Utility of the Housekeeping Genes 18S rRNA, β-Actin and Glyceraldehyde-3-Phosphate-Dehydrogenase for Normalization in Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis of Gene Expression in Human T Lymphocytes

A. Bas*†, G. Forsberg*†‡, S. Hammarström* & M.-L. Hammarström*

*Department of Clinical Microbiology, Immunology; and ‡Department of Clinical Sciences, Pediatrics, Umeå University, Umeå, Sweden

Received 12 April 2004; Accepted in revised form 18 March 2004

Correspondence to: Dr Marie-Louise Hammarström, Department of Clinical Microbiology, Immunology, Umeå University, Umeå, Sweden. E-mail: marie-louise.hammarstrom@climi.umu.se

Abstract

The accuracy of 18S rRNA, β-actin mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as indicators of cell number when used for normalization in gene expression analysis of T lymphocytes at different activation stages was investigated. Quantitative real-time reverse transcriptase-polymerase chain reaction was used to determine the expression level of 18S rRNA, β-actin mRNA, GAPDH mRNA and mRNA for six cytokines in carefully counted samples of resting human peripheral blood mononuclear cells (PBMCs), intestinal lymphocytes and PBMCs subjected to polyclonal T-cell activation. The 18S rRNA level in activated and resting PBMCs and intestinal lymphocytes was essentially the same, while the levels of β-actin and GAPDH mRNAs fluctuated markedly upon activation. When isolated $\gamma\delta TCR^+$, $CD4^+$ and $CD8^+$ subpopulations were studied, 18S rRNA levels remained unchanged after 21 h of activation but increased slightly after 96 h. In contrast, there was a 30–70-fold increase of GAPDH mRNA/cell in these cell populations upon activation. Cytokine analysis revealed that only normalization to 18S rRNA gave a result that satisfactorily reflected their mRNA expression levels per cell. In conclusion, 18S rRNA was the most stable housekeeping gene and hence superior for normalization in comparative analyses of mRNA expression levels in human T lymphocytes.

Introduction

Estimations of gene expression at the mRNA level and comparative analysis of mRNA expression levels of genes of interest in different cells and tissues require methods for accurate normalization. To this end, levels of mRNA for basic structural proteins and genes necessary to maintain basic cellular functions, as well as structural RNA expressed in essentially all cell types have been used as internal standards. Commonly used so-called housekeeping genes are β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA [1]. The development of real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) techniques has generated assays that allow quantitative determination of low concentrations of mRNA of interest [2–5]. Because cell counting is sometimes difficult or impossible, such analysis has to be combined with reliable determinations of RNA for housekeeping genes. A prerequisite for usefulness in normalization is that the expression level of the housekeeping gene does not vary markedly through the cell cycle or in response to different experimental conditions. Studies in experimental animal models clearly indicate significant variations in expression levels of housekeeping genes between tissues and unstable expression in relation to experimental conditions [2, 6–13]. Studies evaluating the expression levels of β-actin mRNA, GAPDH mRNA and 18S rRNA in human cells are few but indicate that GAPDH mRNA levels may vary between different carcinomas, carcinoma cell

†The first two authors contributed equally to this work.

lines as well as normal and cancer tissue biopsies [14–21]. Moreover, one study reported changes in β-actin and GAPDH mRNA levels during epithelial differentiation [22]. Changes in housekeeping gene expression levels may also be expected in lymphocytes upon immune activation. Unawareness of variations in expression levels of the gene used for normalization might even lead to misinterpretations of cellular responses in immune reactions.

The purpose of this study was to determine whether the expression levels of β-actin mRNA, GAPDH mRNA and 18S rRNA are fluctuating during activation of human T lymphocytes. Peripheral blood mononuclear cells (PBMCs) were stimulated by a polyclonal T-cell activator, and the cellular content of the three housekeeping genes was estimated by combination of cell counting and real-time qRT-PCR determinations of β-actin mRNA, GAPDH mRNA and 18S rRNA levels. The cytokines interleukin-2 (IL-2), IL-4, IL-10, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) were chosen as model genes in the analysis of mRNA expression levels.

Materials and methods

Cells and tissues. PBMCs were isolated from 15 healthy adult blood donors (median age 35 years; range 26–50 years) by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. Intestinal lymphocytes were isolated from surgical samples of apparently normal jejunal tissue obtained from six adult patients (median age 64 years; range 58–68 years) undergoing bowel resection for cancer conditions, intestinal bleeding, ulcus or strangi ileus. Intraepithelial and lamina propria lymphocytes were isolated as previously described [23, 24]. All study subjects gave their informed consent.

Antibodies. Anti-CD3 monoclonal antibody (MoAb) OKT3 was purified from culture supernatant of the hybridoma cell line (American Type Culture Collection, Rockville, MD, USA) and used for polyclonal T-cell stimulation.

Paramagnetic beads used for cell fractionation were Dynabeads M-450 coated with goat-antimouse IgG (Dynal, Oslo, Norway) and charged with a mixture of pan δ-chain MoAbs Immun510 (Immunotech, Marseille, France) and 5A6.E9 (Serotec, Kidlinton, Oxford, UK) and pan γ-chain MoAb γ3.20 (Serotec) or Dynabeads M-450 directly coated with anti-CD4 MoAb (Dynal) and anti-CD8 MoAb (Dynal).

In vitro activation of blood T lymphocytes. PBMCs were suspended in HEPES-buffered RPMI-1640 containing 0.4% human serum albumin and antibiotics and incubated at a concentration of 1×10^6 cells/ml in the presence of anti-CD3 MoAb OKT3 (100 ng/ml). Parallel cultures of $2\text{-}4 \times 10^6$ cells were incubated for 4, 7, 21, 45, 72 and 96 h at 37 °C in humid air with 5% CO_2. $\gamma\delta TCR^+$, $CD4^+$ and $CD8^+$ cells were retrieved from PBMCs before stimulation and after 21 and 96 h stimulation with anti-CD3 MoAb OKT3 by sequential positive selection using magnetic beads charged with MoAb specific for TCRγ- and δ-chain, CD4 and CD8 [25]. In order to ascertain accurate estimation of cell numbers, freshly isolated PBMCs and PBMCs activated for various time periods and T-cell subpopulations were counted in triplicate random samples by microscopic inspection in a Bürker chamber. The cell samples were re-suspended and new samples were taken for cell counting if cell clumps were seen. The cell samples were thereafter washed in RNase-free phosphate-buffered solution, snap frozen in liquid nitrogen and stored at −80 °C.

RNA preparation and real-time qRT-PCR. Total RNA was isolated from unstimulated and stimulated PBMCs, freshly isolated and stimulated T-cell subpopulations from blood and intestinal lymphocytes, all by the acid guanidinium–thiocyanate–phenol–chloroform method and suspended in RNase-free water containing 1 kU/ml RNasine ribonuclease inhibitor (Promega, Madison, WI, USA) as described [26].

Real-time qRT-PCR assays for 18S rRNA and GAPDH mRNA were purchased from Perkin-Elmer Applied Biosystems (Foster City, CA, USA). The 18S rRNA analyses were performed in two steps with reverse transcription using random hexamers followed by specific real-time PCR. The GAPDH analyses were performed according to the TaqMan EZ technology with specific reverse transcription using the 3′ primer and *Thermus thermophilus* DNA polymerase followed by PCR using the same enzyme. A real-time qRT-PCR assay was constructed for β-actin. The TaqMan EZ technology was used with sequence-specific primers placed in separate exons and a reporter dye-labelled probe hybridizing over an exon/exon boundary in the amplicon. The sequences were 5′-CCTCGCCTTTGCCGA-3′ for the 5′ primer, 5′-TGGTGCCTGGGGCG-3′ for the 3′ primer and 5′-CCGCCGCCCGTCCACACCCGCC-3′ for the probe. The position was chosen at a site for which no homologous intronless pseudogenes of the β-actin gene are described [27]. The assay did not give any signal when DNA purified from PBMCs was used as template (for IL-2, IL-4, IL-10, TNF-α, TGF-β and IFN-γ real-time qRT-PCR assays see References [28] and [5]). Samples were analysed in triplicate, and emission from released reporter dye was monitored by an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems). Cytokine mRNA levels are expressed as copies/μl as defined from triplicates of serial dilutions of RNA copy standards run in parallel. Levels of 18S rRNA, GAPDH mRNA and β-actin mRNA are expressed as units/μl as defined from triplicate serial dilutions of a pool of total RNA extracted from anti-CD3 MoAb OKT3-stimulated PBMCs. One unit was defined as the signal obtained by 10 pg of this RNA pool. All qRT-PCR assays for different RNA species were run separately. Because of the high concentration of 18S rRNA,

samples were diluted 1/10 before analysis of this RNA species.

Statistics. The significance of differences in expression levels of 18S rRNA and GAPDH mRNA in T-cell subsets was analysed using Student's *t*-test. Correlation analyses were performed using the Spearman rank correlation test. Two-tailed analyses were used throughout. A P value <0.05 was considered statistically significant.

Results

18s rRNA levels are stable in T cells during *in vitro* activation

PBMCs were isolated from seven individuals, and the cell number was estimated by counting the cells in triplicate samples. Parallel cell cultures of PBMCs polyclonally stimulated with anti-CD3 MoAb OKT3 were set up and harvested after 4, 7, 21, 45, 72 and 96 h. At each time point, careful cell counting and extraction of total RNA was performed. Each RNA sample was subsequently subjected to real-time qRT-PCR analyses for determination of 18S rRNA, β-actin mRNA and GAPDH mRNA expression levels. The 18S rRNA (units/cell) was similar in unstimulated and anti-CD3 MoAb-stimulated PBMCs (Fig. 1A), and the concentration of 18S rRNA correlated to the number of cells ($P < 0.0001$, $r = 0.6$; Fig. 2A). In contrast, expression levels of mRNA for both β-actin and GAPDH were affected by T-cell activation in most individuals (Fig. 1B,C), and no correlation was observed between the concentration of either β-actin mRNA or GAPDH mRNA and number of cells (Fig. 2B,C). The poor correlation between cell number and β-actin and GAPDH mRNA concentrations could not be explained as a consistent increase following T-cell activation. Instead, irregular response patterns with regard to both time course and magnitude of change at a given time point were observed. The relative expression levels, calculated as the ratio between the expression level per cell in activated cells and freshly isolated cells, increased markedly at the later time points in most individuals with medians of 3.1 and 4.5 for maximum relative increase for β-actin and GAPDH mRNAs, respectively (Fig. 3B,C). Some individuals had increased expression levels of both mRNA species, while others had unchanged levels of one but not the other. Furthermore, the time point for the peak of change differed between β-actin and GAPDH mRNAs and varied between individuals. In contrast, 18S rRNA levels showed a linear correlation to the number of cells over the entire time period for all but one individual with median values of relative change ranging between 0.8 and 1.6 (Fig. 3A).

Expression levels of 18s rRNA are similar in lymphocytes of blood and small intestine

Expression levels of 18S rRNA were determined in freshly isolated jejunal intraepithelial and lamina propria lymphocytes. The expression levels were almost identical to that of freshly isolated PBMCs, i.e. the median 18S rRNA units per 100 cells were 0.69 (range 0.58–1.70; $n = 5$) and 0.65 (range 0.21–1.38; $n = 6$) for intraepithelial and lamina propria lymphocytes, respectively, compared to 0.72 (range 0.30–1.71; $n = 7$) for PBMCs.

Expression levels of 18s rRNA and GAPDH mRNA in T-cell subsets after *in vitro* stimulation

$\gamma\delta TCR^+$, $CD4^+$, and $CD8^+$ cells were retrieved from freshly isolated PBMCs and PBMC stimulated with anti-CD3 MoAb for 21 and 96 h, respectively, by sequential treatment with magnetic beads charged with a mixture of anti-TCRδ-chain plus anti-TCRγ-chain MoAbs followed by anti-CD4 MoAb and finally anti-CD8 MoAb-charged

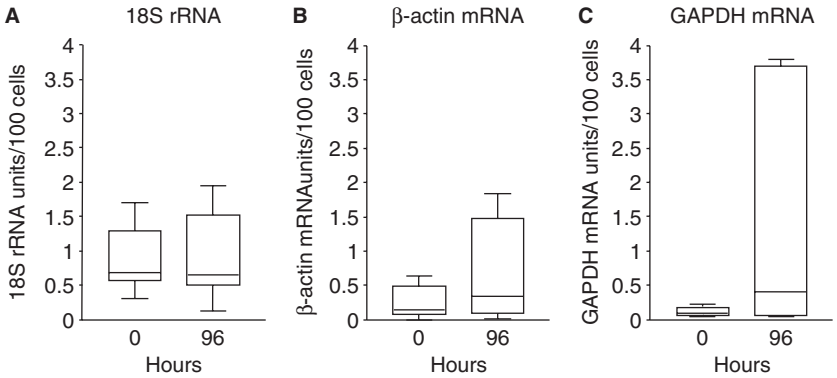

Figure 1 Expression levels of 18S rRNA and mRNA for β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in freshly isolated peripheral blood mononuclear cells (PBMCs) (0 h) and PBMCs stimulated for 96 h with the mitogenic anti-CD3 monoclonal antibody OKT3 (96 h). The cell number was carefully counted at both time points. Thereafter, RNA was extracted, and 18S rRNA, β-actin mRNA and GAPDH mRNA levels were determined by real-time quantitative reverse transcriptase-polymerase chain reaction. Results are expressed as RNA units of the respective housekeeping gene per 100 cells. Whiskers indicate range, boxes the 25th to 75th percentile and horizontal bars inside boxes the median. $n = 7$ at both time points.

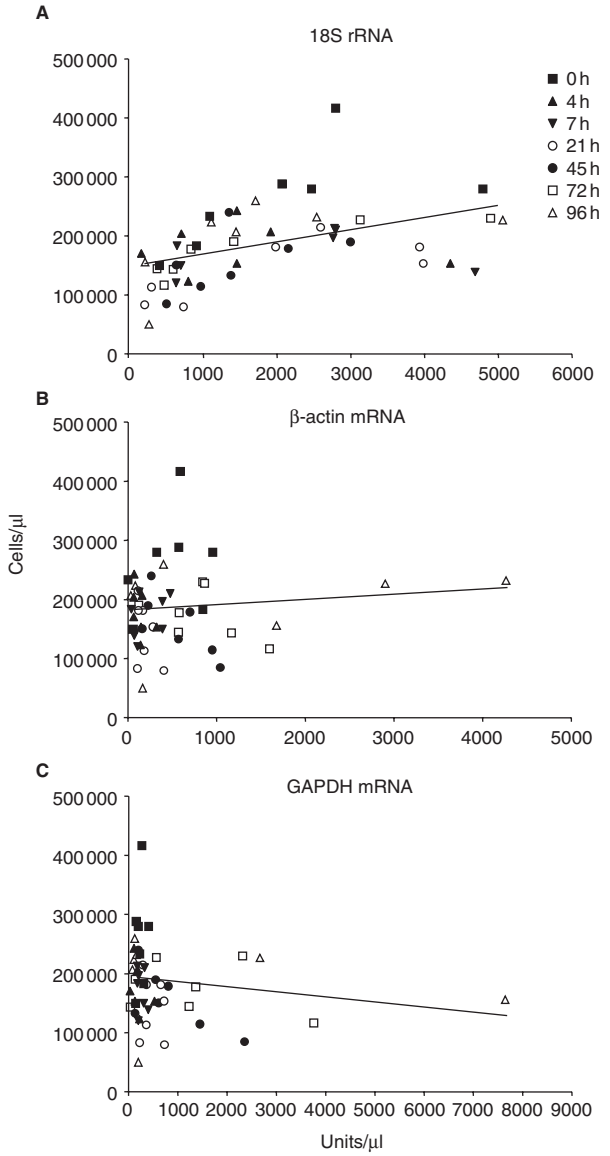

Figure 2 Concentration of cells in individual peripheral blood mononuclear cell (PBMC) samples plotted as a function of concentrations of 18S rRNA. (A) β-actin mRNA. (B) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (C) In the same sample. PBMCs were from seven individuals. Results from both unstimulated PBMCs (■) and PBMCs activated for 4 h (▲), 7 h (▼), 21 h (○), 45 h (●), 72 h (□) and 96 h (△) with anti-CD3 monoclonal antibody OKT3 are included in the graphs.

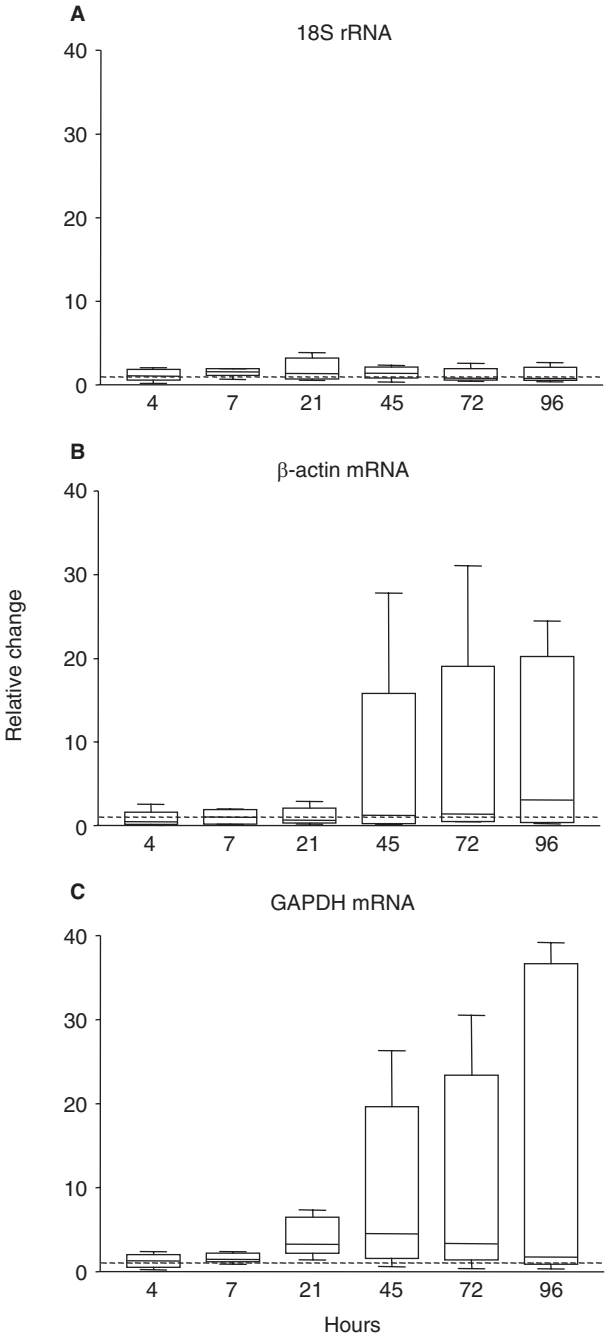

Figure 3 Expression levels of 18S rRNA. (A) β-actin mRNA (B) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (C) In peripheral blood mononuclear cells (PBMCs) after 4-96 h of T-cell activation. Parallel cultures of PBMCs were incubated with anti-CD3 monoclonal antibody OKT3 for the indicated time periods and the expression levels/cell determined by cell counting and real-time quantitative reverse transcriptase-polymerase chain reaction as described. The relative change was calculated as the ratio between the expression level/cell at the individual time point and the expression level/cell in unstimulated PBMCs. Whiskers indicate range, boxes the 25th to 75th percentile and horizontal bars inside boxes the median. The hatched horizontal line indicates no change in expression level compared to unstimulated PBMC. $n = 7$ at each time point.

beads. Expression levels of 18S rRNA and GAPDH mRNA per cell were thereafter determined as described above. After 21 h of stimulation, there was no significant change in the 18S rRNA expression levels in any of the three cell types, whereas there was an average three- to 4.1-fold increase in GAPDH mRNA expression levels (Fig. 4A–C). After 96 h of activation, there was an average 4.4- to fivefold increase in 18S rRNA levels in all three subtypes (Fig. 4A–C), which was statistically significant

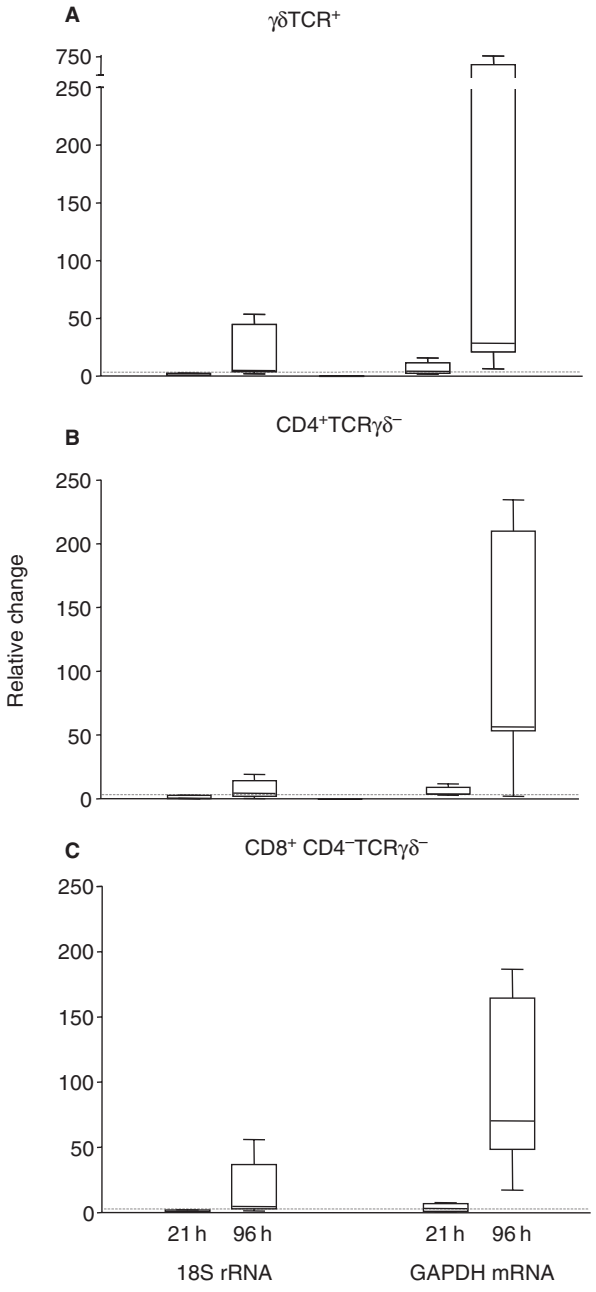

Figure 4 Expression levels of 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in $\gamma\delta TCR^+$. (A) $CD4^+\gamma\delta TCR^-$. (B) $CD8^+CD4^-\gamma\delta TCR^-$. (C) Cells retrieved by sequential positive selection from peripheral blood mononuclear cells stimulated in parallel cultures for 21 and 96 h with the mitogenic anti-CD3 monoclonal antibody OKT3. Cell counting and quantitative reverse transcriptase-polymerase chain reaction were performed as described in Fig. 1. The relative change was calculated as the ratio between the cellular expression level at the indicated time point and the expression level in the unstimulated cells of the respective T-cell subtype before culture. Whiskers indicate range, boxes the 25th to 75th percentile and horizontal bars inside boxes the median. $n = 7$ at each time point.

only in $CD4^+$ cells ($P = 0.05$ compared to unstimulated $CD4^+$ cells; Fig. 4B). This was, however, marginal compared to the marked increase in GAPDH mRNA expression levels at this time point with a median 28-fold increase in $\gamma\delta TCR^+$ cells (Fig. 4A), a median 56-fold increase in $CD4^+$ cells ($P = 0.008$ compared to unstimulated $CD4^+$ cells; Fig. 4B) and a median 70-fold increase in $CD8^+$ cells ($P = 0.003$ compared to unstimulated $CD8^+$ cells; Fig. 4C).

18s rRNA is suitable for normalization of mRNA expression levels in resting and activated T lymphocytes

The utility of 18S rRNA, β-actin mRNA and GAPDH mRNA as housekeeping genes for normalization of gene expression, estimated as mRNA level, was investigated. The average cellular expression levels of mRNA for IL-2, IL-4, IL-10, IFN-γ, TNF-α and TGF-β1 were determined in freshly isolated PBMCs and PBMCs stimulated with anti-CD3 MoAb for 4-96 h by using real-time qRT-PCR with RNA copy standard. The number of cells and the expression levels of 18S rRNA, β-actin mRNA and GAPDH mRNA were also determined in each individual sample. Results are expressed as mRNA copies/cell and mRNA copies/unit 18S rRNA, β-actin mRNA and GAPDH mRNA, respectively. Results normalized to 18S rRNA levels were coherent with results calculated as cytokine mRNA levels/cell at all time points for the five cytokines that were induced by the anti-CD3 MoAb treatment, i.e. IL-2, IFN-γ, TNF-α, IL-4, and TGF-β1 (Fig. 5A–D,F). In contrast, normalization to β-actin or GAPDH mRNA levels gave disparate results compared to cytokine mRNA levels per cell with regard to both magnitude of induction and kinetics (Fig. 5A–D,F). This is most clearly demonstrated by the IFN-γ response, which reaches its peak after 45 h incubation time as determined by calculating cytokine mRNA levels per cell, while normalization to β-actin or GAPDH mRNA levels gave the false impression that the response had already peaked at an earlier time point and was declining at 45 h (Fig. 5B). Correlation analysis between cytokine mRNA levels calculated per cell and cytokine mRNA levels calculated as ratios to the levels of either one of the three housekeeping genes for all individual determinations ($n = 49$ for each cytokine) further emphasized the superiority of 18S rRNA. IL-2, IL-4, IFN-γ, TNF-α and TGF-β1 mRNA levels normalized to 18S rRNA gave r values between 0.81 and 0.96 (median 0.89), suggesting an almost perfect estimation of cellular levels using 18S rRNA. An r value >0.80 was only reached for the most strongly induced cytokines, i.e. IL-2, IFN-γ and TNF-α, when β-actin mRNA was used for normalization and solely for IFN-γ when normalization was done using GAPDH mRNA. IL-10 mRNA expression was only marginally induced by stimulation with anti-CD3 MoAb, which precluded evaluation of housekeeping gene utility using this cytokine (Fig. 5E). However, the correlation analysis suggested that 18S rRNA gives the most reliable results also in this case with

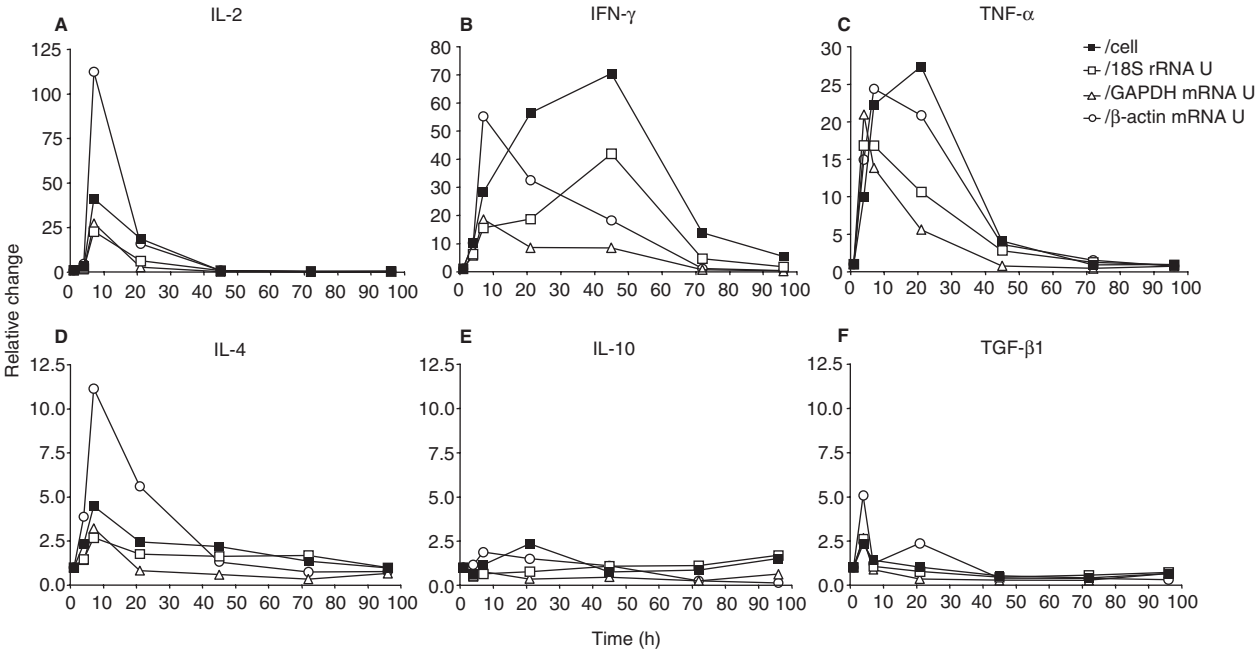

Figure 5 Relative changes in cytokine mRNA levels in peripheral blood mononuclear cells (PBMCs) stimulated by anti-CD3 monoclonal antibody (MoAb) as determined by calculating the number of cytokine mRNA copies per cell (■) compared with the same experimental data normalized to the level of 18S rRNA (□), β-actin mRNA (○) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (△) in the sample. PBMCs were purified from seven individuals, and the number of cells was determined in freshly isolated samples and samples from parallel cultures of PBMCs stimulated with anti-CD3 MoAb for 4-96 h. RNA was extracted from the counted samples, and the concentrations of interleukin-2 (IL-2) (A), interferon-γ (IFN-γ) (B), tumour necrosis factor-α (TNF-α) (C), IL-4 (D), IL-10 (E), transforming growth factor-β1 (TGF-β1) (F), β-actin and GAPDH mRNAs, as well as 18S rRNA were determined by real-time quantitative reverse transcriptase-polymerase chain reaction. The ratios between cytokine mRNA concentration and cell number, 18S rRNA level, β-actin mRNA level and GAPDH mRNA level were calculated for each sample. Thereafter, the relative change was calculated as the ratio between the cytokine mRNA level per cell, per 18S rRNA unit, per β-actin mRNA unit and per GAPDH mRNA unit at the individual time points and the cytokine mRNA level in the unstimulated PBMC before culture calculated in the same way. Graphs show median values from determinations in seven independent samples at each time point.

an r value of 0.59 compared to 0.34 and 0.38 for β-actin and GAPDH mRNA, respectively.

Cytokine mRNA levels in resting and stimulated PBMCs

The experimental set-up also allowed us to compare the average mRNA copy number per cell of the six cytokines in freshly isolated, resting PBMCs and in PBMCs after varying the time of T-cell activation. Generally, the cytokine mRNA levels were low in unstimulated PBMCs (<0.1 mRNA copies/cell; Table 1). TGF-β1 was an interesting exception with as much as 3.6 mRNA copies/cell in resting PBMCs and a poor response to activation (Table 1). IL-2, IFN-γ and TNF-α were all strongly induced in most blood donors (Table 1). However, the kinetics for IFN-γ induction differed from the other two cytokines. While IL-2 and TNF-α showed a maximum increase after 7 h of stimulation, IFN-γ reached maximum expression levels after 45 h (Fig. 5A-C, Table 1). The number of mRNA copies/cell was 10-fold higher

Table 1 Cytokine mRNA expression levels in resting and stimulated peripheral blood mononuclear cells (PBMCs)

Cytokine	Time (h)	Cytokine mRNA* (copies/cell)	Time† (h)	Cytokine mRNA‡ (copies/cell)	Fold increase
Interleukin-2	0	0.008§	7	0.6§	72 §
Interleukin-4	0	0.006	7	0.04	7
Interferon-γ	0	0.1	45	5.7	60
Tumour necrosis factor-α	0	0.09	7	4.5	50
Interleukin-10	0	0.06	7	0.07	1
Transforming growth factor-β1	0	3.6	4	10.5	3

*Average number of cytokine mRNA copies/cell in freshly isolated PBMCs.
†Time point for maximal response to polyclonal T-cell activation for the indicated cytokine.
‡Average number of cytokine mRNA copies/cell in anti-CD3-stimulated PBMCs at the time point for maximal response.
§Median value from independent determinations in PBMCs from seven individuals.

for IFN-γ and TNF-α compared to IL-2, both in resting and in maximally stimulated PBMCs (Table 1). Two individuals had a T helper 2 profile with induction of IL-4 and only a marginal induction of IFN-γ and TNF-α mRNA. However, both IL-4 and IL-10 expression levels were still low after activation (<0.1 copies/cell; Table 1).

Discussion

The present study shows that normalization to 18S rRNA gave the most reliable results followed by β-actin mRNA and last GAPDH mRNA when comparing resting and activated T lymphocytes. 18S rRNA should therefore be the preferred housekeeping gene for normalization when studying gene expression in human lymphocytes. Furthermore, 18S rRNA proved to give an accurate estimation of the cellular levels of mRNA for the six cytokines analysed. The finding that freshly isolated PBMCs and intestinal intraepithelial and lamina propria lymphocytes all had similar 18S rRNA content per cell further supports the conclusion that 18S rRNA is useful for normalization of lymphocytes regardless of the activation state. T lymphocytes in PBMCs are resting with no or low cytokine production (this study and [5]), while intestinal lymphocytes contain T cells producing cytokines at levels similar to polyclonally activated blood T lymphocytes and exhibiting cytotoxic effector functions [5, 25, 29].

To the best of our knowledge, this is the first time β-actin mRNA, GAPDH mRNA and 18S rRNA levels have been compared in normal human lymphocytes using objective and sensitive real-time qRT-PCR. The TaqMan EZ technology was chosen because it includes a reporter dye-labelled probe, which excludes signals from illegitimate amplifications. Only one previous study reported results from comparative analysis of the same three housekeeping genes using real-time qRT-PCR analysis. Although a totally different cell type was studied, i.e. murine fibroblasts, their results are consistent with ours, with significant fluctuations in β-actin and GAPDH mRNA levels and only marginal changes in 18S rRNA levels [30]. β-actin mRNA levels in human immune cells were analysed in a recent study [31]. In line with our results, prominent differences between normal leukocytes, leukocytes of various malignancies and leukaemia cell lines were reported. Furthermore, Hamalainen and coworkers [32] observed significant variations in GAPDH mRNA levels in cultured $CD4^+$ cells.

Although 18S rRNA was the most stable housekeeping gene, it also showed some variations. First, the 18S rRNA level in freshly isolated PBMCs differed up to six times between individuals (range 0.3–1.7 18S rRNA units/100 cells). This might partly be explained by different cellular content of 18S rRNA in the two major cell types in PBMCs, i.e. lymphocytes and monocytes, and variations in cellular composition between PBMC preparations. The cellular content of 18S rRNA in purified monocytes was not determined. However, we have observed that human intestinal epithelial cells contain approximately 10 times more 18S rRNA/cell compared to lymphocytes [4]. Second, there was a slight increase in 18S rRNA levels in all three T-cell subsets analysed after long-time activation (4 days), although 18S rRNA levels remained almost unchanged when the entire PBMC population was analysed. This result may seem conflicting but is most likely explained by the facts that the major T-cell population, i.e. the $CD4^+$ cells, showed the lowest increase and that PBMCs contain additional cells like B cells, natural killer cells and monocytes that do not become activated.

Two previous studies have analysed the cytokine mRNA expression in polyclonally activated human blood T lymphocytes by real-time RT-PCR [33, 34]. They arrive at different conclusions about the kinetics. On one hand, Härtel and coworkers [33] reported that IL-2, IL-4, IL-10, IFN-γ and TNF-α mRNA levels reach the maximum at 4 h and remain high at the last time point analysed (24 h). On the other hand, Stordeur and coworkers [34] who studied IL-10, IFN-γ and TNF-α mRNA expression, reported that expression levels declined after 8 h. Both research groups used β-actin mRNA levels for normalization of the cytokine mRNA and none performed cell counting. We show here that β-actin mRNA expression levels vary significantly between activation stages of lymphocytes and that this can lead to results that are inconsistent with the more accurate estimations obtained by calculating the number of mRNA copies per cell based on cell counting. Errors with regard to both the magnitude of expression levels and the kinetics of cytokine mRNA expression were seen using β-actin for normalization. Hence, the use of β-actin mRNA for normalization is the probable explanation for the inconsistencies between the two studies and between their studies and ours.

The choice of housekeeping genes as internal standard in real-time qRT-PCR is critical for the estimation and comparison of mRNA levels in gene expression studies. From this study, we conclude that 18S rRNA is a reasonably stable housekeeping gene that is suitable for normalization when quantifying mRNA levels of genes expressed in resting and activated human T lymphocytes of blood and tissue origin.

Acknowledgments

This work was financially supported by a grant from the Swedish Research Council, Natural Sciences (B621-2001-1999). We express our sincere gratitude to Ms. Anne Israelsson for preparation of the RNA copy standards and to Dr Åke Öberg and colleagues at the Department of Surgery, Umeå University Hospital for supplying tissue samples.

References

1 Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000;29:332–7.

2 Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23–39.

3 Bas A, Hammarstrom SG, Hammarstrom ML. Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression. J Immunol 2003;171:3359–71.

4 Fahlgren A, Hammarstrom S, Danielsson A, Hammarstrom ML. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. Clin Exp Immunol 2003;131:90–101.

5 Melgar S, Yeung MM, Bas A *et al.* Over-expression of interleukin 10 in mucosal T cells of patients with active ulcerative colitis. Clin Exp Immunol 2003;134:127–37.

6 Matrisian LM, Rautmann G, Magun BE, Breathnach R. Epidermal growth factor or serum stimulation of rat fibroblasts induces an elevation in mRNA levels for lactate dehydrogenase and other glycolytic enzymes. Nucleic Acids Res 1985;13:711–26.

7 Iovanna JL, Dusetti N, Cadenas B, Calvo EL. Changes in growth and pancreatic mRNA concentrations during postnatal development of rat pancreas. Pancreas 1990;5:421–6.

8 Kanayama S, Liddle RA. Influence of food deprivation on intestinal cholecystokinin and somatostatin. Gastroenterology 1991;100: 909–15.

9 Goldsworthy SM, Goldsworthy TL, Sprankle CS, Butterworth BE. Variation in expression of genes used for normalization of Northern blots after induction of cell proliferation. Cell Prolif 1993;26:511–8.

10 Moshier JA, Cornell T, Majumdar AP. Expression of protease genes in the gastric mucosa during aging. Exp Gerontol 1993;28:249–58.

11 Hodin RA, Chamberlain SM, Meng S. Pattern of rat intestinal brush-border enzyme gene expression changes with epithelial growth state. Am J Physiol 1995;269:C385–91.

12 Yamada H, Chen D, Monstein HJ, Hakanson R. Effects of fasting on the expression of gastrin, cholecystokinin, and somatostatin genes and of various housekeeping genes in the pancreas and upper digestive tract of rats. Biochem Biophys Res Commun 1997;231:835–8.

13 Foss DL, Baarsch MJ, Murtaugh MP. Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues. Anim Biotechnol 1998;9:67–78.

14 Tokunaga K, Nakamura Y, Sakata K *et al.* Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res 1987;47:5616–9.

15 Schek N, Hall BL, Finn OJ. Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human pancreatic adenocarcinoma. Cancer Res 1988;48:6354–9.

16 Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 1999;259: 523–6.

17 Ke LD, Chen Z, Yung WK. A reliability test of standard-based quantitative PCR: exogenous vs endogenous standards. Mol Cell Probes 2000;14:127–35.

18 Hsiao LL, Dangond F, Yoshida T *et al.* A compendium of gene expression in normal human tissues. Physiol Genomics 2001;7:97–104.

19 Tricarico C, Pinzani P, Bianchi S *et al.* Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 2002;309:293–300.

20 Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O. Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 2001;295:17–21.

21 Vandesompele J, De Preter K, Pattyn F *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3 [RESEARCH0034].

22 Steele BK, Meyers C, Ozbun MA. Variable expression of some "housekeeping" genes during human keratinocyte differentiation. Anal Biochem 2002;307:341–7.

23 Lundqvist C, Hammarstrom ML, Athlin L, Hammarstrom S. Isolation of functionally active intraepithelial lymphocytes and enterocytes from human small and large intestine. J Immunol Methods 1992;152:253–63.

24 Yeung MM, Melgar S, Baranov V *et al.* Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TCR-gammadelta expression. Gut 2000;47:215–27.

25 Lundqvist C, Melgar S, Yeung MM, Hammarstrom S, Hammarstrom ML. Intraepithelial lymphocytes in human gut have lytic potential and a cytokine profile that suggest T helper 1 and cytotoxic functions. J Immunol 1996;157:1926–34.

26 Lundqvist C, Baranov V, Teglund S, Hammarstrom S, Hammarstrom ML. Cytokine profile and ultrastructure of intraepithelial gamma delta T cells in chronically inflamed human gingiva suggest a cytotoxic effector function. J Immunol 1994;153:2302–12.

27 Kreuzer KA, Lass U, Landt O *et al.* Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin transcripts as quantitative reference. Clin Chem 1999;45:297–300.

28 Forsberg G, Hernell O, Melgar S, Israelsson A, Hammarstrom S, Hammarstrom ML. Paradoxical coexpression of proinflammatory and down-regulatory cytokines in intestinal T cells in childhood celiac disease. Gastroenterology 2002;123:667–78.

29 Melgar S, Bas A, Hammarstrom S, Hammarstrom ML. Human small intestinal mucosa harbours a small population of cytolytically active $CD8^+$ alphabeta T lymphocytes. Immunology 2002;106: 476–85.

30 Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000;46:69–81.

31 Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA. Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. Mol Cell Probes 2002;16:25–30.

32 Hamalainen HK, Tubman JC, Vikman S *et al.* Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Anal Biochem 2001;299:63–70.

33 Härtel C, Bein G, Kirchner H, Kluter H. A human whole-blood assay for analysis of T-cell function by quantification of cytokine mRNA. Scand J Immunol 1999;49:649–54.

34 Stordeur P, Poulin LF, Craciun L *et al.* Cytokine mRNA quantification by real-time PCR. J Immunol Methods 2002;259:55–64.