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Analysis of cytokine gene expression in stimulated T cells of small children by semi-quantitative PCR

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ONLY limited amounts of peripheral blood samples can be obtained from small children. Therefore, a polymerase chain reaction (PCR) aided analysis of cytokine gene expression by PBMC or T cells is a valuable tool. We present a combination of procedures to obtain an accurate estimation of the expression of the cytokines IL-4 and IFN-γ. This can be performed on T cells purified from blood samples of up to 5 ml in volume from children aged 0–4 years with allergic asthma and atopic dermatitis. This procedure includes multiple sampling of PCR products to determine the linear phase of the PCR; inter-experiment correction using a helper T-cell clone, expressing both IL-4 and IFN-γ; inter-patient correction by comparing the expression of a housekeeping gene (*HPRT*); and finally the development of specific software to analyse densitometric data obtained by scanning photographs of agarose gels, separating PCR products. In this way it is possible to study cytokine gene expression from a very small amount of material.

Key words: Children, Cytokine, PCR, Small volumes, T cells

Introduction

Allergic diseases such as allergic asthma and atopic dermatitis are generally characterized by increased IgE levels in the serum.[1–3] Cytokines play an important role in the regulation of IgE synthesis.[4] IL-4 is an inducer of IgE synthesis, while IFN-γ suppresses IgE synthesis.[5,6] Other cytokines play an additional role in the regulation of IgE. IL-5 has an enhancing effect on the stimulatory activity of IL-4[7] and IL-10 has a down-modulating effect on the production of IFN-γ.[8] IL-13 is a recently discovered cytokine, produced by different T-cell subsets, with IgE inducing activities comparable with IL-4.[9,10]

The presence of IL-4 and IFN-γ has been demonstrated in cultured peripheral blood mononuclear cells (PBMC) from healthy individuals, atopic subjects and patients with the hyper-IgE syndrome.[11] Many studies have analysed cytokine production profiles of cultured and stimulated PBMC of allergic patients by ELISA,[12,13] or production of cytokines by cloned T cells.[14,15] However, only a few studies have described cytokine mRNA expression.[16,17] Ehlers *et al.* studied cytokine expression in neonatal T cells, compared with adult T cells, by PCR analysis. These authors showed that cord blood T cells are able, upon stimulation *in vitro*, to transcribe IL-2 mRNA. However, their capacity to transcribe mRNA for IL-3, IL-4, IL-5, IL-6, IFN-γ and GM–CSF was markedly reduced in comparison with that in adults.[16]

Our main interest is the role of cytokines in the development of allergy during infancy, since the immune system of children is generally considered to differ from the system in adults.[19] The number of T cells is relatively low and IFN-γ production by T-cells is decreased, in comparison with IFN-γ production in adults.[18,19]

We chose to analyse cytokine gene expression in young children (0–4 years), who are developing allergic asthma or atopic dermatitis, in order to study the pathogenesis of these diseases. We aim to correlate the mRNA expression levels in PBMC and T cells with the cytokine production profile and clinical manifestations (manuscript in preparation).

To make such studies feasible, T cells were purified from peripheral blood.[20] The amount of peripheral blood obtained from young children is usually small, and the number of T cells that can be purified is therefore limited. Moreover, cytokine gene expression is usually transient and cytokine mRNA levels generally occur at low abundance. Therefore, such analysis requires sensitive procedures.[21] The polymerase chain reaction (PCR)[22,23] is a sensitive method for the detection of gene expression. Here we describe optimizations of the PCR method to analyse cytokine gene expression occurring at low abundance and from a limited number of cells, in a semi-quantitative way.[24,25]

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Materials and Methods

Purification and stimulation of T cells: PBMC from a maximum of 4 ml heparinized blood of children, were purified by density centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden).[26] The children ranged from 0–4 years of age and were healthy or with allergic asthma. These PBMC were incubated (30 min, 4°C) with monoclonal antibodies specific for monocytes (My4, 100 μl (40 μg/ml) per 1 × 10^7 pelleted cells, Coulter Cytometry, FL), B cells (CD19, 100 μl (100 μg/ml) per 1 × 10^7 pelleted cells, Coulter) and natural killer cells (CD16 and CD56PE, both 50 μl (100 μg/ml) per 1 × 10^7 pelleted cells, Becton Dickinson, Mountain View, CA). Subsequently, the samples were incubated (30 mins, 4°C on a roller bank) with sheep–anti-mouse Dynabeads (IgG M450, Dynal, Oslo, Norway). T cells were negatively selected by using a magnet (Dynal). The purity of the T-cell fraction was between 85% and 95%, as determined by FACS analysis, after staining with CD3-specific antibodies (Leu-4-FITC, 50 μl (2.5 μg/ml) per 5 × 10^5 pelleted cells, Becton Dickinson). The contribution of natural killer cells was always less than 3.5%.

T cells (1 × 10^6 cells/ml) were cultured for 16–18 h in Yssel's medium[27] containing 1% human serum with and without the addition of 4-bromo-calcium-ionophore (A23187, final concentration 500 ng/ml, Sigma, St. Louis, MO) and TPA (phorbol-12-myristate-13-acetate, final concentration 1 ng/ml, Sigma) at 37°C, 5% CO_2.

RNA isolation and cDNA reaction: After spinning down the T cells, RNA was isolated from the cells by the RNAzol B (Cinna-Biotecx Laboratories Inc., Houston, TX) method.[28] Briefly, per 1 × 10^6 pelleted cells 200 μl RNAzol (minimum 300 μl) was added as well as chloroform (10 μl per 100 μl RNAzol B). After vigorous shaking and centrifugation, the aqueous phase was collected and an equal volume of phenol:chloroform (1:1) was added. After mixing and centrifugation, an equal volume of chloroform was added to the aqueous phase. Following centrifugation, an equal volume of isopropanol was added to the aqueous phase. After incubation at 4°C for at least 2 h, the RNA was pelleted by centrifugation and washed with 20 μl 70% ethanol per 1 × 10^6 cells (minimum 50 μl). The RNA was resuspended in 10 μl water and the OD_{260}/OD_{280} were determined by spectrophotometry (Ultrospec III, Pharmacia-LKB). Twenty μg glycogen (Boehringer Mannheim, Germany) was added during the phenol:chloroform extraction as a carrier.

cDNA synthesis[29] was performed starting with 1 μg RNA, after heating for 10 min at 65°C. Random hexamer primers were used to ensure all RNA was represented equally in the cDNA pool.[30] The reaction mixture contained 2 μl of 10× AMV–RT buffer (0.5 M Tris–HCl, 0.1 M $MgCl_2$, 0.5 M DTT, 10 mM EDTA and 100 μg/ml BSA; pH 8.3), 0.25 mM of each dNTP, 1 mM salmon spermine HCl (Sigma), 40 units of RNAsin (Promega, Madison, WI), 2.5 OD $(dN)_6$ (Pharmacia), 0.2 μg oligo$(dT)_{12-18}$ (Boehringer) and 5 units reverse transcriptase (from avian myeloblastosis virus; Boehringer). The total reaction volume was 20 μl. Incubation was performed for 1 h at 41°C. Afterwards the cDNA was diluted to 200 μl and stored at –70°C.

Polymerase chain reaction: For the polymerase chain reaction, a mixture was prepared containing 50 ng cDNA (in 10 μl) and 10 μl of 10× Taq polymerase buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM $MgCl_2$ and 1% gelatin; pH 8.3), 0.03 mM of each dNTP and 1 unit of Taq polymerase (Ampli Taq, Perkin Elmer Cetus, Norwalk CT). One μl of the following sense- and anti-sense primers (10 OD/ml) were used:

HPRT	sense:	5′-GTGATGATGAACCAGG-TTTATGACCTT-3′ (exon2),
	antisense:	5′-CTTGCGACCTTGACCAT-CTTTGGA-3′ (exon 6),
	(product size 454 bp)[31]	
IL-4	sense:	5′-ACTCTGTGCACCGAGTT-GACCGTAA-3′ (exon 2),
	antisense:	5′-TCTCATGATCTGCTTTAG-CCTTTCC-3′ (exon 4),
	(product size 300 bp)[32]	
IFN-γ	sense:	5′-TTTAATGCAGGTCATTCA-GATG-3′ (exon 1–2)
	antisense:	5′-CAGGGATGCTTCTTCGA-CCTCGAAAC-3′ (exon 4)
	(product size 388 bp)[32]	

We developed the *HPRT* (human hypoxanthine phosphoribosyl transferase) primerset from the genomic structure, by selecting primers on exon 2 and 6. In this way, the product including the intron sequences will be too large (3.5 kB) to be amplified by PCR. The IL-4 and IFN-γ primersets were kindly donated by Dr R. de Waal Malefijt (DNAX Research Institute, Palo Alto, CA). It was verified by Southern blot analysis that both primersets did not amplify genomic DNA (data not shown).

The total reaction volume was 100 μl. As a negative control 50 ng RNA in 10 μl water, which

$$\text{IL-4/IFN-}\gamma\text{ scan value sample}^{*}\frac{1}{\text{B21 correction factor IL-4/IFN-}\gamma}$$

VII. Calculation of mean values of each sample

IL-4, 40–45 cycles and IFN-γ: 30–35 cycles

VIII. Calculation of the end values corrected for B21 and HPRT

$$\text{Mean sample value IL-4/IFN-}\gamma^{*}\frac{1}{\text{correction factor HPRT}}$$

Southern blotting: Subsequently, the gel was put into an acid solution (0.25 M HCl) for 10 min, a denaturing solution (1 M NaCl, 0.5 M NaOH) twice for a period of 15 min, and a neutralizing solution (0.5 M Tris–HCl, 1.5 M NaCl, pH 7.0) twice for 20 min. After this a Southern transfer[35] was performed, by which the PCR products were transferred overnight to a Nytran filter (0.45 μm, Schleicher and Schuell, Dassel, Germany). DNA was crosslinked to the filter in a UV crosslinker (120 mJ) (UV Stratalinker 2400, Stratagene, La Jolla, CA).

Probes to the inner region of the amplification target were end-labelled with ^{32}ATP (see Reference 37) and hybridization was carried out.[38] The following probes were used:

HPRT: 5′-GAAGAGCTATTGTAATGACCAGTCA-3′ (exon 3–4)[31]

IL-4: 5′-CAGTTCCACAGGCACAAGCAG-3′ (exon 3)[32]

IFN-γ: 5′-TGACTAATTATTCGGTAACTGACTTG-AATG-3′ (exon 3–4)[32]

Results

Semi-quantitative PCR analysis of cytokine gene expression: Initially, three different housekeeping genes, HPRT[31], β-actin[39] and GAPDH (glyceraldehyde-3-phosphate dehydrogenase),[40] were tested on cDNA of different cell populations of healthy control children. The HPRT primer set was considered the most reliable, since it did not amplify genomic DNA, as tested with Southern blot analysis and hybridization. Furthermore, HPRT had a stable expression level in the same range as the cytokine levels studied (data not shown).

Next, T cells of a healthy control child were purified and cultured overnight in medium alone or stimulated polyclonally by the addition of Ca-ionophore and TPA. RT–PCR analysis was performed using primer sets specific for HPRT and IFN-γ. A representative result is shown in Fig. 1. After 25 cycles (HPRT) or 20 cycles (IFN-γ), 10 μl samples were taken from the PCR mixture and subsequently every five cycles up to 45 or 40 cycles, respectively. The PCR products were made visible by means of agarose gel electrophoresis.

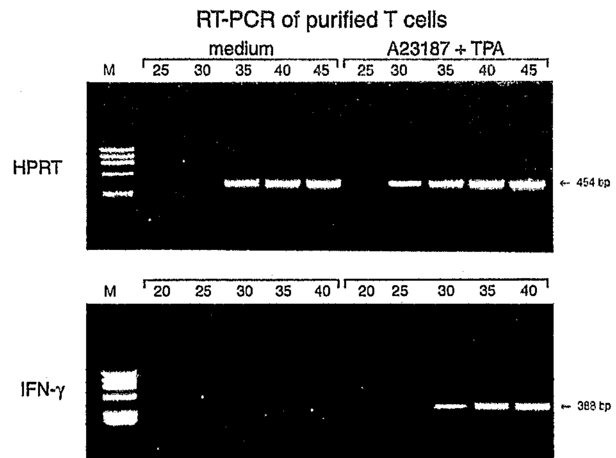

FIG. 1. RT–PCR of purified T cells. RNA was isolated from purified T cells of a healthy child. These cells had been cultured overnight in medium alone or stimulated with A23187 (Ca-ionophore) and TPA. After reverse transcription into cDNA, a PCR was performed for HPRT and IFN-γ. Samples were collected from the reaction tube after different numbers of cycles. For HPRT this was done at 25, 30, 35, 40 and 45 cycles and for IFN-γ at 20, 25, 30, 35, and 40 cycles. The HaeIII digest of PhiX174 was used as a molecular weight marker (M).

Visual analysis enabled qualitative observations like IFN-γ mRNA production by T cells after stimulation with Ca-ionophore and TPA. A differential mRNA expression was observed when comparing IFN-γ expression in T cells cultured in medium alone or stimulated with Ca-ionophore and TPA. The IFN-γ mRNA signal in the stimulated T-cell fraction was higher (already visible after 25 PCR cycles) than in the T-cell fraction cultured with medium alone (visible only after 35 cycles). In the same experiment, the HPRT mRNA appeared to be present in both samples in equal amounts.

It is possible to quantify these products by scanning the photographs and assigning values to the intensities of the different bands. To this end, the photograph of the agarose gel (Fig. 1) was scanned with a scanner and the image was analysed by the described software. The corresponding scan values are shown in Table 1. These results also reflect the differential expression shown in Fig. 1, but now in absolute numbers. For example, the scan values of the IFN-γ mRNA signal after 40 PCR cycles in stimulated T cells *vs*

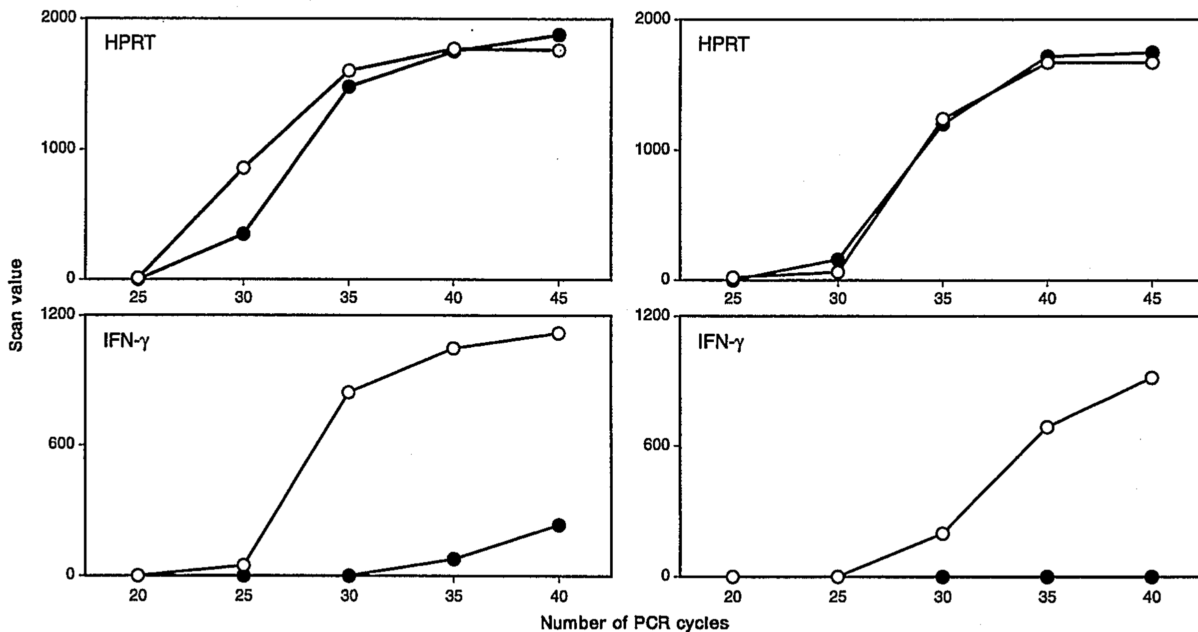

FIG. 3. Analysis of HPRT and IFN-γ mRNA expression in a healthy and an asthmatic child. T cells were purified from peripheral blood of a healthy and an asthmatic child. T cells were cultured overnight in medium alone (●) or with the addition of A23187 (Ca-ionophore) and TPA (○). RT-PCR was performed for: a healthy child for HPRT (upper left), an asthmatic child for HPRT (upper right), a healthy child for IFN-γ (lower left) and an asthmatic child for IFN-γ (lower right).

Table 2. Reproducibility of the hand scanner

Band	Mean	S.D.	*n*
1	665.2	43.57	10
2	1380.8	98.43	10
3	1514.2	169.35	10

One lane of an 1.2% agarose gel with 75 μg/500 ml ethidium bromide was scanned ten times. Arithmetic mean and S.D. were calculated for three bands with different levels of intensity.

ducibility of the PCR of B21 cDNA was high. For this reason, only small inter-experiment corrections (mean 0.77 ± 0.14) were generally necessary. The mean correction factor for HPRT mRNA expression of 20 samples was 0.81 ± 0.21. In case the correction factors for B21 and HPRT were smaller than 0.5, or greater than 1.5, we considered the data not to be reliable because of possible overcorrection. The results of such experiments (in the case of B21) or samples (in the case of HPRT) were excluded from further analysis.

Application of the technique to patient samples: T cells were purified from peripheral blood of healthy and asthmatic children. The T cells were cultured overnight in medium alone or stimulated with a Ca-ionophore and TPA. After RNA isolation and cDNA synthesis, PCR was performed for HPRT, IL-4 and IFN-γ during the described number of PCR cycle times. In Fig. 3 a representative example is shown of one healthy child and one asthmatic child. The shapes of the curves for HPRT were comparable, both for T cells cultured in medium and for stimulated T cells. All curves reached a similar plateau level (1766 ± 74). In both the healthy control (lower left) and the asthmatic child (lower right) there was a marked difference in IFN-γ mRNA expression between stimulated and unstimulated T cells. In stimulated T cells from the healthy control, the plateau phase of IFN-γ PCR was reached earlier (35 PCR cycles) than in the stimulated T cells of the asthmatic child (40 PCR cycles), indicating a higher expression of IFN-γ mRNA. In the unstimulated condition, no IFN-γ expression was found in the T cells from the asthmatic child, whereas after 35 and 40 PCR cycles IFN-γ expression could be detected in T cells from the healthy control. The linear range of the PCR as assessed in B21 cells, was also applicable to HPRT and cytokine gene expression in patient samples of stimulated and unstimulated T cells.

In Table 3 the correction method is applied on the scan values of the healthy and asthmatic children presented in Fig. 3. First, the inter-experiment variation was determined by calculating the B21 correction factors for HPRT and IFN-γ. As shown in Table 3, the inter-experiment correction factor for HPRT was similar for the healthy and the asthmatic child samples (0.76 *vs*

sary. When the variation is too large, over-correction can occur. We consider a correction factor smaller than 0.5 or larger than 1.5 too extreme to detect the cytokine response in the sample reliably.

The analysis developed here is a reliable and simple alternative to other methods based on the use of radioactivity, like phosphor imaging.[45] The variation found in cytokine expression within a patient group of, for example, healthy children of the same age is relatively high (Koning *et al.*, manuscript in preparation). The accuracy of the semi-quantitative method described here has sufficient discernment in relation to this extent of variation.

Recently, true quantitative methods have been developed. The method developed by Gilliland *et al.*[46] uses an internal standard with the same primer requirement, but differing in the size of PCR product. However, the accuracy of quantification may be affected by sequence differences between the DNAs used for the standard and the sample.[47]

When studying the intrinsic capacity of cells to express a particular cytokine gene, it is necessary to analyse highly purified cell populations. However, many studies examine the significance of cytokines in patients by analysing the total PBMC fraction only.[11,13] When endogenous cytokine gene expression is to be studied in purified T cells, it is important to note that the T cells should not be stimulated during purification. Indeed, negative selection during purification of T cells was found to be adequate to avoid stimulation. It is also important that for some cytokines, e.g., IFN-γ, cells need to be stimulated to obtain detectable expression levels. In our study, we used a polyclonal stimulus, Ca-ionophore and TPA, permitting the simultaneous detection of a broad range of cytokines. For such studies allergen specific stimulation can also be applied (manuscript in preparation).

We conclude that it is possible to accurately detect differential cytokine gene expression in T cells isolated from blood of healthy controls and asthmatic children. This can be achieved by using a RT–PCR with differential cycle times for different cytokines and analysing the results with a scanner and specially designed computer software. Cytokine gene expression can thus be studied in a very limited amount of material.

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