Transcriptional and Posttranscriptional Regulation of the Interleukin-4 and Interleukin-3 Genes in Human T Cells

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Human T cells were studied with regard to the regulation of interleukin-4 (IL-4) and IL-3 gene expression. IL-4 and IL-3 mRNA were undetectable in unstimulated T cells. On activation with the lectin concanavalin A (Con A), both IL-4 and IL-3 mRNA were expressed. Accumulation of IL-4 mRNA peaked after 6 to 12 hours, whereas IL-3 mRNA levels peaked after 3 to 6 hours of stimulation with Con A. Nuclear run-on assays showed a low constitutive transcription for both genes. The transcription rates were increased by Con A resulting in a peak for IL-4 after 1 hour (30% increase) and for IL-3 after 3 hours (40% increase) of Con A treatment. mRNA stability studies demonstrated that on activation with Con A both messages decayed with a half-life of approximately 90 minutes. No IL-4 or IL-3 mRNA expression was induced by the protein kinase C activator phorbol myristate acetate (PMA). However, PMA augmented the Con A-induced IL-4 and IL-3 mRNA accumulation. This was shown to be mediated at posttranscriptional level by a large increase in the stability of both messages (t1/2 > 3 hours). The transcription rate of both genes was also enhanced by Con A + PMA and reached peak levels for IL-4 after 1 hour (90% increase) and for IL-3 after 3 hours (70% increase) of stimulation. Furthermore, it appeared that the induction of IL-4 mRNA was dependent on protein synthesis because cycloheximide (CHX) blocked the Con A- and Con A + PMA-induced expression of IL-4 mRNA. In contrast, CHX inhibited, but failed to completely block, the Con A- and Con A + PMA-induced IL-3 mRNA expression, whereas the expression of both genes was completely blocked by cyclosporine A. With regard to the secretion of IL-4 protein it was shown that it closely follows the accumulation of IL-4 mRNA. Taken together, the data show that expression of the IL-4 and IL-3 genes in human T cells is controlled by different activation pathways that affect the gene regulation at transcriptional and posttranscriptional levels.

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T lymphocytes produce several lymphokines after activation in vitro, including interleukin-4 (IL-4) and IL-3.1,2 Much is known about the diverse and sometimes opposing effects of these glycoproteins on the differentiation and activation of hematopoietic cells. With regard to the myeloid lineage it has been shown that IL-4 suppresses the IL-3–supported proliferation, whereas it enhances the granulocyte colony-stimulating factor (G-CSF)–supported granulocytic colony formation.3,5 In contrast, IL-4 suppresses the endotoxin and IL-1–induced expression of G-CSF, tumor necrosis factor (TNF), and IL-1 mRNA in human monocytes.6,7 These data indicate that the secretion of cytokines by T cells may profoundly influence the development of the myeloid lineage. Therefore, it might be supposed that either mechanism exists for the selective production of a certain lymphokine in response to a given activation signal or that the kinetics of cytokine expression are diverse.

Several studies in murine and human T cells have given insight into the process by which different lymphokines are produced. For murine cells, activated type 1 helper T–cell (Th 1) lines synthesize IL-2 and interferon-γ (IFN-γ), while IL-4 and IL-5 are the products of type 2 helper T–cell (Th 2).8,9 Studies in human T-cell subsets showed that the production of IL-4 is highest in the enriched CD4+CD45R− subset,10–13 while the IL-3 gene is only expressed in the CD28+ subset of T cells.14

The mechanisms by which the IL-4 and IL-3 gene expression in T lymphocytes is regulated are poorly understood. To explore the mechanisms, we analyzed the regulation of the expression of both transcripts in human T cells. The results show that the expression of the IL-4 and IL-3 genes is controlled at transcriptional and posttranscriptional levels.

MATERIALS AND METHODS

Preparation of cells. Peripheral blood cells were obtained from volunteer platelet donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density-gradient centrifugation. T lymphocytes were isolated by 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The SRBCs were lysed with 155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA and the remaining cell preparations contained more than 98% T cells as assessed by flow cytometry after immunofluorescent staining with an anti-CD2 monoclonal antibody (MoAb) (Becton Dickinson, Sunnyvale, CA) and less than 1% CD14-positive cells (Becton Dickinson). T cells were cultured in RPMI 1640 media (Flow, Rockville MD) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 6 mg/mL colistine.

Stimulation. Cells (5 × 10⁶/mL) were incubated for various time periods with 25 µg/mL concanavalin A (Con A; Calbiochem, La Jolla, CA) with 50 ng/mL phorbol myristate acetate (PMA; Sigma, St Louis, MO) or with a combination of 25 µg/mL Con A + 50 ng/mL PMA. Cycloheximide (CHX; Sigma) was used at a concentration of 10 µg/mL, actinomycin D (Act D; Boehringer, Mannheim, Germany) at 10 µg/mL, and cyclosporine A (CsA; gift from Sandoz, Basel, Switzerland) at 0.1 µg/mL. Human recombinant IL-4 (gift from Dr P. Trotta, Schering-Plough, Bloomfield, NJ) was used at 15 ng/mL because this dose induced optimal response of myeloid progenitors.5 The specific activity of IL-4 was 10⁶ U/mg.
mRNA extraction and analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate/CSCl method. Nine micrograms of total RNA was electrophoresed in 2.2 mol/L formaldehyde, 1.1% agarose gels, and blotted onto nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK). cDNA probes were labeled with [α-32P]dCTP (3,000 Ci/mol; Amersham) by the random hexamer priming method. The following cDNA probes were used: (1) the 0.3-kb EcoRI/HindIII insert of a human IL-4 cDNA purified from the phMl plasmid (gift from Dr S. Narula, Schering Plough); (2) the 0.3-kb EcoRI/HindIII insert of a human IL-3 cDNA purified from the phMl plasmid (gift from Dr H. Burger, TNO, Rijswijk, The Netherlands); (3) the EcoRI linearized pBR322 plasmid containing a 1.0-kb human actin cDNA insert or a 7.8-kb human 28S cDNA insert.

Hybridization was performed at 65°C for 18 hours in 0.5 mol/L Na2HPO4, pH 7.2, 1 mmol/L EDTA, 7% sodium dodecyl sulfate (SDS). Membranes were washed once in 2 × SSC, 0.1% SDS; once in 1 × SSC, 0.1% SDS; and finally in 0.3 × SSC, 0.1% SDS for 20 minutes at 65°C. The membranes were exposed to Kodak X-Omat intensifying screen. Quantification of mRNA levels was performed by densitometry using a Gel Scan laser densitometer (Pharmacia LKB, Uppsala, Sweden).

Run-on transcription assay. For the nuclear run-on assay, 10⁶ cells were pelleted at 500g for 5 minutes, washed twice with ice-cold phosphate-buffered saline (PBS), and suspended in 4 mL of lysis-buffer (10 mmol/L Tris-HCl, pH 7.5; 3 mmol/L MgCl2; 10 mmol/L NaCl; 0.5% NP-40). After gentle vortexing, the suspension was incubated on ice for 5 minutes. Nuclei were pelleted at 500g for 5 minutes and the lysis described above was repeated. The nuclei were resuspended in 100 μL of glycerol-buffer (50 mmol/L Tris-HCl, pH 8.0, 40% glycerol, 5 mmol/L MgCl2, 0.1 mmol/L EDTA) and incubated at 26°C for 20 minutes with 80 μL transcription-buffer (12.5 mmol/L Tris-HCl, pH 8.0, 6 mmol/L MgCl2, 125 mmol/L KCl, 2 mmol/L dithiothreitol, 1 mmol/L each of ATP, CTP, and GTP) and 12.5 μL [α-35P] UTP (3,000 Ci/mol; Amersham). Transcription was terminated by the addition of 40 U DNAse I, 150 mM RNasin, 40 μg yeast RNA, and 200 μL stop-buffer (10 mmol/L Tris-HCl, pH 7.4, 0.5 mol/L NaCl, 50 mmol/L MgCl2, 2 mmol/L CaCl2), and the solution was incubated at 37°C for 20 minutes. After a proteinase K digestion (750 U proteinase K/mL) in 1% SDS, the nuclear RNA was isolated by phenol-chloroform extractions and then ethanol precipitated two times in 2.5 mol/L ammonium acetate. The RNA was further purified by Sephadex G-50 column separation (Boehringer).

Five micrograms of the following DNAs was immobilized on Hybond N+ membranes: (1) EcoRI linearized pBR322; (2) EcoRI linearized pcD-IL-4 plasmid containing a 0.3-kb human IL-4 cDNA fragment; (3) EcoRI linearized phiM1 plasmid containing a 0.3-kb human IL-3 cDNA fragment; (4) EcoRI linearized plasmid containing a 1.3-kb rat GAPDH cDNA fragment. Hybridization of labeled RNA that was isolated from equal numbers of cells to the membranes was as described above. Washing was as described above except that an extra wash-step with 2 × SSC/1 μg/mL RNase A (Boehringer) was included. Exposure and quantification of transcription rate levels were performed as described above.

Measurement of IL-4 protein. Human T cells per milliliter, 3 × 10⁶, were stimulated with 25 μg/mL Con A, 50 ng/mL phorbol myristate acetate (PMA) or Con A + PMA for 48 hours. Secreted IL-4 protein in supernatants was quantified using a human IL-4 enzyme-linked immunosorbent assay (ELISA) Kit (Genzyme Corp, Cambridge, MA) as recommended by the manufacturer.

RESULTS

Human T cells were stimulated with Con A, PMA, or with Con A + PMA. Total cellular RNA was extracted and IL-4, IL-3, and 28S transcripts were detected by Northern hybridization. Figure 1 shows the time course of IL-4 and IL-3 mRNA accumulation in Con A- and Con A + PMA-stimulated cells. Unstimulated T cells did not express detectable levels of these mRNAs (Fig 1). Con A-stimulated cells peaked for IL-4 mRNA expression after a 6- to 12-hour treatment, whereas IL-3 mRNA levels peaked after 3 to 6 hours of Con A treatment. Costimulation of Con A-stimulated T cells with PMA did not affect the kinetics of IL-4 mRNA expression, but PMA augmented the level of Con A-induced IL-4 mRNA expressed in T cells. The IL-3 mRNA accumulation was also augmented following costimulation with Con A + PMA. Moreover, the kinetics were changed. IL-3 mRNA accumulated maximally at 12 hours and there was still high expression of the IL-3 message after 24 hours of stimulation with Con A + PMA. Stimulation of human T cells with PMA alone did not result in a detectable expression of either IL-4 or IL-3 mRNA (data not shown).

To analyze further the regulation of IL-4 and IL-3 gene expression after stimulation with Con A and Con A + PMA, nuclear run-on assays were performed. Nuclear RNA was isolated from control cells and cells that were treated with Con A or Con A + PMA for 1, 3, 5, and 7 hours. RNA was hybridized to pBR322 (negative control), IL-4, IL-3, and GAPDH DNAs. In Fig 2 a representative experiment is shown. Constitutive transcription of the IL-4 and IL-3 genes was detected in unstimulated T cells. The transcription rate of the IL-4 gene was slightly enhanced by Con A and reached its peak level (30% increase) after 1 hour of Con A treatment. Costimulation with Con A + PMA further augmented the transcription rate to a 90% increase after 1 hour of Con A + PMA treatment. After activation with Con A, the IL-3 transcription was increased and reached its peak level after 3

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**Fig 1.** Kinetics of IL-4 and IL-3 mRNA accumulation in activated human T cells. Northern analysis of total T-cell RNA extracted at various time points (indicated) after activation with Con A or Con A + PMA. Blots were hybridized with a 28S rRNA probe to estimate amounts of RNA in different lanes.
Fig 2. Effect of Con A and Con A + PMA on the transcription rate of the IL-4 and IL-3 genes. (A) Nuclear run-on experiment. Nuclei were prepared from human T cells that were treated with medium (control) and with Con A or Con A + PMA for various time periods (indicated). Transcription in the isolated nuclei was analyzed by hybridization of the 32P transcripts to 5 μg of linearized plasmids containing no insert (pBR322), IL-4, IL-3, and GAPDH cDNA inserts immobilized on nylon membranes. (B) Schematic representation of transcription rates in control and Con A-activated T cells. (C) Schematic representation of transcription rates in control and Con A + PMA-activated T cells. Quantification of transcription rates was performed by densitometry and rates were normalized with respect to the GAPDH signal.

hours (40% increase). Costimulation with Con A + PMA for 3 hours gave rise to a 70% increase as compared with the IL-3 transcription rate in unstimulated cells. These results suggest that regulation of IL-4 and IL-3 gene expression is at least partially regulated at the transcriptional level. However, increases in IL-4 and IL-3 transcription after Con A and Con A + PMA treatment seem inadequate for explaining the large increase in IL-4 and IL-3 mRNA accumulation. Cytoplasmic message stabilization might be the principal mechanism governing IL-4 and IL-3 mRNA levels. Therefore, the stability of IL-4 and IL-3 mRNA was studied.

T cells were activated with Con A or Con A + PMA. After 6 hours Act D was added to inhibit new RNA synthesis. At several time points after inhibiting RNA synthesis, total cellular RNA was prepared and subjected to Northern blot hybridization. The mRNA levels were quantified using densitometry. Figure 3 shows that after activation with Con A, the IL-4 mRNA decayed with a half-life of approximately 90 minutes. In the presence of Con A + PMA, the stability of IL-4 mRNA was increased resulting in a half-life greater than 3 hours. The stability of the IL-3 mRNA was regulated in a similar way. IL-3 mRNA decayed with a half-life of 90 minutes on activation with Con A, while Con A + PMA-induced IL-3 mRNA was more stable and decayed with a half-life exceeding 3 hours.

To study the protein synthesis dependency, human T cells were incubated with CHX alone, a combination of CHX + Con A, or CHX + Con A + PMA. After 6 hours total cellular RNA was extracted and subjected to Northern blot analysis. T cells exposed to CHX did not express detectable levels of IL-4 mRNA (data not shown). Furthermore, the Con A- and Con A + PMA-induced expression of IL-4 mRNA was completely blocked in the presence of CHX (Fig 4). Rehybridization of the filter with an IL-3 probe showed that the Con A- and Con A + PMA-induced transcription of the IL-3 gene was only partially blocked by CHX (Fig 4), demonstrating that the expression of the IL-4 and IL-3 gene is differentially affected by CHX.

The immunosuppressive drug CsA is known to bind to the intracellular protein cyclophilin, thereby inhibiting the transcription of many, but not all, cytokines. To investigate the effect of CsA on the IL-4 and IL-3 mRNA expression, T cells were stimulated with Con A or Con A + PMA in the presence of CsA. As shown in Fig 4, CsA completely blocks the expression of IL-4 and IL-3 mRNA.

To determine whether the Con A- and Con A + PMA-induced IL-4 mRNA accumulation resulted in the production and secretion of protein, we measured IL-4 protein in cell culture supernatants. Cells were activated for 48 hours and supernatants were analyzed for IL-4 protein by ELISA. No IL-4 was measured in supernatants of unstimulated T cells or in T cells activated with PMA (data not shown). As shown in Fig 5, cells activated with Con A or with Con A + PMA secreted IL-4 protein. IL-4, 270 pg/mL ± 141 (mean ± SD, n = 8), was secreted by Con A-activated T cells. After activation with Con A + PMA the secretion of IL-4 increased to 799 pg/mL ± 191 (mean ± SD, n = 8). The secretion of IL-4 varied and was dependent on the donor. However, for every donor the Con A-induced IL-4 protein secretion was enhanced by costimulation with Con A + PMA.

Finally we investigated whether secreted IL-4 in the medium affects the expression of IL-4 and IL-3 transcripts in activated T cells. T cells were stimulated for 6 hours with Con A, IL-4, and Con A + IL-4. Total RNA was isolated and analyzed for IL-4 and IL-3 mRNA by Northern hybridization. As shown in Fig 6, stimulation with IL-4 + Con A resulted in a lower expression of IL-4 and IL-3 mRNA compared with Con A-activated T cells. Densitometry showed a
downregulation of 70% of IL-4 message while the IL-3 message decreased by 30%. IL-4 alone did not induce IL-4 or IL-3 mRNA expression (data not shown).

DISCUSSION

In the present study, we investigated the regulation of IL-4 and IL-3 gene expression in human activated T cells. The results show that expression of both genes is regulated at transcriptional and posttranscriptional levels. Nuclear run-on experiments showed that both the IL-4 and the IL-3 gene are constitutively transcribed in unstimulated human T cells. In contrast, no IL-4 or IL-3 mRNA was detected in unstimulated cells using Northern analysis. This indicates that the IL-4 and IL-3 transcripts in unstimulated T cells are very unstable, or that the run-on assay is more sensitive than the Northern. Because we were not able to detect IL-3 mRNA in unstimulated T cells by the polymerase chain reaction technique (unpublished results, January 1992), we address the first explanation.

IL-4 and IL-3 mRNA accumulated on activation with Con A. The small increase in transcription rate on activation with Con A could not account for the high expression of IL-4 and IL-3 mRNA in total cellular RNA. Therefore, it is likely that Con A also affects the stability of the IL-4 and IL-3 mRNA. PMA, an activator of protein kinase C, was shown to modulate the kinetics of the IL-3 mRNA accumulation and to further stabilize the mRNAs of both cytokines. Control of stability of mRNA is poorly understood, but likely the process involves factors interacting with mRNA sequences.26,27 The AU-rich motifs in the 3' noncoding region of many short-living mRNAs are shown to be the target of a pathway for mRNA degradation and an AUUUA-specific mRNA binding protein has been identified.28-32 Cytokines share these AU-rich sequences in the 3' noncoding region of their messages, which might explain the high turnover rate of the IL-4 and IL-3 mRNAs. However, Iwai et al33 showed that stabilization of the granulocyte-macrophage (GM)-CSF message by Con A is not mediated by sequences within the 3' noncoding region. Other sequences, ie, Con A response elements, located in the 5' noncoding or within the coding region seem to be responsible for mRNA stabilization also.

Recently Shoemaker et al34 reported that the IL-3 gene in T-lymphocyte cell lines is regulated, at least in part, at the level of transcription. Ryan et al35 described transcriptional and posttranscriptional regulation of the IL-3 gene expression in phytohemagglutinin (PHA) + PMA-activated Jurkat cells. Our results demonstrate a similar regulation of the IL-3 gene in normal human T cells. In contrast to the findings of Bohijen et al36 with regard to the IL-4 gene expression in a murine T-helper cell clone, we did observe an inhibitory effect of CHX on the Con A- and Con A + PMA-induced IL-4 mRNA accumulation in human T cells. In activated human T cells, CHX totally blocks the expression of IL-4 mRNA, but only partially blocks the induction of IL-3 mRNA. These results indicate that activation of the human IL-4 gene depends on the synthesis of new protein, while activation of the IL-3 gene is also modulated by pre-existing factors. In addition to previous reports concerning the effect of CsA on the induction of cytokine expression in murine cells and T cell clones,37,38,39 we showed that CsA completely blocks the Con A- and Con A + PMA-induced IL-4 and IL-3 mRNA expression in human T cells.

With regard to the secretion of IL-4 protein, it was shown that Con A-activated T cells secreted IL-4, whereas this cytokine
Fig 4. Effects of CHX and CsA on the Con A- and Con A + PMA-induced IL-4 and IL-3 mRNA expression. Northern analysis of RNA extracted from cells treated for 6 hours with Con A and Con A + PMA in the absence and presence of CHX or CsA. Blots were hybridized with an actine probe to estimate amounts of RNA in both lanes.

was not secreted by unstimulated cells or on activation with the protein kinase C activator PMA. These results are in accordance with the mRNA results. The combination of Con A + PMA enhanced the secretion of IL-4 protein compared with the effects of Con A alone. Moreover, evidence was obtained that secreted IL-4 protein affects negatively the expression of the IL-3 and IL-4 transcripts in human activated T cells. This indicates an important autoregulatory loop for control of IL-4 production by activated T cells. Further experiments have to be performed to see whether IL-4 affects transcriptional activity or the half-life of IL-4 and IL-3 mRNA.

Activation of the protein kinase C-dependent pathway is insufficient to induce IL-4 and IL-3 expression, and requires an additional signal to induce IL-4 and IL-3 expression. This can be related to differences in intracellular phosphorylation induced by Con A and PMA and is supported by the findings that phosphorylation on the serine residues of the CD3γ chain occurs on activation of protein kinase C. However, on activation of the T-cell receptor complex, tyrosine residues of the δ chain were also phosphorylated, which could not be mimicked by addition of phorbol esters or diacylglycerol.

ACKNOWLEDGMENT

We thank Drs S. Narula and H. Burger for providing the IL4 and IL-3 cDNA probes, respectively. We are grateful to Dr P. Trotta for providing IL-4 and to Dr B. Stulp for careful reading of the manuscript.

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