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EXPRESSION OF THE TUMOR NECROSIS FACTOR LOCUS IS NOT NECESSARY FOR THE CYTOLYTIC ACTIVITY OF T LYMPHOCYTES

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In order to test whether tumor necrosis factors α (TNF-α) or β (TNF-β, also known as lymphotoxin) are involved in the lysis of target cells by cytolytic T lymphocytes, we probed for the presence of the TNF mRNAs in several quiescent and activated CTL clones. No TNF mRNA could be found in constitutively cytolytic Lyt-2⁺ clones, and only two out of three clones tested accumulated TNF mRNA after stimulation with phorbol myristate acetate and ionomycin. Of two L3T4⁺ clones that can be induced to become cytolytic by a combination of antigen and IL-1, only one accumulated TNF-β mRNA in the process. The PC60 rat × mouse T cell hybrid, which becomes cytolytic in response to a combination of IL-1 and IL-2, also failed to accumulate TNF mRNA after stimulation with these agents. Our results strongly suggest that TNF-α or -β are not necessary agents of the cytolytic activity exhibited by antigen-specific T lymphocytes.

Tumor necrosis factor β was originally described as a cytotoxic activity present in the supernatants of mitogen-activated T cells (1–3). Subsequent work showed that it has toxic effects on a variety of cultured tumor cell lines and causes necrosis of some tumors, but seems inactive on "normal" cells (4, 5). Recently, it has become evident that TNF-β, beside its toxic properties, is also a lymphokine with pleiotropic effects on cells of various origins, similarly to its close relative, TNF-α (6–10). In fact, in all experimental systems where it has been tested, TNF-β has effects indistinguishable from those of TNF-α, presumably because the receptor for the two proteins is the same (11).

Determination of the primary structure of human TNF-α and TNF-β has established that the two proteins are closely related. The cloning of cDNAs for human, mouse, and rabbit TNF-α, as well as for the human and mouse TNF-β, has made it possible to study the organization of the TNF genes (8). In man as well as in mouse, the genes are tandemly arranged (12, 13), and their structures reveal an common evolutionary origin (14, 15). In both species, the genes are located within the MHC (16, 17), although they do not seem to be related to the class I or II MHC genes.

It has been proposed that TNF-β has a direct role in the cytolytic activity mediated by cytolytic T lymphocytes (18). Indeed, TNF-β has properties that are consistent with those of a putative toxin injected by CTL into their targets: cell culture supernatants containing TNF-β can cause the rapid death of target cells if the cells are subjected to a concomitant osmotic shock (19), and can induce the degradation of target cell DNA, a phenomenon that is also seen in CTL-mediated killing (20, 21). The recent demonstration that T lymphocytes can produce TNF-α as well as TNF-β (22, 23) has opened the possibility that both forms of TNF could be used as mediators of CTL activity.

Having in hand the molecular tools necessary to measure precisely the amounts of TNF-α and TNF-β mRNA present in cell populations, we undertook to re-evaluate the capacity of CTL clones to produce TNF. Our salient conclusion is that, whereas some CTL clones certainly do accumulate TNF mRNA after activation, this is not a prerequisite for their cytolytic activity.

MATERIALS AND METHODS

CTL clones and cell lines. Lyt-2⁺ CTL clones were derived from alloreactive (H-2^b anti-H-2^d) populations. Clones 7 and 11 were derived from peritoneal exudate T lymphocytes isolated from C57BL/6 (H-2^b) mice immunized against P815 (H-2^d) tumor cells by using a micromanipulation technique described elsewhere (24). Clone 75 was derived by micromanipulation of C57BL/6 anti-DBA/2 T cells from a day 5 secondary mixed lymphocyte culture prepared as described (25). The cloned cells were maintained in culture by periodic stimulation with appropriate stimulator cells and a source of interleukin 2. L3T4⁺ CTL clones were derived from lymph node cells of A.TL (H-2^k) mice immunized with the synthetic polymer poly(glu60, ala30, tyr10) (GAT) as described (26). The PC60 cell line, derived from a cell fusion between a mouse CTL clone and a the C58 rat thymoma line (27), was kindly provided by Dr. M. Nabholz (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland).

The P815 mastocytoma line used as a source of target cells, the EL4 thymoma line, and the WEHI 164 clone 13 line used in the TNF assay (28) were maintained in culture using standard methods. The CTLL-2 line was maintained in the presence of exogenous IL-2.

Enzymes and chemicals. Phage SP6 RNA polymerase, RNase-free DNase, and *Escherichia coli* DNA polymerase I Klenow fragment were from Boehringer-Mannheim (Mannheim, FRG). RNase A (bovine pancreatic) was from Sigma Chemical Co. (St. Louis, MO). Lyophilized RNase T1 (from *Aspergillus oryzae*), a Sankyo product, was purchased from Calbiochem (San Diego, CA). Proteinase K was from Merck (Darmstadt, FRG). ^{32}P-labeled UTP (400 Ci/mmol) and ^{125}I-labeled UdR were from Amersham plc (Amersham, United Kingdom). Recombinant human IL-1β was a gift of Dr. J. M. Dayer (University of Geneva). Recombinant human IL-2 was a gift of Biogen SA, Geneva.

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Probes. In most of the experiments described in this paper, we used ^{32}P-labeled RNA probes transcribed by the SP6 phage RNA polymerase from recombinant plasmids constructed in the pSP64 or pSP65 vectors. The inserted fragments, all of murine origin, were derived as follows: TNF-β, a 950-bp *Pvu*II genomic fragment extending from the middle of the first intron 350 bp into the fourth exon (13); TNF-α, a 450-bp *Hin*cII to *Eco*RI fragment entirely contained within the fourth exon (13); IFN-γ, a *Hin*dIII to *Cla*I fragment covering the first exon (subcloned from an IFN-γ genomic clone obtained from W. Fiers, State University of Ghent, Ghent, Belgium); Thy-1, a 600-bp *Pst*I fragment corresponding to the coding portion of the TM8 cDNA clone described by Hedrick et al. (29); β-actin, a 200-bp *Pst*I to *Bgl*II fragment subcloned from the pAL41 β-actin cDNA clone described by Minty et al. (30). To probe for Granzyme A mRNA, we used ^{32}P-labeled overlapping synthetic oligonucleotides as described by Garcia-Sanz et al. (31).

Blots and RNase protection assays. Total cytoplasmic RNA was extracted from the postnuclear supernatant of cells lysed in isotonic buffer containing 0.1% Nonidet P-40 (32). For Northern blots, 5 μg of RNA were separated in a 1.4% formaldehyde-agarose gel (32) and transferred in 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to Hybond N filters (Amersham, United Kingdom). After baking, the filters were prehybridized in 5 × SSC, 50% formamide, 2.5 × Denhardt's solution, 0.2% SDS, 10 mM EDTA, 50 mM sodium phosphate (pH 6.5), and 200 μg/ml of denatured herring sperm DNA for 4 h at 55°C, and hybridized for 16 h in the same solution containing 1 to 5 × 10^7 cpm ^{32}P-labeled RNA probe. Washing was in 0.1 × SSC, 0.1% SDS at 65°C. Slot blots were prepared according to the manufacturer's instructions (Schleicher & Schuell, Keene, NH), using Hybond N filters. Hybridizations were performed as for Northern blots. For densitometric scanning of the autoradiograms, we used a Zeineh soft laser integrating densitometer.

RNase protection assays were performed essentially as described by Zinn et al. (33). A 2-μg sample of cytoplasmic RNA was hybridized to 1 to 5 × 10^5 cpm of RNA probe at 53°C overnight in 30 μl of 80% formamide, 50 mM PIPES (pH 6.4), 0.4 M NaCl, 1 mM EDTA. The hybrids were digested with 100 μg/ml of RNase A and 4 μg/ml of RNase T1 in 300 μl of 0.3 M NaCl, 10 mM Tris · HCl (pH 7.4) for 45 min at 28°C. After digestion with 100 μg/ml of proteinase K in the presence of 0.6% SDS (30 min at 37°C), phenol-chloroform extraction, and precipitation in ethanol, the protected ^{32}P-labeled RNA fragments were separated in 5% acrylamide/urea gels at 55°C and visualized by autoradiography of the fixed and dried gels.

^{51}Cr and ^{125}I release assays. ^{51}Cr release assays were performed as described previously (25). DNA fragmentation assays were carried out as follows; P815 target cells were incubated overnight at 37°C in DMEM 5%[1] containing 4 μCi/ml of [^{125}I]UdR and washed three times in the same medium without [^{125}I]UdR. Ten thousand labeled target cells were mixed with effector cells in a total volume of 100 μl and incubated at 37°C. At the end of the incubation period, Triton X-100 was added to a final concentration of 0.2% and EDTA to 5 mM, the lysed cells were centrifuged at 1000 × G for 5 min and the supernatants were harvested and counted in a gamma counter. Specific release is expressed as 100 × (cpm released − spontaneous release)/(maximum release − spontaneous release). The maximum release value was obtained by incubating Triton-lysed cells with 40 U/ml of micrococcal nuclease (Worthington) and counting the ^{125}I label in the 1000 × G supernatant.

Assays for TNF and IFN-γ activities. The cytostatic activity of TNF on WEHI 164 clone 13 cells was measured using the vital stain 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT; Sigma M2128) (26). 2 × 10^4 WEHI 164/13 cells were incubated with dilutions of the supernatants to be tested in 200 μl of culture medium for 48 h at 37°C. After this period, MTT was added to 1.25 mg/ml, and the incubation was continued for another 4 h. The culture medium was then removed, and the cells were lysed in 100 μl of 3% SDS, 40 mM HCl in isopropyl alcohol. After all of the debris had lysed, the OD_{570} of the wells was read in an ELISA reader.

The macrophage-activating activity of the cell supernatants was measured as described (34). Briefly, bone marrow-derived macrophages were incubated with dilutions of the supernatants to be tested in the presence of 200 ng/ml of LPS, and then tested for their cytolytic activity on ^{51}Cr-labeled P815 target cells. IFN-γ levels were defined as the reciprocal of the supernatant dilution yielding 50% maximal ^{51}Cr release.

[1] Abbreviations used in this paper: DMEM 5%, Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 10 mM HEPES; MTT, 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide.

RESULTS

Detection of TNF mRNA. While testing various cell lines for the presence of TNF mRNA, we discovered that the CTLL-2 line that we commonly use for IL-2 assays (35) contains large amounts of the TNF-β message and lower amounts of the TNF-α message, although it does not seem to secrete biologically active TNF into the culture supernatant (unpublished observation). Therefore, we used CTLL-2 RNA as a positive control for the experiments described in this paper.

Figure 1*A* shows a Northern blot of RNAs extracted from EL4 cells (which do not express either TNF-α or TNF-β), from CTLL-2 cells, and from LPS-activated bone marrow-derived macrophages probed with a TNF-α or a TNF-β probe. Our TNF-β probe detected a 1.4-kb mRNA in the CTLL-2 cells that was not present in macrophages. This mRNA was clearly distinct from that of TNF-α, which had a size of 1.7 kb, and was present in large amounts in LPS-activated macrophages and in much smaller amounts in CTLL-2 cells.

As a more sensitive alternative, we used the RNase protection technique described by Zinn et al. (33), where liquid phase hybridization of a labeled RNA probe to cellular RNA is followed by digestion with RNase A and RNase T1 and separation of the protected fragments on denaturing acrylamide gels. Figure 1*B* shows that using a genomic probe, which contains the second, third, and part of the fourth exon (up to the *Pvu*II site) of the mouse TNF-β (13), and hybridizing it to CTLL-2 RNA, we could detect the predicted protected fragments of 106 (exon 2), 100 (exon 3), and 335 (exon 4, up to *Pvu*II site) bases. The largest protected fragment, which is derived from exon 4, contained the most radioactive label and was used as an indicator of the presence of TNF-β mRNA. Hybridization to the TNF-α probe produced a single band of 450 bases, as expected from the structure of the probe (data not shown). By comparing the signal strengths obtained with probes of identical specific activities specific for TNF-α, TNF-β, β-actin, and the Thy-1 surface antigen, we estimated that TNF-β mRNA was present at 500 to 1000 copies/cell in the CTLL-2 line, and TNF-α at about 100 copies/cell.

TNF mRNA expression in Lyt-2$^+$ CTL clones. In a first series of experiments, we analyzed three independently derived Lyt-2$^+$ CTL clones (clones 7, 11, and 75) for the presence and inducibility of the TNF mRNA. All three clones are directed against H-2^d alloantigens and are maintained in culture by repeated antigenic stimulation in the presence of IL-2. To make sure that the clones had maintained their CTL phenotype, we examined their ability to kill P815 (H-2^d) target cells and to induce rapid DNA degradation in these same targets. The data shown in Figure 2 demonstrate that all three clones were actively cytolytic by these criteria. They were able to induce ^{51}Cr release from P815 targets and to cause extensive DNA degradation (as measured by the release of ^{125}I from the nuclei of [^{125}I]UdR-labeled cells) within 30 min of contact.

We analyzed cytoplasmic RNA extracted from these CTL clones 7 days after the last antigenic stimulation, which is the time at which they were assayed for cytolytic activity, and failed to detect any TNF-β mRNA (Fig. 3, *lanes c* and *f*). From prolonged autoradiographic exposures of our gels, we estimate that TNF-β mRNA is more

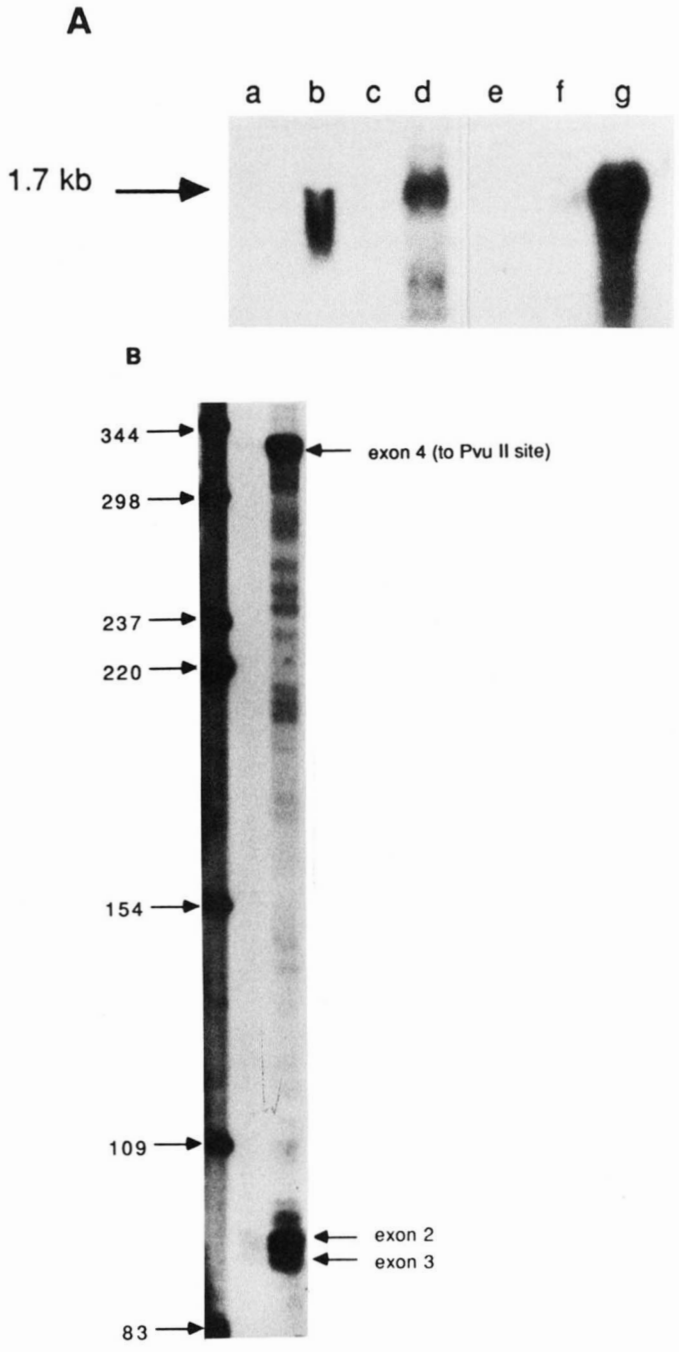

Figure 1. Detection of TNF-β mRNA in Northern blots and RNase protection assays. *A*, 5 μg samples of total cytoplasmic RNA were separated on an agarose/formaldehyde gel and hybridized with a TNF-β (*lanes a, b,* and *c*) or TNF-α (*lanes e, f,* and *g*) probe. *Lanes a* and *e*, EL-4 cells; *lanes b* and *f*, CTLL-2 cells; *lanes c* and *g*, bone marrow-derived macrophages exposed to 10 μg/ml of LPS for 4 h; *lane d*: m.w. markers (the main band visible on this portion of the gel corresponds to a size of 1.7 kb). *B*, 2 μg of CTLL-2 cytoplasmic RNA were hybridized with a genomic probe covering exons 2, 3, and part of exon 4. The RNase protection assay was performed as described in *Materials and Methods*. The sizes of m.w. markers are indicated on the *left*.

than 100-fold less abundant in the CTL clones than in CTLL-2 cells. This would put an upper limit for TNF-β mRNA abundance to 5 to 10 copies/cell. Similar results were obtained with a TNF-α probe in RNase protection assays (data not shown).

In order to test the possibility that TNF-α and/or TNF-β are synthesized in a short burst after stimulation and subsequently stored (e.g., in cytolytic granules), we measured TNF mRNA levels in our CTL clones after stimulation with phorbol myristate acetate, an activator of protein kinase C, and ionomycin, which causes an influx of Ca^{2+} ions into the cells. The combination of PMA and ionomycin has been shown to induce many of the functions specific of activated T cells, and in particular the secretion of IFN-γ and of IL-2 (36).

Figure 3 shows the results of an RNase protection assay performed on RNA extracted before and 24 h after stimulation of CTL clones 7 and 75 with PMA and ionomycin. Although the clone 7 cells accumulated appreciable amounts of TNF-β RNA after stimulation (*lane d*), we failed to detect any TNF-β mRNA in the clone 75 cells 24 h after stimulation (*lane g*). Probing the same RNA preparations with a IFN-γ probe (lower panel), we could easily detect the expected accumulation of IFN-γ mRNA in stimulated cells. The RNA extracted from LPS-activated macrophages (*lane b*), used as a negative control, did not contain messengers for either lymphokine, even though it did contain large amounts of TNF-α mRNA (data not shown).

To obtain the experimental results shown in Figure 4, we stimulated all three CTL clones with PMA and ionomycin and harvested the cells 1, 5, and 24 h later. The abundance of mRNAs for TNF-α, TNF-β, IFN-γ, and Granzyme A was determined by densitometric scanning of "slot blots" hybridized with the relevant probes, and normalized against the signal obtained by rehybridizing the same blot with a β-actin probe. Because the absolute abundances of the mRNAs cannot be estimated with this method, the data are presented in terms of relative signal strength, with the unstimulated cells serving as a reference. Note that the y-axis scales of Figure 4 are different, to accommodate a much larger increase in IFN-γ levels relative to TNF-α, TNF-β, or Granzyme A. The culture media were kept and assayed for the presence of biologic activities associated with IFN-γ and TNF (Table I). IFN-γ was detected by its macrophage-activating activity, whereas TNF was assayed by its cytotoxicity for the WEHI 164 clone 13 line.

The data in Figure 4 show that stimulation of the CTL clones with PMA and ionomycin resulted in the transient accumulation of IFN-γ mRNA in all three, albeit at vastly different levels (>100-fold increase in clone 7, 40-fold in clone 11, and eight-fold in clone 75). Maximum levels of mRNA were reached around 5 h after stimulation. Although a rapid accumulation of TNF-α and TNF-β mRNA (about 15-fold in 1 h for clone 7) could be detected in clones 7 and 11, we observed no hybridization over background in RNA samples from stimulated clone 75 cells. RNase protection assays (data not shown) confirmed that the increased hybridization to TNF-α and TNF-β probes in clone 11 RNA was indeed due to the presence of the corresponding mRNAs.

As a control, we also probed our blots with an oligonucleotide specific for the mRNA of Granzyme A, a serine protease found in cytolytic granules (Fig. 4). Although the mRNA was easily detectable in all CTL clones (the signal strength was much higher than for TNF-β or IFN-γ in unstimulated cells, and approximately equal in all three clones), stimulation of the cells with PMA and ionomycin did not dramatically alter its abundance.

Table I shows the TNF and IFN-γ biologic activities measured in the culture supernatants of the cells from which the RNAs were extracted. For both lymphokines,

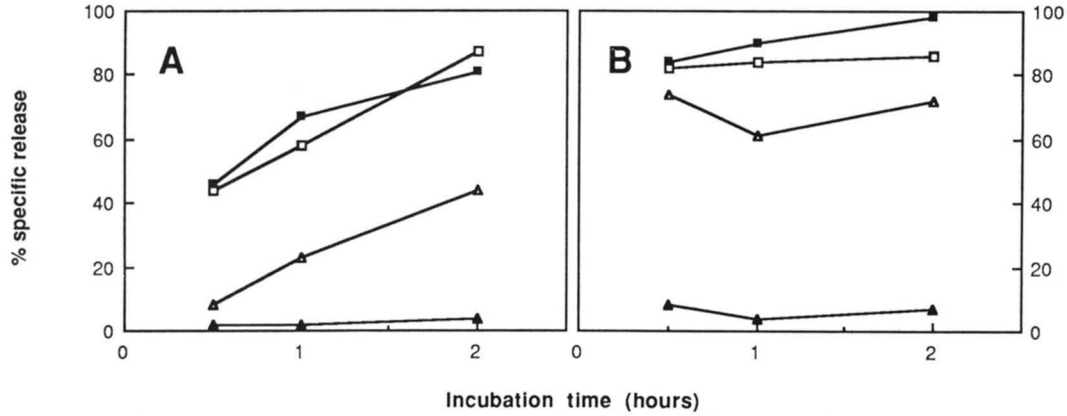

Figure 2. Cytolytic activity of CTL clones 7, 11, and 75 on P815 target cells. *A*, Kinetics of ^{51}Cr release from P815 cells exposed to a 10-fold excess of cloned CTL. *B*, Kinetics of ^{125}I release from the nuclei of P815 cells whose DNA had been labeled with [^{125}I]UdR. ▲, no CTL control; □ CTL clone 7; ■, CTL clone 11; △, CTL clone 75.

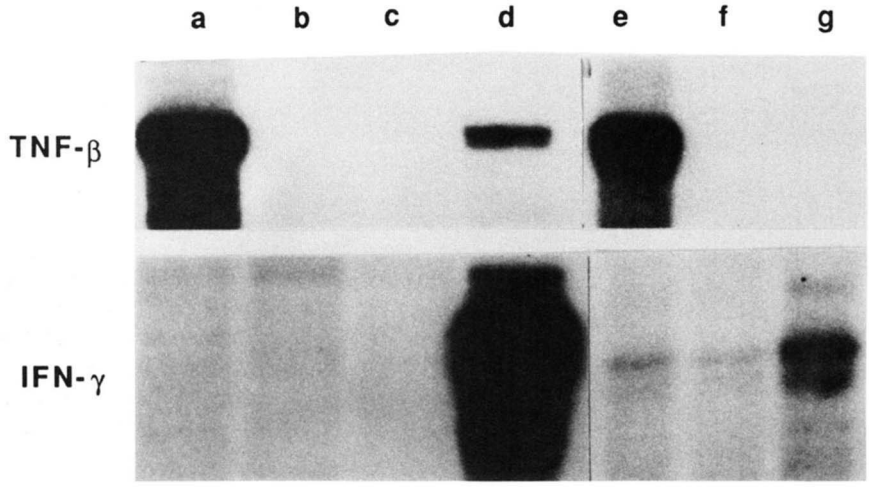

Figure 3. RNase protection assay for TNF-β mRNA in CTL clones. 2 μg of total cytoplasmic RNA were hybridized with ^{32}P-labeled RNA probes specific for TNF-β (*top*) or IFN-γ (*bottom*) as described in *Materials and Methods*, and the protected fragments were separated on a 5% acrylamide gel. *Lanes a* and *e*, CTLL-2 cells; *lane b*, bone marrow-derived macrophages after 4 h of exposure to LPS (1 μg/ml); *lane c*, CTL clone 7 cells 7 days after the last antigenic stimulation; *lane d*, CTL clone 7 cells after 24 h of exposure to PMA (10 ng/ml) and ionomycin (500 ng/ml); *lane f*, CTL clone 75 cells 7 days after last antigenic stimulation; *lane g*, CTL clone 75 cells after 24 h of exposure to PMA and ionomycin.

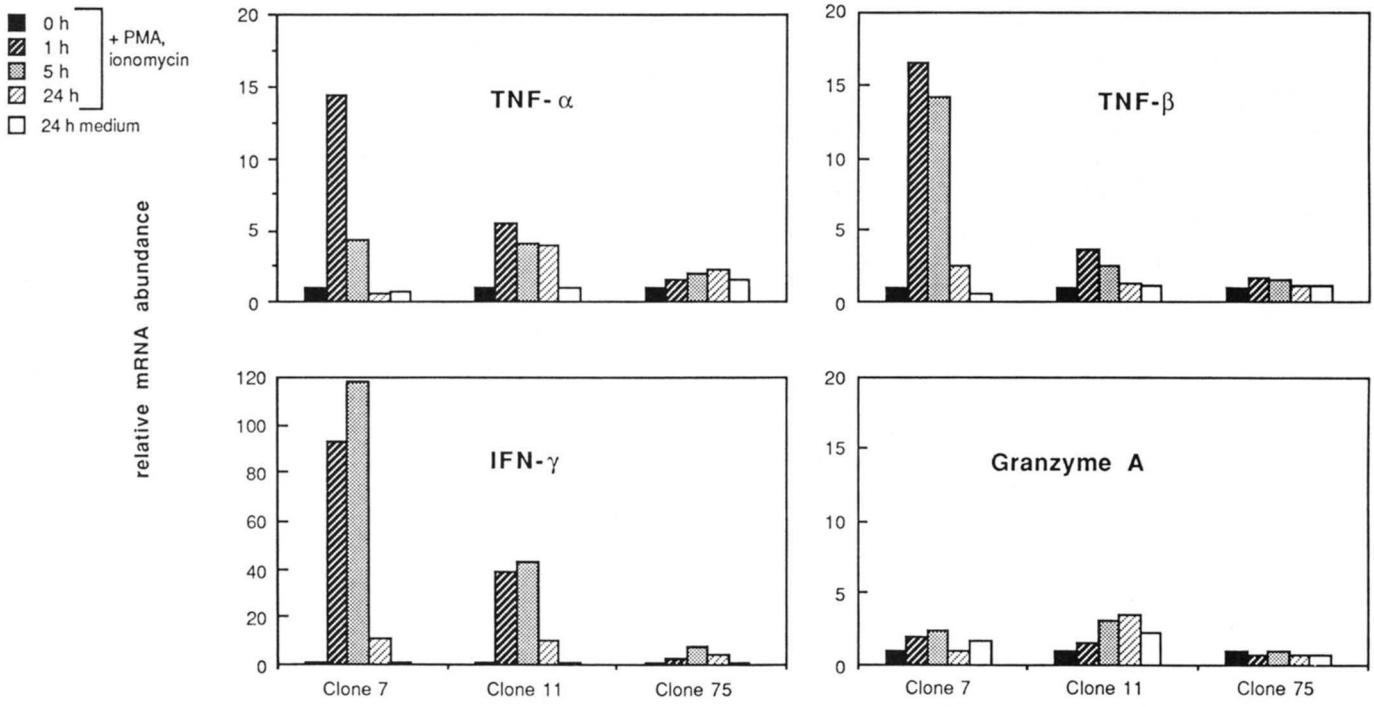

Figure 4. Kinetics of induction of TNF-α, TNF-β, IFN-γ, and Granzyme A mRNAs after exposure of CTL clones to PMA (10 ng/ml) and ionomycin (500 ng/ml). Relative abundances of the mRNAs were determined by blotting serial dilutions of cytoplasmic RNA (0.05 to 5 μg) onto nylon filters, hybridizing with the relevant probes, and scanning the exposed autoradiograms. All results were normalized against the signal obtained by rehybridizing the same filters with a β-actin probe, and are expressed as fold stimulation relative to the hybridization signal obtained from cells before exposure to PMA and ionomycin.

TABLE I
Biologic activities measured in CTL culture supernatants

Cell line and Stimulation[a]	Macrophage-activating Activity[b]	TNF Activity[c]
CTL clone 7		
None (24 h)	5	<8
PMA + ionomycin		
1 h	9	16
5 h	450	>256
24 hr	>1000	>256
CTL clone 11		
None (24 h)	<3	<8
PMA + ionomycin		
1 h	<3	<8
5 h	30	<8
24 h	80	32
CTL clone 75		
None (24 h)	<3	<8
PMA + ionomycin		
1 h	<3	<8
5 h	3	<8
24 h	25	<8

[a] Cells were stimulated by exposure to PMA and ionomycin in DMEM 5% or incubated in DMEM 5% alone (none) for the indicated times. The culture medium was then harvested and assayed for biologic activity.

[b] Macrophage-activating activity of the culture supernatants was measured as described in *Materials and Methods*. The results are expressed as the reciprocal of the dilution yielding 50% maximal cytolytic activity.

[c] TNF cytotoxic activity of the culture supernatants was measured on WEHI 164/13 cells as described in *Materials and Methods*. The results are expressed as the reciprocal of the highest dilution causing more than 50% viability loss as measured in the MTT assay.

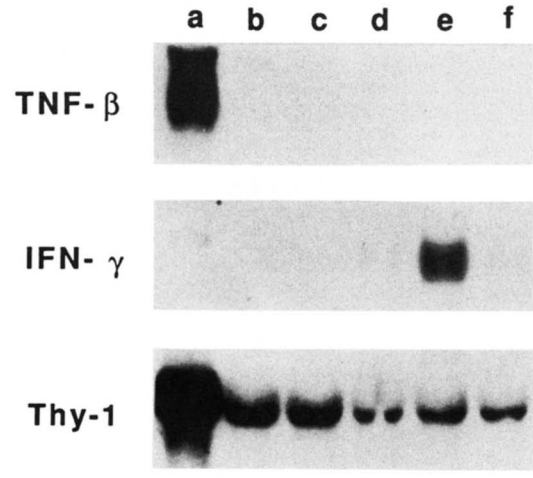

Figure 5. Northern blot of RNA extracted from PC60 cells exposed to different combinations of interleukins. A 5-μg sample of cytoplasmic RNA was separated in a 1.4% agarose/formaldehyde gel, transferred to nylon, and the filter was hybridized successively with the indicated RNA probes. *Lane a*, CTLL-2 cells; *lane b*, uninduced PC60 cells; *lane c*, PC60 exposed to recombinant human IL-2 (100 U/ml) for 4 days; *lane d*, PC60 exposed to recombinant human IL-1β (5 ng/ml); *lane e*, PC60 exposed to IL-1β and IL-2; *lane f*, PC60 exposed to IL-1 and a supernatant from concavavalin A-activated spleen cells.

the biologic activities correlated closely with the RNA levels measured by hybridization. In particular, the supernatants of activated clone 75 cells contained no measurable TNF activity. It should be noted that the WEHI 164/13 cytotoxicity assay does not allow us to distinguish between the effects of TNF-α and TNF-β.

TNF-β mRNA expression in L3T4⁺ clones. Because a strong correlation between cytolytic activity and TNF-β secretion had been reported in L3T4⁺ T cell clones (20, 37, 38), we examined TNF-β expression in two cytolytic L3T4⁺ clones (AT20 and ATX5.3) that are specific for the synthetic polymer GAT and restricted by I-A^k (26). These two clones grow independently of exogenous IL-2, and can be induced to become cytolytic to I-A^k-positive fibroblasts after exposure to their cognate antigen (GAT) and

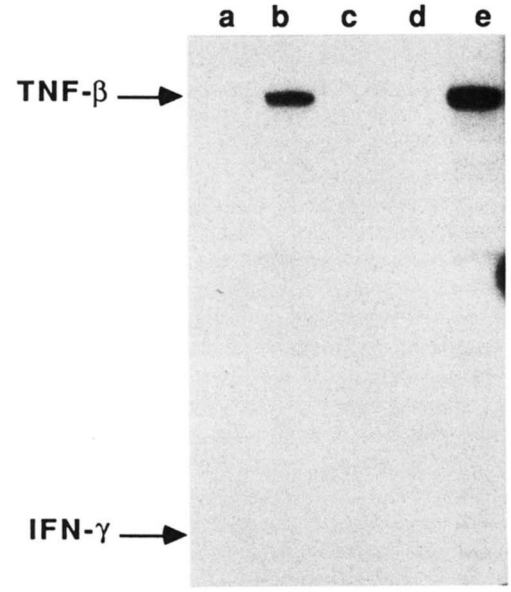

Figure 6. RNase protection assay of RNA extracted from L3T4⁺ T cell clones. A 2-μg sample of cytoplasmic RNA was hybridized to a mixture of TNF-β and IFN-γ RNA probes and processed as before. *Lane a*, Unstimulated AT20 cells; *lane b*, AT20 cells stimulated with GAT and IL-1 for 12 h; *lane c*, unstimulated ATX5.3 cells; *lane d*, ATX5.3 cells stimulated with GAT and IL-1; *lane e*, CTLL-2 cells.

IL-1 (Ph. Naquet, manuscript in preparation). Figure 5 shows the results of an RNase protection assay performed on RNA extracted from these two clones before and after stimulation with GAT and IL-1. Although one of the two clones (AT20) clearly accumulated TNF-β mRNA, the other one did not. No IFN-γ mRNA (Fig. 5) or biologic activity could be detected in either clone. RNase protection assays performed with a TNF-α probe showed a low constitutive level of TNF-α mRNA in both clones, and assays of the culture supernatants in the L929 cell cytotoxicity assay suggested a low level of TNF-α production. Whether this production of TNF-α is necessary for the cytotoxicity of these L3T4⁺ clones remains to be determined.

TNF mRNA does not accumulate in inducible rat × mouse hybrid CTL. The PC60 cell line was derived from a cell fusion between a mouse CTL clone and the C58 rat thymoma line (27). These cells grow in an II-2-independent manner and are not cytolytic. However, when cultured in the presence of IL-1 and IL-2, they accumulate mRNA for IL-2, IL-2 receptor, and Granzyme A, synthesize perforin, the pore-forming protein present in CTL granules, and become cytolytic (39) (G. Plaetinck, unpublished observations).

We assayed for the appearance of TNF mRNA after stimulation of PC60 cells with various combinations of lymphokines. The Northern blot hybridization data presented in Figure 6 clearly show that although IFN-γ mRNA accumulated in PC60 cells 4 days after stimulation by a combination of IL-1 and IL-2, no TNF-β mRNA could be found. Similarly, although TNF-α mRNA could easily be detected in the CTLL-2 cells, none was found in stimulated PC60 cell RNA (data not shown). The level of the mRNA for the T cell-specific Thy-1 surface antigen, which is not regulated under these conditions, remained constant. Similar results were obtained by using an RNase protection assay (data not shown). We also assayed for the presence of TNF-β mRNA at different times

after induction, with similarly negative results (data not shown).

DISCUSSION

In the experiments described in this paper, we have examined the presence and accumulation of TNF-α and TNF-β mRNA in six different murine cell lines: three constitutively cytolytic Lyt-2$^+$ T cell clones, two L3T4$^+$ clones whose cytolytic activity is inducible, and one inducible T cell hybridoma. None of these cell lines expressed TNF mRNA in a constitutive fashion, and only three out of six could be induced to accumulate TNF mRNA by stimuli known to activate other CTL-specific functions. It is significant that one of the two L3T4$^+$ clones as well as the T cell hybridoma failed to accumulate any TNF-β mRNA under conditions where they were acquiring cytolytic activity. We believe that these data provide strong evidence for the lack of involvement of TNF-β in CTL-mediated cytolysis. Although we cannot rule out a role for TNF-α in the cytotoxic activity of the L3T4$^+$ clones, TNF-α mRNA is not produced by the Lyt-2$^+$ clone 75 or by the induced PC60 hybridoma, nor is any TNF biologic activity found in the supernatants of activated clone 75 cells.

It could be argued that very low levels of TNF mRNA, escaping detection by our assay methods, could be sufficient for the synthesis of the TNF required to kill target cells. We think this unlikely, however, because of the high sensitivity of the RNase protection assays, and because no correlation could be found between the ability of T cells to synthesize TNF and their cytolytic activity. In particular, the two L3T4$^+$ clones behave identically in their ability to kill I-A^{k+} target cells in the presence of GAT and IL-1 (Ph. Naquet, in preparation), and yet differed very clearly in their accumulation of TNF-β mRNA.

It should be clearly stated that our data do not challenge the notion that secretion of TNF-α and/or TNF-β can be triggered by contact between a specific CTL and a cell presenting its cognate antigen. In fact, several of the T cell clones that we examined responded to stimulation by synthesizing mRNA for both TNFs, and we could also detect the mRNAs in antigen-stimulated mixed lymphocyte cultures (unpublished results). The important point is that T cells can be cytolytic and induce DNA degradation in their targets without expressing TNF.

The data we obtained with Lyt-2$^+$ CTL clones seemed to show a correlation between the level of expression of IFN-γ and that of TNF-β, which could be interesting in view of the synergistic biologic activities of these two lymphokines. The correlation does not hold true, however. We could not demonstrate any TNF-β mRNA after activation of the CTL clone 75 cells, even though they did synthesize low amounts of IFN-γ. Conversely, the L3T4$^+$ clone AT20 accumulated TNF-β mRNA after activation, but no IFN-γ mRNA. Therefore, it seems unlikely that the two genes are coordinately regulated. In view of the close physical linkage of their genes, it was interesting to compare the levels of TNF-α and TNF-β mRNA in these populations. It has been reported that PMA and ionomycin preferentially induce the expression of TNF-α in T cells, although stimulation with lectins induces mostly TNF-β (22, 23). In our hands, TNF-α and TNF-β mRNAs seemed to appear simultaneously and to be stimulated to approximately the same extent in those Lyt-2$^+$ clones that produced TNF. This correlation did not hold true in the L3T4$^+$ clones or under all conditions of cell stimulation. In general, our data agree with those presented by Cuturi et al. (22) for human peripheral blood lymphocytes.

If TNF is not an obligatory mediator of CTL cytolytic activity, what then is its function? A possible answer can be found in the pleiotropic effects of both TNF-α and TNF-β. Although the two proteins are cytotoxic to a relatively small number of transformed cell lines, they are nontoxic or stimulatory to most nontransformed cells (40, 41), and they have many activities involved in the development of the inflammatory response: activation of granulocytes (42, 43), differentiation of monocyte precursors (44, 45), induction of IL-1 (46), and GM-CSF (47) synthesis, induction of MHC antigens (48) and other surface markers (49), and antiviral activity (50, 51), with new activities described at an accelerating pace. Therefore, it is clear that the role of TNF is not limited to the lysis of target cells (although it may be involved in the destruction of some tumors), but rather that TNF is another member of the growing family of soluble mediators that help increase the efficiency of the imjune response.

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