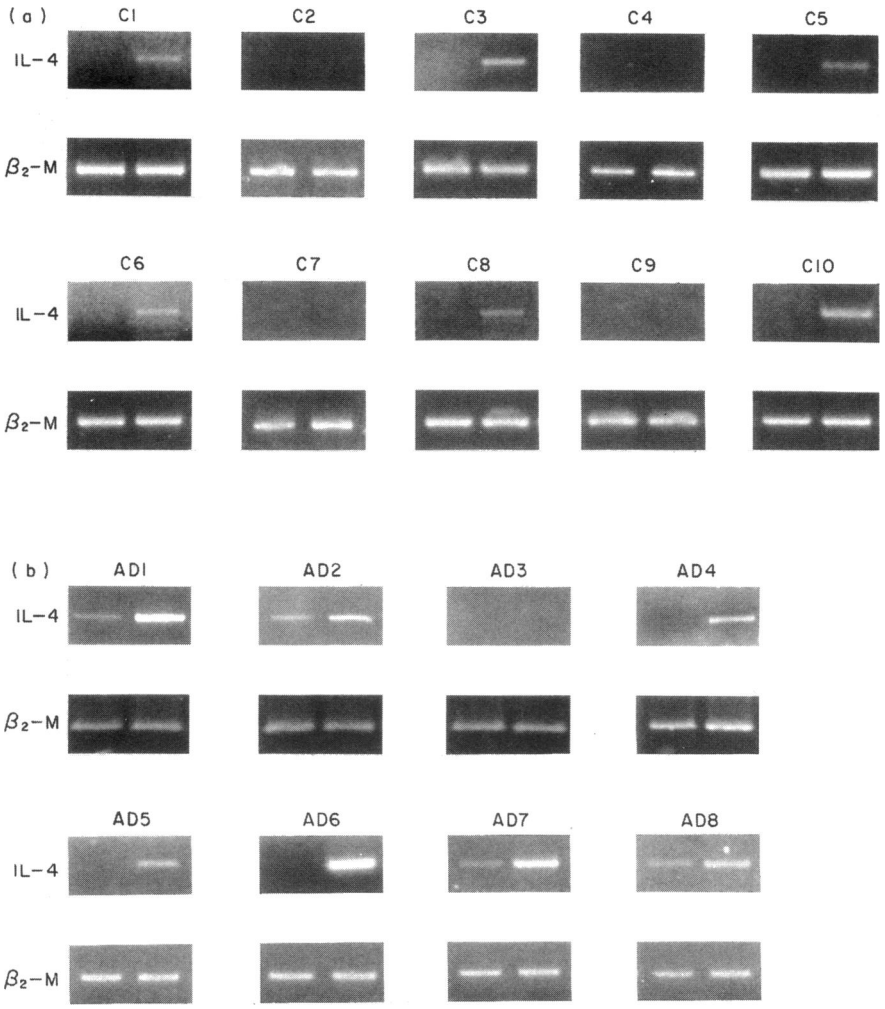

Fig. 2. (a) Polymerase-chain reaction (PCR)-assisted mRNA amplification for IL-4 and β_2 microglobulin (β_2-M) in unstimulated and phorbol myristate acetate (PMA)/Ca-stimulated peripheral blood mononuclear cells (PBMC) from 10 non-atopic controls. Total mRNA was extracted from 4×10^5 PBMC after culture for 6 h in sterile Eppendorf tubes. Expression of β_2-M was similar in unstimulated and stimulated cultures from all controls. IL-4 mRNA expression was detected in six of 10 controls following stimulation *in vitro*. No spontaneous expression of IL-4 mRNA was detected in unstimulated cultures. (b) PCR-assisted mRNA amplification for IL-4 and β_2-M in unstimulated and PMA/Ca-stimulated PBMC from eight children with atopic dermatitis (AD). Total mRNA was isolated from 4×10^5 PBMC after culture for 6 h in sterile Eppendorf tubes. Expression of β_2-M was similar in unstimulated and stimulated cultures from all atopic subjects, and was equivalent to that of controls. Spontaneous expression of IL-4 mRNA was present in four of eight atopic patients. Further increased expression was induced following *in vitro* stimulation with PMA/Ca in these patients. Expression of IL-4 mRNA was detected in stimulated cultures from seven of the eight atopic subjects, and was of greater intensity than that of controls.

by an inhibitor of IL-4 mRNA transcription, 100 ng/ml CsA was added to unstimulated and stimulated cultures from six children with AD and six controls. In all atopic children, presence of CsA resulted in reduced expression of IL-4 mRNA in both 6-h unstimulated and 6-h stimulated cultures. Similar reduction in IL-4 mRNA expression was demonstrated in 6 h stimulated cultures from controls (Fig. 4).

In vitro *IL-4 secretion*

Secretion of IL-4 in unstimulated and PHA-stimulated PBMC cultures from six of the eight children with AD and six of the eight controls was measured at 24 h. Mean levels of secreted IL-4 in stimulated cultures were higher in children with AD than in controls, although this did not reach statistical significance (geometric mean AD 49·5 pg/ml, 95% confidence interval (CI) 40·5–60·7; geometric mean controls 30·0 pg/ml, 95% CI 17·4–50·6; $P > 0{\cdot}05$). No spontaneous IL-4 secretion was detected in unstimulated cultures from atopic children.

DISCUSSION

The major finding in this study was the spontaneous presence of IL-4 mRNA in unstimulated PBMC from a significant number of the atopic subjects which was not found for controls. In previous reports examining IL-4 mRNA expression in PBMC or T cell cultures from healthy adults and neonates by Northern

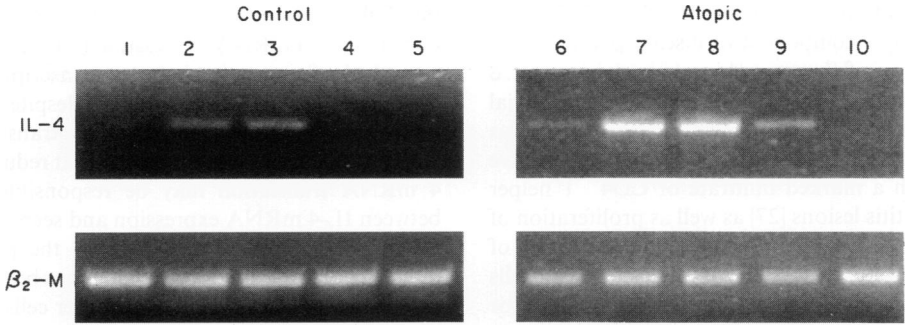

Fig. 3. Representative profiles of the kinetics of IL-4 mRNA expression in children with atopic dermatitis (AD) and controls. Expression of IL-4 mRNA in peripheral blood mononuclear cells (PBMC) from two children with AD and two controls was examined at 0 h and after 3 h, 6 h, 12 h and 24 h stimulation with phorbol myristate acetate (PMA)/Ca. Expression of β_2 microglobulin (β_2-M) mRNA was similar at all time points for patients and controls. IL-4 mRNA in controls was first detected at 3 h, and after transient expression became undetectable after 6 h. In comparison, atopic patients demonstrated constitutive expression of IL-4 mRNA at 0 h. Stimulation *in vitro* induced further increased expression of IL-4 mRNA at 3–6 h which persisted until 12 h before becoming undetectable.

analysis, transcripts have only been detected following stimulation *in vitro* [16–18]. A similar absence of IL-4 mRNA in unstimulated T cells and mononuclear cells was confirmed using the more sensitive technique of PCR-assisted mRNA amplification which we employed in this study [12,13]. The absence of IL-4 mRNA in freshly isolated PBMC or unstimulated cultures from controls is similar to these previous reports. Hence, the spontaneous expression of IL-4 mRNA in PBMC from atopic children suggests that these cells have been previously activated *in vivo*. As IL-4 is predominantly produced by $CD4^+CD45RO$ memory helper T cells [17,19,20], it is likely that the cells which spontaneously expressed IL-4 mRNA represent T cells. Mast cells [21] and non-B non-T cells [22] have also been shown to produce IL-4 *in vitro*, but it is not clear whether these cells represent significant sources of IL-4 *in vivo*, or whether they are present in significant numbers in the peripheral blood. Spontaneous production of intracellular IFN-γ [5] and spontaneous secretion of IL-6 [7] have also been demonstrated in AD. A recent study showed that *in vitro* activation of T cells could induce constitutive expression of mRNA for several cytokines, including IL-1α, IL-2, IL-4, IL-5 and IFN-γ, despite removal of ongoing stimuli [13]. The spontaneous production of several cytokines in unstimulated lymphocytes from AD patients would therefore indicate that previous *in vivo* activation, presumably of T cells, has occurred and implicates a role for T cell activation in AD. Using *in situ* hybridization, Brown *et al.* previously reported increased numbers of IL-4 mRNA-positive lymphocytes in unstimulated PBMC cultures from adults with AD compared with controls; however, the proportion of IL-4 mRNA-positive cells in both patients (1%) and controls (0%) was low, and this difference may be within the limits of normal variation. Our present observation that children with AD have constitutive expression of IL-4 mRNA is consistent with the findings of Brown *et al.* [11], and supports the contention that *in vivo* T cell activation is a feature of AD. Additional evidence of *in vivo* T cell activation in AD is provided by reports of increased serum levels of soluble IL-2 receptors [23–25]. Interestingly, constitutive mRNA expression for multiple cytokines including IFN-γ, IL-4 and IL-6 has also been documented in rheumatoid

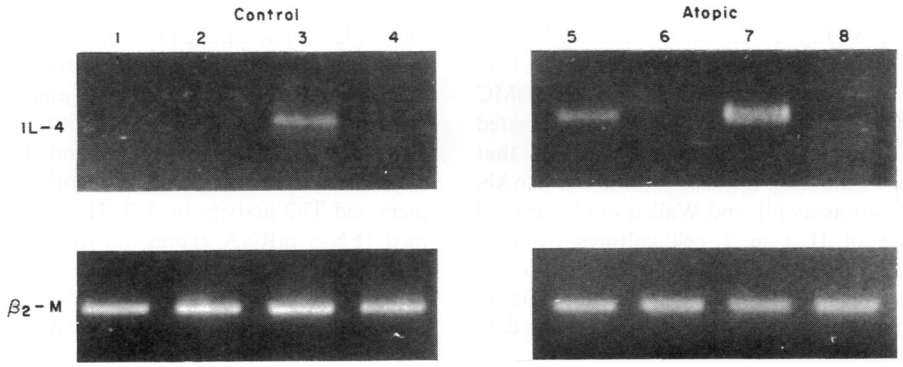

Fig. 4. Effect of cyclosporin A (CsA) on IL-4 mRNA expression. Representative profiles of IL-4 mRNA expression are presented for patients with atopic dermatitis (AD) and controls. Lanes 1–4, unstimulated, unstimulated + CsA, phorbol myristate acetate (PMA)/Ca-stimulated and PMA/Ca + CsA-stimulated peripheral blood mononuclear cells (PBMC) cultured for 6 h from a control. Similar cultures for a child with AD are illustrated in lanes 5–8. Presence of CsA inhibited IL-4 mRNA expression in stimulated cultures from controls. Similar inhibition was evident for both unstimulated and stimulated cultures from atopic children.

arthritis (RA), a condition in which T cell activation is considered to be a major component of disease pathogenesis. Spontaneous expression of these cytokines was demonstrated in freshly isolated mononuclear cells and T cells from synovial fluid and synovial tissue of patients with RA [18,26], and was considered to represent *in vivo* activation in the patients. Active AD is associated with a marked infiltrate of $CD4^+$ T helper cells within the dermatitis lesions [27] as well as proliferation of the paracortical T cell areas in lymph nodes [28]. Activation of T cells could, therefore, occur at either of these sites, with cells entering the circulation from lymph nodes via lymphatics or directly from involved skin. Our finding of increased spontaneous IL-4 mRNA expression in unstimulated cultures from children with AD could facilitate increased spontaneous production of IgE *in vitro*, and possibly *in vivo*.

Previous studies have shown that following *in vitro* stimulation, normal T cells have transient expression of IL-4 mRNA between 4 and 7 h, which returns to undetectable levels after 12 h [12,17]. Comparable transient expression was found for our controls. Examination of the kinetics of IL-4 mRNA expression in the atopic patients, however, showed constitutive expression of IL-4 mRNA at 0 h and an increased duration of expression for up to 24 h after stimulation. Nuclear run-on assays have demonstrated that accumulation of IL-4 mRNA in normal T cells following *in vitro* stimulation occurs as a result of both an increased rate of transcription as well as increased stability of transcripts [29]. The increased constitutive and stimulated IL-4 mRNA expression in the atopic patients could similarly relate to either of these factors. Although the regulation of IL-4 mRNA expression in atopic patients was different from that of controls, gene transcription did not appear to occur in an unrestricted fashion. CsA, which has been shown to inhibit transcription of IL-4 [29], presumably through inhibition of calcineurin and the transcription factor NF-AT [30,31], consistently prevented IL-4 mRNA expression in both unstimulated and stimulated cultures from atopic subjects. In addition, further increased expression of IL-4 mRNA could be induced after 3–6 h by stimulation with PMA/Ca *in vitro*, indicating that IL-4 mRNA expression in the atopic patients remained sensitive to *in vitro* stimuli.

Despite the increased expression of IL-4 mRNA in freshly isolated and unstimulated PBMC from atopic patients, no spontaneous IL-4 secretion was detected. This lack of detectable spontaneous secretion of IL-4 is similar to our previous findings [8] and those of Jujo *et al.* who measured IL-4 in PBMC cultures by bioassay [9]. In contrast, Rousset *et al.* found spontaneous IL-4 secretion in unstimulated PBMC cultures from a small proportion (4/21) of adults with elevated IgE using an ELISA assay with sensitivity of 50 pg/ml that applied the same tracer antibody (mouse anti-huIL-4 MoAb, 11B4) as that used in our assay [4], and Walker *et al.* reported spontaneous secretion of IL-4 in T cell cultures from 15 asthmatic adults using an ELISA assay with sensitivity of 10 pg/ml, although it was not stated whether this was noted for all or only some of the patients tested [10]. These conflicting data may relate to differences in the cell types examined, as the only study which reported significant spontaneous IL-4 secretion examined secretion by purified T cells [10], whilst the other studies investigated IL-4 secretion by PBMC [4,8,9]. In addition, as already discussed, the differing results could reflect the poor sensitivities of the IL-4 assays used. It is also possible that our failure to detect spontaneous IL-4 secretion despite the increased IL-4 mRNA expression in the atopic children represents a lack of correlation between transcription and secretion of IL-4. Indeed, a lack of secreted IL-4 despite the presence of IL-4 mRNA has been documented in EBV-transformed B cell clones [32]. It was suggested in that study that reduced efficiency of IL-4 mRNA translation may be responsible. The discrepancy between IL-4 mRNA expression and secretion in unstimulated PBMC could also be explained by the process of receptor-directed polar release of cytokine. It has been shown that T cells can deliver cytokines directly to other cells without associated release into the surrounding environment [33]. However, this direct cellular transfer of cytokines can be overcome by 'maximal stimulation' resulting in cytokine release into supernatants [33]. Such maximal stimulation may be provided by stimulation *in vitro* with mitogens or a combination of phorbol esters and calcium ionophore, which would account for the detection of secreted IL-4 in stimulated but not unstimulated cultures.

Examination of IL-4 mRNA expression in stimulated cultures revealed an absence of IL-4 mRNA in almost half of the controls, all of whom were $\leqslant 3$ years of age. This reduced ability to express IL-4 in the younger children is compatible with the knowledge that neonatal cord blood T cells do not express IL-4 mRNA [17,34] and that secretion of IL-4 is reduced in normal children until 10 years of age (unpublished data from our laboratory). In comparison, expression of IL-4 mRNA was detected in all but one (the youngest, age 3 months) of the atopic subjects. Although the method of PCR-assisted mRNA amplification used in this study only provides comparative assessment of mRNA expression, expression of IL-4 mRNA in atopic patients appeared to be greater than in controls. This increased expression of IL-4 mRNA in children with AD is consistent with the findings of Brown *et al.*, who reported increased IL-4 mRNA levels by Northern analysis in PMA/Ca-stimulated cultures from adults with AD [11], and with the increased secretion of IL-4 demonstrated for atopic individuals in this and previous studies [4,8,9].

It has been suggested that allergic disease is associated with a predominance of Th2-like T cell activity, with increased IL-4 and reduced IFN-γ production, and that this Th2 predominance is a consequence of selective activation of Th2 type T cells. Previous studies have shown high frequencies of Th2-like allergen-specific T cell clones (TCC) derived from peripheral blood [35–38] or cutaneous patch test lesions [39,40] of atopic patients. A possible caveat of these studies is that the process of cloning in the presence of IL-2 and/or IL-4 preferentially induces expansion of Th2-like TCC [41–43]. Our present finding of increased constitutive and stimulated IL-4 mRNA expression in PBMC from patients with AD supports an increased Th2 activity in AD. However, we have also examined IFN-γ mRNA expression in these patients, and found increased spontaneous IFN-γ expression with detectable mRNA expression in six of the eight atopic patients [6]. A recent study of active atopic dermatitis skin lesions similarly reported more frequent expression of IFN-γ mRNA (13 of 15 patients) compared with IL-4 mRNA (four of 15 patients), as well as greater expression of IFN-γ mRNA than IL-4 mRNA in patients expressing both cytokines [44]. These combined findings do not support a selective activation of Th2-like T cells in atopic disease, and instead suggest activation of both

Th1 and Th2-like T cells. As T cells from patients with atopic dermatitis have been shown to have an intrinsic defect of IFN-γ secretion [9,45], the imbalance of IL-4 and IFN-γ secretion documented in atopic dermatitis [4,8,9,45,46] could reflect general activation of T cells in the presence of an intrinsic defect of IFN-γ secretion.

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