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Reduced interferon-gamma (IFN-γ) secretion with increased IFN-γ mRNA expression in atopic dermatitis: evidence for a post-transcriptional defect

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SUMMARY

Reduced secretion of IFN-γ in atopic individuals has been implicated in the pathogenesis of disease, though the mechanisms leading to this reduced secretion have not been elucidated. As production of IFN-γ has been shown to be predominantly regulated by its rate of transcription, expression of IFN-γ mRNA was examined in atopic children and in age-matched, non-atopic controls by polymerase chain reaction (PCR)-assisted mRNA amplification. Children with atopic dermatitis were found to have constitutive expression of IFN-γ mRNA in freshly isolated peripheral blood mononuclear cells (PBMC) and in unstimulated PBMC cultures which increased further following stimulation with phorbol myristate acetate (PMA)/Ca *in vitro*. In contrast, expression of IFN-γ mRNA in controls was only detected in stimulated cultures, as has been demonstrated previously for normal adults. These findings demonstrate that circulating T cells from atopic children have been activated *in vivo*, and suggest that T cell activation is a significant component of the inflammatory process in atopic dermatitis. Although expression of IFN-γ mRNA was increased in the atopic children, secretion was confirmed to be significantly lower than in controls, indicating that the defect(s) underlying reduced IFN-γ secretion in these individuals lie post-transcriptionally.

Keywords interferon-gamma atopic dermatitis mRNA childhood

INTRODUCTION

Atopic children [1] and adults [2–5] have reduced secretion of IFN-γ *in vitro* which has been implicated to play a role in the pathogenesis of disease [6–8]. The mechanisms underlying this reduced secretion of IFN-γ are unclear. We have recently shown that despite having reduced secretion of IFN-γ, atopic individuals have an increased percentage of IFN-γ-producing cells in unstimulated peripheral blood mononuclear cell (PBMC) cultures compared with controls [9]. These findings indicated that atopic individuals do not have a lack of IFN-γ-producing cells, but rather have reduced secretion of IFN-γ per cell. Previous studies have demonstrated that IFN-γ secretion in atopic patients remained defective despite stimulation with phorbol myristate acetate (PMA) and calcium ionophore, addition of IL-2, presence of anti-IL-4 antibody, or removal of possibly inhibitory monocytes, implicating an intrinsic T cell defect as a cause of reduced IFN-γ secretion [5,9,10]. As the combination of phorbol ester and calcium ionophore are known to act as second messengers allowing direct stimulation of T cells independent of accessory signals [11,12], it is likely that any defect(s) of IFN-γ secretion lie distal to signal transduction, possibly at the level of transcription, translation or secretion.

To localize further the mechanism(s) leading to reduced IFN-γ secretion in atopic individuals, expression of IFN-γ mRNA was examined in children with severe atopic dermatitis and in non-atopic controls. A method of polymerase chain reaction (PCR)-assisted mRNA amplification which allows examination of mRNA expression with increased sensitivity from small numbers of cells was employed [13,14]. Investigation of IFN-γ mRNA expression in peripheral blood lymphocytes may also provide further insight into our previous finding of increased numbers of IFN-γ-producing cells in the presence of reduced IFN-γ secretion in atopic children [9].

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MATERIALS AND METHODS

Reagents

The following reagents were used: human IFN-γ ELISA kit (CSL Ltd, Melbourne, Australia); 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).

Subjects

Five to 10 ml of heparinized venous blood were obtained from: (i) eight children with severe atopic dermatitis (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 (pruritis, erythema, oedema/papulation, lichenification, scaling, and erosion/weeping) [15]. The extent of skin involvement was estimated using the rule of nines [15]. 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 $\leqslant 200$ U/ml). The mean serum IgE level was 10 355 U/ml (61–30 000 U/ml) and the mean age was 4·0 years (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 (5–27 U/ml) and the mean age was 3·7 years (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.

Cell cultures

PBMC were separated from heparinized blood by density gradient centrifugation over Ficoll–Hypaque. For measurement of IFN-γ secretion, 2×10^6 PBMC were cultured in a volume of 1 ml (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. Cultures were incubated in 24-well flat-bottomed culture plates (Costar, Cambridge, MA) 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 72 h, as we have previously found the kinetics of IFN-γ secretion to be similar in atopic and control children, with progressive accumulation of IFN-γ following stimulation to reach maximal levels at 72 h [1].

IFN-γ assay

Supernatants were stored at −70°C. IFN-γ was measured on a human IFN-γ ELISA kit (CSL) using two MoAbs directed to IFN-γ according to the manufacturer's instructions. Purified human IFN-γ supplied with the kit was used as a standard. Sensitivity of the assay was 1 U/ml. All samples were run in duplicate.

IgE assay

Plasma samples were stored at −70°C. 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) were cultured in sterile Eppendorf tubes and stimulated with PMA 50 ng/ml and calcium ionophore 2·0 μM (PMA/Ca). 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 IFN-γ 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 [16]. 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 100 μl 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 IFN-γ were 5′ ATA TCT TGG CTT TTC AGC TC 3′ for the upstream primer and 5′ CTC CTT TTT CGC TTC CCT GT 3′ for the downstream primer. These primer sequences were previously confirmed to amplify specifically IFN-γ cDNA by Southern blot analysis using a digoxigenin-labelled internal probe. Reaction mixtures for IFN-γ 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

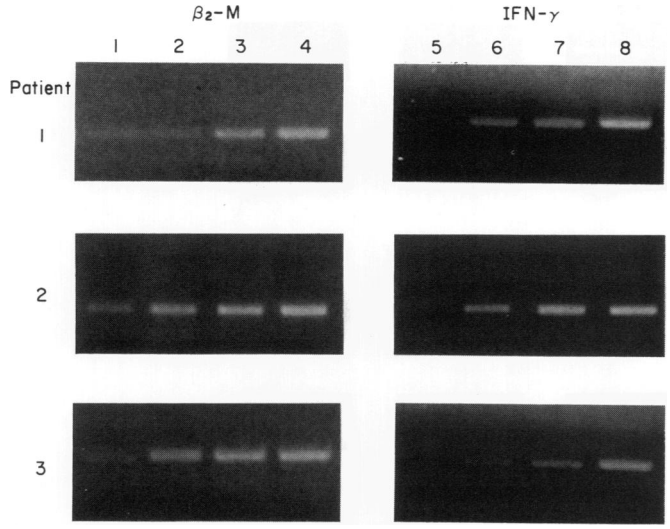

Fig. 1. Polymerase chain reaction (PCR)-assisted amplification of reversed transcribed β_2-microglobulin (β_2-M) and IFN-γ mRNA in three representative patient samples. Lanes 1–4 represent 20, 25, 30 and 35 cycles of amplification for β_2-M cDNA, respectively. Lanes 5–8 represent 25, 30, 35 and 40 cycles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

$MgCl_2$, 50 mM KCl, 0·01% gelatine. Reaction times for IFN-γ 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 visualized by gel electrophoresis in 2% agarose after ethidium bromide staining.

Separate cycle course experiments confirmed linearity of amplification for β_2-M and IFN-γ 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 IFN-γ 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 IFN-γ expression in patients and controls.

Statistical analysis

As data regarding secreted IFN-γ in supernatants were non-parametric, pairwise comparisons between groups were carried out using the Mann–Whitney *U*-test statistic.

RESULTS

Expression of IFN-γ mRNA in PBMC cultures from children with AD and controls

To determine if the reduced IFN-γ secretion in atopic children related to factors which exert their influence at the level of transcription, IFNγ mRNA expression in 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 remained constant in all samples from patients and controls. In all 10 controls, IFN-γ mRNA was not detected in unstimulated cultures, and was only present following stimulation *in vitro* (Fig. 2a). In contrast, in six of eight children with atopic dermatitis, IFN-γ mRNA was detected in both unstimulated and PMA/Ca-stimulated cultures (Fig. 2b). In PMA/Ca-stimulated cultures, IFN-γ mRNA appeared to be expressed in similar quantities for patients and controls based on intensity of ethidium bromide staining. An absence of IFN-γ mRNA in stimulated culture was noted in one control, who was 3 months old. In comparison, all children with AD had detectable IFN-γ mRNA in stimulated cultures. Of note, the corresponding 3-month-old child with atopic dermatitis (AD4) had detectable expression of IFN-γ mRNA in both unstimulated and stimulated cultures.

Kinetics of IFN-γ mRNA expression in children with AD and controls

As IFN-γ mRNA was detected in 6 h unstimulated cultures from atopic subjects, we investigated whether similar expression could be demonstrated in freshly isolated PBMC at 0 h. Expression of IFN-γ mRNA at 0 h was examined in three children with AD who had detectable mRNA in 6-h unstimulated cultures and in eight controls (Fig. 3). Constitutive transcription of IFN-γ mRNA was demonstrated in all three atopic patients. In contrast, no IFN-γ mRNA was detected at 0 h in controls. The kinetics of mRNA transcription in response to *in vitro* stimulation with PMA/Ca was also examined to ascertain whether this may be different in patients and controls. Expression of IFN-γ mRNA was examined after 3 h, 6 h, 12 h, and 24 h stimulation with PMA/Ca in three children with AD who had expressed IFN-γ mRNA in 6-h unstimulated cultures and in two controls (Fig. 3). In the non-atopic subjects, IFN-γ mRNA was first detected at 3–6 h after stimulation *in vitro*, reached maximal levels at 6 h and returned to undetectable levels by 12 h. All three children with atopic dermatitis who demonstrated constitutive transcription of IFN-γ showed increased mRNA expression following 3–6 h of stimulation, but maximal levels were detected from 3 h. Furthermore, two of the three children with AD demonstrated prolonged IFN-γ mRNA expression for up to 12 h after stimulation, with return to undetectable levels by 24 h. The remaining child with AD had undetectable levels of IFN-γ mRNA by 12 h, as seen in the controls.

Effect of CsA on IFN-γ mRNA transcription in children with AD

To determine if the spontaneous IFN-γ mRNA expression in unstimulated cultures from children with AD could be influenced by a known inhibitor of IFN-γ mRNA transcription, 100 ng/ml CsA was added to both unstimulated and PMA/Ca-stimulated cultures from six children with AD and six controls. CsA was added before addition of PMA and calcium ionophore. In all controls, expression of IFN-γ mRNA in 6-h stimulated cultures was inhibited by addition of CsA. In all atopic children, presence of CsA consistently inhibited both the constitutive expression of IFN-γ mRNA in unstimulated cultures and the expression of IFN-γ mRNA in stimulated cultures at 6 h (Fig. 4).

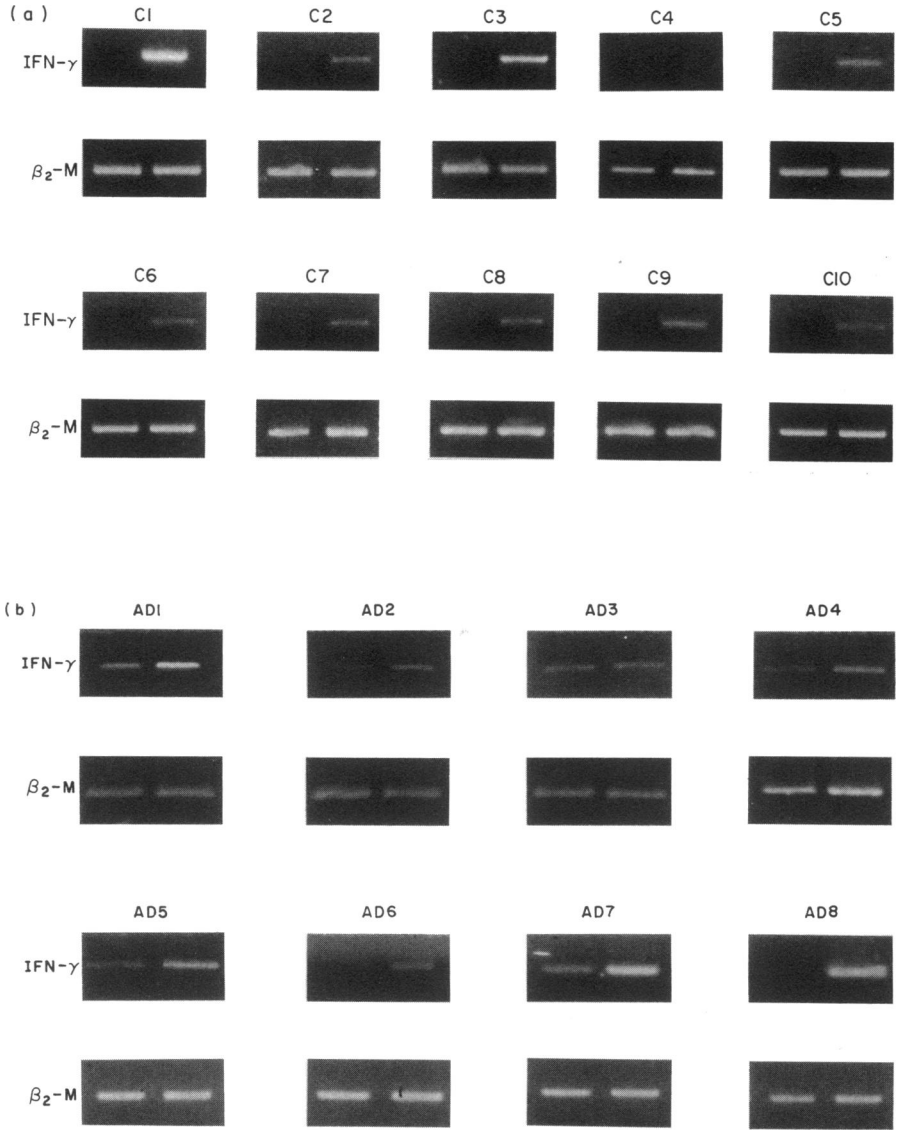

Fig. 2. (a) Polymerase chain reaction (PCR)-assisted mRNA amplification for IFN-γ and β_2-microglobulin (β_2-M) in unstimulated and phorbol myristate acetate (PMA)/Ca-stimulated peripheral blood mononuclear cell (PBMC) cultures from 10 non-atopic controls. Total mRNA was isolated from 4×10^5 PBMC cultured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

In vitro *IFN-γ secretion*

Secretion of IFN-γ in PHA-stimulated PBMC cultures from six of the eight children with AD and six of the eight controls was measured at 72 h. Children with AD were confirmed to secrete significantly less IFN-γ than controls (geometric mean AD 25·9 U/ml, 95% CI 4·4–151·7; geometric mean controls 288·4 U/ml, 95% CI 127·1–654·6; $P = 0{\cdot}037$). No spontaneous IFN-γ secretion was detected in unstimulated cultures from atopic children.

DISCUSSION

The present study aimed to further localize the defect(s) of IFN-γ secretion by examining the expression of IFN-γ mRNA in children with AD and in controls. As IFN-γ secretion is considered to be primarily regulated by the rate of gene transcription [17–19], it was possible that expression of IFN-γ mRNA in atopic individuals would be reduced. A significant finding in this study was the presence of IFN-γ mRNA in

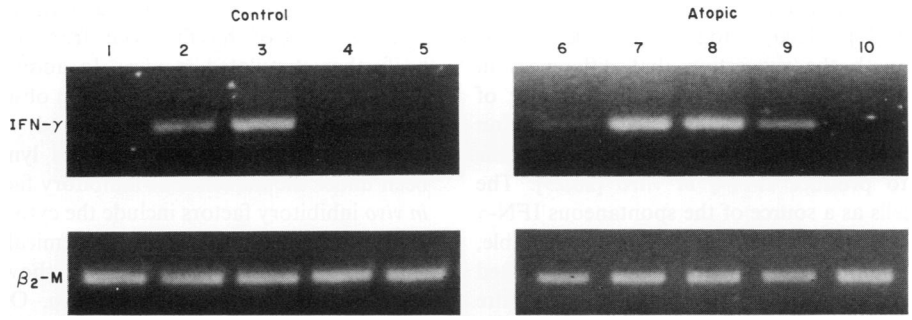

Fig. 3. Representative polymerase chain reaction (PCR)-assisted mRNA amplification illustrating kinetics of IFN-γ mRNA expression in patients with atopic dermatitis and in controls. Expression of IFN-γ mRNA in peripheral blood mononuclear cells (PBMC) from two non-atopic controls and three children with atopic dermatitis was examined at 0 h and after 3 h, 6 h, 12 h and 24 h stimulation with phorbol myristate acetate (PMA)/Ca. Lanes 1–5 show a representative profile for expression of IFN-γ mRNA at 0 h, 3 h, 6 h, 12 h and 24 h, respectively, for non-atopic controls. Lanes 6–10 show a representative profile for IFN-γ mRNA expression at 0 h, 3 h, 6 h, 12 h and 24 h, respectively, for children with atopic dermatitis. Expression of β_2-microglobulin (β_2-M) was similar at all time points in patients and controls. In the controls, IFN-γ mRNA was first detected at 3 h, reached maximal levels at 6 h and became undetectable at 12 h. In comparison, in all three children with atopic dermatitis, IFN-γ mRNA was expressed constitutively at 0 h and increased to maximal levels by 3 h after stimulation. Prolonged expression of IFN-γ mRNA for up to 12 h was also noted in two of the three atopic children.

unstimulated PBMC cultures from atopic subjects. In previous reports examining IFN-γ mRNA expression in PBMC or T cell cultures from healthy adults and neonates by Northern analysis, transcripts have only been detected following stimulation *in vitro* [20–25]. Similar findings have been confirmed using the more sensitive technique of PCR-assisted mRNA amplification which we employed in this study [13,14]. Our finding that controls did not express IFN-γ mRNA in freshly isolated PBMC or unstimulated cultures is consistent with these previous reports. Thus, the presence of IFN-γ mRNA in PBMC from atopic individuals in the absence of stimulation *in vitro* indicates that these cells have been previously activated *in vivo*. Recently, the expression of cytokine mRNA in an *in vitro* model of T cell activation was examined using PCR-assisted mRNA amplification [13]. In that study, T cells were separated into naive and memory populations and then subjected to primary (6 days) followed by secondary (3 days) stimulation with Staphylococcal enterotoxin A. This *in vitro* stimulation was considered to be analogous to activation *in vivo*, as phenotypic analysis of the T cells demonstrated transformation of naive CD45RA cells into memory CD45RO cells following primary activation. No IFN-γ mRNA was detected in freshly isolated naive or memory T cells in the absence of stimulation. However, memory T cells that had been subjected to primary stimulation were found to express IFN-γ mRNA constitutively before secondary stimulation. The spontaneous expression of IFN-γ mRNA by *in vitro* activated memory T cells would support the contention that PBMC from atopic children which constitutively expressed IFN-γ mRNA have been activated *in vivo*.

This constitutive expression of IFN-γ mRNA demonstrated for children with AD substantiates our previous

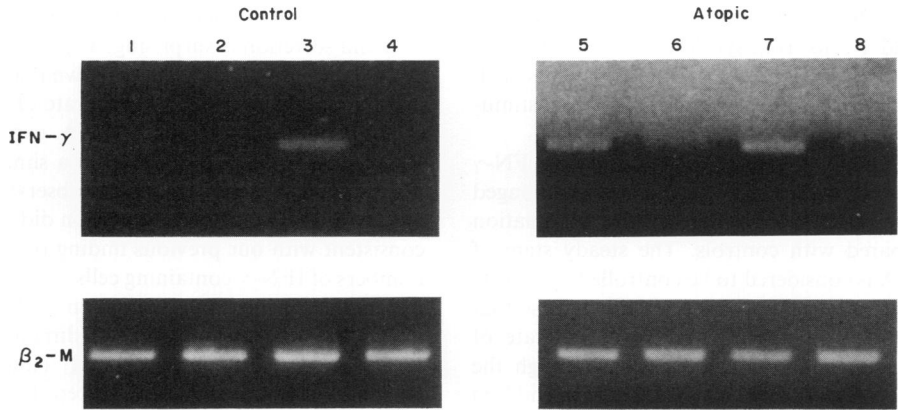

Fig. 4. Effect of cyclosporin A (CsA) on IFN-γ mRNA expression. CsA (100 ng/ml) was added to both unstimulated and phorbol myristate acetate (PMA)/Ca-stimulated cultures from six children with atopic dermatitis and six controls. Lanes 1–4 show representative IFN-γ mRNA expression in unstimulated, unstimulated + CsA, PMA/Ca-stimulated and PMA/Ca + CsA-stimulated peripheral blood mononuclear cells (PBMC) from a non-atopic control, respectively. Lanes 5–8 show representative IFN-γ mRNA expression in unstimulated, unstimulated + CsA, PMA/Ca-stimulated and PMA/Ca + CsA-stimulated PBMC from a child with atopic dermatitis, respectively. Expression of β_2-microglobulin (β_2-M) was similar for all experiment points in patients and controls. In the controls, expression of IFN-γ mRNA in stimulated cultures was inhibited by the presence of CsA. In the children with atopic dermatitis, both the constitutive and the stimulated expression of IFN-γ mRNA was inhibited in the presence of CsA.

finding of IFN-γ-containing cells in unstimulated cultures from children with AD [9]. Taken together, these results are also consistent with the suggestion that differences in cytokine mRNA levels reflect variations in the number of cytokine-producing cells rather than increased transcription in each cell [26,27]. Both T cells and natural killer (NK) cells have been shown to produce IFN-γ *in vitro* [28,29]. The importance of NK cells as a source of the spontaneous IFN-γ mRNA expression in these children with AD is questionable, however, as NK cell number and function have been reported to be reduced in atopic individuals with AD [30,31]. It is more likely that this constitutive expression of IFN-γ mRNA involves T cells, as T cells are the predominant source of IFN-γ [28,32], and T cell activation is associated with early induction of IFN-γ mRNA [18,19]. These observations indicate that *in vivo* T cell activation may be a significant component of the inflammation present in severe AD. Other indicators of *in vivo* T cell activation in patients with AD include increased serum levels of soluble IL-2 receptors [33–35] and spontaneous IL-6 production by T cells [36]. A significant degree of inflammation is present in the children with AD, as they had active dermatitis involving >20% of their skin surface area. Active AD is associated with a prominant infiltrate of $CD4^+$ T helper cells within the dermatitis lesions [37] as well as proliferation of the paracortical T cell areas in lymph nodes [38], so that activation of T cells could occur at either of these sites, with cells entering the circulation from lymph nodes via lymphatics or directly from involved skin. The activating antigens involved are not known, but may include *Staphylococcus aureus*. Skin colonization and infection with this organism is an important factor in exacerbations of AD [39], and recent studies have demonstrated that bacterial antigen-specific T cell clones (TCC) are of the Th1 type producing IFN-γ [40,41], and that several staphylococcus-derived antigens and superantigens are potent inducers of IFN-γ production by T cells [42–45]. Interestingly, constitutive expression of IFN-γ mRNA has also been documented in rheumatoid arthritis (RA), a condition in which T cell activation has been postulated to contribute to the pathogenesis of disease. Spontaneous expression of IFN-γ mRNA was detected in freshly isolated mononuclear cells and T cells from synovial fluid and synovial tissue of patients with RA [24,46]. In addition, patients with RA also have increased IFN-γ mRNA expression in unstimulated peripheral blood T cells [47].

In children with AD, the constitutive expression of IFN-γ mRNA was associated with earlier and more prolonged expression of IFN-γ mRNA following *in vitro* stimulation with PMA/Ca compared with controls. The steady state of cellular IFN-γ mRNA is considered to be controlled by its rate of transcription as well as by its rate of degradation [17], so that these alterations could relate to either an increased rate of transcription or reduced mRNA degradation. Although the kinetics of IFN-γ mRNA expression may be altered in children with AD, the constitutive expression of IFN-γ mRNA in these children appeared to remain under the influence of cellular control mechanisms, as IFN-γ mRNA subsequently became undetectable by 24 h. Furthermore, expression of IFN-γ mRNA in both unstimulated and stimulated cultures was consistently inhibited by CsA, confirming that PBMC from the atopic patients remained responsive to at least one known inhibitor of IFN-γ transcription. The increased mRNA expression seen in PMA/Ca-stimulated compared with unstimulated cultures indicated that these cells from children with AD could be further stimulated *in vitro*. In addition, the spontaneous increase in IFN-γ mRNA expression observed following 6 h of culture in medium alone compared with the constitutive expression at 0 h suggests that the lymphocytes may have been under the influence of inhibitory factors *in vivo*. Possible *in vivo* inhibitory factors include the cytokines IL-4 [48,49] and IL-10 [50,51], as well as any systemically absorbed corticosteroid [52], which have all been shown to inhibit IFN-γ secretion and transcription *in vitro*. Other cytokines, IL-2 [53,54] and IL-12 [55], have been reported to induce and enhance the transcription of IFN-γ *in vitro*, and may contribute to the increased constitutive expression of IFN-γ mRNA in children with AD. Any role for IL-12 is likely to be limited, however, as IL-12-dependent IFN-γ induction has been shown to be insensitive to inhibition by CsA [56].

Examination of IFN-γ mRNA in stimulated cultures revealed equivalent expression in patients and controls. This is similar to the findings of Gauchat *et al.*, who reported equivalent expression of IFN-γ mRNA in pokeweed mitogen (PWM) or allergen-stimulated PBMC from monoallergic patients and controls [27]. Contrary to our finding of spontaneous IFN-γ expression in unstimulated cultures from children with AD, however, the latter study did not detect IFN-γ mRNA in unstimulated cultures from allergic patients. This could relate to the reduced sensitivity of Northern blot analysis which was used in that study compared with PCR-assisted mRNA amplification. Alternatively, the contrasting results may reflect differences in the patient groups studied, as we examined children with severe active dermatitis at the time of testing, whereas the latter study involved individuals reactive to a single allergen, and the activity of disease in those patients was not reported.

Although we have found comparable expression of IFN-γ mRNA in stimulated cultures from patients and controls, secretion of IFN-γ in stimulated cultures was confirmed to be reduced in the children with AD. Furthermore, despite an increased expression of IFN-γ mRNA in unstimulated cultures from atopic patients, no spontaneous IFN-γ secretion was detected. This lack of correlation between mRNA expression and secretion is surprising, as previous studies of lymphocytes from normal adults have shown that secretion of IFN-γ is predominantly controlled by the rate of IFN-γ mRNA transcription, and that IFN-γ mRNA expression correlates to secretion [17–19]. We have found a similar correlation in the non-atopic controls. The present observation that, in children with AD, IFN-γ mRNA expression did not reflect secretion is consistent with our previous finding of a discrepancy between numbers of IFN-γ-containing cells and IFN-γ secretion in such patients [9]. Evidence that secretion of IFN-γ is reduced *in vivo* in AD is provided by reports of diminished NK cell number and activity [30,31], depressed cutaneous cell-mediated responses [57] and increased susceptibility to cutaneous viral infections [58]. Recent studies documenting improvement in severe atopic dermatitis following treatment with recombinant IFN-γ [6–8] would also support this. Thus, the discrepancy between IFN-γ transcription and secretion presently demonstrated for children with atopic dermatitis suggests that reduced secretion of IFN-γ does not relate to abnormal regulation of transcription, but rather to a post-transcriptional defect(s) of IFN-γ production.

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REFERENCES

1 Tang M, Kemp A, Varigos G. IL-4 and interferon-gamma production in children with atopic disease. Clin Exp Immunol 1993; **92**:120–4.

2 Rousset F, Robert J, Andary M, *et al.* Shifts in interleukin-4 and interferon-gamma production by T cells of patients with elevated serum IgE levels and the modulatory effects of these lymphokines on spontaneous IgE synthesis. J Allergy Clin Immunol 1991; **87**:58–69.

3 Reinhold U, Pawelec G, Wehrmann W, Kukel S, Oehr P, Kreysel HW. Cytokine release from cultured peripheral blood mononuclear cells of patients with severe atopic dermatitis. Acta Derm Venereol Stockh 1989; **69**:497–502.

4 Wehrmann W, Reinhold U, Pawelec G, Wernet P, Kreysel HW. *In vitro* generation of IFN-gamma in relationship to *in vivo* concentration of IgE and IgG subclasses and Fc epsilon Rl/CD23 positive circulating lymphocytes in patients with severe atopic dermatitis (AD). Acta Derm Venereol Suppl Stockh 1989; **144**:127–30.

5 Jujo K, Renz H, Abe J, Gelfand EW, Leung DY. Decreased interferon gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. J Allergy Clin Immunol 1992; **90**:323–31.

6 Hanifin JM, Schneider LC, Leung DY, *et al.* Recombinant interferon gamma therapy for atopic dermatitis. J Am Acad Dermatol 1993;**28**:189–97.

7 Reinhold U, Wehrmann W, Kukel S, Kreysel HW. Recombinant interferon-gamma in severe atopic dermatitis (letter). Lancet 1990; **335**:1282.

8 Boguniewicz M, Jaffe HS, Izu A, *et al.* Recombinant gamma interferon in treatment of patients with atopic dermatitis and elevated IgE levels. Am J Med 1990; **88**:365–70.

9 Tang MLK, Kemp AS. Production and secretion of interferon-gamma (IFN-γ) in children with atopic dermatitis. Clin Exp Immunol 1994; **95**:66–72.

10 Reinhold U, Wehrmann W, Kukel S, Kreysel HW. Evidence that defective interferon-gamma production in atopic dermatitis patients is due to intrinsic abnormalities. Clin Exp Immunol 1990; **79**:374–9.

11 Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 1984; **308**:693–8.

12 Truneh A, Albert F, Golstein P, Schmitt Verhulst AM. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 1985; **313**:318–20.

13 Kristensson K, Borrebaeck CA, Carlsson R. Human $CD4^+$ T cells expressing CD45RA acquire the lymphokine gene expression of $CD45RO^+$ T-helper cells after activation *in vitro*. Immunology 1992; **76**:103–9.

14 Pisa EK, Pisa P, Hansson M, Wigzell H. OKT3-induced cytokine mRNA expression in human peripheral blood mononuclear cells measured by polymerase chain reaction. Scand J Immunol 1992; **36**:745–9.

15 Rajka G. Diagnosis and grading (severity). In: Rajka G, ed. Essential aspects of atopic dermatitis. Berlin: Springer Verlag, 1989: 216–22.

16 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; **162**:156–9.

17 Taniguchi T. Regulation of cytokine gene expression. Ann Rev Immunol 1988; **6**:439–64.

18 Efrat S, Pilo S, Kaempfer R. Kinetics of induction and molecular size of mRNAs encoding human interleukin-2 and gamma-interferon. Nature 1982; **297**:236–9.

19 Kronke M, Leonard WJ, Depper JM, Greene WC. Sequential expression of genes involved in human T lymphocyte growth and differentiation. J Exp Med 1985; **161**:1593–8.

20 Lewis DB, Yu CC, Meyer J, English BK, Kahn SJ, Wilson CB. Cellular and molecular mechanisms for reduced interleukin 4 and interferon-gamma production by neonatal T cells. J Clin Invest 1991; **87**:194–202.

21 Rutenfranz I, Kruse A, Kirchner H. *In situ* hybridization of interferon-gamma producing peripheral blood mononuclear cells. Scand J Immunol 1991; **34**:169–78.

22 Sadouk M, Vaquero C, de la Tour B, Amor B, Toubert A. Interferon-gamma mRNA expression upon *in vitro* T lymphocyte activation is decreased in rheumatoid arthritis patients. Clin Immunol Immunopathol 1990; **56**:37–45.

23 Chopra RK, Holbrook NJ, Powers DC, McCoy MT, Adler WH, Nagel JE. Interleukin 2, interleukin 2 receptor, and interferon-gamma synthesis and mRNA expression in phorbol myristate acetate and calcium ionophore A23187-stimulated T cells from elderly humans. Clin Immunol Immunopathol 1989; **53**:297–308.

24 Buchan G, Barrett K, Fujita T, Taniguchi T, Maini R, Feldmann M. Detection of activated T cell products in the rheumatoid joint using cDNA probes to interleukin-2 (IL-2) IL-2 receptor and IFN-gamma. Clin Exp Immunol 1988; **71**:295–301.

25 Gauchat JF, Gauchat D, De Weck AL, Stadler BM. Cytokine mRNA levels in antigen-stimulated peripheral blood mononuclear cells. Eur J Immunol 1989; **19**:1079–85.

26 Qiu G, Gauchat JF, Wirthmuller U, De Weck AL, Stadler BM. Lymphokine production by human peripheral blood lymphocytes: analysis by *in situ* hybridization. Lymphokine Res 1988; **7**:385–92.

27 Gauchat JF, Gauchat D, Qiu G, Mandallaz M, Stadler BM. Detection of cytokine mRNA in polyclonally-, antigen- or allergen-stimulated mononuclear cells. Immunol Rev 1991; **119**:147–61.

28 Farrar MA, Schreiber RD. The molecular cell biology of interferon-γ and its receptor. Annu Rev Immunol 1993; **11**:571.

29 Handa K, Suzuki R, Matsui H, Shimizu Y, Kumagai K. Natural killer (NK) cells as a responder to interleukin 2 (IL2). II. IL2-induced interferon gamma production. J Immunol 1983; **130**:988–92.

30 Jensen JR, Sand TT, Jorgensen AS, Thestrup Pedersen K. Modulation of natural killer cell activity in patients with atopic dermatitis. J Invest Dermatol 1984; **82**:30–34.

31 Wehrmann W, Reinhold U, Kukel S, Franke N, Uerlich M, Kreysel HW. Selective alterations in natural killer cell subsets in patients with atopic dermatitis. Int Arch Allergy Appl Immunol 1990; **92**:318–22.

32 Sandvig S, Laskay T, Andersson J, De Ley M, Andersson U. Gamma-interferon is produced by $CD3^+$ and $CD3^-$ lymphocytes. Immunol Rev 1987; **97**:51–65.

33 Thestrup Pedersen K, Larsen CS, Kristensen M, Zachariae C. Interleukin-1 release from peripheral blood monocytes and soluble interleukin-2 and CD8 receptors in serum from patients with atopic dermatitis. Acta Derm Venereol Stockh 1990; **70**:395–9.

34 Wuthrich B, Joller Jemelka H, Helfenstein U, Grob PJ. Levels of soluble interleukin-2 receptors correlate with the severity of atopic dermatitis. Dermatologica 1990; **181**:92–97.

35 Colver GB, Symons JA, Duff GW. Soluble interleukin 2 receptor in atopic eczema. BMJ 1989; **298**:1426–8.

36 Toshitani A, Ansel JC, Chan SC, Li SH, Hanifin JM. Increased interleukin 6 production by T cells derived from patients with atopic dermatitis. J Invest Dermatol 1993; **100**:299–304.

37 Leung DY, Bhan AK, Schneeberger EE, Geha RS. Characterization of the mononuclear cell infiltrate in atopic dermatitis using monoclonal antibodies. J Allergy Clin Immunol 1983; **71**:47–56.

38 Hurwitt E. Dermatopathic lymphadenitis: focal granulomatous lymphadenitis associated with chronic generalized skin disorders. J Invest Dermatol 1942; **5**:197–204.

39 Hoeger PH, Lenz W, Boutonnier A, Fournier JM. Staphylococcal skin colonization in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. J Infect Dis 1992; **165**:1064–8.

40 Parronchi P, Macchia D, Piccinni MP *et al.* Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. Proc Natl Acad Sci USA 1991; **88**:4538–42.

41 Wierenga EA, Snoek M, de Groot C *et al.* Evidence for compartmentalization of functional subsets of $CD2^+$ T lymphocytes in atopic patients. J Immunol 1990; **144**:4651–6.

42 Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J Clin Invest 1986; **77**:860–7.

43 Damle NK, Leytze G, Klussman K, Ledbetter JA. Activation with superantigens induces programmed death in antigen-primed $CD4^+$ class II^+ major histocompatibility complex T lymphocytes via a CD11a/CD18-dependent mechanism. Eur J Immunol 1993; **23**: 1513–22.

44 Wakasugi N, Virelizier JL. Defective IFN-gamma production in the human neonate. I. Dysregulation rather than intrinsic abnormality. J Immunol 1985; **134**:167–71.

45 Herrmann T, Baschieri S, Lees RK, MacDonald HR. *In vivo* responses of $CD4^+$ and $CD8^+$ cells to bacterial superantigens. Eur J Immunol 1992; **22**:1935–8.

46 Waalen K, Sioud M, Natvig JB, Forre O. Spontaneous *in vivo* gene transcription of interleukin-2, interleukin-3, interleukin-4, interleukin-6, interferon-gamma, interleukin-2 receptor (CD25) and proto-oncogene c-myc by rheumatoid synovial T lymphocytes. Scand J Immunol 1992; **36**:865–73.

47 Maurer D, Felzmann T, Holter W, Petera P, Smolen J, Knapp W. Evidence for the presence of activated CD4 T cells with naive phenotype in the peripheral blood of patients with rheumatoid arthritis. Clin Exp Immunol 1992; **87**:429–34.

48 Peleman R, Wu J, Fargeas C, Delespesse G. Recombinant interleukin 4 suppresses the production of interferon gamma by human mononuclear cells. J Exp Med 1989; **170**:1751–6.

49 Vercelli D, Jabara HH, Lauener RP, Geha RS. IL-4 inhibits the synthesis of IFN-gamma and induces the synthesis of IgE in human mixed lymphocyte cultures. J Immunol 1990; **144**:570–3.

50 Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cells. IV: TH2 clones secrete a factor that inhibits cytokine production by TH1 clones. J Exp Med 1989; **170**:2081–95.

51 Moore KW, Vieira P, Fiorentino DF, Trounstine ML, Khan T, Mosmann TR. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein–Barr virus gene BCRF1. Science 1990; **248**:1230–4.

52 Arya SK, Wong-Staal F, Gallo RC. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. J Immunol 1984; **133**:273–6.

53 Kasahara T, Hooks JJ, Dougherty SF, Oppenheim JJ. Interleukin 2-mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. J Immunol 1983; **130**:1784–9.

54 Pearlstein KT, Palladino MA, Welte K, Vilcek J. Purified human interleukin-2 enhances induction of immune interferon. Cell Immunol 1983; **80**:1–9.

55 Kobayashi M, Fitz L, Ryan M *et al.* Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 1989; **170**:827–45.

56 Chan SH, Kobayashi M, Santoli D, Perussia B, Trinchieri G. Mechanisms of IFN-gamma induction by natural killer cell stimulatory factor (NKSF/IL-12): role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. J Immunol 1992; **148**:92–98.

57 Bos JD, Wierenga EA, Sillevis Smitt H, van der Heijden FL, Kapsenberg ML. Immune disregulation in atopic eczema. Arch Dermatol 1992; **128**:1509–12.

58 Hanifin JM, Lobitz WC. Newer concepts of atopic dermatitis. Arch Dermatol 1972; **113**:663–70.