American Journal of Pathology, Vol. 134, No. 1, January 1989

Rapid Communication

Endotoxin-Induced Cytokine Gene Expression *In Vivo*

I. Expression of Tumor Necrosis Factor mRNA in Visceral Organs Under Physiologic Conditions and During Endotoxemia

Thomas R. Ulich, Kaizhi Guo, and Juan del Castillo

From the Department of Pathology, University of California Irvine School of Medicine, Irvine, California

Tumor necrosis factor (TNF) mRNA was detected by Northern blotting in whole-organ homogenates of the spleen, liver, kidney, lung, and small bowel in naive and saline-injected control rats, supporting the hypothesis that TNF mRNA is present in vivo in a preformed intracellular pool. TNF mRNA in endotoxin-treated rats as quantitated by densitometry of the ratio of TNF mRNA to actin mRNA in Northern blots was present in increased quantity in the liver, kidney, and lung (1.6–2.9 times over time zero levels) at 15 minutes and increased quantity in the spleen, liver, and kidney (1.3–1.9 times over time zero levels) at 30 minutes. The kinetics of endotoxin-induced TNF gene expression are consistent with the relatively transient peak of serum TNF protein levels reported by previous investigators to occur approximately 1 hour after injection of endotoxin. Because TNF mRNA appeared ubiquitous in the organs of control rats examined and because the endotoxin-induced increase in TNF mRNA was relatively small, endotoxin may induce the expression of the TNF protein in serum not only by increasing TNF mRNA levels but perhaps more importantly by a posttranscriptional mechanism. The presence of a preformed pool of TNF mRNA may teleologically be viewed as a mechanism to increase the rapidity of the host's response to sepsis. (Am J Pathol 1989, 134:11–14)

Endotoxin (LPS) is the major cause of the severe systemic toxicity and of the local inflammation associated with gram-negative bacterial infections.[1] Endotoxin, however, does not exert most of its potent biologic effects directly, but rather through the expression of endogenous mediators of inflammation and immunity known as cytokines.[1–3] Cytokines are relatively low molecular weight proteins, many of which were originally discovered to be produced by immune or inflammatory cells, although several mesenchymal and epithelial cells are now implicated as potential sources of cytokines. Several lines of evidence suggest that LPS exerts many of its effects by the release of cytokines. Endotoxin, for example, does not induce the clinical features of endotoxemia in C3H/HeJ mice whose macrophages express very low levels of tumor necrosis factor (TNF) and interleukin-1 (IL-1) as compared with the macrophages of congenic endotoxin-sensitive C3H/HeN mice.[4,5] Endotoxic shock in several experimental animal models is prevented by passive immunization with antisera to TNF[6] and high doses of TNF induce hemorrhagic shock.[7] Even though intradermal injections of LPS induce severe neutrophilic inflammatory infiltrates *in vivo*, endotoxin is not chemotactic for neutrophils *in vitro*.[8] Intradermal injection of certain cytokines reproduces the acute inflammation caused by endotoxin, and some cytokines are chemotactic for neutrophils *in vitro*.[9] Because LPS appears to induce many biologic effects by the release of cytokines in either an endocrine or paracrine fashion, a knowledge of endotoxin-induced cytokine gene expression *in vivo* is of fundamental importance to our understanding of the pathogenesis of endo-

Supported by NIH grant RO1-AI 26551-01.

Accepted for publication November 2, 1988.

Address reprint requests to Thomas R. Ulich, Department of Pathology, UC Irvine School of Medicine, Irvine, CA 92717.

toxin-induced inflammation and morbidity. The purpose of the present study is to assess the presence and quantity of TNF mRNA in the visceral organs of naive and endotoxemic rats.

Materials and Methods

Lewis rats, male, weighing approximately 250 g, either naive or at specified timepoints after the intravenous injection of saline or 100 μg *S. typhosa* lipopolysaccharide (Sigma Chemical Co., St. Louis, MO), were anaesthesized with ether and the organs (spleen, liver, kidney, lung, and small bowel) were removed and placed in a petri dish containing 10 ml of 4 M guanidium isothiocyanate, 25 mM Hepes (pH 7.0) and 0.7 μl β-mercaptoethanol. The organs in an ice bath were immediately minced, crushed, and homogenized for 30 seconds with a Tissumizer tissue homogenizer. The whole organ homogenate was centrifuged in a 50 ml conical tube for 10 minutes at 10,000 rpm at 12 C in a Beckman JA-13 rotor. The RNA-containing supernatant was collected and Sarkosyl was added to a final concentration of 0.5%. The supernatant was heated at 65 C for 2 minutes, and then ultracentrifuged at 25,000 rpm over a CsCl gradient for 20 hours in a Beckman SW-41 rotor. The RNA pellet was resuspended in 10 mM TRIS-HCL, pH 7.4, 5 mM Na-EDTA, 1% SDS at room temperature for 1–2 hours, extracted with phenol/chloroform/isoamyl alcohol, and precipitated with 1/10 volume 3 M sodium acetate and 2.5 volumes absolute ethanol at −30 C overnight. After drying the pellet in a vacuum dessicator, the yield and purity of RNA was quantitated by measuring the ratio of the absorbances at 260 and 280 nm. Successful isolation of undegraded RNA was preliminarily monitored by mini-gel electrophoresis and examination of the sharpness of the 18S and 28S ribosomal RNA bands under ultraviolet light. Northern blotting was performed according to standard methods[10] by electrophoretic separation of total organ RNA (25 μg/lane) in a formaldehyde agarose gel followed by blotting of the RNA onto a nitrocellulose filter paper (Zeta-probe membrane).

A murine TNF cDNA containing plasmid was obtained as a gift from Dr. George Davatelis of the Rockefeller Institute and was transformed in our laboratory into JM101 cells. The TNF cDNA is inserted in the pUC9 plasmid at the Pst I and Bam HI restriction sites. For the purpose of hybridization, however, Pst I and Eco RI were used as restriction enzymes for the purification of the insert from the vector because Eco RI cleaves off a portion of the 3′-untranslated region of the insert that contains a TTATTTATT consensus sequence that is common to IL-1 and other cytokines[11] and might result in unwanted cross-hybridization. The purified insert was labeled with dCTP-α^{32}P by random sequence hexanucleotide priming (prime time "C" biosystem) to a specific activity of approximately 10^9 cpm/μg. A human γ-actin cDNA probe (gift of Dr. Hun Fang) was used as a loading control in a second hybridization of the same Northern blot originally hybridized with the TNF cDNA.[12] After hybridization, the blots were washed at room temperature for 15 minutes, twice with 2× SSC and 0.1% SDS and twice with 0.2× SSC and 0.1% SDS. Laser densitometry (LKB Ultroscan laser densitometer) was used to quantitate the density of the hybridization bands obtained after Northern blotting.

Results

TNF mRNA as detected by Northern blotting was present in all organs examined (spleen, liver, kidney, lung, small bowel) in both a naive uninjected rat killed at time 0 (Fig. 1) and a saline-injected negative control rat sacrificed at 15 minutes (data not shown). Peripheral neutrophil counts, which are probably the most sensitive *in vivo* indicator of endotoxemia or TNF,[13,14] were normal in both control rats. After injection of endotoxin, densitometry demonstrated increases in TNF mRNA expression of 1.8, 1.9, 3.7, 1.3, and 1.3 in the spleen, liver, kidney, lung, and bowel, respectively, comparing the absorbances of the 1.6 kb bands at 15 minutes with time zero. The TNF cDNA probe was then dissociated and the Northern blot was rehybridized with actin cDNA. The so-called "housekeeping gene" actin serves as a control to compensate for any changes in the intensity of hybridization for TNF mRNA that are due to unintentional over- or underloading of total organ RNA in the lanes of the Northern gel. Each organ expresses a unique level of actin mRNA (so that the intensity of hybridization for actin mRNA is not the same in lanes of different organs), but the level of actin gene expression for each organ remains constant after injection of endotoxin. TNF mRNA expression at 15 and 30 minutes was quantitated by laser densitometry of both TNF and actin bands at 0, 15, and 30 minutes with calculation of the change in TNF mRNA expression by the formula: TNF (Abs at 15 or 30 minutes)/TNF (Abs at time zero): actin (Abs at 15 or 30 minutes)/actin (Abs at time zero). On the foregoing assumptions, the liver, lung, and kidney demonstrated TNF mRNA increases of 1.6, 1.6, and 2.9 at 15 minutes, whereas the spleen and bowel actually demonstrated slight decreases in TNF mRNA content to 0.8 and 0.5 of time zero levels. At 30 minutes after the injection, TNF mRNA expression in the spleen, liver, kidney, lung, and bowel was 1.3, 1.3, 1.9, 1.0, and 1.0, respectively, as compared with time zero.

Discussion

The present study documents that TNF mRNA is present within visceral organs under normal physiologic condi-

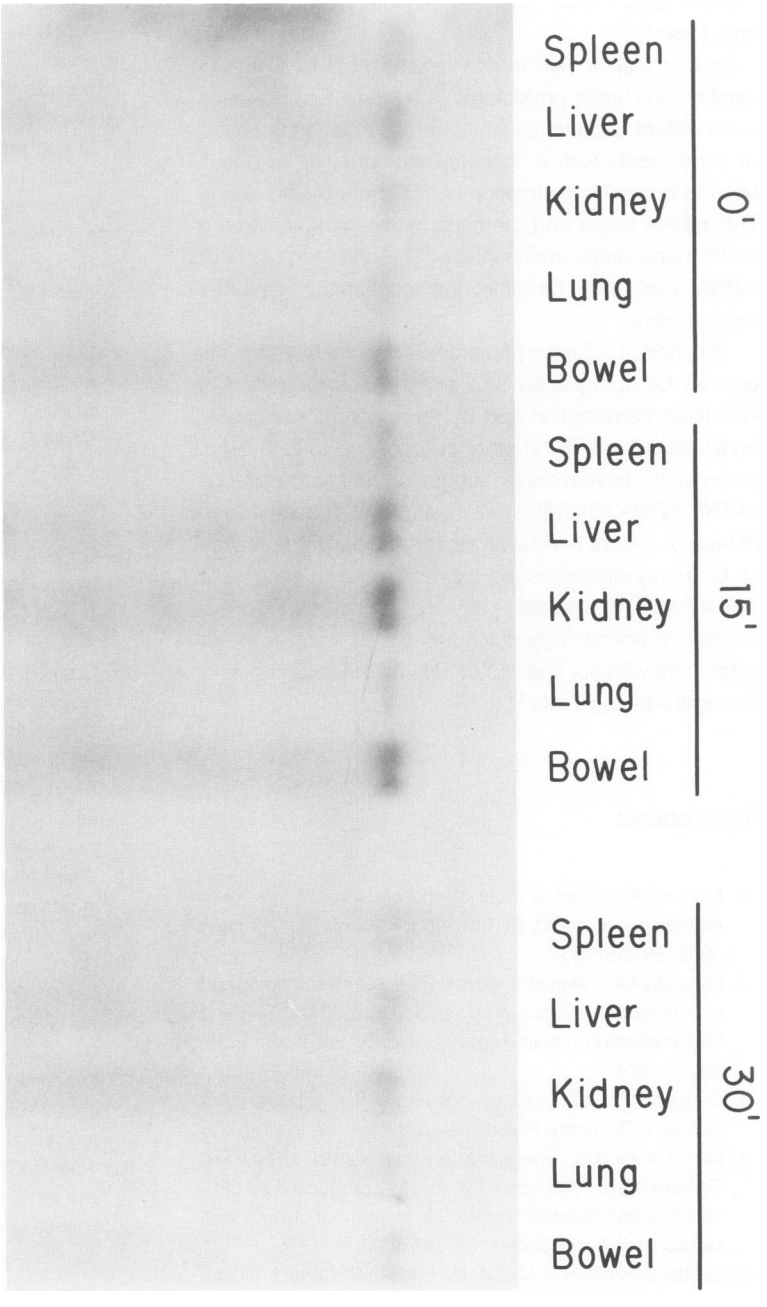

Figure 1. *TNF mRNA (1.6 kb) is detected by Northern blotting in whole-organ homogenates of spleen, liver, kidney, lung, and small bowel in a naive (time zero) rat. TNF mRNA in endotoxin-treated rats as quantitated by laser densitomery of the ratio of TNF mRNA to actin mRNA expression was present in increased quantity in several but not all organs at 15 and 30 minutes.*

tions and that endotoxin can transiently increase TNF mRNA levels in some organs. The seemingly ubiquitous presence of TNF mRNA under physiologic conditions would at first appear paradoxical because TNF is not thought to circulate other than in pathophysiologic conditions.[15] Beutler et al,[5] however, have demonstrated previously that murine thioglycolate-elicited peritoneal macrophages contain a pool of TNF mRNA that is not expressed as protein. After stimulation with endotoxin, the macrophages produced a large additional quantity of TNF mRNA and secreted immunoreactive TNF.[5] The present findings would, therefore, appear to add further evidence to the hypothesis of Beutler and colleagues that the control of TNF synthesis is regulated both at the transcriptional and posttranscriptional levels. Significantly, TNF mRNA has in the present experiments been much more firmly documented to be presented *in vivo* under physiologic "unactivated" conditions because the TNF mRNA in elicited macrophages could conceivably, as mentioned by Beutler et al, be the result of mRNA induction during the isolation process.[5] The kinetics of endotoxin-induced TNF mRNA expression in the rat would appear to correlate well with the kinetics of endotoxin-induced serum TNF in the rat, which peaks in a burst sharply and transiently

approximately 1 hour after the experimental injection of endotoxin.[15]

In conclusion, TNF mRNA is present in multiple visceral organs under physiologic conditions. Endotoxin, as is consistent with the results of Beutler et al[5] on the basis of experiments with monocyte/macrophages, is postulated to control the secretion of TNF both by increasing TNF mRNA levels and, perhaps more importantly, by a posttranscriptional mechanism. The increase in TNF mRNA levels may be either transcriptional or by other mechanisms.

Burchett et al[16] have presented evidence that TNF levels may be upregulated by a change in the duration of increased transcription and by an apparent increase in translation or protein stability in response to LPS. Economu et al[17] have recently suggested that the regulation of TNF mRNA's half-life may occur through a short-lived RNAse. A recent review of as yet unpublished data by M. G. Tovey describes the expression of high levels of TNF mRNA in the spleen, liver, and peripheral blood leukocytes of normal individuals and would seem to be in agreement with our findings of TNF mRNA expression in the organs of naive rats.[18]

References

1. Issekutz AC, Bhinji S: Role of endotoxin in the leukocytic infiltration occupying E. coli inflammation. Infect Immun 1982, 36:558–566
2. Cybulsky ME, Chan MK, Movat HZ: Acute inflammation and microthrombosis induced by endotoxin, IL-1, and TNF and their implication in gram-negative infection. Lab Invest 1988, 58:365–378
3. Oettgen HF, Old LJ: tumor necrosis factor, Important Advances in Oncology. Philadelphia JB Lippincott, 1987, p 105
4. May M, Kunkel SL, Spengler M, Kwon G, Larrick J, Phan SH: Dynamics of LPS-induced TNF and IL-1 production by LPS-sensitive and resistant mice: Analysis at the molecular and cellular level. Fed Proceed 1987, 46:562A
5. Beutler B, Krochin N, Milsark IW. Luedke C, Cerami A: Control of cachectin (tumor necrosis factor) synthesis: Mechanisms of endotoxin resistance. Science 1986, 232:997–980
6. Beutler B, Milsark IW, Cerami AC: Passive immunization against cachectin/TNF protects mice from lethal effects of endotoxin. Science 1985, 229:869–871
7. Tracy KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark JW, Hariri RJ, Fahey TJ, Zentella A, Albert JD, Shires GT, Cerami A: Shock and tissue injury induced by recombinant human cachectin. Science 1986, 234:470–474
8. Snyderman R, Gewurz H, Mergenhagen SE: Interactions of the complement system with endotoxin lipopolysaccharide: Generation of a chemotactic factor for polymorphonuclear leukocytes. J Exp Med 1969, 128:259–272
9. Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA: Acute inflammation and a Schwartzman-like reaction induced by IL-1 and TNF: Synergistic action of the cytokines in the induction of inflammatory and microvascular injury. Am J Pathol 1987, 129:463–476
10. Wahl GM, Meinkoth JL, Kimmel AR: Northern and Southern blots, Guide to Molecular Cloning Techniques. Edited by SL Bergen, AR Kimmel. New York, Academic Press, 1987, p 572
11. Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A: Identification of a common nucleotide sequence in the 3′-untranslated region of mRNA molecules specifying inflammatory mediators. PNAS USA 1986, 83:1670–1674
12. Gunning P, Ponte P, Okayama H, Engel J, Blau H, Kedes L: Isolation and characterization of full length cDNA clones for human α-, β-, and γ-action mRNAs. Mol Cell Biol 1983, 3: 787–795
13. Ulich TR, del Castillo J, Keys M, Granger GA, Ni RX: Kinetics and mechanisms of recombinant human IL-1 and TNFa-induced changes in circulating numbers of neutrophils and lymphocytes. J Immunol 1987, 139:3406–3415
14. Remick DG, Kunkel RJ, Larrick JV, Kunkel SL: Acute in vivo effects of human recombinant tumor necrosis factor. Lab Invest 1987, 56:583–590
15. Waage A: Production and clearance of tumor necrosis factor in rats exposed to endotoxin and dexamethasone. Clin Immunol Immunopathol 1987, 45:348–355
16. Burchett SK, Weaver W, Westall J, Larsen A, Kroheim S, Wilson CB: Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. J Immunol 1988, 140:3473–3481
17. Economu J, Essner R, Rhoades K, McBride W, Morton DL: Tumor necrosis factor and interleukin-1 mRNA half-lives are regulated by a short-lived RNase. J Leuk Biol 1988, 44:287A
18. Tovey MG: The expression of cytokines in the organs of normal individuals: role in homeostasis: A review. J Biol Reg Homeo Agents 1988, 2:87–92

Acknowledgment

The authors thank Dr. George Davatelis for donating the TNF cDNA containing plasmid, Dr. Hung Fan for the actin cDNA containing plasmid, and Drs. Luis de la Maza and Sharon Wilczynski for their advice and for generously sharing the resources of their laboratories.