Title

Hypoxia Induces a Human Macrophage Cell Line to Release Tumor Necrosis Factor-α and Its Soluble Receptors in Vitro

Journal

Journal of Surgical Research, 54(4)

ISSN

0022-4804

Authors

Scannell, Gianna
Waxman, Kenneth
Kaml, Gary J
et al.

Publication Date

1993-04-01

DOI

10.1006/jsre.1993.1044

JOURNAL OF SURGICAL RESEARCH 54, 281–285 (1993)

Hypoxia Induces a Human Macrophage Cell Line to Release Tumor Necrosis Factor-α and Its Soluble Receptors *in Vitro*

GIANNA SCANNELL, M.D.,* KENNETH WAXMAN, M.D.,* GARY J. KAML,† GENE IOLI, B.A.,† TETSUYA GATANAGA, PH.D.,† ROBERT YAMAMOTO, B.S.,† AND GALE A. GRANGER, PH.D.†

*Departments of *Surgery and †Molecular Biology and Biochemistry, University of California, Irvine, California 92717*

Presented at the Annual Meeting of the Association for Academic Surgery, Montreal, Quebec, Canada, November 18–21, 1992

Tissue hypoxia following hemorrhage and trauma is a possible initiating factor of the generalized inflammatory response seen after shock. The role of hypoxia in the release from a human monocyte cell line (THP-1) of tumor necrosis factor-α (TNFα) and its soluble membrane receptors (TNFαR) *in-vitro* is investigated in this study. Flat-bottom plates with 500,000 THP-1 cells/ml were placed in air-tight sealed boxes and exposed to hypoxia (O_2 = 1%) or controls (O_2 = 9%) for up to 24 hr. Supernatants were tested for TNFα, as well as 55- and 75-kDa soluble receptors for TNFα, by ELISA. Cell viability was assessed by vital dye uptake and was found to be maintained throughout hypoxic exposure. Medium pH levels were within normal range. In eight experiments conducted in duplicate, minimal change over 24 hr occurred in control samples. Control mean and SD were: TNFα = 12.0 ± 4.2, 55-kDa R = 34.6 ± 2.03, and 75-kDa R = 38.88 ± 9.68 pg/ml. During hypoxia, TNFα was released as early as the first 30 min of exposure (41.3 ± 2.3 pg/ml) with a small peak at 1 hr (52 ± 5.0 pg/ml) and a later more pronounced peak at 18 hr (526 ± 48 pg/ml). Both 55- and 75-kDa R were released by the hypoxic monocytes; release was progressive and was maximal at 24 hr in this study. Maximal release value of 55-kDa R was 236 ± 15 pg/ml, while for 75-kDa R it was 2450 ± 63 pg/ml. By one-way analysis of variance followed by the Student–Newman–Keuls test for multiple comparisons, P values were <0.01 at all but the 15-min time period, where P > 0.05. We conclude that when exposed to complete or nearly complete absence of oxygen, human monocytes spontaneously release TNFα, followed by TNFαR.

INTRODUCTION

Prolonged periods of reduced microcirculatory blood flow and vasoconstriction are associated with shock states; during shock, tissue oxygen tension may reach very low levels [1]. The mechanisms of cellular response to acute hypoxia during shock are unclear; in particular, the effect of hypoxia upon lymphoid cells has not been defined [2]. Hypoxia may cause direct cellular damage [3], which may then secondarily induce activation of phagocytes [4] or directly cause macrophagic activation. Recent research implicates the role of the monocyte–macrophage and cytokines in septic shock [5–8]. Tumor necrosis factor-α (TNFα) produced in the blood stream by macrophages in response to bacterial lipopolysaccharide (LPS) affects endothelial cells resulting in increased vascular permeability. TNFα also elicits production of procoagulant activity and promotes the adhesion of neutrophils to endothelial cells via the expression of adhesion molecules. High levels of circulating TNFα have been demonstrated 90 to 120 min after intravenous infusion of LPS in rabbits and endotoxin in humans [7]. The appearance of TNFα is sequentially followed by IL-1 and IL-6. In septic shock, IL-1β levels peak about 3 hr after injection, and IL-6 levels continue to rise for up to 8 hr [8]. It is now clear that these cytokines have a major role in the pathophysiology of septic shock. This evidence is supported by the observation that receptors which block IL-1 and TNFα can block septic shock in animal models [9, 10]. However, the presence and importance of cytokines after acute traumatic and hemorrhagic shock is not as clearly understood.

Recently, two soluble membrane receptors (R), each with a different capacity for binding and activating recombinant human TNFα and TNFβ, have been identified. These soluble receptors have been found in serum of traumatized patients, in serum and urine of patients with chronic renal failure, as well as serum and ascites of patients with cancer [11–14]. These TNFR are shed from the extracellular segments of the 55- to 60-kDa and 75- to 80-kDa TNFα membrane receptors and may be important in the regulation of TNFα activity [14, 15]. The TNFαR are capable of binding TNFα and prevent its attachment to cellular membrane receptors, thus blocking TNFα's biologic effects. In fact, TNFR have been found to decrease TNFα cytotoxicity to tumor cells *in vitro* and *in vivo*, as well as to decrease mortality in animals with septic shock [14, 16, 17]. TNFαR may be

0022-4804/93 $4.00

important regulators of TNFα activity, but their release during hypoxia has not been studied.

The purpose of this study was to determine if hypoxia, comparable to that seen *in vivo* during traumatic and hemorrhagic shock, stimulates macrophage release of TNFα and TNFR.

MATERIALS AND METHODS

Culture of the Human Myelocytic Leukemia Cell Line THP-1

Human monocytic leukemia cell line THP-1 cells were derived from the peripheral blood of a 1-year-old male with acute monocytic leukemia [18]. THP-1 cells express surface receptors and can perform most functions associated with human macrophages, including release of various monokines, such as IL-1 and TNFα under stimulation *in vitro* [19]. THP-1 cells were established as monolayers isolated according to the method of Armstrong [20]. They were placed in T-25 flat-bottomed culture flasks (Corning, NY) at a concentration of 500,000 cells per milliliter in 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY) at 37°C and 5% CO_2. They were passaged by trypsinization biweekly.

Exposure of THP-1 Cells to Hypoxia

Oxygen-permeable plastic petri plates (60 × 15 mm) containing THP-1 cells were placed in air-tight modified anaerobic glass jars (volume of 10 cm^3), incubated at 37°C, and exposed to a controlled hypoxic atmosphere, consisting of a mixture of oxygen and nitrogen with partial pressures reflecting almost complete anoxia (8 mm Hg or 1% oxygen), or oxygen tensions present under normal circumstances in capillaries (68 mm Hg or 9% oxygen). The cells were exposed for 15 and 30 min and 1, 4, 12, 16, 18, and 24 hr. Equilibration of oxygen in the media with the atmosphere was nearly instantaneous [21]. Oxygen concentrations were verified with a portable oxygen analyzer (Novametrix Medical Systems, Orange, CA). The pH of medium was monitored with a needle pH electrode (Microelectrodes, Inc., Londonderry, NH) at the beginning and end of each experiment.

Cell viability was assessed by 0.05% trypan blue stain using an Easy Reader 400 AT microtiter plate absorbance detector (SLT 6 Labinstruments, GEs.m.b.H., Austria). Viability was unchanged and remained over 90% in all experiments.

Collection of Supernatants

Supernatants were collected at the end of each incubation period after exposure to hypoxia and from controls and cleared of cells and debris by centrifugation at 450g for 4 min in a IEC-HN-S 2 centrifuge. Supernatants were then stored at −70°C and tested for TNFα and TNFαR levels with enzyme-linked immunosorbent assay (ELISA) techniques.

ELISA Assay of Human TNFα

Commercially available TNF ELISA kits were obtained from Genzyme Corp. (Boston, MA). These kits consisted of a monoclonal capture antibody, a polyclonal anti-TNF, and a biotin-linked anti-polyclonal third antibody that binds a streptavidin-linked peroxidase. The substrate hydrogen peroxide was allowed to react with the peroxidase until a faint yellow color was visible in the least concentrated TNF standard well, or for a maximum time of 5 min. The reaction was stopped with 1 *M* H_2SO_4, and the plate was read at an absorbance of 490 nm on an Easy Reader 400 AT microtiter plate absorbance detector (SLT 6 Labinstruments). A standard absorbance/concentration curve at 490 nm was obtained.

ELISA Assay of Soluble TNFα Receptors

A polyclonal sandwich ELISA was utilized. Human recombinant 55- and 75-kDa TNFR were obtained from Synergen (Boulder, CO).

Immunoglobulins (IgG) against the TNFR were produced by the method of Yamamoto *et al.* [22]; 96-well flat-bottom microtiter plates were coated with rabbit IgG against 55- or 75-kDa TNFR and incubated at 4°C overnight. Plates were washed with 0.2% Tween (Bio-Rad Laboratories, Richmond, CA) in phosphate-buffered saline. Standard preparations of 55- or 75-kDa TNFR were diluted in phosphate-buffered saline with 1% bovine serum albumin. TNFR standards and human serum samples were added to the plates. Plates were again incubated at 4°C overnight and then washed with Tween. Horseradish peroxidase-bound rabbit anti-TNFR IgG was added, followed by incubation for 1 hr at 37°C and washing with Tween. Substrate containing one 2,2'-azinobisdiammonium salt tablet (Pierce, Rockford, IL) and 3 μl of 30% H_2O_2 in 10 ml of 1 *M* acetate buffer (pH 4.2) was added to the plate. The final incubation was at room temperature for 20 min. Absorbance at 405 nm was read with a microplate ELISA reader. A standard absorbance/concentration curve was generated and concentrations of TNFR in samples were calculated from the curve.

Statistical Analysis

Each experiment was performed in duplicate with a sample size of eight in each group. Data were analyzed using a one-way analysis of variance and the Student–Newman–Keuls test for multiple comparisons.

RESULTS

TNFα and TNFα Receptor Release by Human THP-1 Cells Exposed to Capillary Levels of Oxygen in Vitro

Samples of the media collected from cultures placed in anaerobic jars and exposed to partial pressures of 68 mm

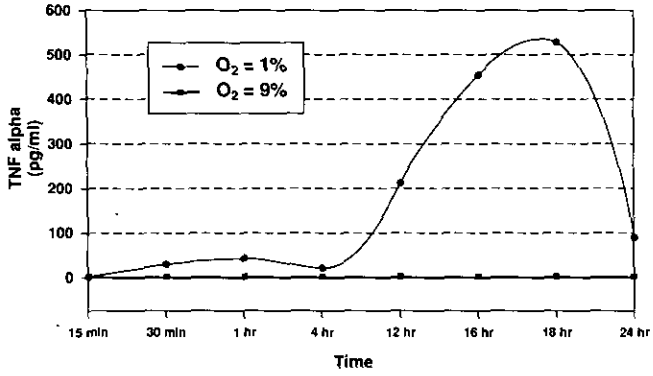

FIG. 1. Levels of TNFα were minimal at all times in supernatants of human THP-1 leukemia cells maintained at an ambient oxygen concentration of 9% (—). At 1% O_2 levels peaked at 1 hr (52 ± 5 pg/ml) and 18 hr (526 ± 48 pg/ml) (—).

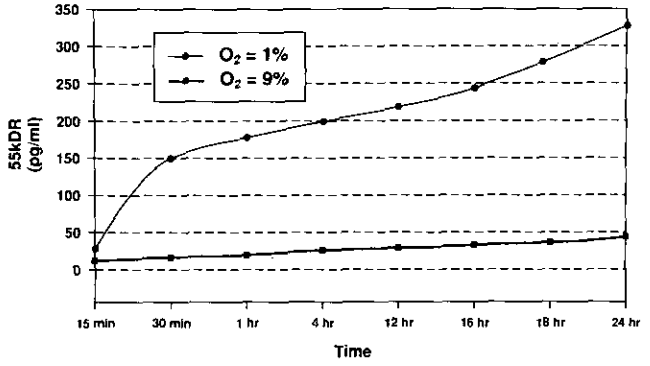

FIG. 3. Levels of the 55-kDa soluble receptor for TNFα were minimal over time at 9% O_2 (34.6 ± 2.03 pg/ml) (—). Peak levels at 24 hr were lower than those of the 75-kDa R (236 ± 15 pg/ml) (—) at an oxygen concentration of 1%.

Hg or 9% oxygen for the various time periods were tested for TNFα and TNFαR by ELISA as described under Materials and Methods. Constant levels of TNFα (12.0 ± 4.2 pg/ml) were found at all time periods in supernatants of cultures grown at 9% oxygen (Fig. 1). From 15 min to 24 hr, mean concentration of 55-kDa soluble receptor for TNFα was 34.6 ± 2.03 pg/ml; mean concentration of 75-kDa receptor was 38.88 ± 9.68 pg/ml (Figs. 2 and 3). No statistically significant increase of either TNFα or of its soluble receptors was seen over the 24 hr examined in this study. Media from cell cultures grown at 12% oxygen were also tested for TNFα and TNFαR by ELISA, with similar results (values not reported). Cell viability averaged 95% at the conclusion of each experiment, and pH of supernatants remained at values between 7.40 and 7.58.

TNFα and TNFα Receptor Release by Human THP-1 Cells Exposed to Hypoxia

Samples of the media collected from cultures placed in anaerobic jars and exposed to partial pressures of 8 mm Hg or 1% oxygen for the various time periods were tested for TNFα and TNFαR by ELISA as described under Materials and Methods.

Elevated levels of TNFα above control values were first detected after 30 min of exposure to a pO_2 of 8 mm Hg (41.3 ± 2.3 pg/ml); a second peak of TNFα release was documented at 18 hr (526 ± 48 pg/ml); at 24 hr levels began to decline (102 ± 10.2 pg/ml) (Fig. 1).

Concentrations of both 55- and 75-kDa soluble TNFα receptors began rising within 30 min to 1 hr of exposure to hypoxia, and rose continuously throughout the 24 hr period of this study to peak values of 236 ± 15 pg/ml and 2,450 ± 63 pg/ml, respectively (Figs. 2 and 3). Cell viability was tested by vital dye uptake at the beginning and conclusion of each experiment and again averaged 95%; pH of supernatants remained at values between 7.40 and 7.82.

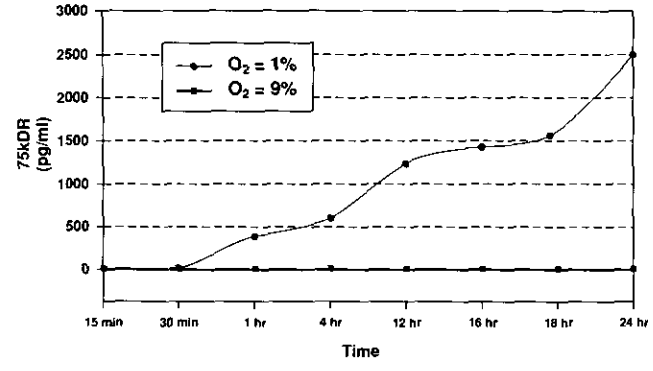

FIG. 2. Levels of 75-kDa soluble receptor for TNFα were minimal over time in supernatants of THP-1 maintained at an ambient oxygen concentration of 9% (38.88 ± 29.68 pg/ml) (—). At 1% O_2 levels steadily increased for the duration of the experimental (24 hr) to 2450 ± 63 pg/ml (—).

DISCUSSION

During shock, hypoperfusion leads to tissue hypoxia [1]. Cellular damage under these conditions is perpetuated by a complex series of events which occur secondarily, including vasoconstriction, activation of the clotting and complement systems, microcirculatory thrombosis, and increased leukocyte aggregation to capillary endothelium [23].

In septic shock, clinical and basic studies have documented an association between activated macrophages, elevated levels of the cytokines TNFα, IL-1, IL-6, and the presence of infection [7, 8, 24]. TNFα appears to be the first cytokine to be released into the blood stream, subsequently inducing a cascade of cytokine release: IL-1 and then IL-6. TNFα is an important mediator of septic shock. This is demonstrated by the finding that shock can be reversed both in animals and in humans by administration of monoclonal antibodies directed against TNFα [10, 25, 26] and in animals by infusion of soluble TNFαR [16, 17]. When 20 μg of TNFαR-IgG (55 kDa)

was administered to mice prior to an LD_{100} dose of endotoxin, a survival of 100% resulted. TNFαR-IgG continued to have a protective effect, albeit partial, when administered up to 3 hr after injection of endotoxin [17].

While animal studies have demonstrated increased levels of TNFα in the blood stream as early as 2 hr after hemorrhagic shock [27], no study to date has found elevated systemic levels of TNFα after trauma and hemorrhage in humans. Recently, however, we detected consistently high serum levels of 55- and 75-kDa TNFα soluble receptors within 1 hr in seriously injured trauma patients [11]. The role and source of these receptors is presently under investigation. It is known that monocytes express primarily 75-kDa receptors [28], and when exposed to recombinant TNFα, release of TNFαR occurs, both *in vitro* and *in vivo* [15, 29].

The purpose of this study was to determine the effect of hypoxia on TNFα and TNFαR release *in vitro*. Exposure of THP-1 cells to nonlethal levels of hypoxia caused rapid *in vitro* release of TNFα and TNFαR. The release of TNFα was biphasic. TNFαR were released at a constant rate which increased with the time of exposure. The 75-kDa receptor was released in the supernatant in larger quantities than the 55-kDa receptor. This may be due to the fact that the 75-kDa receptor is the predominant TNFα receptor in the cell. This study suggests that TNFα can be released locally by hypoxic monocytes. The significance of receptor release is speculative at the present time. It is possible that release may represent an attempt to counteract TNFα activity. Hypoxia may be a primary stimulator of macrophages in shock and other ischemic states, resulting in local release of TNFα and its soluble receptors.

REFERENCES

1. Schumaker, P. T., and Samael, R. W. Oxygen delivery and uptake by peripheral tissues: Physiology and pathophysiology. *Crit. Care Clin.* **5:** 255, 1989.
2. Chaudry, I. H., Ayala, A., Ertel, W., and Stephan, R. N. Hemorrhage and resuscitation: Immunological aspects. *J. Physiol.* **259**(4, Pt. 2), R663, 1990.
3. Schlag, G., Redl, H., and Hollstrom, S. The cell in shock: The origin of multiple organ failure. *Resuscitation* **21:** 137, 1991.
4. Santilli, S. M., Fiegel, V. D., Aldridge, D. E., and Knighton, D. R. Rabbit aortic endothelial cell hypoxia induces secretion of transforming growth factor beta and augments macrophage adhesion *in vitro*. *Ann. Vasc. Surg.* **5:** 429, 1991.
5. Abraham, E. Physiologic stress and cellular ischemia: Relationship to immunosuppression and susceptibility to sepsis. *Crit. Care Med.* **19:** 613, 1991.
6. Baigrie, R. J., Lamont, P. M., Dallman, M., and Morris, P. J. The release of interleukin-1 beta (IL-1) precedes that of interleukin 6 (IL-6) in patients undergoing major surgery. *Lymphokine Cytokine Res.* **10:** 253, 1991.
7. Hesse, D. G., Tracey, K. Y., Fong, Y., Manogue, K. R., Palladino, M. A., Jr., Cerami, A., Shires, T., and Lowry, S. F. Cytokine appearance in human endotoxemia and primate bacteremia. *Surg. Gyn. Obstet.* **166:** 147, 1988.
8. Fong, Y., Moldawer, L. L., Marano, M., Wei, H., Tatter, S. B., Clarick, R. H., Santhanan, V., Sherris, D., May, L. T., and Sehal, P. B. Endotoxemia elicits increased circulating beta 2-IFN/IL-6 in man. *J. Immunol.* **142:** 2321, 1989.
9. Ohlsson, K., Bjork, P., Bergenfeldt, M., Hageman, R., and Thompson, R. C. Interleukin-1 receptor antagonists reduces mortality from endotoxin shock. *Nature* **348**(6301): 550, 1990.
10. Exley, A. R., Cohen, J., Buurman, W., Owen, R., Hanson, G., Lumley, J., Aulakh, J. M., Bodmer, M., Riddell, A., Stephens, S., and Perry, M. Monoclonal antibody to TNF in severe septic shock. *Lancet* **335**(8700): 1275, 1990.
11. Tan, L. R., Waxman, K., Scannell, G., Ioli, G., and Granger, G. A. Trauma causes early release of soluble receptors for Tumor Necrosis Factor. *J. Trauma,* in press.
12. Olsson, I., Lantz, M., Nillson, E., Peetre, C., Thysell, H., Grubb, A., and Adolf, G. Isolation and characterization of a tumor necrosis factor binding protein from urine. *Eur. J. Haematol.* **42:** 270, 1989.
13. Cappuccini, F., Yamamoto, R. S., Disaia, P. J., Grosen, E. A., Gatanaga, M., Lucci, J. A., Ininnis, E. K., Gatanaga, T., and Granger, G. A. Identification of tumor necrosis factor and lymphotoxin blocking factor(s) in the ascites of patients with advanced and recurrent ovarian cancer. *Lymphokine Cytokine Res.* **10**(3): 225, 1991.
14. Gatanaga, T., Hwang, C., Kohr, W., Cappuccini, F., Lucci, J. A., Jeffes, E. W. B., Lentz, R., Tomich, J., and Yamamoto, R. S. Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultra-filtrates of human cancer patients. *Proc. Natl. Acad. Sci. USA* **87:** 8781, 1990.
15. Lantz, M., Gullberg, U., Nillson, E., and Olsson, I. Characterization *in vitro* of a human tumor necrosis factor-binding protein: A soluble form of a tumor necrosis factor receptor. *J. Clin. Invest.* **86:** 1396, 1990.
16. Ashkenazi, S., Marsters, S. A., Carpon, D. J., Chamow, S. M., Figari, I. S., Pennica, D., Goeddel, D. V., Palladino, M. A., and Smith, D. H. Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesion. *Proc. Natl. Acad. Sci. USA* **88**(23): 10535, 1991.
17. Lesslauer, W., Tabuchi, H., Gentz, R., Brockhaus, M., Schlaeger, E. J., Grau, G., Piquet, P. F., Pointaire, P., Vassalli, P., and Loetscher, H. Recombinant soluble tumor necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality. *Eur. J. Immunol.* **21**(11): 2883, 1991.
18. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* **26:** 171, 1980.
19. Chantry, D., Turner, M., Brennan, F., Kingsbury, A., and Feldmann, M. Granulocyte-macrophage colony stimulating factor induces both HLA-DR expression and cytokine production by human monocytes. *Cytokine* **2**(1): 60, 1990.
20. Armstrong, C. A., Klostergaard, J., and Granger, G. A. Isolation and initial characterization of tumoricidal monokine(s) from the human monocytic leukemia cell line THP-1. *J.N.C.I.* **74:** 1, 1985.
21. Koch, C. J. A thin-film culturing technique allowing rapid gas-liquid equilibration (6 sec) with no toxicity to mammalian cells. *Radiat. Res.* **97:** 434, 1984.
22. Yamamoto, R. S., Hiserodt, J. C., Lewis, J. E., Carmack, C., and Granger, G. A. The human LT system: II. Immunological relationships of LT molecules released by mitogen activated human lymphocytes *in vitro*. *Cell. Immunol.* **38:** 403, 1978.
23. Solomkin, J. S., Cotta, L. A., Satoh, P. S., Hurst, J. M., and

Nelson, R. D. Complement activation and clearance in acute illness and injury: Evidence for C5a as a cell-directed mediator of the adult respiratory distress syndrome in man. *Surgery* **97:** 668, 1985.

24. Fong, Y., Moldawer, L. L., Shires, G. T., and Lowry, S. F. The biologic characteristics of cytokines and their implication in surgical injury. *SG&O* **170:** 363, 1990.
25. Lucas, R., Heirwegh, K., Neirinck, A., Remels, L., Van Heuverswyn, H., and De Baetselier, P. Generation and characterization of a neutralizing rat anti-rmTNF-alpha monoclonal antibody. *Immunology* **71:** 218, 1990.
26. Fong, Y., Tracey, K. J., Moldawer, L. L., Hesse, D. G., Managua, K. B., Kenney, J. S., Lee, A. T., Kuo, G. C., Allison, A. C., and Lowry, S. F. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin 6 appearance during lethal bacteremia. *J. Exp. Med.* **170:** 1627, 1989.
27. Ayala, A., Perrin, M. M., Meldrum, D. R., Ertel, W., and Chaudry, I. H. Hemorrhage induces an increase in serum TNF which is not associated with elevated levels of endotoxin. *Cytokine* **2:** 170, 1990.
28. Gatanaga, T., Hwang, C., Gatanaga, M., Cappuccini, F., Yamamoto, R. S., and Granger, G. A. The regulation of TNF receptor mRNA synthesis, membrane expression, and release by PMA- and LPS-stimulated human monocytic THP-1 cells *in vitro*. *Cell. Immunol.* **138**(1): 1, 1991.
29. Landz, M., Malik, S., Slevin, M. L., and Olsson, I. Infusion of tumor necrosis factor (TNF) causes an increase in circulating TNF-binding protein in humans. *Cytokine* **2**(6): 402, 1990.