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Characterization of cytokine gene expression in $CD4^+$ and $CD8^+$ T cells after activation with phorbol myristate acetate and phytohaemagglutinin

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SUMMARY

Cytokines are important mediators involved in the development of effector cells and in the regulation of immune responses. The gene expression of these mediators in T cell subset has yet to be fully elucidated. Using sensitive reverse transcription-polymerase chain reaction (RT-PCR), the kinetics of cytokine gene expression in human $CD4^+$ and $CD8^+$ T cells were examined. $CD4^+$ T cells were more readily activated by phorbol myristate acetate (PMA) and phytohaemagglutinin (PHA) than $CD8^+$ T cells in terms of the IL-2 receptor (IL-2R) mRNA expression. Quantitative differences in cytokine gene expression between $CD4^+$ and $CD8^+$ T cells were confirmed and higher levels of cytokine mRNAs were induced in $CD4^+$ than in $CD8^+$ T cells. Early induction of IL-2 mRNA was observed in both T cell subsets. The demonstration of different kinetics of cytokine gene expression illustrates one of the examples of the complexity of immunoregulation. The differential response of cytokine gene expression in different T cell subsets should be taken into consideration when clinical studies in cytokine production by peripheral blood mononuclear cells are interpreted.

Keywords cytokine gene $CD4^+$ T cells $CD8^+$ T cells mRNA time-course reverse transcription polymerase chain reaction

INTRODUCTION

Polymerase chain reaction (PCR) provides the most sensitive means of detecting gene expression [1, 2]. cDNA is synthesized by reverse transcription (RT) of total RNA and is then subjected to enzymatic amplification by PCR. RT-PCR is particularly useful in studying cytokine gene expression since most cytokine genes are expressed transiently at low levels.

Subdivision of mature human peripheral T cells is mainly based on the expression of the CD4 or CD8 accessory molecules. Recently, it has been suggested that $CD4^+$ T cells could be further subdivided into TH1, TH2, and TH0 based on their distinct patterns of cytokine secretion [3, 4]. However, the existence of similar subsets of human $CD8^+$ T cells is still a matter of controversy [5]. It is recognized that both $CD4^+$ and $CD8^+$ T cells can synthesize combinations of cytokines based on *in vitro* studies of T cell clones, T cell lines and peripheral blood mononuclear cells (PBMC) [6, 7]. Investigations into the cytokine profile of T cell subpopulations can provide valuable information for the understanding of T cell functions in various autoimmune disorders. In this study, we report the results of a time-course study on the expression of IL-2 receptor (IL-2R) gene and a panel of cytokine genes in $CD4^+$ and $CD8^+$ T cell subsets after activation with phorbol myristate acetate (PMA) and phytohaemagglutinin (PHA). The specific gene expression level was measured by semi-quantitative PCR and information gained from the present study will be useful in optimizing the experimental conditions for further investigation of cytokine gene expression in disease.

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MATERIALS AND METHODS

Isolation of $CD4^+$ and $CD8^+$ T cell subsets

Venous heparinized blood (40 μl) was obtained from each of the three healthy volunteers. PBMC were separated by Isopaque–Ficoll (Lymphoprep, Nycomed AS, Oslo, Norway) density gradient centrifugation and washed with PBS. Isolated PBMC were resuspended in culture medium consisting of RPMI 1640 (GIBCO, Chargrin Falls, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Magnetizable polystyrene beads coated with MoAb specific for CD4 or CD8 (Dynabeads M-450 CD4 or Dynabeads M-450 CD8, Dynal AS, Oslo, Norway) were used to select $CD4^+$ or

$CD8^+$ cells from PBMC. Briefly, PBMC suspension was initially cooled to 4°C. Dynabeads M-450 CD4 were added using a bead-to-cell ratio of 1·5:1 and incubated at 4°C for 1 h with gentle tilting and mixing rotation. After incubation, the rosetted $CD4^+$ cells were isolated by the Dynal Magnetic Particle Concentrator (Dynal MPC, Dynal AS). Dynabeads M-450 CD8 were then added to the remaining cell population using a bead-to-cell ratio of 1·5:1 after the depletion of $CD4^+$ cells and the mixture was incubated at 4°C for 10 min. The rosetted $CD8^+$ cells were similarly collected by the Dynal Magnetic Particle Concentrator. The rosetted $CD4^+$ or $CD8^+$ cells were separately resuspended with 500 μl of complete culture medium. Five units of DETACHaBEAD (Dynal AS) were added to the rosetted cells and incubation was carried out at room temperature for 45 min. The detached $CD4^+$ or $CD8^+$ cells previously collected by Dynal Magnetic Particle Concentrator were washed with culture medium and adjusted to a final concentration of 1×10^6/ml with complete culture medium. Isolated $CD4^+$ or $CD8^+$ cells were assayed by flow cytometry (EPICS, Coulter Corp., FL) using fluorescein conjugated anti-human Leu-3a (CD4) and anti-human Leu-2a (CD8) MoAbs (Becton Dickinson, CA). The purity was found to be greater than 99·8% and 99·2% respectively. Cell viability was greater than 98% as determined by trypan blue dye exclusion test.

Culture of $CD4^+$ and $CD8^+$ T cells

Isolated $CD4^+$ or $CD8^+$ cells, adjusted to 1-ml aliquots of 1×10^6 cells, were dispensed into 24-well culture plates. The cells were incubated at 37°C in humidified atmosphere with 5% CO_2 in the presence or absence of PHA (10 μg/ml) and PMA (0·5 ng/ml). Preliminary experiments indicated that these concentrations induce maximal cytokine production with lowest toxicity [8]. At time 0, 1, 2, 4, 6, 8, 24 and 48 h after incubation, cells were harvested for total RNA preparation.

Preparation of total RNA

A microadaptation of the guanidium thiocyanate/caesium chloride (GuSCN/CsCl) procedure [9] was used to prepare total RNA from $CD4^+$ or $CD8^+$ T cells. Cells were pelleted at 5500 ***g*** for 5 min with a microcentrifuge before being lysed with 100 μl of 4 M GuSCN solution. They were then layered over 100 μl of 5·7 M CsCl and centrifuged at 247 000 ***g*** for 2 h at 20°C with a TLA-100 rotor in a Beckman TL-100 bench-top ultracentrifuge (Fullerton, CA). After removal of the supernatant, the RNA pellets were washed with 70% cold ethanol and dissolved with diethylpyro-carbonate-treated distilled water (DEPC-dH_2O). RNA was precipitated twice at −70°C for 3 h with 1/10 volume of 3 M sodium acetate (pH 5·2) and 2·5 volume of cold absolute ethanol. The RNA pellets were dried and redissolved in DEPC-

Table 1. Primers used in polymerase chain reaction (PCR)

Cytokine	Primer sequences	PCR product (bp)	Concentration used (μM/PCR)
IL-1 β	5′ATGGCAGAAGTACCTAAGCTCGC 3′ACACAAATTGCATGGTGAAGTCAGTT	802	0·15
IL-2	5′ATGTACAGGATGCAACTCCTGTCTT 3′GTTAGTGTTGAGATGATGCTTTCAC	458	1
IL-3	5′ATGAGCCGCCTGCCCGTCCTG 3′GCGAGGCTAAAGTCGTCTGTTG	449	0·15
IL-4	5′ATGGGTCTCACCTCCCAACTGCT 3′CGAACACTTTGAATATTTCTCTCTCAT	456	0·5
IL-5	5′GCTTCTGCATTTGAGTTTGCTAGCT 3′TGGCCGTCAATGTATTTCTTTATTAAG	293	0·5
IL-6	5′ATGAACTCCTTCTCCACAAGCGC 3′GAAGAGCCCTCAGGCTGGACTG	628	1
IL-8	5′ATGACTTCCAAGCTGGCCGTGGCT 3′TCTCAGCCCTCTTCAAAAACTTCTC	289	0·15
IFN-γ	5′ATGAAATATACAAGTTATATCTTGGCTTT 3′GATGCTCTTCGACCTCGAAACAGCAT	494	1
TNF-α	5′ATGAGCACTGAAAGCATGATCCGG 3′GCAATGATCCCAAAGTAGACCTGCCC	695	1
GM-CSF	5′ATGTGGCTGCAGAGCCTGCTGC 3′CTGGCTCCCAGCAGTCAAAGGG	424	1
TGF-β	5′GCCCTGGACACCAACTATTGCT 3′AGGCTCCAAATGTAGGGGCAGG	161	0·15
IL-2R	5′GAATTTATCATTTCGTGGTGGGGCA 3′TCTTCTACTCTTCCTCTGTCTCCG	398	0·15
B-actin	5′ATGGATGATGATATCGCCGCG 3′CTAGAAGCATTTGCGGT GGACGATGGAGGGGCC	1126	0·15

TNF-α, Tumour necrosis factor-alpha; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF-β, transforming growth factor-beta; IL-2R, IL-2 receptor.

dH_2O. All RNA samples were stored at −70°C until assay. The quality of RNA was checked by formaldehyde agarose gel electrophoresis.

Oligonucleotide primers

Specific primers of IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor-beta 1 (TGF-β1), IL-2R α-chain and β-actin for PCR were obtained from Clontech (Palo Alto, CA). Table 1 shows the sequence and related information.

Detection of cytokine gene expression by PCR

Cytokine gene expression in CD4+ and CD8+ cells was studied using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). mRNAs of each sample were first reverse-transcribed into cDNAs, which in turn were subjected to PCR amplification using specific primers for individual cytokines. In brief, first-strand DNA was synthesized in a final volume of 20 μl with the following components: 2 μl total RNA from CD4+ or CD8+ cells in DEPC-dH_2O, 2·5 μM Oligo d(T)16 primers, 5 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 8·3, 50 mM KCl, 1 mM dNTP, and 10 U of RNase inhibitor. The mixture was incubated in a Perkin-Elmer Cetus DNA Thermal Cycler at 42°C for 30 min, followed by 5 min at 99°C and flash cooling to 4°C. The cDNA preparations were stored at −70°C until PCR. PCR was performed using GeneAmp PCR system 9600 (Perkin Elmer Cetus). cDNA (10 μl) was mixed with 2 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 8·3, 50 mM KCl, 0·15 to 1 μM specific PCR primers, 1·25 U AmpliTaq DNA polymerase, and 10 μCi [α-^{32}P]-dCTP (Amersham, UK) in a final volume of 50 μl. Amplification was repeated for 25–35 cycles. Each cycle consisted of 15 s at 94°C for denaturation, 30 s at temperatures between 55°C and 62°C for annealing (according to different cytokine genes), and 1 min at 72°C for extension. The final extension lasted 7 min at 72°C in all instances.

Negative controls for PCR consisted of: (i) samples in which the reverse transcriptase was omitted to detect any contamination by previously amplified cDNA; and (ii) reagents control in which RNA was replaced by DEPC-dH_2O. Positive control DNAs for each cytokine were obtained from Clontech Laboratory.

Agarose gel electrophoresis of the PCR products

The PCR mixture (20 μl) was added to 5 μl of loading dye mix and electrophoresed in a 90 V constant-voltage field in 1·6% agarose (Sigma, St Louis, MO) containing ethidium bromide until the bromophenol blue dye front had migrated for a distance of 6 cm. φX174/*Hae*III digested DNA (Gibco BRL, NY) was used to determined the size of the PCR products.

Semi-quantification by measurement of ^{32}P incorporated into PCR products

After electrophoresis, the agarose gel was slightly dried by blotting filter papers and autoradiography was performed using Kodak X-OMAT AR X-ray film (Eastman Kodak, NY) and intensifier screens at −70°C for 8 h. After autoradiography of the gel, gel slides corresponding to the radioactive bands of PCR product were excised and counted by liquid scintillation. ^{32}P incorporated into the PCR products of respective cytokines or IL-2R were normalized with that of the amplified β-actin PCR products. The result was expressed as a ratio calculated from the ct/min of amplified cytokine or IL-2R gene PCR product over the ct/min of amplified β-actin PCR product.

RESULTS

RT-PCR

Preliminary studies were performed to optimize RT-PCR conditions for amplification of IL-2R, cytokines and β-actin mRNAs. The size of the PCR products was confirmed by agarose gel electrophoresis (Fig. 1a). The magnesium ion concentration, primer concentration and the number of cycles used for amplification were adjusted to ensure that the PCR

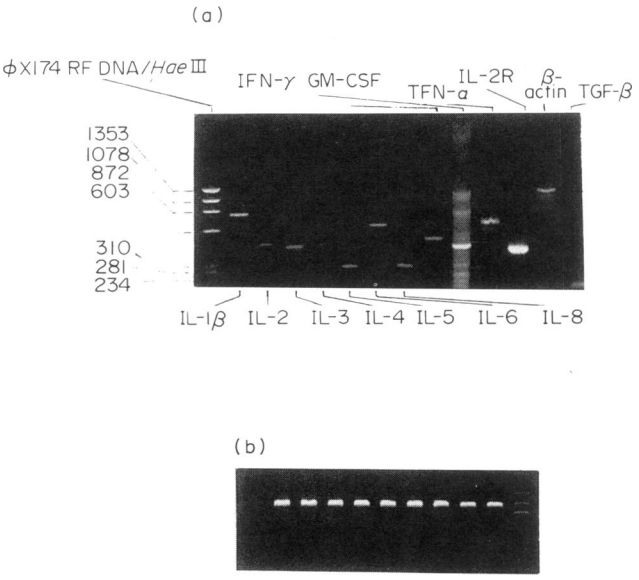

Fig. 1. (a) Demonstration of polymerase chain reaction (PCR) products by agarose gel electrophoreis. (b) Amplified β-actin reverse transcription (RT)-PCR products of nine replicated runs using total RNA from CD4+ T cells. The average amount of ^{32}P-dCTP incorporated into the excised bands was 54 354 ct/min with a coefficient of variation of 5·4%.

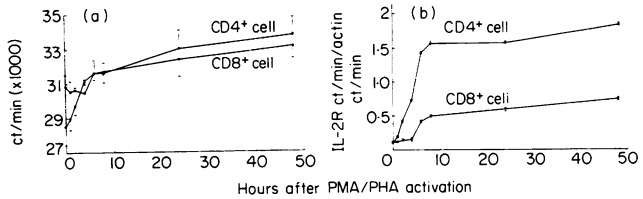

Fig. 2. (a) β-actin mRNA induction in CD4+ and CD8+ cells after stimulation with phorbol myristate acetate (PMA) (0·5 ng/ml) and phytohaemagglutinin (PHA) (10 μg/ml). The results (mean ± s.d.) from three donors were expressed as ^{32}P-dCTP incorporated into the excised polymerase chain reaction (PCR) bands in ct/min. (b) IL-2 receptor (IL-2R) mRNA induction in CD4+ and CD8+ cells after stimulation with PMA (0·5 ng/ml) and PHA (10 μg/ml). The results (mean ± s.d.) from three donors were expressed as ratio of ^{32}P-dCTP incorporated into IL-2R and that of β-actin.

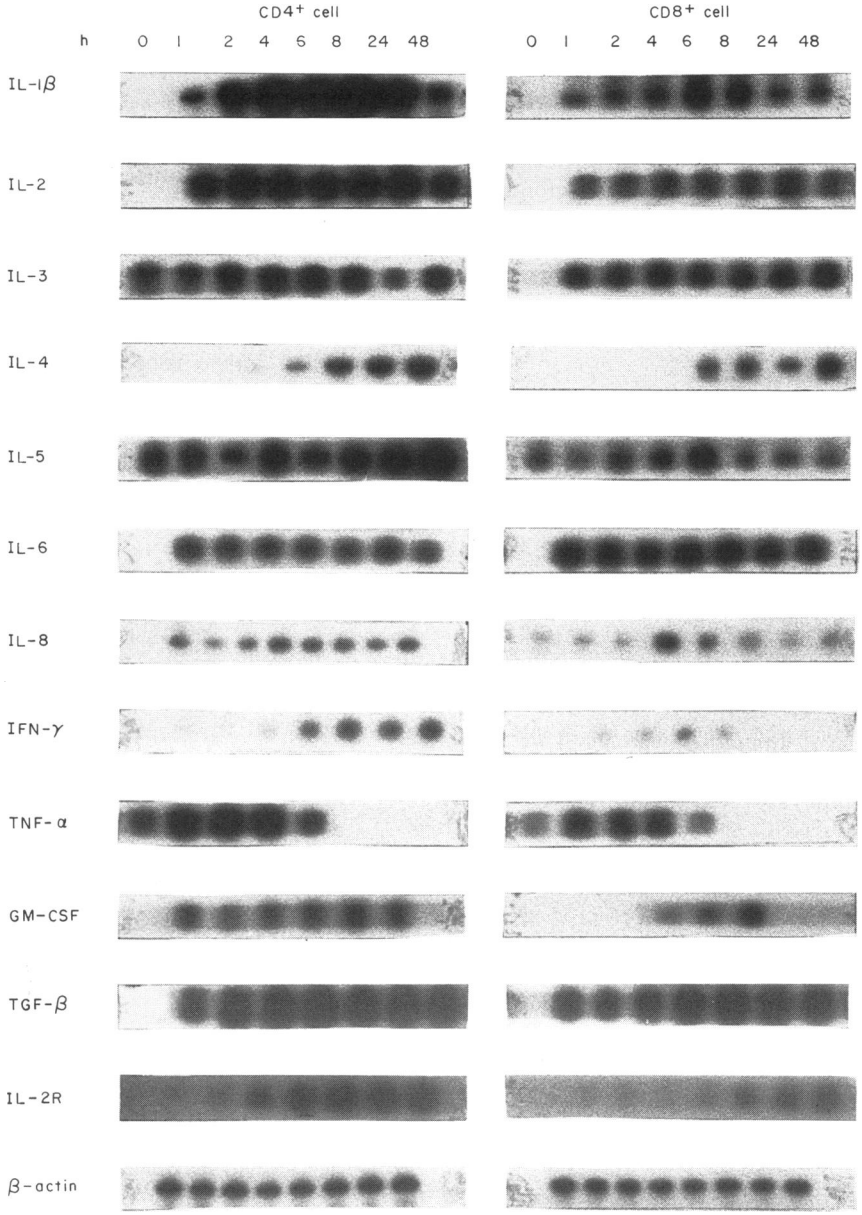

Fig. 3. Autoradiograms demonstrating the kinetics of different cytokines, IL-2 receptor (IL-2R) and β-actin mRNAs induction in $CD4^+$ and $CD8^+$ cells after stimulation with phorbol myristate acetate (PMA) (0·5 ng/ml) and phytohaemagglutinin (PHA) (10 μg/ml).

products were analysed before the copies of PCR products reached saturation. The differences in RNA isolation and cDNA synthesis were normalized using a housekeeping gene, β-actin. All RNA samples were RT-PCR at the same time to avoid interbatch variation or imprecision. Reproducibility of the RT-PCR was demonstrated in Fig. 1b. Same RNA sample was RT-PCR in nine different tubes and the average ^{32}P-dCTP incorporated into excised band of β-actin PCR product is 54 354 ct/min with a coefficient of variation (C.V.) of 5·4%.

β-actin and IL-2R mRNA levels

As illustrated in Fig. 2a, β-actin mRNA expression in $CD4^+$ and $CD8^+$ T cells showed a similar profile. β-actin mRNA levels increased gradually following activation by PMA and PHA, and reached a maximum level at 48 h. β-actin mRNA expression at 48 h after stimulation increased by 9·6% in $CD4^+$ cells and 16·5% in $CD8^+$ cells from the respective values of unstimulated cells measured at 0 h. IL-2R mRNA was induced earlier in $CD4^+$ T cells than in $CD8^+$ T cells (Fig. 2b). IL-2R mRNA first appeared at 2 h in $CD4^+$ T cells but only at 6 h in $CD8^+$ T cells. Higher levels of IL-2R mRNA were expressed in $CD4^+$ T cells than in $CD8^+$ T cells throughout the time course. The peak IL-2R mRNA level increased by 1827% in $CD4^+$ cells and by 577% in $CD8^+$ cells compared with the baseline IL-2R mRNA levels at 0 h.

Cytokine mRNA levels

Figure 3 depicts the kinetics of different cytokine mRNA expression in $CD4^+$ or $CD8^+$ T cell subsets after activation by PMA and PHA. mRNA levels of the cytokines examined in this study were generally higher in $CD4^+$ T cells than in $CD8^+$ T cells. In $CD8^+$ T cells, mRNAs for IL-1β, IL-2, IL-5, IL-8, IFN-

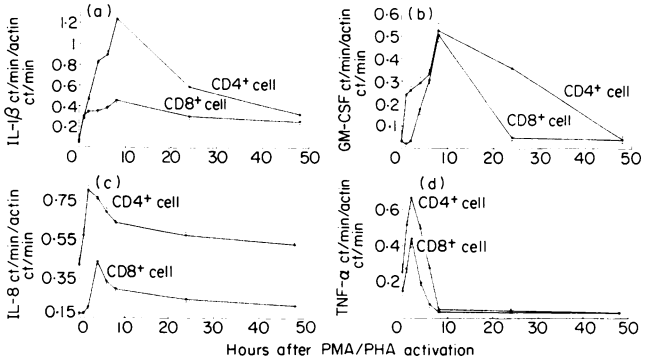

Fig. 4. Kinetics of (a) IL-1β, (b) GM-CSF, (c) IL-8 and (d) tumour necrosis factor-alpha (TNF-α) mRNAs induction in CD4$^+$ and CD8$^+$ cells after stimulation with phorbol myristate acetate (PMA) (0·5 ng/ml) and phytohaemagglutinin (PHA) (10 μg/ml). The results (mean $\pm$ s.d.) from three donors were expressed as ratio of ^{32}P-dCTP incorporated into the cytokine and that of β-actin.

γ were much less readily induced in CD8$^+$ T cells than those of CD4$^+$ T cells. Nonetheless, the time-course patterns of mRNA expression of IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α, GM-CSF and TGF-β1 were comparable between CD4$^+$ and CD8$^+$ T cells except in magnitude.

IL-1β and GM-CSF mRNA levels (Fig. 4) in both T cell subsets reached a peak at 8 h after stimulation and declined to control levels at 48 h. IL-8 and TNF-α mRNA levels peaked earlier at approximately 2–4 h following stimulation and thereafter decreased rapidly to background levels at 8 h.

IL-2, IL-3 and IL-6 mRNAs were induced rapidly, reaching a peak at 4–8 h and the high levels of gene expression of these cytokines were maintained up to 48 h (Fig. 5). IL-4 and TGF-β1 showed similar patterns of mRNA induction, except that the process was more gradual during the first few hours after activation (Fig. 6).

The IL-5 and IFN-γ gene expression in T cell subsets showed a markedly different pattern of regulation compared with other cytokines (Fig. 7). In CD4$^+$ T cells, IL-5 and IFN-γ mRNA increased gradually after induction and reached a maximum at 48 h. In contrast, the levels of IL-5 and IFN-γ mRNAs in CD8$^+$ T cells were lower at all time points. Furthermore, the IL-5 mRNA peaked at 6 h while IFN-γ mRNA peaked at 4 h. Gene expression of these two cytokines in CD8$^+$ T cells declined rapidly to baseline levels after 8 h.

DISCUSSION

In this study, we have examined the kinetics of cytokine gene expression in CD4$^+$ and CD8$^+$ T cells using a highly sensitive method of RT-PCR. Previous studies of cytokine gene expression often involved techniques such as Northern blotting, *in situ* hybridization and ribonuclease protection analysis. Not infrequently, these methods suffer from the drawbacks of low sensitivity and requirement of a huge amount of RNA, and are technically demanding. Recently, the high sensitivity and specificity of RT-PCR make it an extremely useful method for the analysis of cytokine gene expression [10, 11]. Gilliland *et al* [12] reported a useful approach for quantification of gene expression by RT-PCR using 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 the sequence difference between the DNAs used for the standard and in experiment [13]. We have demonstrated in this report a simpler approach which can effectively compare relative amounts of cytokine gene expression in different RNA samples. A housekeeping gene, β-actin, was successfully used to normalize the differences occurring during RT and PCR. Using this method, we are able to semi-quantify the kinetics of cytokine gene expression in T cell subsets with satisfactory reproducibility.

It has been reported that differences in cytokine profile between CD4$^+$ and CD8$^+$ T cells are quantitative rather than qualitative [14, 15]. While other studies encourage the view that the functional dichotomy of both human CD4$^+$ and CD8$^+$ T cells can be discriminated and predicted based on their cytokine production profiles [16, 17], the understanding of such a complicated network is far from complete. Our data support the notion that CD4$^+$ and CD8$^+$ cells are not precommitted to follow distinct patterns of cytokine gene expression. Instead, cytokine genes are expressed more strongly in CD4$^+$ than in CD8$^+$ T cells and the kinetics for these genes' expression are also different between the two T cell subsets. These differences probably reflect the functional properties of these cells and their mediators and further highlight the complicated regulatory mechanisms of the cytokine gene expression.

Sander *et al.* [18] have demonstrated that both MHC class I- and class II-restricted T cells could regulate immune response by secreting the same array of cytokines. Indeed, our results also indicated that both CD4$^+$ and CD8$^+$ T cells could express the same array of cytokines under PMA and PHA activation, although differences in magnitude and time-course were demonstrated for individual cytokines. Our data also revealed that

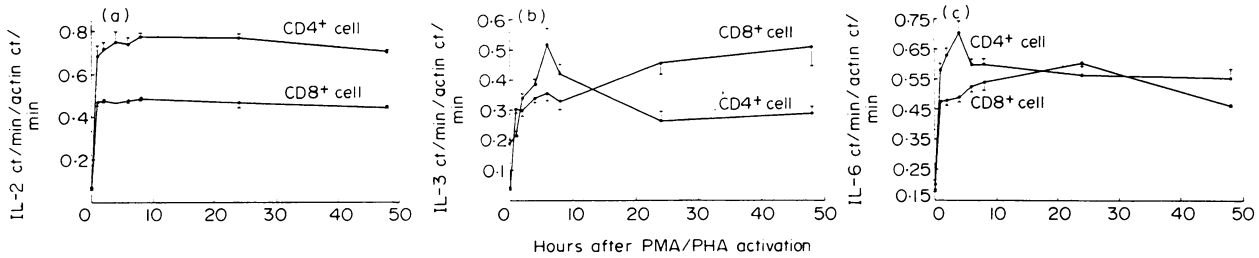

Fig. 5. Kinetics of (a) IL-2, (b) IL-3, and (c) IL-6 mRNAs induction in CD4$^+$ and CD8$^+$ cells after stimulation with phorbol myristate acetate (PMA) (0·5 ng/ml) and phytohaemagglutinin (PHA) (10 μg/ml). The results (mean $\pm$ s.d.) from three donors were expressed as ratio of ^{32}P-dCTP incorporated into cytokine and that of β-actin.

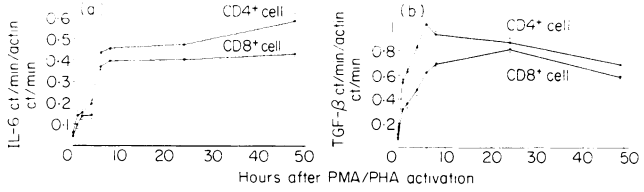

Fig. 6. Kinetics of (a) IL-4 and (b) transforming growth factor-beta 1 (TGF-β1) mRNAs induction in CD4$^+$ and CD8$^+$ cells after stimulation with phorbol myristate acetate (PMA) (0·5 ng/ml) and phytohaemagglutinin (PHA) (10 μg/ml). The results (mean $\pm$ s.d.) from three donors were expressed as ratio of ^{32}P-dCTP incorporated into cytokine and that of β-actin.

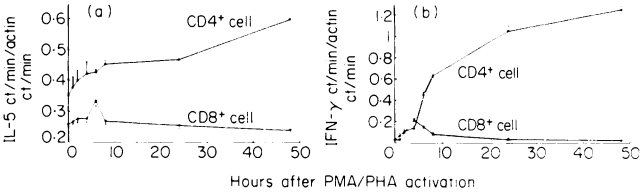

Fig. 7. Kinetics of (a) IL-5 and (b) interferon-gamma (IFN-γ) mRNAs induction in CD4$^+$ and CD8$^+$ cells after stimulation with phorbol myristate acetate (PMA) (0·5 ng/ml) and phytohaemagglutinin (PHA) (10 μg/ml). The results (mean $\pm$ s.d.) from three donors were expressed as ratio of ^{32}P-dCTP incorporated into cytokine and that of β-actin.

induction of IL-2R mRNA was earlier and stronger in CD4$^+$ than in CD8$^+$ T cells. Gauchat *et al.* [19] have demonstrated a good correlation between the expression of IL-2R mRNA in PBMC and the cells that entered the G_1 cycle. This suggested that CD4$^+$ T cells were activated more effectively than CD8$^+$ T cells under stimulation by PMA and PHA. Although we cannot exclude the possibility that these quantitative differences were genetically determined, we have observed that the cytokine gene expression is similarly stronger in CD4$^+$ than CD8$^+$ T cells after stimulation by PMA and PHA. Furthermore, there are distinct differences in the kinetics of cytokine gene expression amongst the T cell subsets. Early induction of IL-2 mRNA supports the notion that IL-2 initiates the induction of a cascade of other cytokine mRNAs [20, 21]. The persistent expression of IL-2, IL-4, IL-5, IL-6 and IFN-γ may be the result of stabilization of cytokine mRNA by PMA as Carter & Malter [22] have reported stabilization of the labile cytokine mRNAs by phorbol ester (TPA) for the presence of a stretch of AUUUA in their sequence. Our findings in IL-5 and GM-CSF are somewhat different from those in PBMC reported by Staynov & Lee [23]. They have demonstrated that transcription of both genes started about 3 h after activation by PMA and continued to increase until 15 h (with a peak at approximately 10 h), after which time it remained stable for another 24 h. We have observed transcription of IL-5 mRNA in resting CD4$^+$ and CD8$^+$ cells. The transcription of GM-CSF started as early as 1 h after activation in CD4$^+$ cells but only occurred at 4 h in CD8$^+$ cells. The levels of mRNAs peaked at 8 h but persisted for another 24 h only in CD4$^+$ cells. The observed variations in kinetics of cytokine gene expression suggest that cytokine gene expression is regulated in diverse manners.

Abnormalities in T cell activation and cytokine release have been reported in various pathological conditions [23–29]. Since the majority of these studies involved the use of mixed population of cells (mainly PBMC) with different stimuli, abnormal patterns of cytokine production or IL-2R release may be due to the different ratios of activated CD4$^+$ and CD8$^+$ cells [24,28,29]. Preferential stimulation of T cell subsets should therefore be taken into consideration in the interpretation of those results and for designing future investigation of cytokine profiles in those pathological entities.

We have confirmed that cytokine gene expression in CD4$^+$ and CD8$^+$ T cells following activation by PMA and PHA are, quantitatively rather than qualitatively, different. Differences in the kinetics of mRNA induction suggest a complicated regulation of these cytokines in T cell subsets.

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