

PRODUCTION OF TUMOR NECROSIS FACTOR/CACHECTIN
BY HUMAN T CELL LINES AND PERIPHERAL BLOOD
T LYMPHOCYTES STIMULATED BY
PHORBOL MYRISTATE ACETATE AND
ANTI-CD3 ANTIBODY

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TNF/cachectin is made up of 17-kD polypeptides and has diverse biological effects (reviewed in references 1–3). The gene encoding TNF is located on chromosome 6 in tandem with the gene coding for lymphotoxin (LT)¹ (4, 5). Although TNF has been considered to be a product of cells in the monocyte/macrophage lineage, evidence has accumulated that TNF is produced by other cells. In mouse, a TNF-resistant clone was shown to produce TNF constitutively (6). In man, NK cell-enriched populations when incubated with NK target cell lines secreted an inhibitor for colony formation (7). This inhibitor had characteristics resembling TNF. With anti-TNF antibodies as a blocking agent for cytotoxicity assays on L cells, purified NK cells, mitogen-stimulated cytotoxic T cell clones, and a subclone of HUT-102 were shown to secrete TNF-like cytotoxins (8–10). In addition, a T-T hybridoma was induced to secrete TNF (11). In a recent study of lymphocyte populations isolated from peripheral blood and extensively depleted of monocytes, Cuturi et al. (12) provided convincing evidence that T lymphocytes were shown to respond to mitogens to secrete TNF.

Anti-CD3 mAbs and tumor promotor PMA have been used to activate Jurkat cells to secrete IL-2 (13). This combination induces resting T cells to proliferate in the absence of monocytes (14). In these systems, the activation of the phosphoinositol pathway by anti-CD3 mAbs via the CD3/Ti complex and the activation of protein kinase C by PMA are required. In the present study, the stimulation of TNF mRNA accumulation in leukemic T cell lines and the potentiation of this stimulation by an anti-CD3 mAb in selected lines were examined. Although there were differences in the amounts of TNF mRNA accumulated, all T cell lines were shown to synthesize TNF mRNA in response to these stimuli. The accumulation of LT mRNA under identical experimental

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¹ *Abbreviations used in this paper:* LT, lymphotoxin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

conditions was compared. Similar approaches were extended to isolated peripheral blood T cells. In both cases, it was shown that both anti-CD3 mAb and PMA were required for the maximal induction of mRNA accumulation for TNF and LT. These stimuli were capable of inducing TNF secretion by T leukemia cells and freshly isolated peripheral blood T cells.

Materials and Methods

Cell Lines. All cells were maintained in RPMI 1640 (Gibco, Grand Island, NY) with 10% FCS (HyClone Laboratories, Sterile Systems Inc., Logan, UT), 2 mM glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin, except L-929 cells (from American Type Culture Collection, Rockville, MD) which were maintained in DMEM (Gibco) with 10% FCS.

The T cell line KE-4 was derived in our laboratory from a patient with T cell lymphoma. All other cell lines were generous gifts of Dr. Jun Minowada (Fujisaki Cell Center, Hayashibara Biochem. Labs. Inc., Fujisaki, Okayama, Japan). The T cell lines CEM, Jurkat, Molt-4, HSB-2, MT-1, KE-4, and SKW-3 were cured of mycoplasma by several cycles of BM-Cycline (Boehringer Mannheim Biochemicals, Indianapolis, IN) treatment and subsequently cloned in soft agar (15) and characterized by Dr. Wei Chen of our laboratory. Fast-growing clones, free from mycoplasma contamination, were used in this study. The surface markers of these T cell lines are as follows. (a) CEM: CD2⁻, CD3⁺, CD4⁺, CD8⁺; (b) Jurkat: CD2⁺, CD3⁺, CD4⁻, CD8⁻; (c) Molt-4: CD2⁻, CD3⁺, CD4⁻, CD8⁻; (d) HSB-2: CD2⁻, CD3⁻, CD4⁻, CD8⁻; (e) MT-1: CD2⁻, CD3⁻, CD4⁺, CD8⁻; (f) KE-4: CD2⁺, CD3⁻, CD4⁻, CD8⁺; (g) SKW-3: CD2⁺, CD3⁻, CD4⁺, CD8⁺.

Isolation of Peripheral Blood T Cells. Peripheral blood T cells were prepared from buffy coat preparations obtained from the Oklahoma Blood Institute (Oklahoma City, OK). After Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) centrifugation, the mononuclear cells were fractionated by counterflow centrifugal elutriation in an elutriator rotor (model JE-6; Beckman Instruments Inc., Palo Alto, CA) as described (16). The lymphocyte fractions with <0.1% monocyte contamination as shown by nonspecific esterase staining (17) were pooled and further depleted of non-T cells by E-rosette sedimentation (18) followed by nylon wool columns (19). The final T cell preparations were 98–99% CD2⁺, and contained <1% CD20⁺ and <1% surface Ig⁺ cells as shown by flow cytometry analysis. Less than 0.01% nonspecific esterase-staining monocytes were detected in these preparations.

Monoclonal Antibodies. Anti-CD3 mAb (T3-II, IgG1) was used (14) for activation of leukemic T cell lines and peripheral blood T cells. mAbs C16 and E43 were made against isolated human TNF (Wang, C. Y., manuscript in preparation). mAb C16 was used in Western blot analysis. It reacted with the isolated 17-kD TNF. mAb E43 was chosen for its ability to block TNF activity as assayed on L cells. Neither of the anti-TNF antibodies reacted with LT.

cDNA and Oligonucleotide Probes. The mouse TNF cDNA probe in pUC8 was a generous gift of Dr. B. Beutler at the Howard Hughes Medical Institute at Dallas, TX (20). This mouse probe was used to isolate a human genomic TNF clone from an EMBL-3 library (Clontech, Palo Alto, CA). A 3.4-kb Pst I fragment of the TNF gene comprising exons 1–3 and part of exon 4 was used to confirm the Northern blot results obtained using the TNF oligonucleotide probe. The chicken β actin cDNA probe originated from Dr. M. Kirschner at the University of California at San Francisco, CA (21) and were subcloned into pT3/T7–19 (Bethesda Research Laboratories, Bethesda, MD). The human T cell receptor β chain cDNA clone Jur β 1 of Yoshikai et al. (22) was obtained from Dr. L. Pickering (T-Cell Sciences, Cambridge, MA). The IL-2-R cDNA clone (pIL-2R2) was donated by Drs. Warner Greene (Howard Hughes Medical Institute, Durham, NC) and Warren Leonard (National Institutes of Health, Bethesda, MD) (23). The IL-2 probe of Clark et al. (24) was obtained from Drs. S. Clark (Genetics Institute, Cambridge, MA) and A. Granelli-Piperno (The Rockefeller University, New York, NY). The oligonucleotide 24mer probes 5'-TACAGGCTTGTCACCTCGGGGTTTCG for human TNF (TNF-I; ref-

erence 25), and 5'-CATCTTGGGGTCTGACGGGCAGT for human lymphotoxin (LT-I; reference 26), and the 30mer 5'-GTGCAATGGTGTGAATCCGAGAGT-GAAGTG for the putative human X-linked chronic granulomatous disease gene (379 probe; reference 27) encoding the β chain of cytochrome b₋₂₄₅ were synthesized by phosphoramidite chemistry and purified by C-18 reverse-phase HPLC by Syntek (Umea, Sweden). The CD2 24mer probe 5'-TGTCTGACCCAAGGCACCCAGG (28), the second TNF 25mer probe (TNF-II) 5'-AGGAAGGAGAAGAGGCTGAGGAACA, and the second LT 24mer oligonucleotide probe (LT-II) 5'-GTGGGGACCAGGAGA-GAATTGTTG were synthesized and purified by the same methods by Dr. Kenneth Jackson at the Molecular Biology Resource Facility at the St. Francis Medical Research Institute in Oklahoma City, OK. The CGD 379 probe detected a 5-kb major band in Northern blot analysis of myelomonocytic cell lines as described (27). The CD2 oligonucleotide probe detected the two RNA bands at 1.7 and 1.3 kb in T cell lines (28). Homology search of the oligonucleotide 24mer probes TNF-I and LT-I in Genbank using default values of the Ifind program of Bionet revealed no homologous sequence with a ranking <12 and thus no known crosshybridizing human mRNA species. Similar results were obtained for homology searches for the TNF-II and LT-II oligonucleotide probes. Methods for plasmid preparation and labeling by nick translation were performed as described (29), as were the kinasing of oligonucleotide probes by [γ -³²P]-ATP (New England Nuclear, Boston, MA).

RNA Preparation and Analysis. RNA from cell pellets was prepared by ultracentrifugation of the guanidinium thiocyanate cell lysate on a CsCl cushion as described (29). Poly(A)⁺-RNA were isolated by oligo(dT)-cellulose columns (29). For Northern blot hybridizations, RNA was fractionated in 1.4% agarose gels containing 2.2 M formaldehyde, as described (29). Transfer of RNA to Genescreen Plus (New England Nuclear) hybridization, washing, and stripping of probes from blots for re-use followed the recommendations of the supplier. Hybridization of β actin, T cell receptor β chain, IL-2, and IL-2-R probes to the RNA blots were performed at 42°C in 50% formamide, 1% SDS, 1 M NaCl, 10 mM EDTA, 10% dextran sulfate (Pharmacia Fine Chemicals), 50 mM sodium phosphate, pH 7.0, and 100 μ g/ml sheared and heat-denatured salmon sperm DNA (Sigma Chemical Co., St. Louis, MO). Mouse TNF cDNA and all oligonucleotide probes were hybridized to the blots at 41°C in the same solution containing only 35% formamide. After overnight hybridization, the blots were washed in three changes of 2 \times SSC at room temperature (5 min each), followed by two 30-min washes at 60°C in 2 \times SSC and 1% SDS. The blots hybridized with actin, T β , IL-2, and IL-2-R probes were washed for an additional 30 min at 60°C in 0.2 \times SSC and 1% SDS. Blots were exposed to X-Omat XAR-5, XK-1 (Kodak), or Cronex-4 (DuPont Co., Wilmington, DE) X-ray films.

Western Blotting. Supernatant concentrates were subjected to electrophoresis with Rainbow prestained molecular weight markers (Amersham Corp., Arlington Heights, IL) in 12.5% SDS-polyacrylamide gels under reducing conditions and electrophoretically transferred to genescreen plus nylon membranes (New England Nuclear) as described (30). Blocking of nonspecific binding was performed with Carnation nonfat dry milk at 50°C overnight. Reactions of blots with primary and secondary antibody were performed as described (31). The goat anti-mouse IgG secondary antibody was affinity purified and labeled with ¹²⁵I using iodogen as described by the supplier (Pierce Chemical Co., Rockford, IL).

Cytotoxicity Assay. TNF cytotoxic activity was performed essentially as described (32) using actinomycin D-treated L-929 cells as target. Serial dilutions in duplicates of T cell supernatants or supernatant concentrates were incubated with L-929 cells for 16–20 h followed by the addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) for 4 h. The cells were then washed once with PBS, the MTT formazan was solubilized with acidified isopropanol, and the absorbance at 570 nm was read with a microELISA autoreader (model MR580; Dynatech Corp., Alexandria, VA). For anti-TNF blocking experiments, E43 was incubated with serially diluted supernatant solutions for 4–18 h before addition to the L cells. The number of cytotoxic units is defined as the reciprocal of the supernatant dilution causing 50% decrease in absorbance.

Results

Specificities of TNF and LT Oligonucleotide Probes. Two oligonucleotide (24mer and 25mer) probes for TNF (TNF-I and TNF-II) and two 24mer oligonucleotide probes for LT (LT-I and LT-II) were made for the purpose of Northern analysis of TNF and LT mRNAs accumulated in unstimulated or PMA-treated leukemic T cell lines. Fig. 1 shows the specificities of TNF-I and LT-I. In this experiment, a myeloid leukemia cell line ML-1 and a leukemic T cell line HSB-2 were treated with 30 ng/ml of PMA for 4 h. The poly(A)⁺-mRNA was isolated from control and PMA-treated cultures. Northern blot analysis was carried out under high stringency hybridization and washing conditions. A mouse TNF cDNA probe which crosshybridized with human TNF cDNA was also used. Poly(A)⁺-mRNA from unstimulated ML-1 failed to hybridize with any of the probes. With PMA treatment, mRNAs that hybridized with both mouse TNF cDNA and TNF-I probes were synthesized. Both probes identified the same 1.7-kb major and 2.9-kb minor bands. This is in agreement with the results of Northern analyses of myeloid cell line using human TNF cDNA probes (3). As expected, LT-I did not hybridize to any band (Fig. 1A, lanes 5 and 6). Similar results were obtained

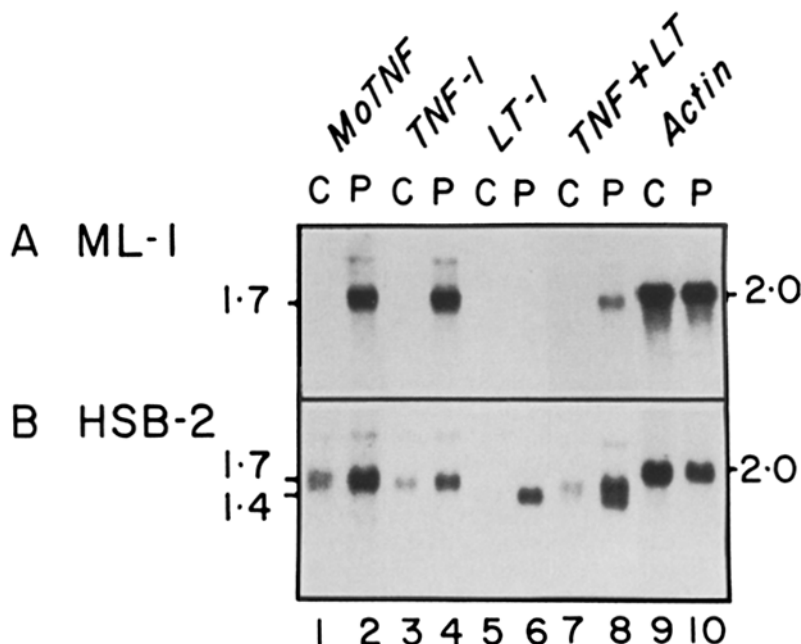


FIGURE 1. Hybridization of TNF and LT probes to myeloid (ML-1) and T (HSB-2) leukemic cell lines. Poly(A)⁺-RNA was isolated from control (C lanes) or PMA-treated (4 h, 30 ng/ml; P lanes) ML-1 (A) or HSB-2 (B) cell lines as described in Materials and Methods. A total of 5 μ g of ML-1 and 10 μ g of HSB-2 Poly(A)⁺-RNA was loaded in each lane. Northern blot analysis was performed as in Materials and Methods. Mouse TNF plasmid DNA was labeled by nick translation to a specific activity of $1-4 \times 10^8$ cpm/ μ g, heat denatured, and added to the blots at $0.5-1 \times 10^6$ cpm/ml. Oligonucleotide probes TNF-I and LT-I were labeled by kinasing to a specific activity of $4-8 \times 10^8$ cpm/ μ g and used at $0.5-1 \times 10^6$ cpm/ml. End-labeled Hind III cut phage λ DNA were heat denatured and used as molecular weight markers. The sizes of the hybridized bands are calculated molecular weights for RNA. The blots were hybridized with mouse TNF (lanes 1 and 2), TNF-I (lanes 3 and 4), LT-I (lanes 5 and 6), TNF-I plus LT-I (lanes 7 and 8), and β actin (lanes 9 and 10).

with two other myelomonocytic leukemia cell lines, KG-1 and U-937. In the case of the leukemic T cell line HSB-2, the same 1.7-kb and 2.9-kb bands hybridized to the mouse TNF cDNA and TNF-I probes in both control and PMA-treated cells. However, LT-I hybridized to a 1.4-kb mRNA. This 1.4-kb mRNA band was identified in all seven leukemic T cell lines as discussed later and three EBV-transformed B lymphoblastoid lines. The molecular weight of this mRNA is identical to that of human LT mRNA. The differences in TNF and LT expressions between ML-1 and HSB-2 were most evident when the Northern blots were probed with a mixture of TNF and LT oligonucleotides (Fig. 1, lanes 7 and 8). Although not shown, similar results were obtained when TNF-II and LT-II were used. Northern blots results obtained with the oligonucleotide and mouse TNF probes have also been confirmed by the use of a human genomic TNF Pst I fragment. As with all Northern blot analyses presented in this study, the blots were probed with β actin cDNA. In Fig. 1, β actin probing shows that similar amounts of mRNA were loaded in each lane for electrophoresis. From the above analyses, the specificities of TNF and LT oligonucleotide probes were established.

Expression of TNF and LT mRNA in T Cell Lines. Seven T cell lines were studied for their responses to PMA stimulation to make TNF and LT mRNA. In dose-response studies with CEM cells, it was determined that 10–30 ng/ml was the optimal dose range without toxicity to the cells. For subsequent experiments, 30 ng/ml of PMA was used. As shown in Fig. 2, all control cell cultures accumulated low-to-undetectable levels of TNF mRNA except HSB-2, which accumulated high TNF mRNA levels. With PMA treatment, TNF mRNA was detected in all seven cell lines. The accumulations were severalfold over that in unstimulated cells. CEM, HSB-2, and SKW-3 cells expressed the highest level of TNF mRNA, while Jurkat and Molt-4 cells expressed it at barely detectable levels. When the same blots were probed by LT-I, the 1.4-kb LT mRNA was detected in all cell lines without stimulation, and this mRNA was markedly increased upon PMA stimulation (Fig. 2B). Thus, there were considerable differences among T cell lines in their expressions of TNF and LT mRNAs. Qualitatively, the accumulations of TNF and LT mRNAs were similar in CEM, HSB-2, and MT-1 cells whereas more LT mRNA accumulation was detected in Jurkat, KE-4, Molt-4, and SKW-3 cells. In addition to cell surface marker analysis, these cell lines were shown to be of T cell origin by using cell type-specific probes. These cell lines either expressed the correct-sized rearranged T β mRNA as shown in Fig. 2C or expressed CD2 mRNA (data not shown). None of these lines made mRNA that hybridized with the myeloid-specific chronic granulomatous disease 30mer oligonucleotide probe (27).

Potentiation of PMA-induced TNF and LT mRNA Accumulation by A23187 and Anti-CD3 mAb. The effects of PMA and calcium ionophore A23187 on the induction of TNF and LT mRNA accumulations were investigated in detail in selected T cell lines. As shown in Fig. 3, lane 1, small but detectable amounts of TNF and LT mRNAs were made by the cell line CEM without the addition of either PMA or A23187. A23187 by itself in doses between 0.3 and 3 μ M had minimal effects on TNF or LT mRNA accumulation. Doses >10 μ M were toxic to the cells. At 30 ng/ml of PMA, an increase in TNF mRNA level was detected

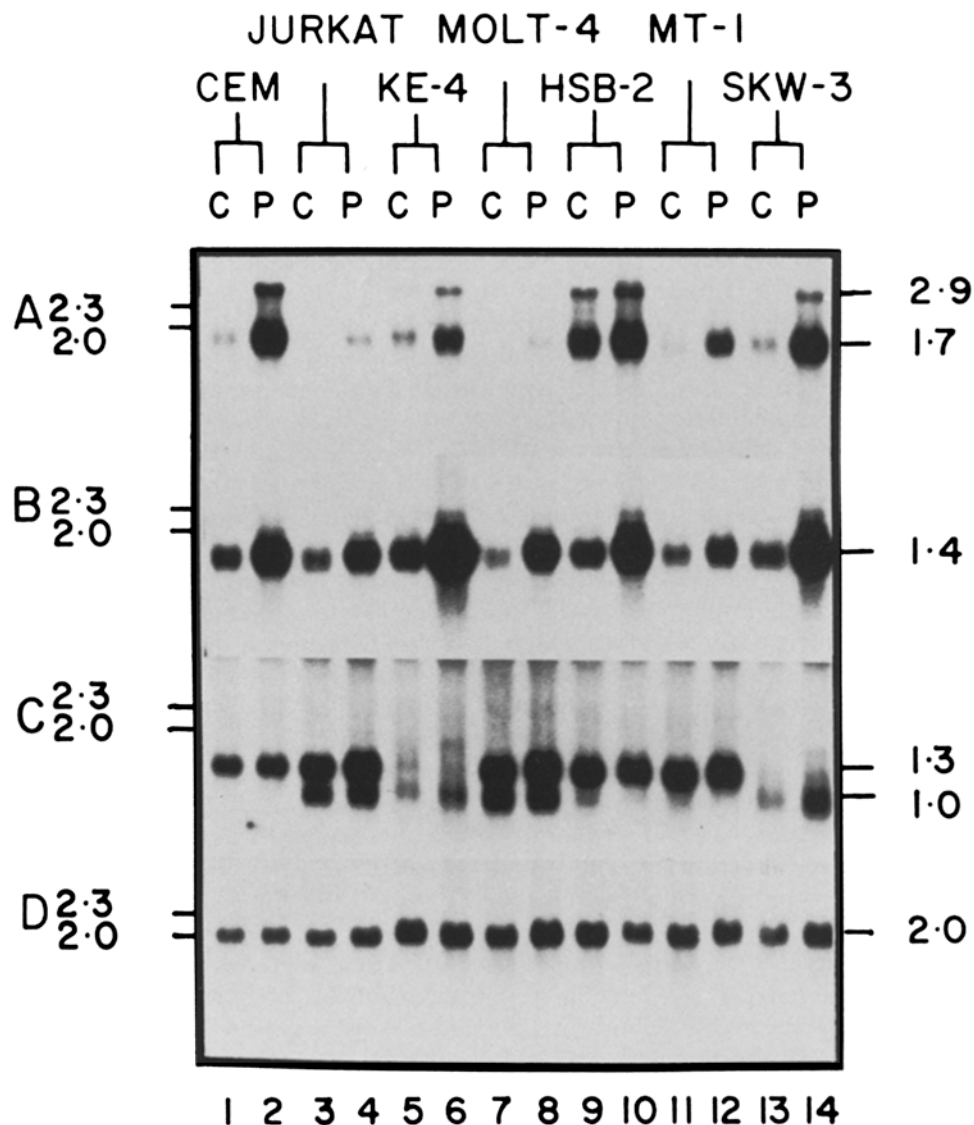


FIGURE 2. TNF and LT mRNA expression in human T cell lines. Poly(A)⁺-RNA (10 μ g per lane) from the human T cell lines CEM (lanes 1 and 2), Jurkat (lanes 3 and 4), KE-4 (lanes 5 and 6), Molt-4 (lanes 7 and 8), HSB-2 (lanes 9 and 10), MT-1 (lanes 11 and 12), and SKW-3 (lanes 13 and 14) were fractionated and hybridized to TNF-I (A), LT-I (B), human T cell receptor T β chain (C), and chicken β actin (D) probes as in Fig. 1. TNF-I and LT-I oligonucleotide probes were labeled to the same specificity and used in the same concentration to hybridize to duplicate blots, respectively. After exposure to XK-1 films, the blots were washed for 20 min at 100°C in 0.1 \times SSC and 1% SDS to remove the probes and reused to hybridize with the T β and actin probes. The exposure time for TNF- and LT-probed blots was 36 h. The molecular weight on the left were from Hind III cut DNA markers and the size markers on the right designated calculated RNA sizes. RNA from control cells (C lanes) and PMA-treated cells (4 h, 30 ng/ml; P lanes) were analyzed for each cell line.

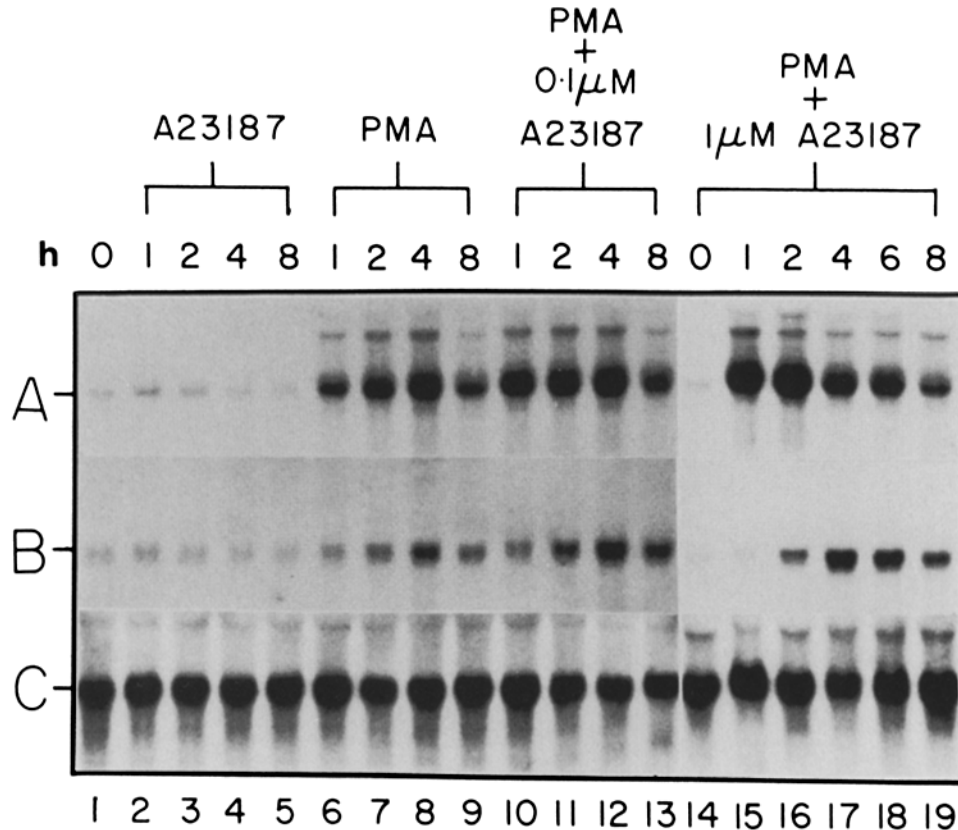


FIGURE 3. Stimulation of the expression of TNF and LT mRNA in CEM by PMA and A23187. CEM cells were stimulated with 1 μ M A23187 (lanes 2-5), 30 ng/ml PMA (lanes 6-9), 30 ng/ml PMA plus 0.1 μ M A23187 (lanes 10-13), and 30 ng/ml PMA plus 1 μ M A23187 (lanes 15-19) at 2×10^6 cells/ml in RPMI-1640 with 10% FCS for 1-8 h, chilled on ice, and harvested for RNA extraction. Northern blot hybridization to TNF-I (A), LT-I (B), and β actin (C) probes were performed as in Fig. 1. A total of 20 μ g of total RNA was loaded in each lane. Identical blots were probed with TNF-I or LT-I, exposed to XK-1 films, and stripped of the probes by boiling. The same blots were then rehybridized to the β actin probe. The exposures for panels A and B were 24 h.

1 h after incubation (Fig. 3, lane 6). The maximal TNF mRNA accumulation was reached by 4 h of incubation (Fig. 3, lane 8). Similar kinetics were seen regarding LT mRNA. A23187 at 1-3 μ M in combination with 3-100 ng/ml PMA at 4 h stimulated both TNF and LT mRNA expression three- to fourfold above the level achieved by PMA stimulation alone (not shown). With an increased dosage (1 μ M) of A23187 in combination with 30 ng/ml PMA, the peak of TNF mRNA accumulation was shifted to 1-2 h after the addition of these agents (Fig. 3). In contrast, the peak of LT mRNA accumulation remained at 4 h. Thus, for the stimulation of cellular TNF and LT mRNA accumulations, there was synergy between PMA and A23187. Similar results were obtained in the case of SKW-3.

mAb T3-II (IgG1 anti-CD3) at 1 μ g/ml was examined for its ability to potentiate PMA in stimulating TNF and LT mRNA accumulations in CEM cells.

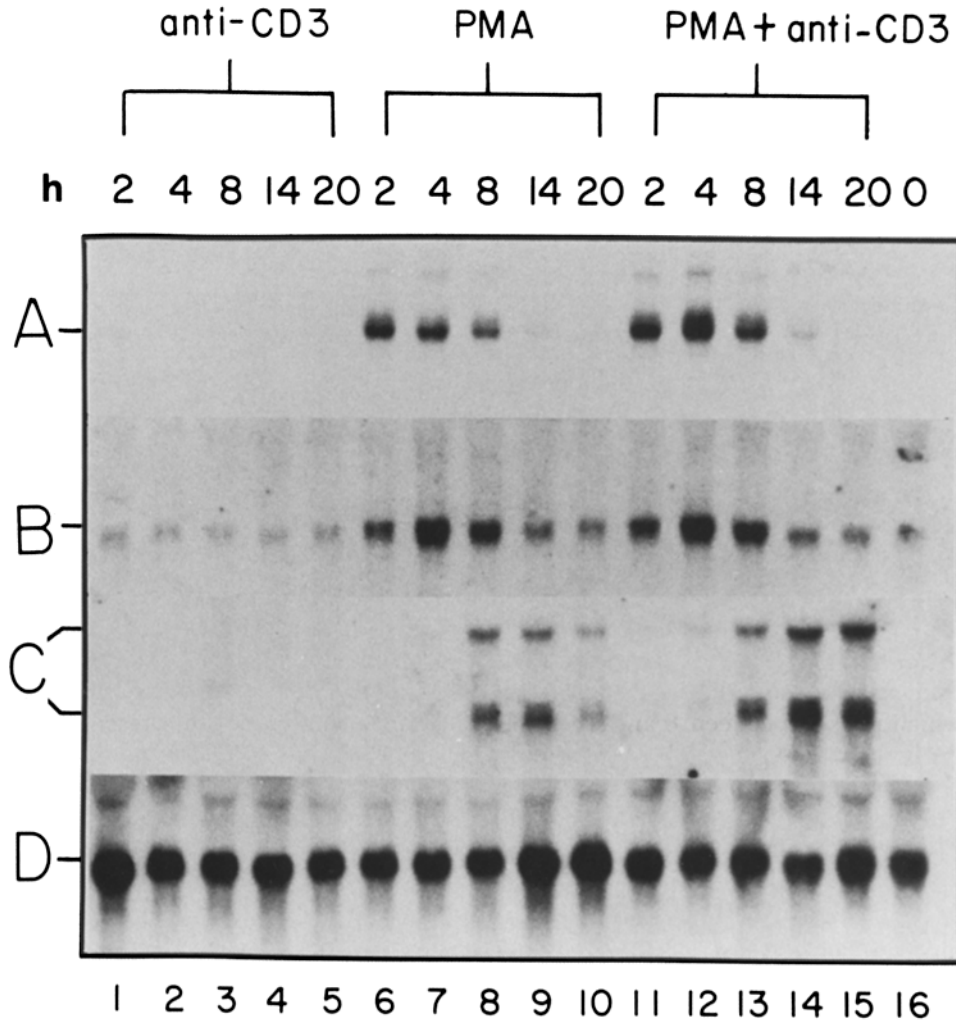


FIGURE 4. Effects of PMA and anti-CD3 on TNF and LT mRNA accumulation by CEM cells. Cells at $2 \times 10^6/\text{ml}$ were treated with $1 \mu\text{g}/\text{ml}$ anti-CD3 (T3-II) (lanes 1-5), $30 \text{ ng}/\text{ml}$ PMA (lanes 6-10), or the combination of the two reagents (lanes 11-15) for the indicated period of time. $20 \mu\text{g}$ of total RNA were loaded in each lane and Northern blot hybridizations to TNF-I (A), LT-I (B), and β actin (D) were performed as in Figs. 1 and 4. Conditions of hybridization and washing of blots probed with IL-2-R (C) were identical to that used for β actin hybridization. The exposure time of blots to the films were 24 h for A, 57 h for B, and 5 d for C.

mAb T3-II was chosen because it induced significant increases in intracellular free $[\text{Ca}^{++}]$ (data not shown). As shown in Fig. 4, mAb T3-II had no discernible effect on TNF and LT mRNA levels in CEM cells. In conjunction with PMA, T3-II induced more TNF mRNA accumulation. This combination also sustained TNF mRNA accumulation for a longer period of time. However, the peak of TNF mRNA accumulation remained at 4 h. For comparison, the cDNA of IL-2-R was used to probe the same blot. As shown in Fig. 4C, PMA induced CEM cells to make IL-2-R mRNA. T3-II was synergistic with PMA to cause increased

and more prolonged accumulation of IL-2-R mRNA. mRNA for IL-2-R peaked at 14 h. mAb T3-II was not synergistic with PMA in inducing TNF mRNA accumulation in the CD3⁻ cell line SKW-3 (not shown). The accumulation of IL-2 mRNA could be readily detected in SKW-3 stimulated with 30 ng/ml PMA and 1 μ M A23187. This expression peaked at 2–4 h after stimulation (not shown).

Of the seven T cell lines studied, Jurkat and Molt-4 cells synthesized little TNF mRNA when PMA was added. Although both cell lines were positive for CD3, they showed little response to mAb T3-II in the presence of PMA. In the presence of 1 μ M A23187, a synergistic response to PMA was detected in Molt-4 but not in Jurkat cells, resulting in an approximately fivefold increase in TNF mRNA accumulation (not shown).

Induction of TNF and LT mRNAs by PMA and mAb T3-II in Peripheral Blood T Cells. Highly purified peripheral blood T cells, which contained <0.01% monocytes and <1% B cells, were used to demonstrate the induction of TNF and LT mRNA syntheses by PMA and mAb T3-II. These T cell populations did not proliferate in the presence of mAb T3-II alone, indicating the absence of monocytes. As shown in Fig. 5A, PMA stimulated the increase in TNF mRNA accumulation at both 4 and 18 h while mAb T3-II did not have detectable effects. However, mAb T3-II increased PMA-induced TNF and LT mRNA accumulation by 5–10-fold. The maximal response for TNF mRNA accumulation occurred at ~4 h (Fig. 5B), in contrast to the LT mRNA accumulation which peaked between 8 and 18 h. IL-2 mRNA accumulation in these T cells peaked between 4 and 8 h when stimulated with PMA plus anti-CD3 (not shown). The amounts of β actin mRNA increased with time when cells were treated with PMA and mAb T3-II. This was due to an increase in mRNA synthesis upon activation. It is of note, however, that TNF and LT mRNA syntheses peaked before total mRNA accumulation per cell reached its highest level at 18 h.

The increase in TNF mRNA at 4–18 h in response to PMA and mAb T3-II was not due to contaminating monocytes because of the purity of the T cell preparations used in these experiments. As an added control, studies of isolated monocytes were carried out. Monocyte populations isolated according to Wright and Silverstein (33) were shown to reach peak TNF mRNA accumulation within 1 h after the addition of either PMA or IFN- γ plus LPS. In addition, PMA at doses >10 ng/ml was toxic to freshly isolated monocytes. With 10 ng/ml of PMA, 40% of the monocytes were stained by trypan blue after 4 h of incubation and little TNF mRNA was detected. By 18 h, all monocytes were trypan blue positive. Neither TNF mRNA nor β actin mRNA could be detected by Northern blot analysis. An experiment in which monocytes were added to the T lymphocyte cultures was further carried out. The addition of increasing numbers of monocytes (0.1–1% of total cell number) had no appreciable effect on TNF mRNA accumulation at 18 h in peripheral blood T cell populations stimulated by PMA and mAb T3-II (not shown).

Secretion of TNF by Activated CEM Leukemic T Cells and Peripheral Blood T Cells. The secretion of TNF by CEM cells was analyzed by Western blot analysis and by assaying cytotoxicity to L cells. mAb C16 was used for Western blot analysis. It reacted with isolated 17-kD TNF as well as rTNF but did not bind

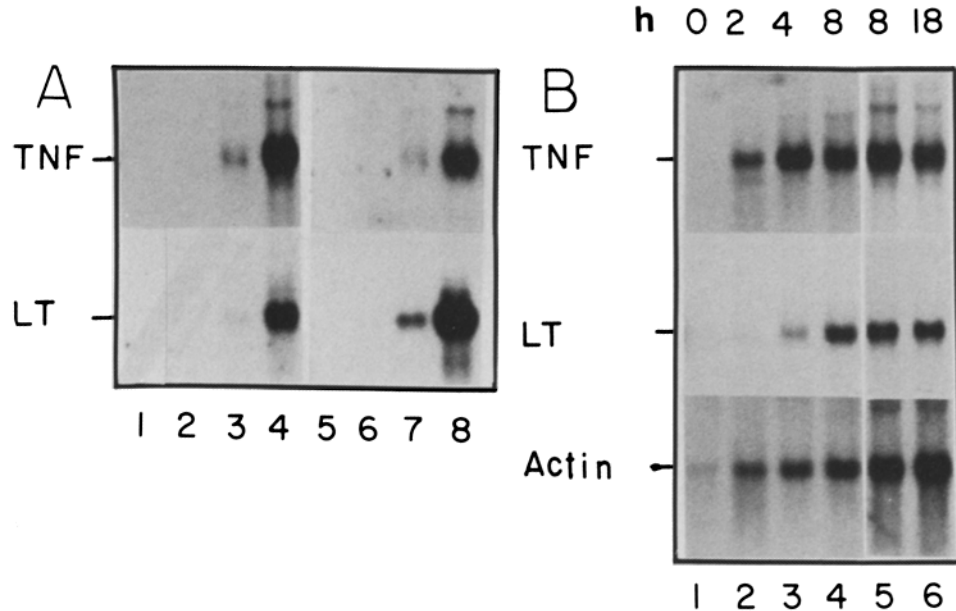


FIGURE 5. Expression of TNF and LT mRNA in peripheral blood T cells stimulated with PMA and anti-CD3. (A) Extensively purified T cells at 10^6 cells/ml in medium (50×10^6 per sample) were treated with control medium (lanes 1 and 5), $1 \mu\text{g/ml}$ T3-II anti-CD3 antibody (lanes 2 and 6), 10 ng/ml PMA (lanes 3 and 7), or the combination of PMA and anti-CD3 (lanes 4 and 8) for 4 (lanes 1–4) or 18 h (lanes 5–8). The total RNA extracted as described in Fig. 1 was quantitated by using an aliquot (5% of the sample) for absorbance measurement at 260 nm and then split into two halves and loaded into two gels. Northern blot hybridization to TNF-I and LT-I were performed as in Fig. 1. (B) The time course of TNF and LT mRNA accumulation by purified T cells stimulated with 10 ng/ml PMA plus $1 \mu\text{g/ml}$ T3-II anti-CD3 antibody. The procedure was the same as A. Samples in lanes 1–4 were from one experiment and samples in lanes 5 and 6 were from a different experiment due to the limitation of cell numbers.

isolated LT. CEM cells were grown in serum-free medium for 24 h with various stimuli. As shown in Fig. 6, unstimulated CEM cells secreted little stainable TNF (Fig. 6, lane 2). The addition of A23187 enhanced TNF secretion (Fig. 6, lane 4). However, a more marked increase in TNF was detected when PMA was added (Fig. 6, lane 6). With the combination of PMA and A23187, maximal TNF secretion was detected (Fig. 6, lane 8). Similar results were obtained in two additional experiments.

Actinomycin D-treated L-929 cells were used as targets for cytotoxicity assays. To distinguish between TNF and LT activities, mAb E43 was chosen because it blocked TNF activity in the L cell assay and it had no effect on LT activities. In a typical experiment, the supernatant of CEM treated with 30 ng/ml PMA and $0.1 \mu\text{M}$ A23187 was shown to have 17.6 U/ml cytotoxic activity while the unstimulated supernatant had no cytotoxic activity. In the presence of $1 \mu\text{g/ml}$ of mAb E43, the cytotoxic activity was reduced to 1.7 U/ml . The serum-free supernatant of stimulated CEM cells was passed through a Con A column to absorb LT activity. The flow-through remained active at 14.4 U/ml . Similar results were obtained with two additional experiments.

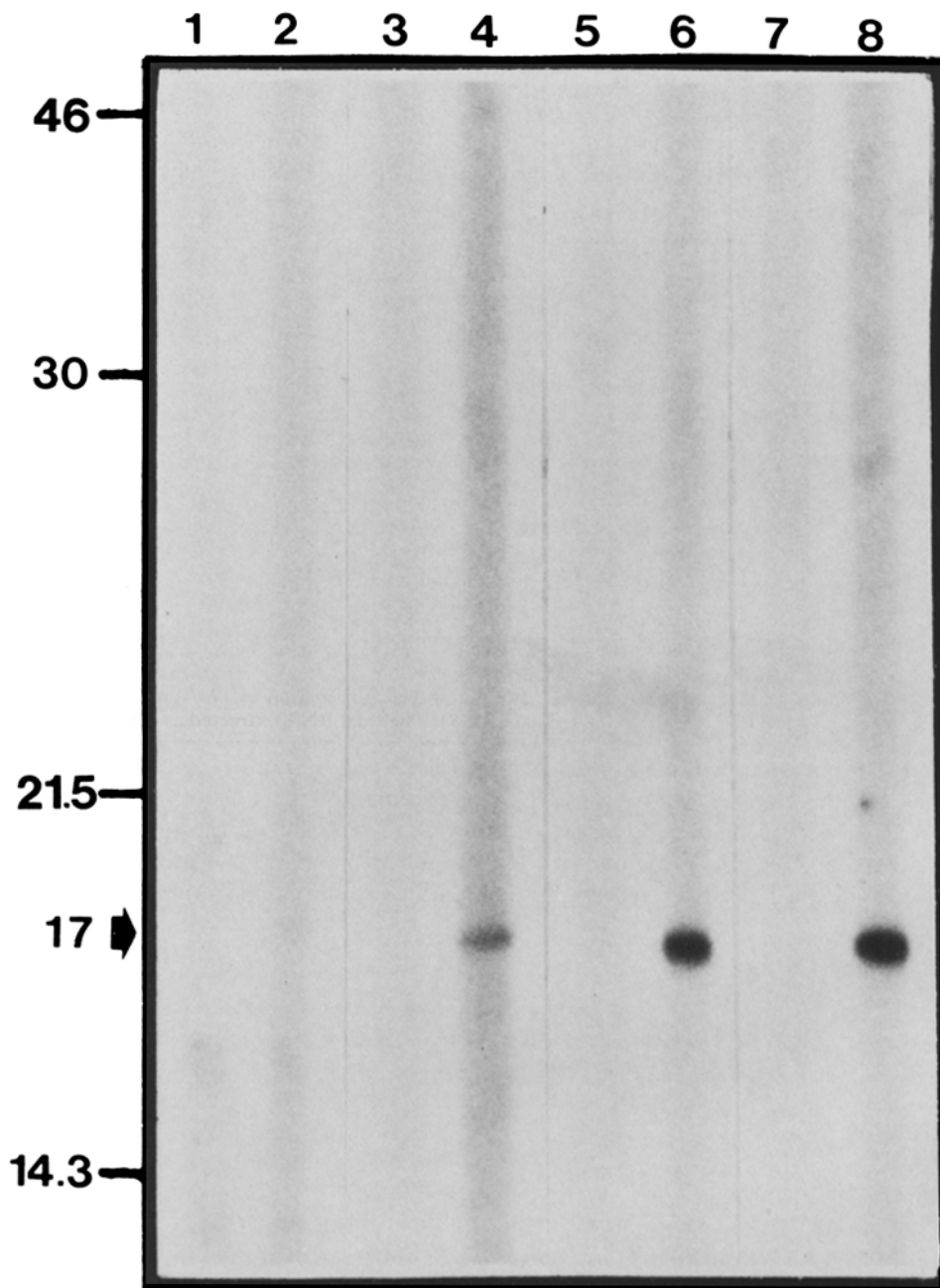


FIGURE 6. TNF production by T cell line stimulated with PMA and A23187. CEM cells were washed twice with PBS and resuspended in RPMI-1640 with 10 mM Hepes, pH 7.4, at 2.5×10^6 /ml. 50-ml samples were treated with no addition (lanes 1 and 2), 0.1 μ M A23187 (lanes 3 and 4), 30 ng/ml PMA (lanes 5 and 6) or 0.1 μ M A23187 plus 30 ng/ml PMA (lanes 7 and 8) for 24 h. The supernatants were concentrated to 300 μ l, made 1% in octyl glucoside, and 50- μ l samples were subjected to electrophoresis in 12.5% SDS polyacrylamide gels under reducing conditions. Transfer of proteins to nylon membranes (30) and blocking of nonspecific binding by nonfat dry milk (31) have been described. The lanes were cut into 3-mm strips and reacted with 1:5 dilution of control hybridoma supernatant (HDP-1, IgG1 anti-DNP Ab; lanes 1, 3, 5, and 7) or anti-TNF hybridoma supernatant (C16, IgG1 anti-TNF antibody; lanes 2, 4, 6, and 8) for 4 h, washed, and reacted with 10 μ g/ml 125 I-labeled affinity-purified goat anti-mouse IgG antibody (sp act $\approx 4 \times 10^6$ cpm/ μ g). After washing, the blots were mounted and exposed to Cronex 4 films on lightning plus intensifying screens. Positions of Rainbow prestained molecular weight markers are shown on the left.

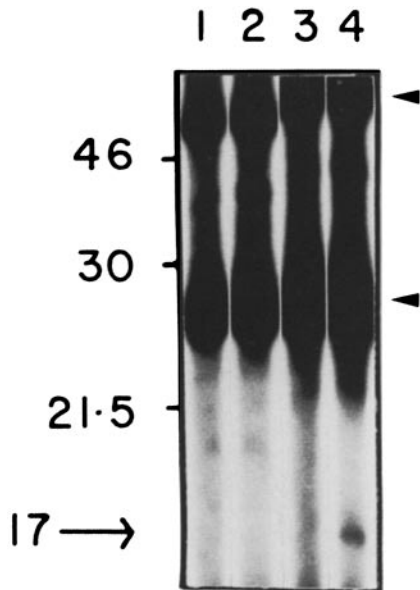


FIGURE 7. TNF production by peripheral blood T cells stimulated with anti-CD3 plus PMA. Purified peripheral blood T cells were treated with PMA at 10 ng/ml and anti-CD3 (T3-II) at 2 μ g/ml and incubated for 8 h (lanes 1 and 2) or 18 h (lanes 3 and 4). Western blot analysis was performed as in Fig. 8, except that proteins were transferred to nitrocellulose membranes. Lanes 1 and 3 were reacted with control primary antibody (HDP-1) and lanes 2 and 4 were reacted with anti-TNF antibody (C16). The positions of the Rainbow molecular mass markers ($\times 10^{-3}$) and the position of the 17-kD TNF monomer is shown on the left. The arrows on the right indicate the position of the H and L chains of the anti-CD3 mouse mAb used in the T cell stimulation. The Ig chains reacted strongly with the iodinated secondary goat anti-mouse antibody.

Experiments were carried out with peripheral blood T cells stimulated with PMA at 10 ng/ml and mAb T3-II at 2 μ g/ml. With 18 h of incubation, Western blot analysis revealed a 17-kD band by mAb C16 (Fig. 7). At 8 h, this band was not detected. In cytotoxicity assays, the supernatant from day 1 culture revealed 60.2 U/ml. In the presence of anti-TNF mAb E43, 5.0 U/ml remained. There was 74.4 U/ml cytotoxic activity in day 2 supernatant and 22.5 U/ml was detected in the presence of mAb E43. In day 3 supernatant, 78.0 U/ml cytotoxic activity was detected. 34 U/ml remained in the presence of mAb E43. Without stimuli, no cytotoxic activity was detected. Similar data were obtained in one additional experiment.

Discussion

The expression of TNF and LT mRNAs by leukemic cell lines was studied by Northern blot analysis. The specificities of the oligonucleotide probes were documented extensively. Both TNF and LT oligonucleotide probes identified mRNA species of expected sizes; i.e., 1.7 kb and 1.4 kb, respectively. The results obtained with the TNF oligonucleotide probes were similar to those obtained with a murine cDNA probe and finally confirmed with a human genomic TNF Pst I segment. In myeloid cell lines ML-1, KG-1, and U-937, only the 1.7-kb TNF mRNA was identified. There were no LT mRNA detected in these three lines. The lack of homologous sequences in known genes registered with GenBank further supports the specificity of the probes.

Results from the TNF probes showed that all T cell lines studied synthesized TNF mRNA. All lines except HSB-2 expressed little TNF mRNA when unstimulated, but could be induced to accumulate varying levels of TNF mRNA. The degree of accumulation of TNF mRNA by PMA stimulation did not correlate with presence of the CD4 or CD8 surface markers. This result is consistent with

peripheral blood T cell studies which showed that both CD4⁺ and CD8⁺ T cells synthesize comparable levels of TNF (12). Though Molt-4 and Jurkat cells accumulated little TNF mRNA during PMA stimulation, one of the lines, Molt-4, could be further stimulated by A23187 to express a higher level of TNF mRNA. This response of Molt-4 cells to the two signals is similar to that of a Jurkat cell line studied by Weiss et al. in IL-2 mRNA synthesis (13). The low TNF mRNA levels in PMA-stimulated Jurkat and Molt-4 cells probably led Goeddel et al. (3) to conclude that T lymphocyte cell lines do not synthesize TNF mRNA. HSB-2 behaved anomalously in that it accumulated high levels of TNF mRNA without stimulation. The bases of this constitutive mRNA synthesis and of the varying level of mRNA accumulation in different T cell lines after PMA stimulation are of interest. There may be a heterogeneity in the human T cell population, in that subpopulations of T cells may synthesize LT and TNF at different rates in response to the same stimuli. This difference in responsiveness may be directly related to their immune functions. Our results concerning the T cell leukemic cell lines and those regarding monocytes offer further evidence that TNF and LT are expressed independently. This is remarkable because the two genes have been localized to be 1.2 kb apart on chromosome 6 and were shown to share considerable homology (3, 5).

Similar approaches were used to show that peripheral blood T cells could be induced to make TNF. The T cell preparations were extensively depleted of monocytes, as demonstrated by cell marker analysis and nonspecific esterase staining. Functionally, these T cell preparations did not proliferate in response to IgG1 or IgG2a anti-CD3 mAb or PHA. With these T cell populations, anti-CD3 mAb was shown to markedly augment PMA in the induction of TNF mRNA synthesis and accumulation. This responsiveness to anti-CD3 mAbs is a unique property of T cells. Thus, this observation provides further evidence that the cell population responsible for the TNF synthesis measured in our experiment is indeed of the T cell lineage. Other supportive evidence includes experiments in which the addition of monocytes to the T cell populations did not substantially alter the experimental results. This can in part be accounted for by our demonstration that monocytes do not survive the prolonged exposure to the level of PMA used in our experiment. Thus, our results indicate that both freshly isolated T cells and leukemic T cell lines are inducible to make TNF. TNF and LT mRNA expression in peripheral blood T cells is different from that in T cell lines in several features. Resting peripheral blood T lymphocytes do not synthesize TNF or LT mRNA (Fig. 5). Though PMA stimulates TNF and LT mRNA expression, anti-CD3 further increases the accumulation of TNF and LT mRNA 5–10-fold in peripheral blood T cells, in contrast to the small increase caused by anti-CD3 in the PMA-stimulated cell line, CEM. The maximum accumulation of LT mRNA in peripheral blood T cells occurs at 8–18 h, in comparison to that in cell lines which occurs at 4 h. These results extend and add support to the recent observation by Cuturi et al. (12).

The time course of TNF, LT, and IL-2 mRNA accumulations in peripheral blood T lymphocytes stimulated by PMA plus anti-CD3 are different. TNF and LT mRNA accumulations occurred maximally at 4 h and 8–18 h after stimulation, respectively. The maximal IL-2 mRNA accumulation in these stimulated

cells occurred at 4–8 h, intermediate between the peak times of TNF and LT mRNA accumulations. Studies by Granelli-Piperno et al. (34) on IFN- γ and IL-2 mRNA accumulations in PHA- and PMA-stimulated T cells showed that the accumulations of both mRNAs occurred maximally at ~12 h after stimulation in unprimed peripheral blood T cells. These studies and ours show that maximal accumulation in stimulated unprimed T cells occurs the earliest for TNF mRNA, followed by IL-2, IFN- γ , and LT mRNAs.

Although the stimulation of TNF and LT mRNA accumulations in T cell lines and peripheral blood T cells by PMA, anti-CD3, or A23187, alone or in combination, were examined in this study, the effects of each of the stimulants on TNF and LT gene transcription, mRNA degradation, and rate of translation have not been elucidated. TNF and LT mRNAs contain a canonical AUUA sequence that occurs in a number of growth factor mRNAs and is believed to be responsible for the rapid degradation of these messages (35). The results that cycloheximide elevates TNF mRNA level support the notion that such degradation occurs (3). TNF and LT syntheses may also be under the translational control which occurs in the synthesis of mouse TNF (36). The relative proportion of LT and TNF produced in a T cell hybridoma also varies with the agent used to stimulate the T cells (12). The separate effects of different T cell-activating agents on each of these processes will need to be studied in order to more fully understand the control mechanisms of TNF and LT syntheses in T cells.

Although monocytes have been thought to be the major source of TNF, these studies and others (6–12) establish that T cells, NK cells, as well as certain nonhematopoietic cells, are capable of producing TNF. Preliminary data in our laboratory indicate that B cells under appropriate stimuli do make TNF. It is likely that TNF is produced by even a wider range of tissues and cells. In addition to the established diverse effects of TNF, newer biological functions will undoubtedly be discovered. In particular relevance to this paper, it would be of considerable interest to determine whether lymphocytic infiltrates in chronic inflammatory lesions are a major source of TNF. This is particularly germane in view of the finding by Dayer et al. (37) that TNF is a potent stimulant of synovial cells and dermal fibroblasts for the production of collagenase and prostaglandin E₂.

Summary

The induction of mRNA synthesis and accumulation of TNF/cachectin and lymphotoxin (LT) mRNAs in T leukemic cell lines and freshly isolated T cells were studied by Northern blot analyses. Without stimulation, TNF mRNA was barely detected in four T cell lines (CEM, KE4, MT-1, and SKW-3) and not detectable in Molt-4 and Jurkat cells, while a considerable amount of TNF mRNA was observed in HSB-2 cells. When stimulated by PMA, these T cell lines accumulated varying levels of TNF mRNA. All seven T cell lines expressed LT mRNA when unstimulated and responded well to PMA by increased accumulation of LT mRNA. The calcium ionophore A23187 by itself had no effect on TNF and LT mRNA accumulations in these cell lines. The CD3⁺ T cell lines did not respond to anti-CD3 mAb T3-II alone. However, A23187 and mAb T3-II further elevated TNF and LT mRNA accumulations in PMA-treated T cell

lines. Synergism between PMA and mAb T3-II was modest in the CD3⁺ cell lines. A slight difference in kinetics of TNF and LT mRNA accumulations was noted. In addition, heterogeneities in TNF and LT expressions by these cell lines in responses to PMA and other stimuli were observed. In monocyte-depleted peripheral blood T cell populations, PMA was able to induce both TNF and LT mRNA syntheses. This effect was potentiated markedly by the addition of anti-CD3 mAb T3-II. This synergistic response to anti-CD3 mAb and PMA provided further evidence that T cells were the source of TNF synthesis in these cultures. There was a difference in the kinetics of TNF mRNA accumulation and that of LT mRNA. Maximal accumulation of TNF mRNA occurred at 4 h while 8–18 h was required for maximal LT mRNA accumulation. IL-2 mRNA accumulated at an intermediate peak time of 4–8 h. Western blot analyses and cytotoxicity assays with L cells as targets indicated that these T cell lines and peripheral blood T cells secreted TNF. These results provide further evidence that human T cells are capable of making TNF as well as LT under appropriate stimulations. Their productions are an integral part of T cell response to activation signals. In addition, it appears that the production of these two closely related molecules is independently regulated.

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