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Mechanisms of differential regulation of interleukin-6 mRNA accumulation by tumor necrosis factor alpha and lymphotoxin during monocytic differentiation

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In the present report we compare the capacity of two related cytokines, tumor necrosis factor (TNF) alpha and lymphotoxin (LT), to modulate mRNA levels of interleukin-6 (IL-6) in cells representing different stages of monocytic differentiation including the human leukemia cell lines HL 60, U 937, THP-1, MonoMac 1 and peripheral blood monocytes. We show that the capacity of TNF alpha and LT to induce IL-6 mRNA accumulation increases as monocytic differentiation proceeds with TNF alpha being more potent than LT, suggesting that alternate pathways may be used by differentiating cells to control expression of IL-6. In contrast, in monocytes which constitutively synthesize IL-6 transcripts, TNF alpha and LT treatment had opposite effects on levels of IL-6 mRNA accumulation. In these cells TNF alpha enhanced steady state levels of IL-6 transcripts due to mRNA stabilization, whereas LT shortened IL-6 mRNA half-life, most likely due to induction of a RNA destabilizer since LT-mediated downregulation of levels of IL-6 mRNA in monocytes could be prevented by inhibition of protein synthesis. Neither TNF alpha nor LT altered IL-6 mRNA accumulation by interfering with preexisting transcription factors since both TNF alpha and LT required de novo protein synthesis to exert their effects.

Gene expression; Interleukin-6; Tumor necrosis factor; Lymphotoxin

1. INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine with numerous synonyms reflecting its distinct biological activities such as B-cell stimulatory factor 2, interferon beta-2 and hybridoma/plasmacytoma growth factor [1]. IL-6 is known to play a central role in the immune response, in acute phase reactions and in hematopoiesis [1,2]. Dysregulation of IL-6 expression is involved in the pathogenesis of various chronic inflammatory diseases, autoimmune states, and lympho/hematopoietic disorders [3]. The molecular cloning of the IL-6 gene has revealed a 5-kb gene consisting of 5 exons and four introns, containing three transcriptional initiation sites and three TATA-like sequences [4]. A c-*fos* serum response element, a recognition site for signals mediated by cAMP and for binding of transcriptional enhancer elements such as AP-1 have been identified in the highly conserved 5′ flanking region of the IL-6 gene. Two AU-rich islands have been detected in its 3′ untranslated region [4]. These sequences largely contribute to the instability of the IL-6 mRNA [5] and have also been identified in many other cytokine genes or proto-oncogenes whose expression is transiently regulated by external stimuli [6]. Many different cell types such as monocytes, fibroblasts, keratinocytes, endothelial cells, T-cells and B-cells synthesize IL-6 either constitutively or following induction by a variety of stimuli [1]. However, cells of the monocyte/macrophage lineage are believed to represent the major physiologic source of IL-6 [7–9]. Signal transduction pathways controlling IL-6 gene expression involve activation of protein kinase C as well as cAMP [1]. A tissue specific preferential utilization of distinct initiation sites suggests distinct regulatory mechanisms controlling IL-6 gene expression in different cell species [4,10,11]. Mechanisms that account for differential regulation of the IL-6 gene during ontogeny of a distinct cell type are, however, still unknown. In the present study we have analyzed the regulation of IL-6 gene expression in various monocytic cells belonging to different stages of monocytic differentiation including the human leukemia lines HL 60, U937, THP-1, and MonoMac 1 and normal peripheral blood monocytes. As inducers of IL-6 expression in these cells two other cytokines have been instrumental because of their known stimulatory effects on IL-6 transcript synthesis in other cell species, namely tumor necrosis factor

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(TNF) alpha and lymphotoxin (LT) [12]. Comparison of the capacity of these cytokines to induce IL-6 expression was of particular interest because of their known ability to exert differential effects on expression levels of other genes despite sharing the same receptor [12–14].

2. MATERIALS AND METHODS

2.1. *Biological reagents and cDNAs*

Recombinant human (rh) tumor necrosis factor alpha (spec. act. 5×10^7 U/mg of protein) and rh lymphotoxin (spec. act. 10^8 U/mg of protein) were kindly provided by G. Adolf, Boehringer, Vienna, Austria. The monoclonal antibody anti-CD 14, kindly provided by J. Griffin, Dana Farber Cancer Institute, Boston, MA, was used to control purity of monocyte preparations and to assess the stage of monocytic differentiation of the respective leukemia cell line. The cDNA probe for IL-6 was produced by two of us (T.H. and T.K.) and was derived from a *Taq*I/*Ban*II fragment of pBSF 2.38; the beta-actin probe, a 2.2 *Pst*I fragment of the pA1 plasmid, was kindly provided by J. Ramadori, 1. Medical Department, University of Mainz, FRG.

2.2. *Cell lines*

All cell lines were grown in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 10% low endotoxin FCS (Hazelton Laboratories, Vienna, VA), 4 mM L-glutamine, 100 ng/ml streptomycin, 100 U/ml penicillin, and 10 mM sodium pyruvate (Gibco) (= standard culture medium) at a cell density of 10^6 cells/ml. In selected experiments culture medium was also supplemented with polymyxin B (10 μg/ml; Sigma, Munich, FRG) to prevent effects of possibly contaminating lipopolysaccharide. From each cell type, 50×10^6 cells were exposed to either increasing concentrations of TNF alpha (10^1, 10^2, 10^3 or 5×10^3 U/ml) or LT (10^1, 10^2, 10^3 or 10^4 U/ml) for 4 h or to a maximum stimulating dose of TNF alpha (5×10^3 U/ml) or LT (10^4 U/ml) for 4, 8 and 12 h and then subjected to RNA analysis. HL 60, U 937 and THP-1 cells were purchased from the American Type Culture Collection, the MonoMac 1 line was kindly provided by H.W.L. Ziegler-Heitbrock, Institute for Immunology, University of Munich, FRG [15].

2.3. *Preparation of peripheral blood monocytes*

Peripheral blood-derived mononuclear cells (PBMC) were isolated from platelet phoresis bags obtained from consenting healthy volunteer donors by Ficoll-Hypaque (Seromed, Berlin, FRG) density separation (specific density of 1.078 kg/l). A fraction enriched for monocytes was obtained by a second density separation (specific density of 1.062 kg/l) of E-rosette-negative PBMCs as described elsewhere [16]. Individual cell fractions were assessed by morphology (Wright/Giemsa and alpha naphthyl acetate esterase staining) and immunofluorescence analysis employing monoclonal anti-CD 14 antibody [17]. This procedure reveals monocyte preparations of more than 95% purity.

2.4. *Culture of monocytes*

Monocytes were plated in standard culture medium in plastic Petri dishes (Falcon, Oxnard, CA) at a density of 2×10^6 cells per ml for 3–12 h with or without TNF alpha or LT. In selected experiments cycloheximide (CHX; Sigma, Munich, FRG) (10 μg/ml) was added either 45 min prior to incubation with TNF alpha or LT for 4 h or for a 3-h incubation period that was preceded by 3 h of culture in the presence or absence of TNF alpha or LT. In additional experiments monocytes were exposed to actinomycin D (Sigma, Munich, FRG) (5 μg/ml) for various periods of time (30, 60, 90 and 120 min) following a 4-h incubation period with or without TNF alpha or LT. In a third set of experiments, monocytes were incubated with actinomycin D (5 μg/ml) with or without cycloheximide (10 μg/ml) for 1 or 3 h, respectively, following a 4-h incubation period in standard culture medium.

2.5. *Extraction of cellular RNA and Northern blot analysis*

Total cellular RNA was isolated as previously described [18,19] by lysing cells in guanidinium isothiocyanate followed by recovery of RNA by centrifugation through cesium chloride. RNA was subsequently denatured by treatment with a buffer containing formaldehyde/formamide/morpholinopropanolsulfonic acid (Serva, Heidelberg, FRG) at 65°C. Samples of 20 μg were fractionated on a 1% formaldehyde agarose gel and blotted onto synthetic membranes (Schleicher and Schuell, Dassel, FRG). Messenger RNA of interest was detected using an IL-6-specific probe. The probe was labelled by primer extension with [α-^{32}P]dCTP (3000 Ci/mmol). Membranes were prehybridized overnight at 42°C in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml salmon sperm. Hybridization was performed in an identical solution supplemented with 1×10^5 cpm/ml labelled probe. The membranes were washed in decreasing concentrations of SSC at 65°C, dried and exposed to Kodak X-Omat films with intensifying screen for 2–3 days. To exclude incomplete RNA transfer in single lanes, all membranes were reprobed for beta-actin. In selected experiments relative signal intensity of hybridization was determined by laser densitometry [12].

3. RESULTS

3.1. *Stage of monocytic differentiation of cells to be studied*

The CD 14 molecule is expressed on the surface of approximately 90% of peripheral blood monocytes and is progressively acquired during monocyte ontogeny. Table I indicates surface expression of CD 14 on all cell species studied by immunofluorescence analysis with anti-CD 14 monoclonal antibody and demonstrates a maturation with HL 60 cells being more immature than U 937 > THP 1 > MonoMac 1 > blood monocytes.

3.2. *Constitutive IL-6 expression during monocytic differentiation*

In contrast to monocytes which constitutively synthesized stable IL-6 transcripts, IL-6 mRNA was undetectable in HL 60, U 937, THP-1 and MonoMac 1 cells (Figs 1A,B and 2).

3.3. *TNF alpha-mediated regulation of IL-6 gene expression during monocytic differentiation*

In the promyelocytic cell line HL 60, known to be induced by TNF alpha to differentiate along the

Table I

CD 14 expression of cells studied

Cell species	Surface expression of CD 14 (%)
HL 60	0
U 937	5–8[a]
THP-1	24–26
MonoMac 1	58–63
Blood monocytes	89–96

[a] Range of percent assessed by three different occasions

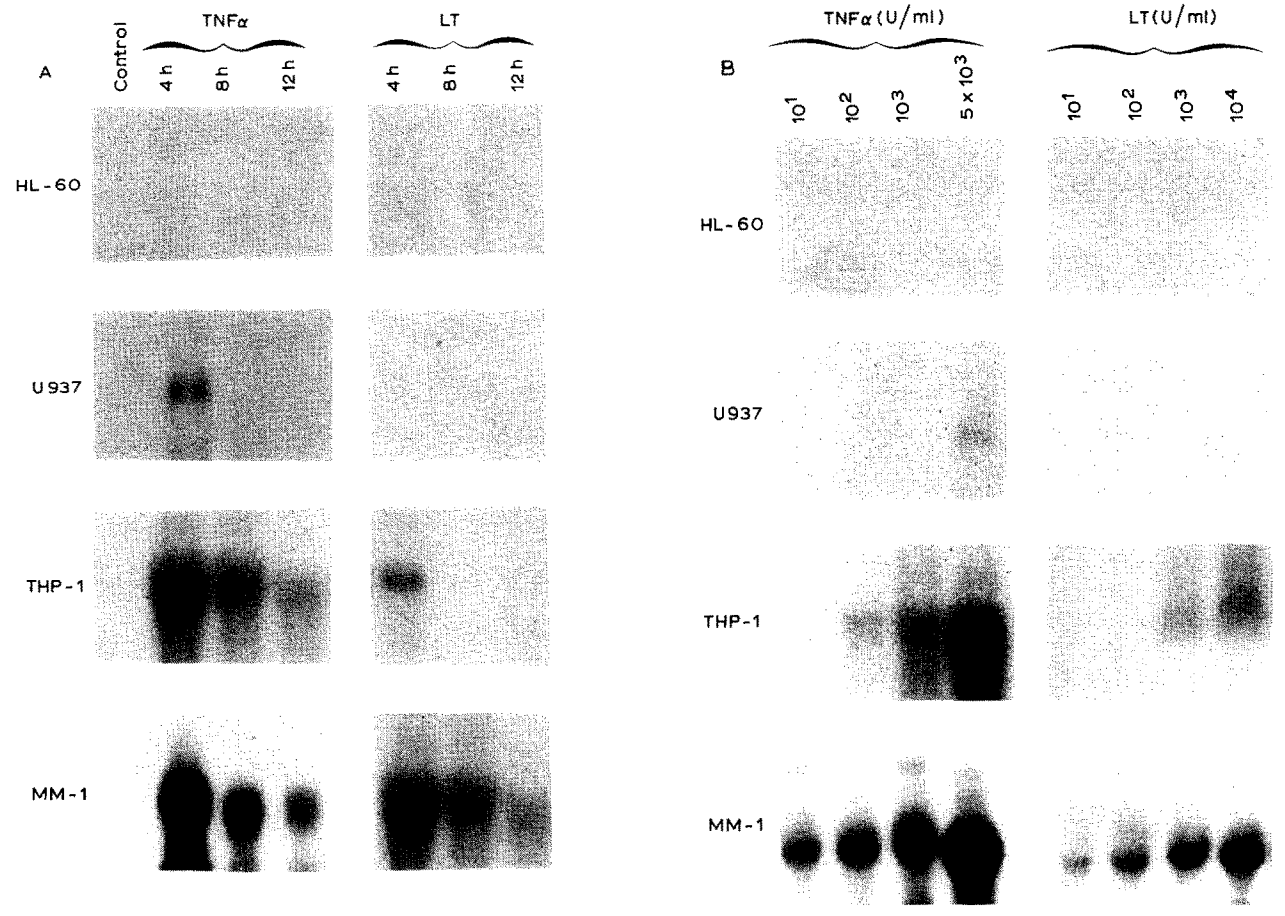

Fig. 1. (A) Time-dependent effect of TNF alpha and LT on levels of IL-6 mRNA in the cell lines HL 60, U 937, THP-1 and MonoMac (MM) 1). Cells were incubated in the presence or absence of TNF alpha (5×10^3 U/ml) or LT (10^4 U/ml) for various periods of time. Cytoplasmic RNA (20 μg/lane) was hybridized with an IL-6-specific cDNA (1.6 kb). Rehybridization of the filters with beta-actin-specific cDNA confirmed identical RNA loading in single lanes (not shown). (B) Dose-dependent effect of TNF alpha and LT on levels of IL-6 mRNA in the cell lines HL 60, U937, THP-1 and MM 1. Cells were incubated in the presence or absence of various concentrations of TNF alpha and LT for 4 h. Cytoplasmic RNA (20 μg/lane) was hybridized with an IL-6-specific cDNA (1.6 kb). Rehybridization of the filters with beta-actin-specific cDNA confirmed identical RNA loading in single lanes (not shown).

monocytic pathway [20], TNF alpha failed to induce detectable IL-6 mRNA at all concentrations investigated (Fig. 1B). In U937 cells only exposure to an otherwise maximum dose of TNF alpha (5×10^3 U/ml) for 4 h resulted in detectable IL-6 mRNA levels (Fig. 1B) that declined within a further 4–8 h to baseline levels (Fig. 1A). In the THP-1 cell line TNF alpha stimulated IL-6 mRNA accumulation already when given at a concentration of 10^2 U/ml for 4 h, leading to a dose-dependent stronger signal when the TNF alpha dose was increased to 5×10^3 U/ml (Fig. 1B). Again, after prolongation of the culture period IL-6 mRNA levels declined but were still detectable (Fig. 1A). In the more mature monocytic cell line MonoMac 1, IL-6 mRNA accumulation was induced in a dose-dependent fashion by all TNF alpha concentrations used (Fig. 1B), with maximum hybridization signals detectable at 4 h of culture (Fig. 1A). In monocytes which constitutively expressed IL-6 mRNA, TNF alpha treatment resulted in an enhancement of IL-6 transcript levels at 4 h that declined back to starting levels when monocytes were exposed to TNF alpha for a further 4 h (Fig. 2).

3.4. *LT-mediated IL-6 regulation of IL-6 gene expression during monocytic differentiation*

Similar to TNF alpha, LT failed to induce IL-6 mRNA accumulation in the promyelocytic HL 60 line, but failed also to induce IL-6 transcripts in U 937 cells (Fig. 1B). Low levels of IL-6 mRNA were stimulated in THP-1 cells by LT, as compared to IL-6 levels induced by TNF alpha. In the more mature MonoMac 1 cells, LT was even more potent in stimulating IL-6 expression. In these cells IL-6 mRNA accumulated at all LT concentrations investigated after 4 h of culture (Fig. 1A). In contrast to all cell lines investigated, LT downregulated constitutively expressed IL-6 mRNA levels in monocytes after 4 h of culture (Fig. 2).

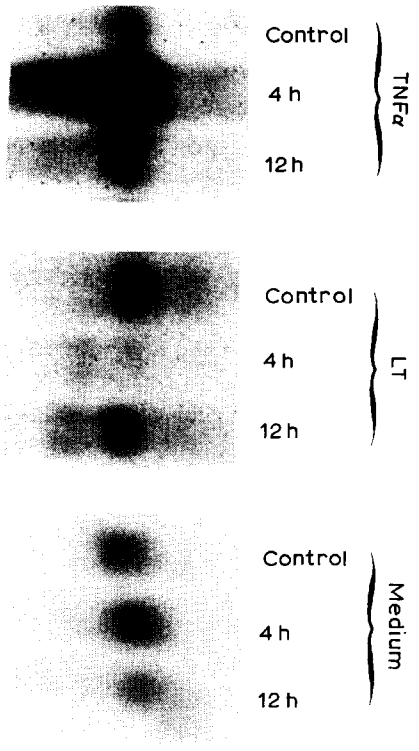

Fig. 2. Time-dependent effect of TNF alpha and LT on levels of IL-6 mRNA in peripheral blood monocytes. Freshly separated monocytes were either directly subjected to mRNA analysis (control) or were incubated in the presence or absence of TNF alpha (5×10^3 U/ml) or LT (10^4 U/ml) for 4 and 12 h. Identical experiments were also performed with polymyxin B as culture supplement and gave comparable results, thus ruling out possible influences of contaminating lipopolysaccharide. Cytoplasmic RNA (20 μg/lane) was hybridized with an IL-6-specific cDNA (1.6 kb). Rehybridization of the filters with beta-actin-specific cDNA confirmed identical RNA loading in single lanes (not shown).

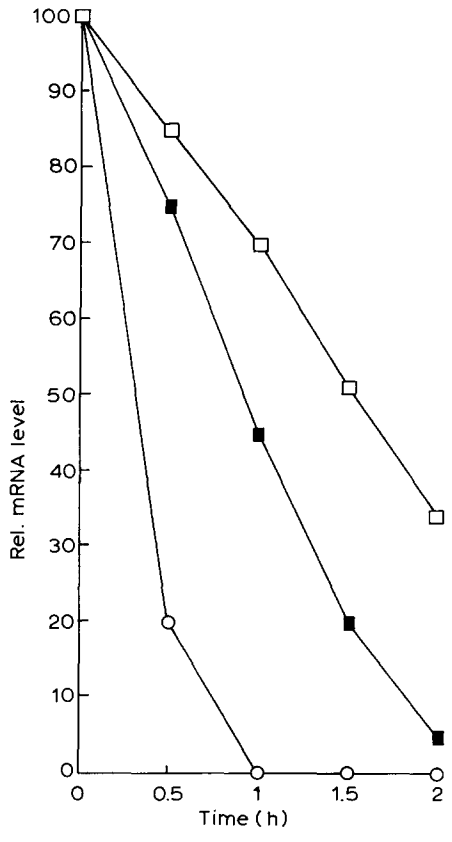

Fig. 3. Stability of IL-6 mRNA levels in peripheral blood monocytes cultured with or without TNF alpha or LT. Monocytes were cultured in the presence or absence (closed squares) of TNF alpha (open squares) (5×10^3 U/ml) or LT (open circles) (10^4 U/ml) for 4 h and then actinomycin D (5 μg/ml) was added to the cultures for 0.5–2 h. Cytoplasmic RNA (20 μg/lane) was sequentially hybridized to IL-6 and beta-actin-specific cDNA. Blots were analyzed by laser densitometry. Values are expressed as actual mRNA levels relative to baseline mRNA levels (= 100%).

After a 12-h culture period in the presence or absence of LT (10^4 U/ml), IL-6 mRNA levels were indistinguishable regardless of whether LT was present in the culture or not, suggesting that a short acting LT-induced mechanism may have led to a decay of IL-6 mRNA in these cells.

3.5. *Regulation mechanisms governing IL-6 mRNA accumulation in monocytes in response to TNF alpha or LT*

High levels of constitutively expressed IL-6 mRNA in monocytes declined to undetectable levels when monocytes were exposed to actinomycin D for 2 h (5 μg/ml), suggesting an IL-6 mRNA half-life of less than 1 h. In monocytes exposed to TNF alpha (5×10^3 U/ml) for 4 h prior to actinomycin D, however, Northern blot analysis still revealed abundant IL-6 message after exposure to actinomycin D for 2 h, suggesting a prolongation of the half-life of IL-6 mRNA by TNF alpha. In contrast, a more rapid decay of accumulated IL-6 mRNA occurred in LT (10^4 U/ml)-stimulated monocytes as compared to untreated controls (Fig. 3), thus suggesting an LT-mediated destabilization of constitutively expressed IL-6 mRNA. Inhibition of protein synthesis by CHX (10 μg/ml) for 3 h was followed by an increase of IL-6 message in uninduced and induced monocytes (Fig. 4), suggesting that de novo protein synthesis interferes with IL-6 mRNA stability in each cell sample. Incubation with cycloheximide for 45 min that was followed by stimulation with TNF alpha (5×10^3 U/ml) or LT (10^4 U/ml) abolished TNF alpha- and LT-mediated IL-6 mRNA modulation (Fig. 4), suggesting that both LT and TNF alpha require de novo protein synthesis to exert their effects. To show whether mRNA accumulation in monocytes is regulated by labile proteins, monocytes were exposed to actinomycin D either alone or in combination with CHX. As shown in Fig. 5, CHX delayed decay of IL-6 mRNA. When transcription of the IL-6 gene was blocked by actinomycin D treatment for 1 h, levels of IL-6 mRNA declined to 50% of baseline. Prolongation of the actinomycin D treatment to 3 h

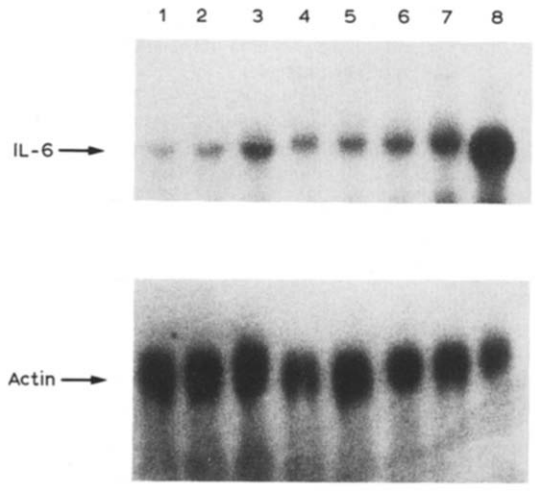

Fig. 4. Effect of CHX treatment on levels of IL-6 mRNA in monocytes induced by TNF alpha (5×10^3 U/ml) and LT (10^4 U/ml). Monocytes were cultured in the presence of TNF alpha or LT for 4 h and then either CHX (10 μg/ml) or fresh medium was added to the culture for a further 3 h. In lane 1 the primary culture was with LT, the secondary culture with medium. Lane 2 shows the treatment sequence medium/medium; lane 3: TNF/medium; lane 6, LT/CHX; lane 7: medium/CHX; lane 8: TNF/CHX. In the experiments depicted in lanes 6 and 8, LT or TNF were not present during the incubation period with CHX. In other experiments the effects of CHX treatment that was followed by exposure of cells to either TNF or LT on steady-state levels of IL-6 mRNA were investigated. The primary culture was performed with CHX for 45 min followed by a 4-h culture with LT (lane 4) or TNF alpha (lane 5). Cytoplasmic RNA (20 μg/ml) was sequentially hybridized to IL-6 and beta-actin-specific cDNA.

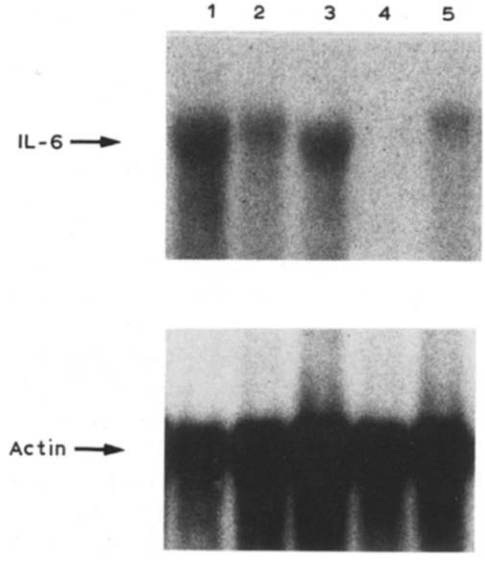

Fig. 5. Effect of CHX treatment on the stability of IL-6 mRNA levels in peripheral blood monocytes. Monocytes were cultured in the presence of medium and then actinomycin D (act D; 5 μg/ml) was added to the cultures either alone or in combination with CHX (10 μg/ml) for 1 or 3 h. Lane 1: monocytes cultured in medium alone; lane 2: exposure to act D for 1 h; lane 4: exposure to act D for 3 h. In lanes 3 and 5 act D was combined with CHX either for 1 (lane 3) or 3 h (lane 5). Cytoplasmic RNA (30 μg/ml) was sequentially hybridized to IL-6 and beta-actin-specific cDNA.

resulted in complete decay of IL-6 mRNA. However, when blockade of transcription was combined with inhibition of protein synthesis by CHX, IL-6 mRNA was still detectable.

4. DISCUSSION

Despite considerable structural identity of 36% and homology of 52% of the amino acid sequence [21] and functional similarities [20,22] TNF alpha and LT are also known to differ in their capacity to induce certain cytokines [12–14]. Both polypeptides share a common receptor [23], although they probably interact in a different fashion with this receptor [24]. Distinct receptor affinities have been accounted for the distinct biological capacities of both cytokines [23,24]. In the present paper we demonstrate that the potential of LT to induce IL-6 message in the less-differentiated cell lines U937 and THP-1 is reduced as compared to the action of TNF alpha, although LT has been shown to share with TNF alpha the ability to spur differentiation of THP-1 cells [20,25]. In monocytes, however, both cytokines display even opposite effects on IL-6 mRNA accumulation, which cannot simply be explained by different receptor binding alone. It is well known that posttranscriptional mechanisms govern accumulation of certain mRNAs such as mRNA of some proto-oncogenes [26] or growth factors [27]. Alternate pathways in growing and differentiating cells have recently been reported to selectively control c-*myc* mRNA accumulation [28]. It has been shown for instance that c-*myc* expression in plasma cells is under transcriptional control of short-lived repressor molecules which are, however, inactive in early B-lineage cells [29]. Therefore, the apparent rationale for regulating gene expression of a multifunctional biological molecule such as IL-6 in cells maturing along the monocytic line by distinct cytokines is to provide alternate transcriptional or posttranscriptional pathways as labile proteins acting as short-lived repressors/derepressors or interfering with mRNA stability. As presented here, neither cytokine modulates accumulation of the IL-6 mRNA by interacting with preexisting transcription factors (either repressors or activators) since both TNF alpha and LT require de novo protein synthesis to exert their effects. Induction of transcriptional activators such as AP-1, which has recently been shown to mediate TNF alpha-induced collagenase gene expression in fibroblasts [30] or of G-binding proteins known to mediate effects of TNF alpha on endothelial cells [31], might also be involved in LT and TNF alpha-modulated IL-6 gene expression. In uninduced as in induced monocytes, IL-6 mRNA is under control of short-lived destabilizers leading to superinduction of IL-6 message as soon as protein synthesis is inhibited. Preexisting nucleases, which interact with the AU-rich islands and thus cause rapid degrada-

tion of mRNA by random endonucleolytic cleavage, have been proposed to cause rapid turnover of the c-*fos* mRNA [32]. A similar mechanism could account for regulation of IL-6 mRNA. However, apart from that, LT also induces a destabilizer or a short-lived repressor, leading to shortening of the IL-6 half-life and downregulation of the IL-6 message as compared to untreated controls. Since ongoing transcription does not necessarily correlate with mRNA accumulation [26,27], LT-induced downregulation might well be due to a LT-induced destabilizer rather than an inhibition of IL-6 gene transcription. On the other hand, a different expression pattern of labile proteins in response to LT may account for the increase of IL-6 mRNA mediated by LT in less mature MonoMac 1 cells. The recent observation that TNF alpha-stimulated IL-6 expression is downregulated in THP-1 cells when protein synthesis is inhibited [33] but upregulated in monocytes, as shown here, also suggests alternate post-transcriptional pathways mediated by cytokine-induced short-lived proteins which then selectively regulate IL-6 gene expression along the monocytic pathway. So far, mechanisms leading to shut-off of induced transcription of genes are poorly understood. Nuclear factors such as AP-1, however, may possibly become active only transiently after cellular induction through specific modifications such as phosphorylation or allosteric changes [34]. Studies are now under way to further determine to which extent either transcriptional or posttranscriptional mechanisms account for TNF alpha- and LT-modulated IL-6 gene expression in monocytes and whether nuclear transcription factors such as the c-*jun*/AP-1 complex or G-proteins are involved.

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