Tumor necrosis factor (TNF) and lymphotoxin (LT; TNF-β) are major cytokines produced by B lymphocytes. Stimulation by okadaic acid, a phosphatase 1 and 2A inhibitor, markedly increased TNF mRNA accumulation and cytokine production. On the other hand, the accumulation of LT mRNA was not affected by okadaic acid despite structural and functional similarities between TNF and LT. The increase in TNF mRNA accumulation was due to the stimulation of gene transcription and a marked stabilization of this mRNA. The binding activities of the transcription factors AP-1 and AP-2 and NF-κB, which regulates TNF gene transcription, were also stimulated by okadaic acid. In addition, okadaic acid was shown to increase TNF production at the protein level. These results show the importance of protein phosphatases in the regulation of cytokine production in B cells, and further identifies differences in the regulation of TNF-α and LT production.

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MATERIALS AND METHODS

Reagents. OA and PMA were from Calbiochem (San Diego, CA). Actinomycin D was from Sigma Chemical Co (St Louis, MO).

Cell lines. 32Al (α+β+λ+) and RPMI 1788 (μ-δ-ε-) are EBV-transformed lines. They were cultured in RPMI 1640 (Whittaker, Walkersville, MD) with 10% fetal calf serum (FCS) (HyClone, Logan, UT).

Isolation of tonsillar B cells. Tonsillar B cells were prepared essentially as described. Briefly, single-cell suspensions from tonsils were centrifuged on a Ficoll-Hypaque cushion. The interface cells were incubated on plastic plates for 1 hour to remove adherent cells. The final cell preparation contained less than 2% T cells plus monocytes by flow cytometry analysis with monoclonal antibody (MoAb) specific for T cells (CD2) and monocytes (ML143).

Northern blot and mRNA measurements. Total cellular RNA was prepared according to the method of Chomczynski and Sacchi. Northern blot was performed essentially as described. The

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PROTEIN PHOSPHATASES AND TNF AND LT SYNTHESIS

**Fig 1.** Kinetics of OA stimulation of TNF and LT mRNA expression. 32a1 cells at 10^5/mL were stimulated by PMA (10 ng/mL) or OA (50 ng/mL) for the time period indicated. Total cellular RNA was extracted and Northern blot analysis was performed as described in the Materials and Methods section with TNF, LT, and β-actin probes. The results are representative of three similar experiments.

signal intensities were quantitated by densitometry. For mRNA stability studies, cells were stimulated by phorbol myristate acetate (PMA) (10 ng/mL) for 1 hour or OA (50 ng/mL) for 4 hours. After washing in cold phosphate-buffered saline (PBS), the cells were suspended in prewarmed medium containing 10 μg/mL actinomycin D and the appropriate drugs. At the indicated time points, cells were chilled by the addition of 4 to 5 volumes of cold PBS, collected by centrifugation, and their RNA extracted as described. After Northern blot analysis, the intensities of TNF, LT, and β-actin mRNA signals of underexposed films were measured by densitometry using an LKB Ultrascan XL scanner (Pharmacia LKB, Piscataway, NJ). The intensities of signals in these films were within the linear range of the absorbance versus radioactivity curve. TNF and LT intensities were normalized against that of β-actin and expressed as relative intensities in arbitrary units that were plotted against time in a semilogarithmic plot. Decay curves were fitted by linear regression using the computer program Sigmaplot (Jandel Scientific, Corte Madera, CA).

**Nuclear run-on measurements.** Transcription rate was measured as described. The AP-1, AP-2, xB-2, xB-3, and MyoD double-stranded DNA probes have been described. Electrophoretic mobility shift assays were performed essentially as described using 2 μg per lane of nuclear extract.

**Immunoprecipitation.** 32a1 cells (2 × 10^7/sample) were resuspended at 1 × 10^9/mL in cysteine-free RPMI 1640 (GIBCO BRL, Gaithersburg, MD) containing 10% dialyzed FCS and labeled for 10 hours with 50 μCi/mL of [35S]cysteine (DuPont/NEN, 6,000 Ci/mmoll. Immunoprecipitation using rabbit anti-TNF antibody was performed essentially as described. Immuno-precipitates were analyzed on a 12.5% sodium dodecylsulfate–polyacrylamide gel. Pharmacia low molecular weight markers were used as standards.

**RESULTS**

*Induction of TNF-α and LT mRNA in B-cell lines by OA.* The induction of TNF and LT mRNA by the protein phosphatase 1 and 2A inhibitor OA was examined. The two B-cell lines RPMI 1788 and 32a1 that showed marked increases in TNF and LT mRNA accumulation on PMA stimulation were chosen for these studies. At concentrations of 10 to 100 ng/mL, OA stimulated TNF mRNA accumulation in these cell lines in a dose-dependent manner (not shown). The time course of TNF and LT mRNA accumulation in 32a1 cells is shown in Fig 1. PMA stimulated TNF mRNA accumulation, which peaked at 0.5 to 1.0 hour. OA stimulation was more delayed, with peak accumulation at 4 hours. In contrast to PMA stimulation of LT mRNA accumulation, little increase was observed with OA stimulation. Similar results were found in RPMI 1788 cells.

Because PMA and OA stimulate cellular functions by different mechanisms, the combined effects of these two agents on TNF and LT mRNA increases were examined. At suboptimal doses of PMA and OA that stimulated little TNF mRNA accumulation, the combination of the two drugs caused a marked increase in TNF mRNA accumulation (Fig 2, lanes 2 through 4). This stimulation was at least twofold higher than the sum of the individual stimulation (Fig 2B). At a higher OA dose, slightly less synergy was found. As observed in Fig 1, OA stimulated little LT mRNA increase and no synergy with PMA in stimulating LT mRNA accumulation was detected. OA also stimulated little LT mRNA accumulation in tonsillar B cells. Thus far, OA effects on B cells were shown on B lymphoblastoid cell lines. Its effects on primary B cells were also studied. OA at 50 ng/mL by itself potently stimulated TNF but not LT mRNA expression in tonsillar B cells (Fig 3, lane 5). At 20 ng/mL, OA weakly stimulated TNF mRNA accumulation. A suboptimal concentration of
Fig 2. Effects of OA and PMA on the induction of TNF and LT mRNA in the B-cell line 32a1 showing a synergistic effect of OA and PMA on TNF mRNA accumulation. In (A), 32a1 cells were suspended at 10^5/mL in medium alone (lane 1) or medium containing 5 ng/mL PMA (lanes 2, 4, and 6) or OA at 20 ng/mL (lanes 3 and 4) or 50 ng/mL (lanes 5 and 6) for 4 hours. Twenty micrograms of total cellular RNA were loaded in each lane for Northern blots. The intensities of TNF, LT, and β-actin mRNA in (A) were determined by densitometry. The relative absorbance of TNF (○) and LT (■) mRNA plotted in (B) for lanes 1 through 6 in (A) was obtained by dividing the intensity of cytokine mRNA band by the intensity of β-actin mRNA band. One of three similar experiments is shown.

OA stimulation of TNF gene transcription rate. The stimulation of TNF mRNA accumulation by OA may also be because of the stimulation of TNF gene transcription rate. This was examined in tonsillar B cells and was found to be indeed the case, as shown by nuclear run-on studies (Fig 5). This increased TNF transcription rate may in part be because of the stimulation of the transcription rates of c-fos and c-jun (Fig 5), whose gene products form dimers and activate TNF transcription by binding to the AP-1 binding site. A low level of LT gene transcription rate stimulation

Fig 3. The induction of TNF and LT mRNA by OA and PMA in tonsillar B cells. Purified tonsillar B cells at 2 x 10^5/mL were stimulated for 4 hours in control medium (lane 1), medium containing combinations of 5 ng/mL PMA (lanes 2, 4, and 6), and 20 ng/mL OA (lanes 3 and 4), or 50 ng/mL OA (lanes 5 and 6) as indicated. Total cellular RNA from 5 x 10^6 cells was used for Northern blot analysis. The blots were probed with TNF, LT, or β-actin cDNA inserts. Two additional experiments yield similar results.
PROTEIN PHOSPHATASES AND TNF AND LT SYNTHESIS

A

PMA OA
(min) 0 15 30 45 60 90 0 15 30 45 60 90
TNF
LT
Actin

B

OA stimulation of TNF translation in B cells. OA stimulated the production of TNF by the cell line 32a1. TNF secretion was affected by OA, as shown in Fig 7. The highest level of secreted TNF was found in supernatants of B cells stimulated with both PMA and OA (lane 8), although OA in the presence or absence of PMA synthesized comparable amounts of cell-associated precursor and mature TNF. A role of PMA seems to be the facilitation of the processing of precursor TNF or the secretion of mature TNF. Unlike IL-1β, the precursor form of TNF was not secreted by B cells into the medium.

DISCUSSION

TNF and LT are two major cytokines produced by B lymphocytes. It is interesting to observe that the protein was found. Similar run-on results for TNF and LT transcription rate measurements were obtained with the cell line 32a1.

OA stimulation of transcription factor binding activities. AP-1, AP-2, and NFκB binding is important for the induction of TNF gene transcription. AP-2 and NFκB may also be important for LT transcription. Electrophoretic mobility shift assays using 32a1 nuclear extracts showed that OA was comparable with PMA in stimulating AP-2 and NFκB binding activities (Fig 6B,C). However, for AP-1 binding activities, OA was a better stimulant (Fig 6A). The combination of OA and PMA induced the highest level of binding activities for all three transcription factors. Competition studies showed that the binding to all three probes was specific. Specific probes competed for binding activities well, whereas a nonspecific probe did not appreciably affect the binding (Fig 6D,E).

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phosphatase inhibitor OA stimulates TNF but not LT mRNA accumulation, although the two genes are highly homologous and contiguous in the genome. This is the most significant difference between the regulation of TNF and LT mRNA expression besides their difference in cell type-specific occurrence. Differential OA stimulatory effects on cytokine mRNA expression has also been observed in monocytes whose TNF and IL-1/β but not IL-1α and IL-6 mRNA accumulation is stimulated by OA. OA stimulates TNF production not only in B cells but also in monocytes and T cells (S.-S.J. Sung, J.A. Walters, and S.M. Fu, unpublished results), which indicates that this stimulation is not cell-type dependent. While our studies were in progress, Rieckmann et al. reported that OA induced TNF mRNA accumulation and TNF secretion in tonsillar B cells. No mRNA stability or transcription rate was determined. In addition, OA effects on LT gene expression were not studied. Thus, our results not only confirm but also greatly extend their observations.

One reason for the preferential stimulation of TNF but not LT mRNA expression by OA was the difference in transcriptional regulation of these two genes. TNF transcriptional stimulation by OA may be mediated by the increased binding activities of NFκB, AP-1, and AP-2. Although the LT promoter contains AP-2 and NFκB binding sites and a 16-bp TNF-α/β homology domain also present in the TNF promoter, it lacks the TNF-CRE and AP-1 binding sites that may be crucial for OA transcriptional stimulation. In addition, the LT promoter contains negative regulatory elements that may override the stimulation mediated by OA. Recently, an AT-rich sequence located 580 bp upstream of the LT mRNA start site important for transcription initiation has been shown to bind high-mobility group I proteins. The role of this AT-rich sequence in OA responsiveness is presently unclear.

An important mechanism for OA stimulation of TNF mRNA accumulation is the stabilization of TNF mRNA (Fig 4), which contains tandem repeats of the destabilizing AUUUA motif. This motif, present in the 3'-untranslated region of many cytokine and oncogene mRNA, has been shown to confer instability to many mRNAs and bind a number of AU-binding proteins. The binding of these proteins to mRNA is increased by phosphorylation. This regulation by phosphorylation agrees with the role of protein kinase C in stabilizing TNF mRNA and suggest a mechanism by which mRNA stability is regulated. OA...
would be expected to stabilize TNF mRNA if phosphatase 1 or 2A regulates the activities of these binding proteins in the cell as well. Besides the AUUUA motifs, other mRNA structures may be responsible for TNF mRNA destabilization. Although LT, IL-1α, IL-1β, and IL-6 mRNA also contain multiple AUUUA motifs in their 3′-untranslated region, these mRNAs were not similarly stabilized by OA (Fig 4). Studies on the regulation of c-fos and c-myc mRNA degradation indicate that their destabilizing sequences also lie elsewhere than the multiple AUUUA motif-containing region. Other experimental evidence also suggests that binding proteins other than the AU-binding proteins may be responsible for TNF mRNA stabilization. Although anti-CD3 stimulates AU-binding protein activities, TNF mRNA is stabilized much more markedly by T-cell treatment with anti-CD28 than by anti-CD3. Thus the mechanism of TNF mRNA stabilization by protein kinase C, OA, and anti-CD28 remains to be determined.

Although PMA plus OA stimulated more mature TNF secretion than OA alone (Fig 7), it is interesting to note that OA stimulated comparable levels of cell-associated TNF as the PMA and OA combination. A similar result was obtained in human peripheral blood monocytes with lipopolysaccharide (LPS) and OA. LPS and PMA possibly stimulate the processing of the precursor TNF and the secretion of the mature TNF.

The stimulation of TNF production by OA in B cells indicates that phosphatase 1 or 2A plays critical roles in the regulation of transcription, mRNA degradation, and protein processing and secretion in B cells. OA has also been shown to increase plasminogen activator and urokinase receptor transcription and neuroepitope and neurotransmitter release. Our work emphasizes that protein phosphatases are crucial in maintaining and restoring the resting states in cells. The regulation of biochemical processes by phosphatases is complex, and the elucidation of the mechanism of this regulation in lymphoid cells is important to our full understanding of the regulation of B-cell functions.

Protein phosphatase 1 and 2A activities have been shown to be regulated by protein kinases. Tyrosine phosphorylation of protein phosphatase 2A catalytic subunit by pp60c-src, p56Lck, epidermal growth factor receptor, and insulin receptor at Tyr507 inactivates its phosphatase activity. Protein phosphatase 2A is also inactivated when its threonine residues are phosphorylated by autophosphorylation-activated protein kinase. Likewise, protein phosphatase 1 is activated by its tyrosine phosphorylation by pp60c-src. Many of these kinases function along signal transduction pathways and are activated during receptor stimulation. This suggests that an important mechanism for increasing protein ser/thr phosphorylation in the signal transduction pathway may be the inhibition of protein phosphatase 1 and 2A activities by kinases. In this regard, the synergy between suboptimal concentrations of PMA and OA in stimulating TNF production may be physiologically very relevant. Signal transduction molecules such as diacylglycerol usually exhibit weaker and more subtle effects than their analogous pharmacologic agents. The inhibition of PPI and 2A by phosphorylation are also only in the range of about 80%. Thus, the liberation of diacylglycerol and the inactivation of protein phosphatase 1 and 2A by kinase activation during receptor stimulation may provide similar synergism in stimulating TNF production as suboptimal doses of PMA plus OA.

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Differential regulation of human B-lymphocyte tumor necrosis factor-alpha (TNF-alpha) and lymphotoxin (TNF-beta) production by protein phosphatase 1 and 2A inhibitor

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