Role of Lymphotoxin in Expression of Interleukin 6 in Human Fibroblasts

Stimulation and Regulation

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Abstract

IL-6 is a cytokine with a number of biological functions, including stimulation of immunoglobulin synthesis and proliferation of early hematopoietic stem cells. We showed that lymphotoxin stimulated accumulation of IL-6 mRNA in human fibroblasts (W138) in a dose-responsive fashion; tumor necrosis factor-alpha (TNF-α) was about threefold more potent than lymphotoxin. Further experiments suggested that stimulation by lymphotoxin was independent of protein kinase C activity, did not require new protein synthesis, and was at least in part a result of increased stabilization of IL-6 mRNA. $t_{1/2}$ of the IL-6 transcripts increased from 0.3 h in unstimulated cells to 0.85 h in cells stimulated with lymphotoxin. In addition, stimulators of protein kinase C, including phorbol esters and teleocidin, enhanced accumulation of IL-6 mRNA. Cycloheximide (CHX), inhibitor of protein synthesis, also markedly increased levels of IL-6 mRNA. Both CHX and activators of protein kinase C increased by > 16-fold the stability of IL-6 mRNA. Further, dose-response studies showed that sodium fluoride (NaF), activator of G-binding proteins, and ouabain, inhibitor of Na^+/H^+ pump, increased levels of IL-6 mRNA. NaF stimulated IL-6 mRNA levels independent of protein kinase C activity. These results suggest that stimulators of several pathways of signal transduction increase levels of IL-6 mRNA and posttranscriptional stabilization is, in part, the mechanism that many of these signals, including lymphotoxin, use to increase levels of IL-6 RNA. (*J. Clin. Invest.* 1990. 85:121–129.) messenger RNA • posttranscriptional stabilization

Introduction

IL-6 is a 21-kD glycoprotein containing 184 amino acid residues derived from a precursor peptide of 212 amino acids. The amino-terminal of IL-6 shares homology with granulocyte-colony stimulating factor (G-CSF)[1] (1). IL-6 contains five exons (2) and is located on chromosome 7 (3). This cytokine was previously known as IFN-β-2, 26K protein, B-cell stimulating factor-2, hybridoma growth factor, or hepatocyte-stimulating factor (4). IL-6 appears to have a variety of functions, including stimulation of proliferation of early hematopoietic stem cells and megakaryocytes, induction of differentiation of B lymphocytes, and stimulation of the liver to produce acute phase-reactive proteins.

Tumor necrosis factor-alpha (TNF-α), also known as TNF, is produced predominately by myeloid cells, especially macrophages (5, 6). Lymphotoxin, also known as TNF-β, is synthesized predominately by activated lymphocytes (5, 7, 8). Lymphotoxin has structural similarities to TNF and both are cytolytic or cytostatic for transformed cells (9, 10). Previously, Kohase et al. (11) reported that TNF-α and several other agents, including a phorbol ester, stimulated normal fibroblasts to synthesize IL-6, and their data suggested that the stimulation was probably mediated through activation of the protein kinase C pathway (12). However, little is known of the role that lymphotoxin plays in modulation of IL-6 expression. In this study, we show that stimulators of several pathways of signal transduction elevate levels of IL-6 mRNA and stabilization of these transcripts is, in part, the mechanism that many of these signals, including lymphotoxin, use to increase levels of IL-6 mRNA.

Methods

Reagents. Recombinant human TNF-α (5.6×10^7 U/mg protein) and recombinant lymphotoxin (2×10^8 U/mg protein) were generously supplied by M. Shephard, Genentech Inc. (South San Francisco, CA); both were expressed in *Escherichia coli.* Both contained low levels of endotoxin (TNF-α preparation, < 0.03 EU/mg protein; lymphotoxin preparation, 3.13 EU/mg protein). Specific activity of each was assayed twice; once at Genentech and then at UCLA by examining their cytolytic activity on actinomycin-D–treated L929 fibroblasts (6). Results were nearly identical. 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 4-0-methyl TPA, phorbol 12,13-didecanoate (PDD), 4α-PDD, and phorbol 12,13-dibutyrate (PDB) were purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in acetone, and stored at −20°C until use. All other reagents were also of the best grade available from Sigma Chemical Co.

Cells and cell culture. Normal human embryonic lung fibroblast (WI38, obtained from the American Type Tissue Culture Collection) and a human lung adenocarcinoma cell line (Lu-CSF-1) (13) were cultured in α medium (Flow Laboratories, Inc., McLean, VA) supplemented with 10% FCS in a humidified atmosphere containing 5% CO_2. Cells were harvested by treatment with 0.05% trypsin, 0.02% EDTA (wt/vol) in PBS. Conditioned media (CM) from confluent cultures containing WI38 fibroblasts and either TNF-α or lymphotoxin were prepared by centrifuging the supernatants at 1,000 *g* for 10 min,

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1. *Abbreviations used in this paper:* CHX, cycloheximide; CM, conditioned media; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte monocyte-colony stimulating factor; PDB, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-decanoate; TNF-α and -β, tumor necrosis factor-α and -β, respectively; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

putting them through filters (0.22 μm; Millipore Corp., Bedford, MA), and storing at −20°C until use.

Measurement of IL-6 bioactivity. IL-6 levels were measured using the IL-6–dependent murine hybridoma clone, MH60.BSF2, in a bioassay (14). These hybridoma cells are absolutely dependent on IL-6; IL-1α and β, IL-2, IL-3, IL-4, IL-5, IFN-α or -γ, and G-CSF do not induce their growth (12). 1×10^4 cells/well were cultured in RPMI-1640 medium containing 10% FCS (lot 5121103; Irvine Scientific, Santa Ana, CA), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol with test samples or varying concentrations of recombinant IL-6 (5×10^6 U/mg protein; generous gift of Cetus Corp., Emeryville, CA [14]). Each sample and standard were studied at different dilutions in triplicate, round-bottom, 200 μl microtiter wells (Nunclon® Delta, InterMed Nunc, Roskilde, Denmark) for 48 h in a 5% CO_2 humidified incubator at 37°C, pulsed with 2 μCi [^{3}H]thymidine (20.0 Ci/mmol; New England Nuclear, Boston, MA) for the last 6 h, harvested onto glass filter paper, and prepared for liquid scintillation. Radioactivity was measured on a scintillation counter (Beckman Instruments, Inc., Fullerton, CA), and data were analyzed and graphed on a Macintosh II personal computer using StatWorks® (Version 1.0, Datametrics, Inc., Philadelphia, PA) and Cricket Graph (Version 1.2, Cricket Software, Malvern, PA) software.

Studies of antibody neutralization of IL-6 activity in CM used a heterogenous rabbit antisera raised against recombinant IL-6 (Genzyme Corp., Boston, MA). It was used at 1 μg/ml. Aliquots of fibroblasts CM were preincubated for 2 h at 37°C with IL-6 antibody and then added to the MH60.BSF2 cells.

DNA probes. Human IL-6 cDNA (1.2 kb, Xho I) was derived from plasmid pXM (generous gift of S. Clark, Genetics Institute, Cambridge, MA). β-actin DNA probe (0.7 kb, Eco RI-Bam HI) was from pHF-β A-3′ at plasmid (15). These probes were ^{32}P-labeled by random priming method (16). The specific activity was $5–8 \times 10^8$ cpm/μg.

Isolation and blotting of RNA. For cytoplasmic RNA, WI38 cells were suspended in hypotonic buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM KCl, 3 mM $MgCl_2$, and were lysed with 0.3% NP-40. Cytoplasmic RNA was extracted by phenol/chloroform method as previously described (17). After denaturation at 65°C, RNA was electrophoresed in an agarose-formaldehyde gel (1%) and transferred to a nylon-membrane filter (Biotrans®, ICN, Irvine, CA) (18). Hybridization with labeled probe was for 16–24 h at 42°C in 50% formamide, 2× SSC (1× = 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's solution, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml salmon sperm. Filters were washed to a stringency of 0.1× SSC, 65°C and exposed to XAR film (Eastman Kodak Co., Rochester, NY). Autoradiograms were developed at different exposures.

Blots were usually sequentially hybridized with ^{32}P-labeled IL-6 and β-actin DNA. The actin band of hybridization was used to help confirm that similar amounts of RNA were added to each lane. Modulation in levels of IL-6 RNA were quantified by initial standardization to the amount of β-actin–specific transcripts. The relative density of β-actin–specific and IL-6–specific transcripts in the different lanes was first determined by laser densitometry using multiple exposures of the blot, and the ratio of IL-6/β-actin in the control lane was assigned to be the baseline level. The fold stimulation in the experimental lanes was calculated by multiplying the ratio of density of (IL-6/β-actin) transcripts by the reciprocal of the ratio of the baseline level.

[^{3}H]Uridine and [^{35}S]methionine incorporation. Fibroblasts were exposed to protein kinase C inhibitors or cycloheximide (CHX) in culture dishes (Falcon®, Becton Dickinson Labware, Oxnard, CA) in triplicate per experimental point for 2 h. Cells were pulsed with 2 μCi of [^{3}H]uridine (43 Ci/mmol sp act) or 4 μCi of [^{35}S]methionine (200 mCi/mM) for 1 h at 37°C, washed twice in PBS, precipitated in 5% TCA in 30 mM Na_2HPO_4 at 4°C for 1 h, filtered onto a glass microfiber membrane (Whatman Inc., Clifton, NJ; GF/F), washed in 3% TCA (30 mM Na_2HPO_4), and heated at 80°C for 1 h. Each sample was counted by liquid scintillation. Results were compared with those of untreated cells.

Protein kinase C assay. The activity of protein kinase C was assayed as described previously (19). Briefly, soluble (cytosol) and solubilized particulate (membrane) fractions of cells were prepared, and both fractions were assayed for protein kinase C activity using histone H1 as substrate. The amounts of protein kinase C in the cytosolic (22 μg) and solubilized membrane (5 μg) fractions were determined in the reaction mixtures (0.2 ml) containing 25 mM Tris-HCl, pH (7.5), 10 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP, and 40 μg of histone.

Results

Lymphotoxin induction of IL-6 protein in fibroblasts. Lymphotoxin was added to subconfluent cultures of WI38 lung fibroblasts for 4 d; CM were harvested for IL-6 activity (Fig. 1). In the absence of lymphotoxin, fibroblasts constitutively produced IL-6 protein. Lymphotoxin enhanced production of IL-6 in a dose-dependent manner. 10^4 U/ml lymphotoxin stimulated approximately five times greater concentration of IL-6 than was constitutively produced. The activity of IL-6 in the CM cells cultured with 10^3 U/ml lymphotoxin was almost neutralized by IL-6 antibody (78±1%). Additional control experiments showed that fresh culture media containing 10^3 or 10^4 U/ml lymphotoxin inhibited [^{3}H]Tdr incorporation in the IL-6 responder cells (MH60.BSF2) by < 5% of untreated control cultures (data not shown).

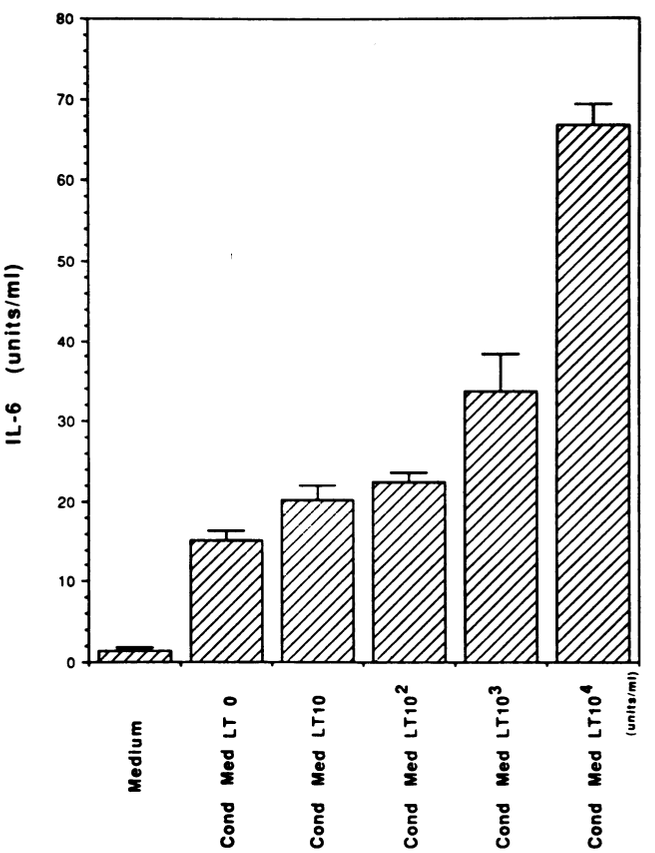


Figure 1. IL-6 production after exposure of fibroblasts to lymphotoxin (LT). Human lung fibroblasts were cultured with 0–10^4 U/ml lymphotoxin for 4 d; conditioned media (CM) were harvested and added (50% vol/vol) to IL-6–dependent murine hybridoma clone, MH60.BSF2 as described in Methods. Results represent mean and standard error of triplicate cultures.

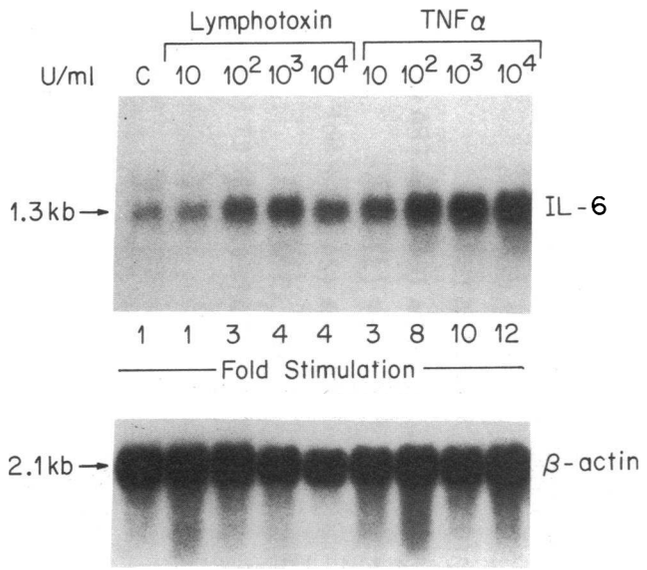


Figure 2. Dose-dependent effect of lymphotoxin and TNF-α on levels of IL-6 mRNA in human lung fibroblasts. Fibroblasts were cultured with either lymphotoxin or TNF-α for 8 h. Cytoplasmic RNA (15 μg/lane) was prepared and analyzed by formaldehyde–agarose gel electrophoresis and transferred to a nylon membrane as described in Methods. Hybridization was with ^{32}P-labeled IL-6 cDNA (1.3 kb; *top*) and β-actin DNA (2.1 kb; *bottom*). Fold stimulation of level of IL-6 mRNA as compared with levels in untreated cells was equalized for levels of β-actin (see Methods).

Dose-dependent effect of lymphotoxin and TNF-α on levels of IL-6 mRNA. Fibroblasts constitutively contained a low concentration of mRNA coding for IL-6 (Fig. 2). After exposure to 10–10^4 U/ml of either lymphotoxin or TNF-α for 8 h, levels of IL-6 mRNA increased in a dose-dependent manner. TNF-α was more potent than lymphotoxin. 10 U/ml TNF-α stimulated an equivalent accumulation of IL-6 mRNA as 10^2–10^3 U/ml lymphotoxin; and at maximally stimulatory concentrations, TNF-α was threefold more potent than lymphotoxin. Time-response experiments (0–8 h) found that both TNF-α (3,000 U/ml) and lymphotoxin (3,000 U/ml) stimulated accumulation of IL-6 mRNA with a similar rapidity, with maximal stimulation achieved within 1 h (data not shown). As was found in the dose-response studies, TNF-α was fourfold more potent than lymphotoxin at each time point (data not shown).

Effect of teleocidin and various derivatives of phorbol ester on expression of IL-6 mRNA. Teleocidin is a nonphorbol tumor promoter which can activate protein kinase C (20). Treatment of cells with 5 or 50 μg/ml teleocidin for 2 h increased accumulation of IL-6 mRNA about fourfold compared with untreated cells (Fig. 3 *A*). TPA, PDD, and PDB are phorbol esters that are potent activators of protein kinase C; their derivatives, 4-*O*-methyl TPA and 4-α-PDD are unable to activate protein kinase C (21). Fibroblasts were exposed to each compound (50 nM) for 2 h and levels of IL-6 mRNA measured. Potency of the phorbol ester to stimulate accumulation of IL-6 mRNA in fibroblasts paralleled their known abilities to activate protein kinase C (Fig. 3 *B*).

Effect of inactivation of protein kinase C on expression of IL-6 mRNA stimulated by either NaF or lymphotoxin. NaF is an activator of several G-binding proteins (22). To determine whether NaF stimulates IL-6 mRNA accumulation, fibroblasts were cultured with NaF at different concentrations for 4 h. 5mM NaF stimulated a 28-fold increased accumulation of IL-6 mRNA as compared with untreated control cells (Fig. 4 *A*).

Cells exposed for prolonged durations to TPA reduce their protein kinase C activity, thus making them resistant to repeated exposure to TPA (23). We showed that treatment with 100 nM TPA for 24 h decreased protein kinase C activity by > 80% and decreased cell membrane-binding of [^{3}H]PDB by > 90%, and reexposure to TPA did not stimulate the activity of protein kinase C (data not shown). Fibroblasts incubated with TPA (50 nM) for 4 h contained markedly increased levels of IL-6 mRNA compared with untreated cells (Fig. 4 *B*). Pro-

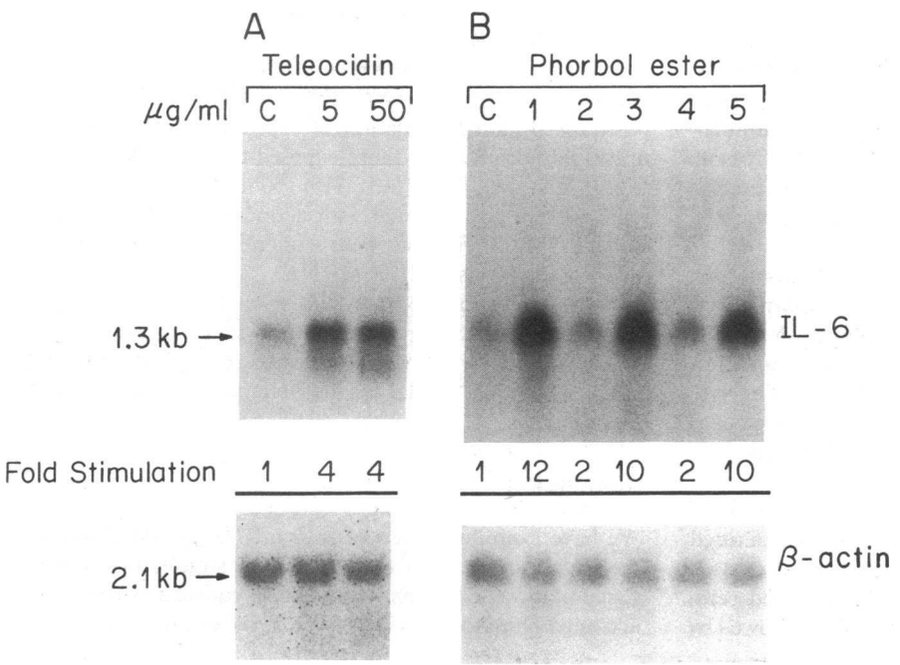


Figure 3. Effect of teleocidin (*A*) and various derivatives of phorbol ester (*B*) on expression of IL-6 mRNA in fibroblasts. (*A*) Cells culture with either 5 or 50 nM teleocidin for 2 h. Lane *C*, untreated control. (*B*) Cells were exposed to each compound (50 nM) for 2 h: lane *1*, TPA; lane *2*, 4-0-methyl TPA; lane *3*, PDD; lane *4*, 4-α-PDD; lane *5*, PDB. Lane *C*, untreated control. Northern blot analysis of mRNA was performed by blotting cytoplasmic RNA (15 μg/lane). The 1.3- and 2.1-kb hybridizing bands are consistent with mRNA coding for IL-6 and β-actin, respectively. Fold stimulation of levels of IL-6 mRNA as compared with levels in untreated cells was calculated as described in Methods.

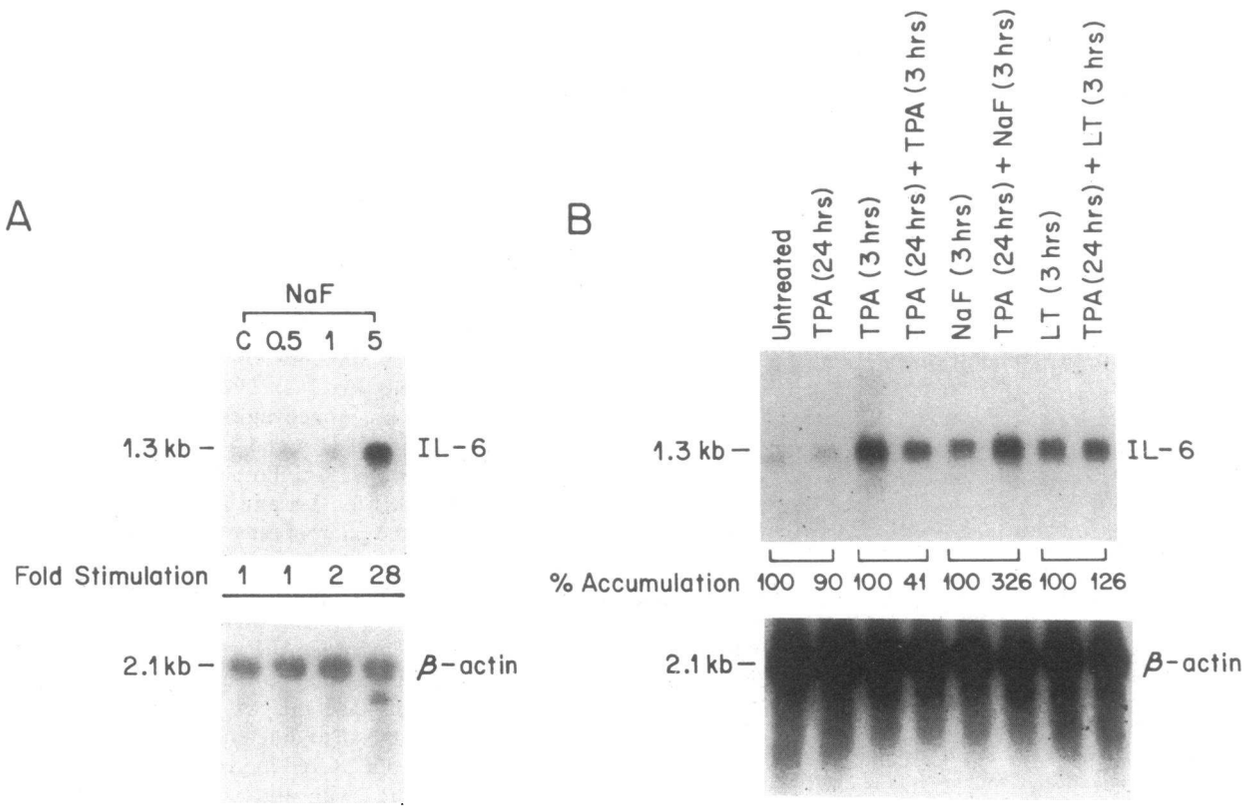


Figure 4. Effect of prolonged exposure of a phorbol ester on expression of IL-6 mRNA stimulated by NaF or lymphotoxin. (*A*) Fibroblasts were cultured with different concentrations (0.5–5 mM) of NaF (4 h). (*B*) Cells were pretreated with TPA (100 nM, 24 h), washed three times, and exposed to either TPA (50 nM), NaF (5 mM), or lymphotoxin (4,000 U/ml) for 3 h. As positive control, cells were cultured only with TPA (50 nM, 3 h), NaF (5 mM, 3 h) or lymphotoxin (4,000 U/ml, 3 h). Cytoplasmic RNA was extracted and each blot (15 μg/lane) was sequentially hybridized with IL-6 cDNA (1.3 kb) and β-actin DNA (2.1 kb). For percent accumulation, each positive control (3 h exposure to TPA, NaF, lymphotoxin) was compared with cells pretreated with TPA (24 h) and then cultured (3 h) with TPA, NaF, or lymphotoxin.

longed exposure (24 h) of cells to TPA did not stimulate their accumulation of IL-6 mRNA; reexposure of these cells to TPA for 4 h had 60% lower levels of IL-6 mRNA as compared with those cells cultured with TPA for 4 h alone. In contrast, the enhanced levels of IL-6 mRNA mediated by NaF or lymphotoxin were not blocked by preexposure of the cells to TPA for 24 h (Fig. 4 *B*). The combination of 24-h exposure to TPA plus an additional 4 h of TPA and NaF stimulated the accumulation of IL-6 mRNA slightly more than NaF alone. The reason for this enhanced stimulation is not clear. These experiments were repeated three times with similar results (data not shown).

Effect of ouabain on expression of IL-6 mRNA. Fibroblasts were cultured with ouabain (inhibitor of Na^+/H^+ pump; 24); cytoplasmic RNA was extracted and analyzed for IL-6 mRNA levels. Exposure to ouabain markedly increased accumulation of IL-6 mRNA in a dose-dependent manner (Fig. 5). Time-response experiments showed that within 4 h, ouabain increased IL-6 mRNA levels by 20-fold, and a 100-fold increase occurred at 24 h (data not shown).

Effect of lymphotoxin on expression of IL-6 mRNA in the absence of protein synthesis in fibroblasts. Fibroblasts cultured with 20 μg/ml CHX (inhibitor of protein synthesis) decreased protein synthesis by 93% as compared with untreated cells. Cells cultured initially with CHX (20.0 μg/ml) and followed by the addition of lymphotoxin had a 98-fold enhanced accumulation of IL-6 mRNA as compared with untreated control cells (Fig. 6). This enhancement was greater than those observed when the cells were cultured with either lymphotoxin (fivefold) or CHX (59-fold) alone.

Stability of steady-state IL-6 mRNA in fibroblasts exposed to either lymphotoxin, TNF, TPA, or CHX. To examine for posttranscriptional regulation of expression of IL-6 mRNA, untreated fibroblasts or those exposed to lymphotoxin (4,000 U/ml), TNF-α (1,000 U/ml), TPA (50 nM), or CHX (5 μg/ml) for 4 h were also cultured with actinomycin D for an additional 0.5–4.0 h. Samples were sequentially harvested and examined for levels of IL-6 mRNA. $t_{1/2}$ of steady state IL-6 mRNA in unstimulated fibroblasts was 0.3 h (Fig. 7). $t_{1/2}$ of IL-6 mRNA was 0.85 h after culture with either lymphotoxin or TNF-α (Fig. 7) and was > 4 h after exposure to either TPA or CHX (Fig. 8). Similar results were observed in three more experiments.

Discussion

We have examined levels of IL-6 mRNA in fibroblasts after their exposure to either lymphotoxin or stimulators of several signal pathways. Lymphotoxin is a glycoprotein produced by activated lymphocytes (7, 8, 25–27); it can inhibit the growth

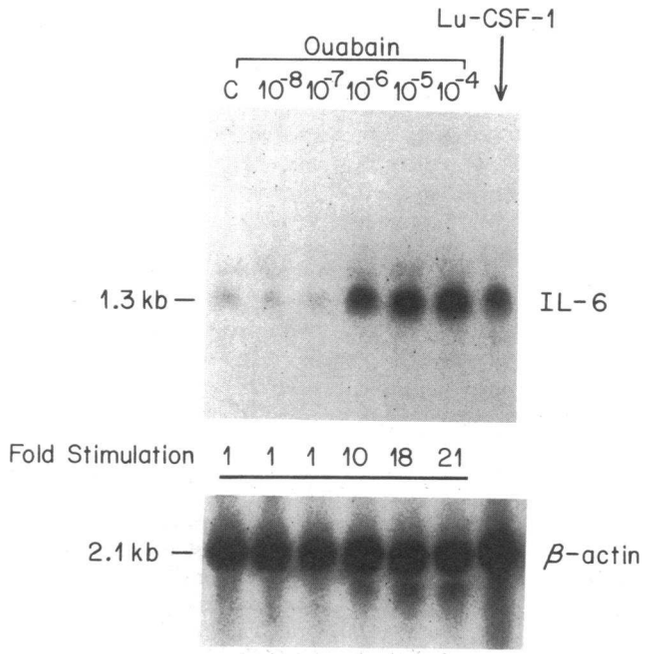


Figure 5. Effect of ouabain on expression of IL-6 mRNA. Fibroblasts were exposed to different concentrations of ouabain for 24 h (10^{-8}–10^{-4} M). Northern blot analysis of IL-6 and β-actin mRNA were performed by blotting cytoplasmic RNA (15 μg/lane) from cells as described in Methods. Fold-stimulation was calculated as stated in Methods. Lu-CSF-1 mRNA (lung adenocarcinoma cell line) was used as a positive control (13). Fold stimulation of IL-6 mRNA levels determined as stated in Methods. The minor bands seen at ~ 1.3 kb in the β-actin panel represent radioactivity that was not removed after hybridization with the IL-6 probe.

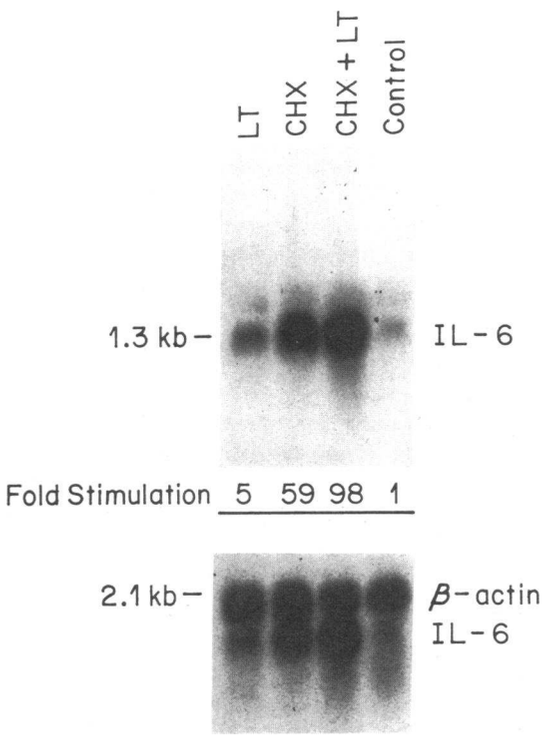
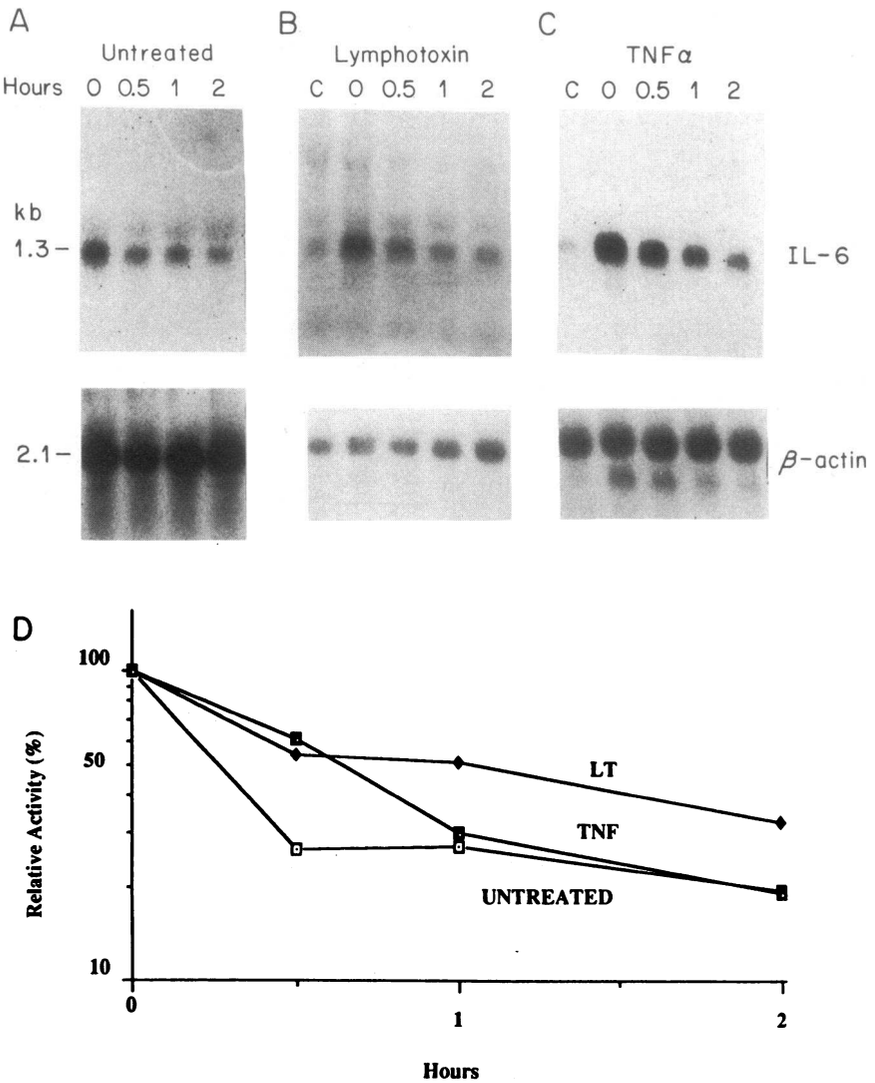


Figure 6. Ability of lymphotoxin to stimulate accumulation of IL-6 mRNA in fibroblasts in absence of protein synthesis. Cells were either treated with lymphotoxin (2.5 h, 3,000 U/ml), (CHX, 2.5 h, 20 μg/ml) or pretreated with CHX (0.5 h) and then cultured with CHX and lymphotoxin (2.0 h). Analysis was performed by blotting cytoplasmic RNA (15 μg/lane) and hybridizing with ^{32}P-labeled IL-6 (1.3 kb) or β-actin (2.1 kb) DNA probes as described in Methods. Fold stimulation of bands of IL-6 mRNA was determined as described in Methods. The bands seen at ~ 1.3 kb in the panel of β-actin represent radioactivity that was not removed after previous hybridization with the IL-6 probe.

of a variety of cells transformed by chemicals or viruses (28, 29). Many of the actions of lymphotoxin are similar or identical to those observed with TNF-α. Lymphotoxin and TNF-α probably share the same cell surface receptors (30). Previously, we have observed that lymphotoxin stimulates normal fibroblasts to produce several hematopoietic CSFs (31). In this study, we have found that normal lung fibroblasts constitutively produced IL-6 mRNA. After exposure to lymphotoxin, levels of IL-6 mRNA increased in these cells more than fourfold. TNF-α was about three times more potent than lymphotoxin at equivalent concentrations of these cytokines.

Protein kinase C is involved in signal transduction by coupling receptor-mediated inositol phospholipid turnover with a variety of cellular functions (32). We have observed that both phorbol diesters and nonphorbol compounds that activate protein kinase C stimulated accumulation of IL-6 mRNA in the fibroblasts. In contrast, phorbol esters incapable of activating protein kinase C were unable to stimulate increased concentrations of IL-6 mRNA. In addition, we took advantage of the fact that prolonged exposure of cells to TPA leads to inactivation of protein kinase C (33, 34). Prolonged (24 h) exposure to TPA partially blocked accumulation of IL-6 mRNA after reexposure of cells to TPA. However under these same conditions, accumulation of IL-6 mRNA was not blocked after reexposure of the TPA-treated cells to lymphotoxin. Zhang et al. reported that TNF and IL-1 stimulated IL-6 mRNA levels by a protein kinase C–independent mechanism in human fibroblasts using similar methods (35). Taken together, these results also suggest that lymphotoxin probably does not mediate accumulation of IL-6 mRNA through protein kinase C. Another study using putative inhibitors of protein kinase C suggested TNF-α stimulated accumulation of IL-6 mRNA through the protein kinase C pathway (12). We also have found that the same inhibitors (H-7 and H-8, 50 mM for 2 h) could block the stimulation mediated by both TNF-α and lymphotoxin; but these agents also inhibited the stimulation mediated by NaF, CHX, and ouabain, and decreased total RNA synthesis by 70% (data not shown). In view of the very short $t_{1/2}$ of IL-6 mRNA, we believe that the ability of H-7 to inhibit the accumulation of IL-6 mRNA mediated by lymphotoxin is probably a nonspecific effect.

A recent study suggested that G-binding proteins may be involved in transducing the signal mediated by TNF after binding to its receptors on leukemia cells (36). In this regard, we have found that NaF stimulated the accumulation of IL-6 mRNA. F^- ion in combination with Al^{3+} (usually present in trace amounts in tissue culture) binds and activates G-binding proteins. Preexposure of the fibroblasts to pertussis toxin (10–1,000 ng/ml for 4–24 h) was unable to block the action of



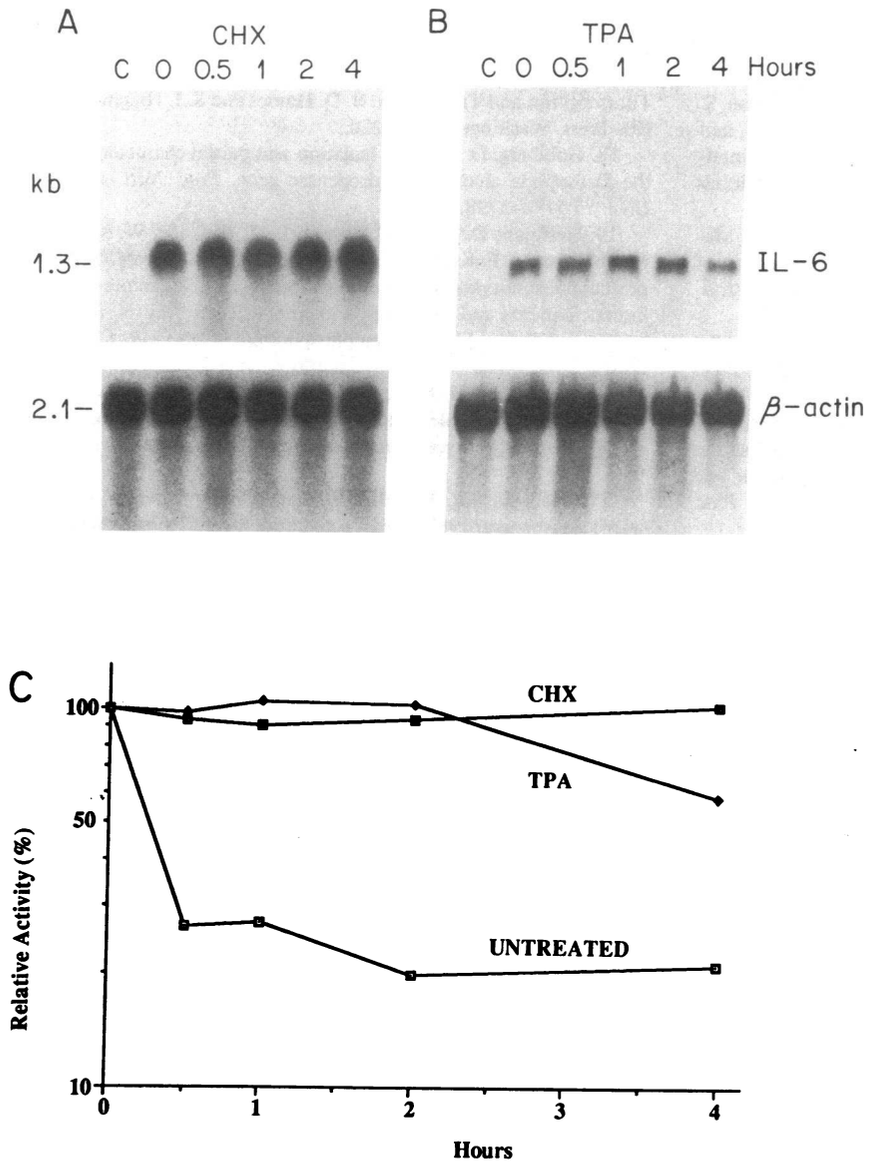
Figures 7 and 8. Stability of steady-state IL-6 mRNA in human fibroblast exposed to lymphotoxin, TNF-α, TPA, or CHX. Untreated cells (Fig. 7 *A*) or those exposed to lymphotoxin (4,000 U/ml, Fig. 7 *B*); TNF-α (1,000 U/ml, Fig. 7 *C*); CHX (5 μg/ml, Fig. 8 *A*) or TPA (50 nM, Fig. 8 *B*) for 4 h were then also cultured with actinomycin D (5 μg/ml) for 0.5–4.0 h. Cytoplasmic RNA (30 μg/lane in untreated cells and 15 μg/lane in lymphotoxin, TNF, CHX, or TPA-treated cells) was extracted and analyzed by RNA blotting as described in Methods. Blots were sequentially hybridized with ^{32}P-labeled IL-6 cDNA (1.3 kb bands, *top panels*) and with β-actin DNA (2.1 kb bands, *bottom panels*). Intensity of hybridization was determined by densitometry of several different exposures of the autoradiograms. Plots of RNA stability are shown on Fig. 7 *D* and Fig. 8 *C*. Untreated cells of each experiment were assumed to have 100% activity.

NaF (data not shown). Several G-binding proteins are not ribosylated and therefore inactivated by pertussis toxin (37). NaF may modulate levels of IL-6 mRNA in fibroblasts through a G-binding protein pathway that is insensitive to pertussis toxin. In further experiments, our results suggest that the increase of levels of IL-6 mRNA mediated by NaF probably does not require protein kinase C because partial inactivation of this enzyme did not blunt the response of NaF.

We observed that ouabain markedly increased levels of IL-6 mRNA in a dose-dependent manner. Ouabain blocks the Na^+/K^+ pump, causing a rise of intracellular Na^+ (24). Increased intracellular Na^+ levels can increase the cytoplasmic Ca^{++} concentration. Prior studies showed that a Ca^{++} ionophore can enhance levels of IL-6 mRNA (12). Further studies are in progress to determine the importance of Na^+ and/or Ca^{++} cellular fluxes in mediating the accumulation of IL-6 mRNA.

The IL-6 mRNA level increased in the fibroblasts when new protein synthesis was nearly stopped by CHX. Seghal et al. also showed that exposure of human diploid fibroblasts to CHX increased their concentrations of IL-6 mRNA (9). In addition, we have found that lymphotoxin was able to cause the accumulation of IL-6 mRNA in the absence of new protein synthesis.

To pursue further how lymphotoxin stimulated the increase in levels of IL-6 mRNA, we studied stability of IL-6 mRNA. In untreated fibroblasts, the $t_{1/2}$ of IL-6 mRNA was 0.3 h. Exposure of the cells to either CHX or TPA markedly stabilized the message, with little decrease in levels at 4 h of exposure. IL-6 mRNA is a transiently expressed gene having an AU-rich 3′ region, which may be a target for short-acting RNAses. Studies of Shaw and Kamen (38) suggested that TPA and CHX stabilized another cytokine (GM-CSF) by altering the response of the cell to this AU-rich region of the mRNA. TNF-α and lymphotoxin also consistently increased the stability of IL-6 mRNA, both increasing the $t_{1/2}$ of IL-6 mRNA to ~ 0.85 h as compared with the untreated, control fibroblasts. Further studies are now determining if the stabilization of mRNA by lymphotoxin requires the AU canonical sequences in the mRNA. We previously have found that TNF-α can increase levels of G- and GM-CSF mRNA by stabilization of these RNAs (13). A posttranscriptional regulation by various



Figures 7 and 8 (Continued)

signals, including TNF-α and lymphotoxin, may be a common mechanism for rapid and sensitive change in levels of IL-6 and other cytokines. Because both TNF-α and lymphotoxin equally stabilized the IL-6 mRNA, another explanation is required to account for the threefold increased potency of TNF-α as compared with lymphotoxin in stimulating levels of IL-6 mRNA. Experiments to measure rates of IL-6 transcription in fibroblasts exposed to lymphotoxin and TNF-α are now in progress.

Fibroblasts constitute a major element of bone marrow stroma, as well as submucosal and subcutaneous tissue. Our data suggest that in vivo, lymphotoxin produced by activated lymphocytes can stimulate these mesenchymal cells to synthesize IL-6. Under normal conditions, IL-6 can have numerous functions, including the stimulation of the proliferation of early hematopoietic stem cells (39) and the differentiation of B lymphocytes (40). Lymphotoxin and IL-6 may also be involved in pathologic conditions. For example, affected joint spaces in rheumatoid arthritis often contain high levels of IL-6, lymphotoxin, TNF-α, and IL-1 (41–43). These factors at least in part are capable of causing bone resorption, as well as collagen and cartilage destruction (42–45). Furthermore, the B and T lymphocytes, macrophages, and synovial cells within these joints may inappropriately develop a paracrine network through production of these cytokines, helping to maintain the chronic inflammatory rheumatoid joint. Fundamental understanding of cytokines and their interactions may lead to novel approaches to several diseases.

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