INDEPENDENT REGULATION OF TUMOR NECROSIS FACTOR AND LYMPHOTOXIN PRODUCTION BY HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Tumor necrosis factor (TNF)[1] and lymphotoxin (LT) are two partially homologous factors originally described on the basis of their cytotoxic or cytostatic effects on tumor cell lines (1–3). Purification studies and cloning of genes for TNF and LT have allowed more detailed studies of the biology of these factors (4–8). In addition to their cytotoxic activity, TNF and LT exert regulatory effects on various cell types, e.g., they act as growth factors and exert antiviral activity on fibroblasts (9–11), activate endothelial cells (12), and regulate proliferation, differentiation, and functions of myelomonocytic cells at all stages of differentiation (13–18).

LT is a product of activated lymphocytes, both B and T cells, whereas TNF has been considered exclusively a product of monocytes/macrophages (3, 19). However, the possibility that at least some lymphocyte subsets can produce TNF was suggested by our observation that supernatants from human peripheral blood natural killer (NK) cells cocultured with NK-sensitive target cells or with normal bone marrow cells contain a factor that inhibits colony formation and that has antigenic and physicochemical characteristics of TNF (14). NK cells also produce a cytotoxic factor (NKCF) active on NK-sensitive target cell lines (20). There is evidence to suggest that the cytotoxic activity of NKCF can be attributed to more than one protein, because anti-TNF antibodies inhibit the activity of NKCF when assayed on TNF-sensitive target cells such as the macrophage line U937 (14, 21), but not on the TNF-resistant erythromyeloid line K562 (21, 22). Thus, the cytotoxic activity mediated by NK cells cannot be attributed solely to TNF, with the possible exception of lysis of TNF-sensitive target cells (22). The possibility that lymphocytes are producers of TNF was also suggested by studies showing that IL-2-dependent NK and T cell lines and a T cell tumor–derived line, HUT-102, produce cytotoxins that are inhibited by anti-TNF antibodies (23, 24). Aderka et al. (25) showed that mitogen-stimulated lymphocytes produce, in addition to LT, a cytotoxic molecule with characteristics of TNF, although

participation of monocyte/macrophages was not ruled out. Rubin et al. (26) presented evidence that LT-resistant L cells constitutively produce TNF, showing that TNF is not a specific product of monocyte/macrophages.

In this study, we show that human peripheral blood lymphocytes, carefully depleted of contaminating monocytes, rapidly accumulate message for TNF and produce high levels of TNF protein when stimulated with phorbol diester and calcium ionophore, or with mitogen. The message for LT is also detected in these cells, but the regulation of LT gene expression appears to be independent of the TNF gene.

Materials and Methods

Cell lines. The human promyelocytic HL-60 cell line, the murine antibody-secreting somatic cell hybrids, and the murine L-929 α subline cells were maintained in RPMI-1640 culture medium (Flow Laboratories, Rockville, MD) supplemented with 10% FCS (Flow Laboratories). All cell lines used were free of mycoplasma contamination on testing.

Antibodies. Antibodies B36.1 (anti-CD5 antigen on T cells, IgG2b) (27, 28); B73.1 (anti-CD16 antigen on NK cells and polymorphonucleated granulocytes, IgG1) (27, 29); B66.6 (anti-CD4 antigen on helper/inducer T cells, IgG1) (27); B116.1 (anti-CD8 antigen on suppressor/cytotoxic T cells and a proportion of NK cells, IgG1) (27); B52.1 (anti-CD14 antigen on monocytes, IgM) (18); B33.1 (anti-HLA-DR, nonpolymorphic determinant, IgG2a) (27); B154.7 and B154.9 (non-crossreacting antibodies directed against two distinct determinants of TNF and not reacting with LT, both IgG1) were produced and characterized in our laboratory. Antibody OKT3 (anti-CD3 antigen on T cells, IgG2a) (30) was produced from cells purchased from the American Type Culture Collection (Rockville, MD). Either culture supernatant fluids or Ig antibodies purified from ascitic fluids were used at predetermined optimal concentrations. Antibodies to human LT (antibody LT-B, 5 × 10^2 neutralizing U/μg IgG), to human TNF (antibody TNF-D, 2.7 × 10^4 neutralizing U/μg, TNF-E 6 × 10^3 neutralizing U/μg), and a rabbit immune serum anti-LT (2.9 × 10^7 neutralizing U/ml) were kindly provided by Dr. H. M. Shepard (Genentech Inc., South San Francisco, CA). The goat F(ab')$_2$ anti–mouse IgG used to prepare the erythrocytes for indirect rosetting (see below) was produced in our laboratory, absorbed on a column of human IgG coupled to Sepharose 4 B (Pharmacia Fine Chemicals, Uppsala, Sweden) and further affinity purified on a mouse IgG-Sepharose 4 B column (Pharmacia Fine Chemicals).

Human Peripheral Blood Leukocyte Populations. Peripheral blood was obtained by venipuncture from adult healthy donors and was anticoagulated with heparin. Mononuclear cells were prepared by centrifugation on a Ficoll/Hypaque (F/H) density gradient (1.077 g/cm^3). Monocytes were prepared by adhering the mononuclear cells to plastic flasks (3024, Falcon Labware, Oxnard, CA) for 45 min at 37°C in RPMI 1640 medium with 10% FCS and recovered by scraping with a rubber policeman after careful removal of nonadherent lymphocytes with several gentle washes with PBS. These cell preparations were composed of >95% α-naphthyl-acetate-esterase (ANAE)-positive monocytes, and no effort was made to eliminate a minor lymphocyte contamination. Nonadherent mononuclear cells, obtained after two cycles of adherence to plastic flasks for 45 min at 37°C, were depleted of residual contaminating monocytes after sensitization with antibodies B52.1 and B33.1 (supernatant fluid 1:4 final dilution, 20 μl per 10^6 cells), and indirect rosetting with CrCl$_3$-treated sheep erythrocytes coated with goat F(ab')$_2$ anti–mouse IgG; rosetting (antibody-positive) and nonrosetting (antibody-negative) cells were separated using F/H density gradient centrifugation, as previously described (27, 29, 31). The nonrosetting cell populations (nonadherent HLA-DR$^-$/B52.1$^-$ lymphocytes) were composed mostly of T and NK cells, as judged by surface phenotyping in indirect immunofluorescence, and <1 in 10,000 ANAE$^+$ cells could be detected on cytocentrifuge smears. Several lymphocytes subsets (CD3$^-$, CD3$^+$, CD8$^-$/CD16$^-$, CD4$^-$/CD16$^-$, CD16$^-$, CD16$^+$) were obtained from this lymphocyte population by indirect rosetting after sensitization

with the appropriate antibodies (OKT3, B36.1, B73.1, B66.6, and B116.1), as previously described (29, 31).

Leukocyte Stimulation. Monocyte and lymphocyte preparations were cultured at 37°C for the indicated periods of time in RPMI-1640 supplemented with 10% FCS (10^6 cells/ml) in the presence of predetermined optimal concentrations of various inducers. Phorbol-12,13-dibutyrate (PDBu) (Chemicals for Cancer Research, Inc., Eden Prairie, MN) was used at 100 ng/ml final concentration; LPS from *E. coli* (serotype 0127:B8, Sigma Chemical Co., St. Louis, MO) at 10 µg/ml; A23187 calcium ionophore (free acid form, Sigma Chemical Co.) at 0.5 µg/ml; PHA (Wellcome S.A., Dartford, England) at 2%; Con A (Pharmacia Fine Chemicals) at 0.5 µg/ml; rIL-2 (Takada Chemical Industry, Inc., Osaka, Japan, a generous gift of Dr. T. Taguchi of Osaka University, Japan) at 1,000 U/ml.

Recombinant Cytotoxins. Human rTNF (10^7 U/mg on L929 cells) and rLT (1.2×10^8 U/mg) were kindly supplied by Dr. H. M. Shepard (Genentech, Inc.).

Assay for LT Activity. Serial dilutions of cell-free supernatant fluids from the leukocyte cultures induced as above were added to 3×10^4 mouse L-929 cells (α subline) per well in 96-well flat-bottomed microtiter plates in the presence of 0.5 µg/ml actinomycin D (Calbiochem Behring Corp., La Jolla, CA). Cytotoxicity was assessed by microscopic examination after 48-h incubation at 37°C. The concentration (U/ml) of LT for each sample was defined as the reciprocal of the dilution that produced 50% cytotoxicity. To ascertain that cytotoxicity was due to LT, the cell-free supernatant fluids from PBL induced with different stimuli were separately incubated with the anti-LT or anti-TNF monoclonal (5 µg/ml) or polyclonal antibodies (1:1,000 dilution) for 6 h at 4°C before being tested in the Lα cell cytotoxic assay.

TNF Radioimmunoassay (RIA). Anti-TNF antibody B154.7, purified IgG1, was labeled with Na ^{125}I (1 mCi ^{125}I per 20 µg protein, sp act 13–17 mCi/µg; Amersham International, Amersham, U.K.) using the chloramine T method. Antibody B154.9 (5 g IgG1/ml in 0.1 M carbonate buffer, pH 9.5) was absorbed (50 µl/well, 48 h at 4°C) to 96-well vinyl plates (Serocluster, Costar, Cambridge, MA). The plates were washed three times with cold PBS containing 0.05% Tween 20 (Bio-Rad, Richmond, CA) (PBS-Tween), and 50 µl of test samples were added to triplicate wells and incubated 18 h at 4°C. Plates were washed three times with PBS-Tween, ^{125}I-labeled antibody B154.7 was added (0.1 µg/ml, 50 µl/well), and the plates were incubated for 18 h at 4°C. The plates were then washed six times with cold PBS-Tween, dried, and bound radioactivity was measured in a Packard Auto-Gamma 800 automated gamma counter (Packard Instrument Co., Downers Grove, IL). The TNF concentrations in the test samples were determined on the basis of a standard curve, constructed in each experiment using a rTNF standard (Fig. 1). The sensitivity of this RIA is at least 0.01–0.02 ng/ml, corresponding to 0.1–0.2 U/ml in the Lα cell assay. Two sources of natural TNF, obtained from HL-60 cells (induced by phorbol ester, LPS and A23187, as described in Wang et al. [8]) and from human lymphocytes (stimulated with PDBu and A23187) were detected in this RIA with a sensitivity comparable to that observed with rTNF. Up to 10^4 U/ml of natural human LT, purified for the supernatant fluid of PDBu-stimulated RPMI-8866 B cell line (18) and up to 10^4 U/ml of rLT were not detected in the RIA (Fig. 1). rIFN-α, -β, and -γ, up to 10^5 U/ml, were also not detected (not shown).

IFN-γ Radioimmunoassay. RIA for IFN-γ was previously described (15). The sensitivity of this RIA is 0.2 U/ml of IFN-γ, and no crossreaction with TNF, LT, or other species of IFN is observed.

cDNA Probes. TNF and LT cDNA probes (kindly provided by Dr. H. M. Shepard, Genentech, Inc.) were grown in *E. coli* DH5-competent cells. Both probes are Eco RI restriction fragments from the coding region of human TNF and LT cDNA. IFN-γ cDNA probe pSWIF (grown in HB101-competent cells) was kindly provided by Dr. Steven C. Clark, Genetics Institute, Boston, MA. ^{32}P-labeling of all probes was by nick translation (32).

Northern Blot Hybridization. Total RNAs were prepared from uninduced and induced cells using the phenol method. Briefly, cells were harvested by centrifugation, extensively
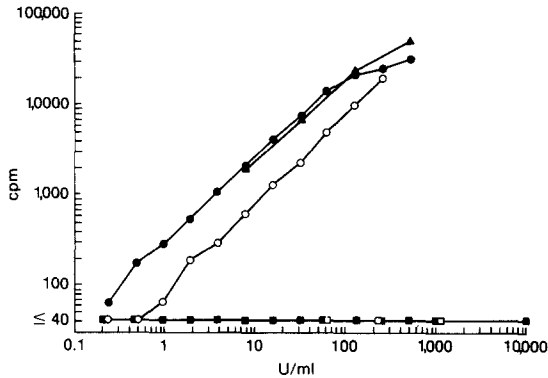
FIGURE 1. RIA for TNF. Different preparations of TNF and LT were titrated for cytotoxic activity on $L\alpha$ cells and serial dilutions (containing the indicated concentrations of cytotoxic units on $L\alpha$ cells) were tested in the TNF RIA, as described. (●) rTNF (sp act, 10^7 U/mg), (▲) natural TNF from PDBu and A23187–stimulated lymphocytes, (○) natural TNF from phorbol diester, A23187 and LPS–stimulated HL-60 cells, (■) rLT (sp act 1.2×10^8 U/mg), (□) natural LT purified from supernatant fluid of PDBu-stimulated RPMI 8866 cells.

washed with cold PBS, and resuspended in hypotonic buffer (Tris-EDTA containing 0.65% [vol/vol] NP-40). The cell lysate was centrifuged, and the nuclei-free cytoplasmic fraction was treated with 0.5% SDS before the phenol-chloroform extraction. Total cytoplasmic RNA was precipitated for 18 h at $-20\,^\circ$C with 2.5 volumes of 95% ethanol. RNA samples were fractionated in a 1% agarose-formaldehyde gel (32). Briefly, total RNA was transferred to nitrocellulose paper and hybridized with nick-translated purified DNA fragments bearing human TNF, LT, or IFN-γ sequences. Filter-bound RNA was hybridized with nick-translated probe in 50% formamide, $5 \times$ SSC ($1 \times = 0.15$ M NaCl, 0.015 M $Na_3C_6H_5O_4$, pH 7.0), $1 \times$ Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% BSA, 0.02% Ficoll 400), 20 mM sodium phosphate buffer, pH 6.5, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS at $42\,^\circ$C for 16 h. Filters were prehybridized for 3–6 h at $42\,^\circ$C in 50% formamide, $5 \times$ SSC, $5 \times$ Denhardt's, 50 mM phosphate buffer, 250 μg/ml denatured salmon sperm DNA, and 0.1% SDS. After hybridization the filters were washed at $50\,^\circ$C with $0.5 \times$ SSC, 0.2% SDS for 1 h and autoradiographed using X-Omat AR film (Eastman Kodak, Rochester, NY) and an intensifying screen (Dupont Co., Wilmington, DE).

Characterization of TNF by Western Blot Analysis.   Cell-free supernatants were obtained from serum-free cultures of cells 18 h after induction. The supernatants were concentrated fourfold and 5 μl aliquots (equivalent to the supernatant from 2×10^4 cells) was electrophoresed in a 12.5% SDS-polyacrylamide gel under nonreducing conditions. Western blotting onto nitrocellulose paper (Schleicher and Schuell, Keene, NH) was performed according to Towbin (33).

Blots were first blocked using 1% dry milk, 0.05% Tween-20 in 10 mM Tris, 150 mM NaCl, pH 7.5. After incubation with ^{125}I-labeled anti-TNF antibodies B145.7 and B145.9 (0.1 μg/ml in Tris-NaCl, 0.05% Tween 20) for 18 h at $4\,^\circ$C, blots were washed five times with Tris-NaCl containing 0.25% NP-40, 0.25% Tween 20, dried and autoradiographed for 92 h. rTNF, supernatants from unstimulated cells, and controls using ^{125}I–anti-IL-2 monoclonal antibodies were always run in parallel.

Results

Production of TNF by Lymphocytes and Monocytes.   Highly enriched preparations of lymphocytes (nonadherent HLA-DR⁻ peripheral blood mononuclear cells, containing almost exclusively T and NK cells, and with <0.01% contamination of ANAE⁺ monocytes) and of monocytes (adherent peripheral blood mononuclear cells containing >95% ANAE⁺ monocytes) were cultured for 6 h with various inducers, and the concentration of TNF in the cell-free supernatant fluids was measured by RIA. The RIA detects natural TNF and rTNF with similar sensitivity, but does not detect LT or other lymphokines (Fig. 1). Table I gives results obtained using matched lymphocyte and monocyte preparations

TABLE I
*TNF Production by Human Peripheral Blood Lymphocytes
and Monocytes*

Inducer	TNF produced by:	
	Lymphocytes	Monocytes
	ng/ml	
None	<0.02	0.20 ± 0.20
PDBu	0.80 ± 0.80	3.50 ± 3.40
A23187	0.40 ± 0.40	0.40 ± 0.50
PDBu + A23187	20.28 ± 15.91	2.80 ± 1.90
PHA	0.60 ± 0.40	0.30 ± 0.40
PDBu + PHA	4.20 ± 1.30	3.70 ± 2.40
LPS	0.20 ± 0.20	9.80 ± 13.60
PDBu + LPS	1.00 ± 0.90	10.20 ± 5.40
Con A	0.70 ± 0.80	0.30 ± 0.40
IL-2	0.10 ± 0.20	0.40 ± 0.50

Lymphocytes (nonadherent B52.1⁻/B33.1⁻ peripheral blood mononuclear cells) and monocytes (plastic-adherent peripheral blood mononuclear cells) from six donors were cultured (6 h, 37°C) in the presence of the various inducers as described. Supernatant fluids were collected and tested for TNF content using RIA.

from six donors. Both the phorbol diester PDBu and the calcium ionophore A23187 stimulated TNF production from lymphocytes; using both inducers together, more than an additive effect was observed. The mitogens PHA and Con A (6-h incubation) induced moderate levels of TNF from lymphocytes (<1 ng/ml on average), and PHA-induced TNF production was potentiated by PDBu. rIL-2 or LPS induced only minimal levels of TNF production from lymphocytes. Monocytes but not lymphocytes constitutively produced low levels of TNF, possibly due to a low endotoxin contamination (<2 ng/ml by the Limulus assay) of the culture medium. Production of TNF in monocyte cultures was maximally induced by LPS and by PDBu. PDBu, however, did not synergize with LPS, A23187, or PHA in inducing TNF production by monocytes.

The kinetics of TNF production in cultures of lymphocytes stimulated with PDBu, A23187, a combination of PDBu and A23187, or PHA was analyzed with lymphocytes from four donors and compared with the production of IFN-γ and of cytotoxic activity on L929α cells (Fig. 2). Significant TNF titers were induced by PDBu and by PDBu and A23187 within 6 h, and those values were maximum at 24 h of incubation (Fig. 2A). No significant TNF production was observed in supernatants from 6-h PHA-induced lymphocytes, but levels of TNF comparable to those observed upon PDBu-induction were detected after 24-h or longer induction. The production of IFN-γ in the same cell-free supernatants paralleled that observed for TNF, although the kinetics of induction with PDBu or PHA was slower and no significant production was measured at 6 h (Fig. 2B). High levels of both IFN-γ and TNF were detected in the supernatant fluids from lymphocytes stimulated for 6 h with PDBu and A23187.

The cytotoxic activity of the supernatants was tested on L929α cells (Fig. 2C). Cytotoxic activity in supernatants from PHA-induced lymphocytes, especially at incubation times longer than 24 h, was higher than that detected in supernatants
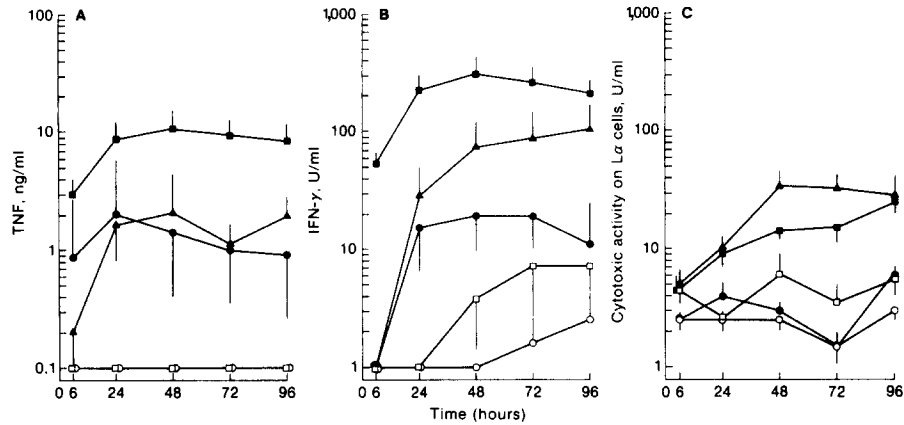
FIGURE 2. Production of cytotoxins and IFN-γ by lymphocytes. Lymphocytes (nonadherent, B52.1⁻/B33.1⁻ peripheral blood mononuclear cells) were cultured in the presence of the different inducers, cell-free supernatant fluids were collected at the indicated time, dialyzed extensively and assayed for TNF (A) and IFN-γ (B) by RIA and for cytotoxins (C) in a Lα cytotoxic assay. Cells were cultured in RPMI 10% FCS with no inducer (○) or in the presence of (●) PDBu, (□) A23187, (■) PDBu + A23187, (▲) PHA. Experimental points are mean ± SE of four experiments.

from PDBu- or PDBu and A23187-induced cells, suggesting that another cytotoxin(s) active on L929α cells was produced in PHA-stimulated cultures, in addition to the TNF measured by RIA (Fig. 2A). Therefore, the cytotoxic activity on L929α cells in the supernatant fluids obtained at 24 and 72 h from lymphocyte preparations cultured in the presence of various inducers was further characterized in neutralization experiments using inactivating antibodies specific for TNF or LT (Table II). In all cases, the greatest inhibition was observed when both anti-TNF and anti-LT antibodies were added to the supernatant fluids, suggesting the presence of both LT and TNF in the supernatant fluids. The remaining cytotoxic activity observed when both anti-TNF and anti-LT antibodies were used could be due to a cytotoxin different from both TNF and LT or, more likely, to incomplete activation of TNF and LT by the antibodies. In most preparations from cells stimulated with PDBu and A23187 for 24 h (Table II) or less (not shown) an almost complete inhibition was, however, observed with anti-TNF antibodies alone, suggesting that TNF is the predominant source of cytotoxin produced during early times of culture. Inhibition of cytotoxic activity was almost never observed using an inactivating monoclonal antibody against IFN-γ (antibody B133.3).

TNF Production by Lymphocyte Subpopulations. TNF production was evaluated in the supernatant fluids from lymphocyte preparations enriched for specific lymphocyte subpopulations and stimulated with PDBu and A23187 for 6 h (Table III). T cells (>95% pure), obtained by either negative or positive selection using anti-CD16 antibody B73.1 or anti-CD3 antibody OKT3, respectively, produced the same or higher TNF levels than the total HLA-DR⁻ lymphocytes. The T cells positively selected with the anti-CD3 antibody produced significant TNF levels even in the absence of PDBu and A23187, probably due to the stimulatory effect of the antibody used for the separation. Both T cell prepara-

TABLE II

Cytotoxic Activity on Lα Cells Mediated by Supernatant Fluids from Stimulated Lymphocytes: Inactivation by Anti-LT and Anti-TNF Antibodies

Exp.	Inducer	Time	Cytotoxic activity on Lα cells in supernatants treated with:				
			Nothing	Anti-TNF	Anti-LT	Anti-TNF + anti-LT	Anti-IFN-γ
		h			U/ml		
A	PDBu + A23187	24	4	0	4	0	4
	PHA	24	8	8	4	0	8
	PDBu + A23187	72	64	32	4	0	64
	PHA	72	128	32	32	4	128
	PDBu + PHA	72	16	8	8	4	32
B	PDBu + A23187	24	8	4	4	0	8
	PHA	24	32	8	8	0	32
	PDBu + PHA	24	8	4	4	0	8
	PDBu + A23187	72	64	32	8	0	64
	PHA	72	256	64	64	0	128
	PDBu + PHA	72	32	16	16	4	32
C	PDBu + A23187	24	8	2	8	0	8
	PHA	24	16	8	8	8	16
	PDBu + PHA	24	16	8	8	4	16
	PDBu + A23187	72	16	4	8	0	16
	PHA	72	64	64	32	16	64
	PDBu + PHA	72	128	32	64	16	128

Lymphocytes (nonadherent B52.1⁻, B33.1⁻ peripheral blood mononuclear cells) from three donors were cultured in the presence of the various inducers as described. Supernatant fluids were collected at the indicated time and tested for cytotoxicity on Lα cells after 6-h preincubation at 4°C with the indicated antibodies. In Exp. A and B, monoclonal antibody TNF-E and a rabbit antiserum anti-LT were used; in Exp. C monoclonal antibodies TNF-D and LT-B were used.

TABLE III

Production of TNF by Lymphocyte Subpopulations

PBL subset	TNF produced in PBL induced by:	
	Nothing	PDBu + A23187
	ng/ml	
HLA-DR⁻	0.02 ± 0.02	7.52 ± 5.80
HLA-DR⁻, CD16⁻	<0.01	7.62 ± 6.47
HLA-DR⁻, CD3⁺	3.30 ± 0.30	19.00 ± 4.00
HLA-DR⁻, CD8⁻, CD16⁻	0.10 ± 0.17	10.15 ± 4.65
HLA-DR⁻, CD4⁻, CD16⁻	0.02 ± 0.03	3.70 ± 2.21
HLA-DR⁻, CD3⁻	0.01 ± 0.02	1.60 ± 1.45
HLA-DR⁻, CD16⁺	<0.01	3.00 ± 1.40

The indicated lymphocyte subsets from four donors were obtained by indirect rosetting and cultured in the presence or absence of PDBu and A23187 as described. Cell-free supernatant fluids (n = 4) were collected after 6-h culture and tested for TNF content using RIA.

TABLE IV

TNF Production by Lymphocytes and NK Cell–enriched Preparations

Inducer	TNF produced by:	
	Lymphocytes	NK cells
	ng/ml	
None	0.03 ± 0.04	0.03 ± 0.04
PDBu	1.20 ± 0.90	<0.02
A23187	<0.2	<0.02
PDBu + A23187	13.16 ± 4.73	1.80 ± 2.26
PHA	3.03 ± 2.67	<0.02
PDBu + PHA	21.33 ± 8.73	0.43 ± 1.69
LPS	0.55 ± 0.60	<0.02
PDBu + LPS	3.66 ± 2.62	0.06 ± 0.09

Lymphocytes (nonadherent, B52.1⁻, B33.1⁻ peripheral blood mononuclear cells) and NK cell–enriched preparations obtained after T cell depletion using antibodies B36.1 and OKT3 were cultured in the presence of the various inducers as described. Supernatant fluids ($n = 3$) were collected at 24 h and tested for TNF content using RIA.

tions enriched (>90% pure) for CD4⁺ (helper/inducer T cells) and for CD8⁺ cells (suppressor/cytotoxic T cells) by negative selection produced TNF upon stimulation with PDBu and A23187, although, on average, CD4⁺-enriched cells produced approximately threefold higher TNF levels than CD8⁺-enriched cells (Table III). Lymphocyte preparations enriched (>90% pure) for NK cells by negative (CD3⁻ cells) or positive (CD16⁺ cells) selection consistently produced TNF levels lower than total lymphocytes or T cell–enriched preparations. The ability of lymphocyte preparations highly enriched in NK cells [nonadherent HLA-DR⁻/CD3⁻/CD5⁻ lymphocytes, containing >95% CD16 (B73.1)⁺ NK cells] to produce TNF in response to various stimuli was compared to that of nonadherent HLA-DR⁻ lymphocytes (Table IV). NK cells produced TNF only in response to PDBu plus A23187 and to PDBu plus PHA, although, on average, at levels 7- and 50-fold lower than lymphocytes, respectively. Unlike total lymphocytes, NK cells failed to produce TNF in response to PDBu or to PHA used alone. NK cells induced with LPS or with LPS and PDBu also did not produce TNF.

Transcription of TNF and LT Genes in Lymphocytes. The presence of TNF, LT, and IFN-γ mRNA was analyzed by Northern blotting in lymphocytes stimulated with PDBu and A23187, PHA, or LPS (Fig. 3). No mRNA for any of the lymphokines tested was detected in cultured unstimulated lymphocytes or in lymphocytes cultured in the presence of LPS. The TNF and IFN-γ mRNA, and, to a lesser extent, the LT mRNA were detected in PBL upon 6-h induction with PDBu and A23187. LT and IFNγ mRNA and, at a much lower level, TNF mRNA were detected at 24 h, but not at 6 h after PHA-induction. Similar levels of mRNA were present in all preparations as monitored by hybridization to a human β-actin probe (not shown). The levels of mRNA detected in Fig. 3 correlate well with the amounts of TNF and IFN-γ proteins detected by RIA and the LT cytotoxic activity in the Lα assay.

Characterization of the TNF Produced by Lymphocytes. The TNF, as identified

A   **TNF**   **B**   **IFN-γ**   **C**   **LT**
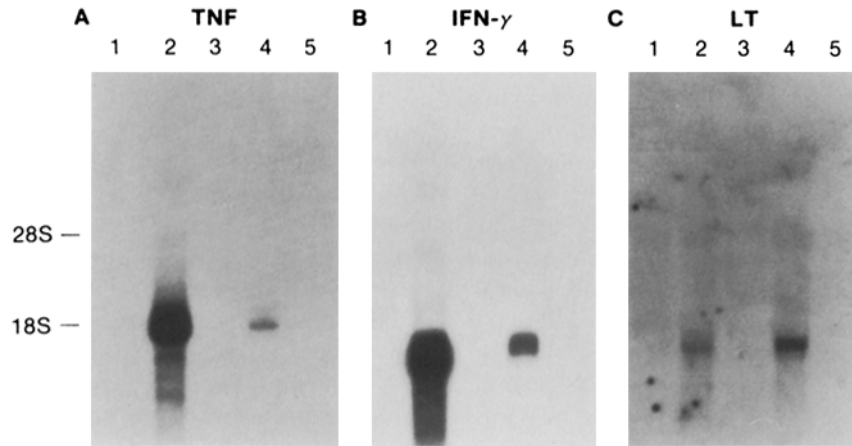 1 2 3 4 5   1 2 3 4 5   1 2 3 4 5

28S —

18S —

FIGURE 3. Induction of TNF (A), IFN-γ (B), and LT (C) mRNA in activated human lymphocytes. Total cytoplasm RNA was extracted from lymphocytes (nonadherent B33.1⁻/B52.1⁻ mononuclear peripheral blood cells) cultured in the presence of various inducers and electrophoresed on a 1% agarose-formaldehyde gel (20 μg per lane). After transfer to nitrocellulose, RNA was hybridized to the indicated cDNA probes. Lane 1, lymphocytes cultured for 6 h in control medium in the absence of any inducer; lane 2, lymphocytes cultured for 6 h with PDBu and A23187; lanes 3 and 4, lymphocytes cultured for 6 h and, for 24 h, respectively, in the presence of PHA; lane 5, lymphocytes cultured for 6 h in the presence of LPS.
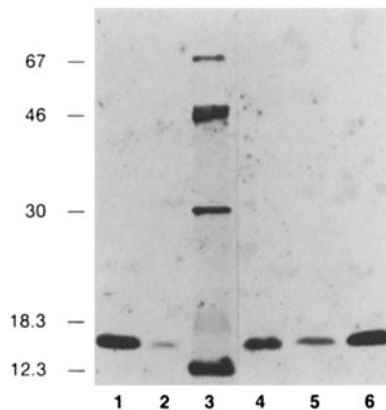
67 —

46 —

30 —

18.3 —

12.3 —

 1   2   3   4   5   6

FIGURE 4. Western blotting of TNF-containing supernatants using ¹²⁵I-labeled monoclonal anti-TNF antibodies B145.5 and B145.7. Lanes 1 and 6, rTNF, 10 ng; lane 2, cell-free supernatant fluid from serum-free PDBu and A23187-stimulated lymphocytes; lane 3, ¹⁴C-labeled markers (× 10⁻³); lane 4, cell-free supernatant fluid from PDBu, A23187, and LPS-stimulated HL-60 cells; lane 5, cell-free supernatant fluid from LPS-stimulated monocytes.

by RIA, produced by lymphocytes induced with PDBu and A23187, has physicochemical characteristics identical to the TNF produced by monocytes or HL-60 cells, and to rTNF, when analyzed by gel filtration or by anion-exchange chromatography (data not shown). The same comparison by Western blotting using ¹²⁵I-labeled anti-TNF antibodies reproducibly revealed a band with identical migration, and with M_r of 17,000 in supernatant fluids from stimulated lymphocytes, monocytes, or HL-60 cells and in a preparation of rTNF (Fig. 4). When supernatant fluids from unstimulated lymphocytes were analyzed by Western blotting, no polypeptides reacting with anti-TNF antibodies were detected (not shown).

Discussion

The results presented in this report show that lymphocytes are able to produce TNF. This conclusion is based on analysis, in highly purified lymphocyte preparations, of both TNF secretion, using a sensitive and highly specific RIA, and transcription of the TNF gene. The RIA for TNF is at least five times more sensitive than the biological assay and, unlike the latter, does not crossreact with LT or other cytotoxins. The RIA simultaneously used two monoclonal antibodies specific for two separate antigenic determinants on the TNF molecule; thus, false positive results due to a crossreacting specificity of only one of the antibodies is unlikely. Up to 10^4 U of rLT or 10^3 U of B cell line–derived natural LT gave no signal in the RIA, whereas <1 U of rTNF or natural TNF was easily detected. The possibility that the cells producing TNF in our lymphocyte preparations were contaminating monocytes was excluded both by the careful depletion procedures and by the differential response to the stimuli that induce production of TNF from lymphocyte and monocyte preparations. LPS, a potent inducer of TNF production by monocytes, did not induce any significant production of TNF from lymphocytes, confirming that contaminant monocytes were not the producers of TNF in our lymphocyte cultures. Phorbol diesters induced TNF production in both lymphocytes and monocytes, but the synergistic induction of TNF using phorbol diester and calcium ionophore in combination was observed only with lymphocyte preparations. Mitogenic lectins, alone or synergistically with phorbol diesters, were efficient inducers of TNF from lymphocytes, but not from monocytes. These results suggest that both protein kinase C activation and increase in intracellular calcium may play a role in the production of TNF by lymphocytes. Similar observations have been reported for induction of the human IFN-γ and IL-2 genes (35).

Analysis by Northern blotting showed that accumulation of TNF, LT, and IFN-γ mRNA was demonstrable in lymphocyte preparations stimulated with phorbol diester and calcium ionophore, or with PHA. Interestingly, when the ability of phorbol diester and calcium ionophore to induce TNF, LT, and IFN-γ was compared with that of the mitogen PHA, the first combination of inducers maximally induced accumulation of mRNA for TNF and IFN-γ. In contrast, PHA, although with slower kinetics than PDBu and A23187, induced accumulation preferentially of LT mRNA, although TNF and IFN-γ mRNA were also detected. Thus, accumulation of the IFN-γ and TNF messages responds to stimuli in a manner discordant with the LT gene, suggesting a different regulatory mechanism. These results only document steady-state accumulation of mRNA, and do not allow us to distinguish whether this results from increased transcription or decreased mRNA degradation. We consider the former possibility more likely, because no message can be detected in unstimulated lymphocytes and because no spontaneous production of any of the three lymphokines is observed in short-term cultures of fresh lymphocytes. The independent regulation of the TNF and LT genes was confirmed by analyzing the kinetics of synthesis of the two cytotoxins. These semiquantitative data, based on inactivation of biological activity on L929α cells using specific antibodies, show that phorbol diesters and calcium ionophore at early times induce mostly or exclusively TNF, whereas LT, which is the major cytotoxin species induced by PHA,

is produced at later times in culture. The temporal regulation of production of IFN-γ by lymphocytes closely paralleled the production of TNF, consistent with the results obtained by analysis of mRNA expression. TNF and LT have almost identical biological activity and are encoded by two closely linked genes in the major histocompatibility complex region (36). Although they share almost 50% nucleotide homology in the protein coding region (7), the two genes differ in their flanking regions (7). The close linkage between TNF and LT genes suggests that, rather than differences in chromatin organization, promoter-specific factors and upstream regulatory sequences might be responsible for the differential regulation of expression of these two genes.

To completely eliminate monocytes from our lymphocyte preparations, we used treatments (i.e. depletion of HLA-DR$^+$ cells) that also remove B cells, and therefore, the preparations used contained almost exclusively T and NK cells. T cells, both CD4$^+$ enriched and CD8$^+$ enriched, were the most efficient producers of TNF, especially upon mitogen stimulation. The observation that most of the TNF production is associated with T cell preparations, obtained using positive selection, excludes the possibility that contaminant cell types such as basophils, previously reported to produce TNF (34), account for most of the TNF production.

Our previous data (14) clearly indicate that NK cells are needed for TNF production in NK cell/target cell cultures. The demonstration of production of TNF in coculture of NK cells with cell lines (14, 20) or with normal bone marrow cells (14), though suggestive of a role for TNF in NK cell functions such as suppression of hematopoietic colony inhibition, remains difficult to interpret because of the possibility that the cells used as stimulators act as accessory cells or even directly participate in TNF production. The inducers used in the present study are probably not optimal for NK cell stimulation. Lectins such as PHA and Con A are not mitogenic for and do not activate NK cells (37). Phorbol diesters and calcium ionophore, although affecting NK cell functions (38), are much better inducers of IFN-γ production in T cells than in NK cells (38), although the latter cell type is preferentially induced to produce IFN-γ when stimulated by IL-2 (39). However, the present data confirm the ability of NK cells to produce TNF, although at a lower level than T cells. The possibility that T cells contaminating the enriched NK cell preparations are responsible for TNF production is unlikely, not only because careful cell purification procedures were used, but also because the mitogen PHA, a powerful inducer of TNF in T cells, was inactive on NK cell–enriched preparations. We have also obtained preliminary results demonstrating transcription of the TNF gene in stimulated preparations of pure NK cells (our unpublished observation). The complete understanding of the regulation of TNF production by NK cells and of the role of TNF in NK cell functions awaits a better analysis of the effect of NK cell–specific stimuli and of the role of accessory cells in cytokine production by different cell types.

Summary

We present evidence that human peripheral blood lymphocytes, free of contaminating monocytes, rapidly produce high levels of tumor necrosis factor

(TNF) when stimulated with phorbol diester and calcium ionophore, and lower but significant levels of TNF when stimulated with mitogens. These two types of inducers act preferentially on T cells, both $CD4^+$ and $CD8^+$. NK cells produce TNF only when stimulated with phorbol diester and calcium ionophore, and they do so at a much lower level than T cells. The procedures used in the purification of lymphocytes and the differential ability to respond to various inducers allow us to exclude that monocytes or basophils contaminating the lymphocyte preparation participate in the production of TNF. In particular, LPS, a potent inducer of TNF production from monocytes, is unable to induce significant levels of TNF in the lymphocyte preparations. The TNF produced by lymphocytes has antigenic, physicochemical, and biochemical characteristics identical to those of the TNF produced by myeloid cell lines or monocytes upon stimulation with LPS. LT is also produced by lymphocyte preparations. Production of TNF and LT proteins in response to the different inducers is paralleled by accumulation of cytoplasmic TNF and LT mRNA. Both at mRNA and at protein levels, stimulation of T lymphocytes with phorbol diester and calcium ionophore preferentially induces TNF, whereas mitogen stimulation preferentially induces LT. Our data suggest that the TNF and LT genes, two closely linked genes encoding two partially homologous proteins with almost identical biological functions, are independently regulated in lymphocytes.

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