JOURNAL OF SURGICAL RESEARCH 49, 350–353 (1990)

Lactic Acidosis Increases Tumor Necrosis Factor Secretion and Transcription *in Vitro*[1]

J. CHRISTIAN JENSEN, M.D., CAROLYN BURESH, B.S., AND JEFFREY A. NORTON, M.D.

Surgical Metabolism Section, Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Submitted for publication November 20, 1990

Lactic acid has been shown to affect numerous biologic processes. We investigated the role of lactic acidosis as a signal for the production of TNF by macrophages *in vitro*. Male F344 rats were administered thioglycolate media intraperitoneally. Macrophages were recovered 7 days later, cultured for 24 hr in complete media (CM), or CM with L-lactic acid (5, 10, or 15 m*M*), or with endotoxin (LPS) (10 μg/ml). TNF levels were measured in the supernatants. Female C57BL/6 mice were similarly treated, and macrophages were harvested and cultured in CM or CM containing lactic acid (15 m*M*), or LPS (10 μg/ml). RNA was extracted after 24 hr, separated by electrophoresis, and transferred to nitrocellulose. Human ^{32}P-cDNA TNF and actin probes were used to determine relative TNF gene expression. Gel densitometry was used to calculate the TNF expression index (EI) in lactic acid and LPS treated cells as described.

pH levels of the supernatant indicated that increasing concentrations of lactic acid caused increasing acidosis. Trypan blue exclusion demonstrated that lactic acidosis did not reduce cell viability. LPS significantly increased secretion of TNF relative to control ($P < 0.001$). Each concentration of lactic acid significantly increased TNF secretion ($P < 0.05$), but not in a dose-dependent manner. TNF gene transcription was elevated in macrophages cultured with lactic acid and LPS relative to control (EI = 1.13 and 1.18, respectively). This suggests that lactic acid concentration can regulate TNF secretion at the level of transcription, and is consistent with the hypothesis that local levels of lactic acid (lactic acidosis) may be a regulator of cytokine secretion.

INTRODUCTION

Lactic acidosis is common in the local environment of injured, infected, and neoplastic tissue. High local levels of lactate have been implicated in initiating the synthesis of collagen by fibroblasts [1], enzymes important in wound healing [2], and the secretion of factors important for new capillary growth [3]. Lactic acid may be an immune activator *in vitro*, as studies have demonstrated that lactate can cause macrophage activation from a resting state [4].

The macrophage is intimately involved in the host response to infection, injury [5, 6], vascular proliferation [7], and cancer [8, 9]. Furthermore, macrophages secrete cytokines, molecules that appear to have important paracrine effects in the body's system to repair injured tissue and control infections or neoplastic invasion.

Tumor necrosis factor (TNF) is one such protein. TNF is markedly elevated in endotoxemia and sepsis, and recent evidence suggests that circulating levels of TNF [10] increase in animals with increasing tumor burden. Furthermore, TNF is a known angiogenic factor [11]. This investigation examines the role that lactic acidosis has on the production and secretion of TNF by macrophages.

METHODS AND MATERIALS

Ten male F344 rats, of 150 to 200 g, were injected with 10 ml thioglycolate media (Difco) intraperitoneally (ip). Peritoneal exudative cells were obtained 7 days later by lavage with calcium- and magnesium-free Hanks' balanced salt solution (HBSS). The cells were washed and resuspended in HBSS. Morphologic analysis revealed the cells to be predominately macrophages. The cells were then aliquoted to 24 well plates at 10^6 cells per well, and cultured for 2 hr at 37.5°C, and 5% CO_2. The HBSS was removed, and the monolayer was washed extensively with HBSS. The adherent cells were cultured at the same conditions in 1 ml of complete media alone (NO TX), or complete media containing L-lactic acid (Sigma) at 5, 10, or 15 m*M*, or endotoxin (LPS) (Sigma) at 10 μg/ml. The pH of each condition was measured by a pH meter and recorded. The supernatant was recovered after 24 hr and frozen at −70°C for later analysis. Cell viability was assessed at 24 hr in each of the experimental groups by trypan blue exclusion.

TNF levels were determined by the L929 assay [12].

[1] Presented at the Annual Meeting of the Association for Academic Surgery, Louisville, KY, November 15–18, 1989.

0022-4804/90 $1.50

Results were recorded in units per milliliter. Protein A-purified New Zealand White rabbit antibody raised to recombinant murine TNF (rmuTNF; Genentech) was used to inhibit activity seen in the L929 assay, and served as a specificity control.

Data are expressed as means ± SEM and differences from the no-treatment control group were evaluated for statistical significance by two-tailed, independent Student's *t* test.

Twenty female C57BL/6 mice were injected with thioglycolate media, 3 ml each ip, and 72 hr later, peritoneal exudative cells were recovered as above. The cells were aliquoted to sterile petri dishes (Costar) and incubated at 37°C and 5% CO_2, and the nonadherent cells were removed 2 hr later by extensive washing with sterile HBSS. The adherent cell monolayer was incubated at the same conditions as above with complete media alone or complete media containing 15 m*M* L-lactic acid or LPS (10 μg/ml). Twenty-four hours later, the adherent cells were recovered with a sterile rubber policeman, lysed in 4 *M* guanidine isothiocyanate, and centrifuged through a 5.7 *M* cesium chloride gradient. The RNA pellet was suspended in autoclaved deionized distilled water. Twenty-five micrograms of each sample was separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane using standard techniques [13]. Human cDNA, TNF-α ^{32}P-labeled probe was then used to ascertain the level of TNF-α gene transcript in each sample. The blot was stripped by standard methods and rehybridized with a similarly labeled probe for actin. Following 16–24 hr of exposure to Kodak X-Omat AR film (Eastman Kodak), with screen intensifier (DePont), gel densitometry (Beckman) was performed to quantitate image density. To correct for RNA loading differences, a tumor necrosis factor gene expression index (EI) was calculated according to the following formula:

$$EI = (\text{TNF experimental density/TNF control density})/(\text{actin experimental density/actin control density}).$$

RESULTS

pH Levels and Cell Viability

The pH of complete media alone was 7.43. When lactic acid at concentrations of 5, 10, and 15 m*M* was added to complete media, pH levels dropped to 6.75, 6.34, 5.91, respectively. Cell viability measured at the end of 24 hr by trypan blue exclusion remained greater than 90% in all experimental groups.

Secretion of TNF

When PEC were incubated with 10 μg/ml of LPS, supernatant levels of TNF activity were markedly increased compared to levels seen with complete media alone ($P < 0.001$) (Fig. 1). When PEC were incubated in three different concentrations of L-lactic acid, there was a significant increase in TNF secretion detected at each concentration. Supernatant levels of TNF activity increased from a mean level of 151 units/ml in the no-treatment group to a mean level of 256, 291, and 258 units/ml in the 5, 10, and 15 m*M* lactic acid treatment groups, respectively. Each of these differences was significant ($P < 0.05$ vs NO TX control). Lactic acid itself did not demonstrate inherent activity in the L929 assay and anti-rmuTNF antibody completely abrogated activity observed in each treatment group.

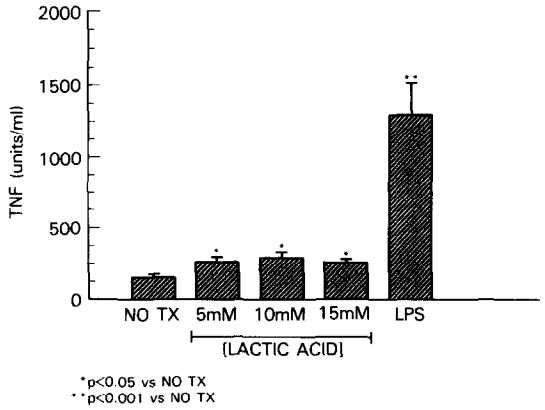

FIG. 1. Effect of lactic acid concentration on TNF secretion by peritoneal exudative cells. Endotoxin (LPS) markedly increases secretion of TNF compared to media alone (NO TX) ($P < 0.001$). Each concentration of L-lactic acid (5, 10, and 15 m*M*) also increases secretion of TNF compared to complete media alone ($P < 0.05$).

TNF Gene Expression

As expected, Northern blot analysis of RNA from PEC stimulated with LPS demonstrated an increase in TNF gene expression compared to cells incubated with complete media alone (EI = 1.18; Fig. 2). PEC incubated in 15 m*M* lactic acid also demonstrated an increase in TNF gene expression compared to cells incubated in complete media alone (EI = 1.13; Fig. 2).

DISCUSSION

New data continue to accumulate implicating cytokines in important biologic processes. TNF and IL-1 share many of the same biological effects that appear to be important in host defense mechanisms against tumor and infectious organisms. These include activation of T-cells, cytotoxic and cytostatic effects on tumor cells, and cachectic and pyrogenic effects [14]. In addition, each can initiate changes important in wound repair including an increase in prostaglandin and collagenase synthesis, induction of procoagulant activity, and stimulation of neutrophil migration and adherence to endothelial cells [14]. Tumor necrosis factor appears to be essen-

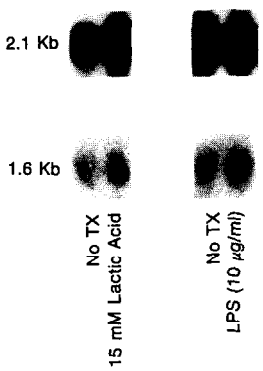

FIG. 2. Northern blot for TNF gene expression of peritoneal exudative cells incubated with 15 m*M* lactic acid, endotoxin (LPS), or conditioned media (NOTX) alone. TNF gene expression index (EI) is calculated relative to NOTX alone for 15 m*M* lactic acid and LPS (see Materials and Methods). TNF gene expression appears to increase 18% by LPS (an index of 1.0 would be the same as NOTX alone) and 13% by lactic acid.

tial in the pathogenesis of gram-negative and endotoxin shock, as evidenced by the findings that endotoxemia and bacteremia induce circulating levels of TNF [15]; the administration of recombinant human TNF results in the hemodynamic profile of septic shock [16]; and passive immunization of mice against TNF protects against the lethal effects of endotoxemia [17].

The data implicating TNF in the immune response to cancer are controversial and contradictory [18–20]. Tumor-bearing animal data have suggested circulating levels of TNF mediates cancer cachexia, because serum levels of TNF correlated with other parameters of cachexia [10]. These findings have been confused by a recent report that passive immunization against TNF did not prolong survival or improve food intake in the same cachectic tumor-bearing rat model [21]. Other studies in two different mouse models of cancer cachexia suggested that administration of specific anti-murine TNF antibodies can reverse the anorexia and host compositional changes associated with progressive tumor growth even though serum levels of TNF were not measurable [22].

Tumor necrosis factor, IL-1, and other cytokines have been difficult to detect and quantitate in the circulation. It appears from various studies that when detectable, circulating levels of these cytokines have negative effects and poor prognostic value for the host [10, 16, 19]. The dichotomy arising from the presence of highly conserved though potentially detrimental proteins may be better understood by recent work suggesting that while the systemic (endocrine) actions of these cytokines may be detrimental, the local (paracrine) functions of these cytokines can be beneficial [23]. This appears to be an important distinction. The present investigation studies whether lactic acidosis, a potential local environmental signal for tumor, infection and injury, can regulate the secretion of cytokines by macrophages.

Our data establish a link between L-lactic acid concentration and cytokine secretion by macrophages. Supernatant pH became progressively acidic as L-lactic acid concentration increased. While acidity was not controlled, studies of the effects of pH on secretion of macrophage angiogenesis factors found lactate to be most important with pH as low as 6.2 to be unimportant [3]. L-lactic acid, the biologically active form of this molecule, increases TNF secretion by macrophages at all concentrations between 5 and 15 m*M*. This influence on TNF secretion is statistically significant, and does not appear to be a dose–response phenomenon. In addition, trypan blue exclusion demonstrated similar cell viability in all groups despite high lactate concentrations and acidosis. These findings imply that the changes measured in TNF secretion are real and specific to lactic acidosis and not a result of an artificial *in vitro* condition. Moreover, the anti-TNF-α antibody control completely neutralized all TNF activity in the L929 assay supporting the validity of these measurements. Northern blot analysis suggests that lactic acid exerts its effect on TNF secretion by enhancing gene transcription (Fig. 2). This finding further supports the notion that lactic acid concentration can regulate cytokine synthesis by macrophages.

Lactic acid and endotoxin have vastly different effects on cytokine production, and the effects may be exerted by separate pathways. This is consistent with the observation that lactic acidosis may be a distinct mechanism for macrophage activation [4].

These data are consistent with reports suggesting a possible role for locally produced TNF as an important factor in initiating angiogenesis and other steps in wound repair. Anaerobic metabolism, with an elevated level of lactate and lactic acidosis in the environment of tumors, may also induce macrophages to synthesize and secrete TNF, a peptide that is capable of tumor cell destruction. Lactic acid appears to be a local signal to induce transcription and secretion of active TNF. Finally, alterations of lactic acid concentration may participate more generally in the host response to inflammation and cancer by the local perturbation of cytokine homeostasis.

REFERENCES

1. COMSTOCK, J. P., AND UDENFRIEND, S. Effect of lactate on collagen proline hydroxylase activity in cultured L929 fibroblasts. *Proc. Natl. Acad. Sci. USA* **66:** 552, 1970.
2. HUNT, T. K., CONOLLY, W. B., ARONSON, S. B., AND GOLDSTEIN, P. Anaerobic metabolism and wound healing: An hypothesis for the initiation and cessation of collagen synthesis. *Amer. J. Surg.* **135:** 329, 1978.
3. JENSEN, J. A., HUNT, T. K., SCHEUENSTUHL, H., AND BANDA, M. J. Effect of lactate, pyruvate and pH on secretion of angiogenesis and mitogenesis factors by macrophages. *Lab. Invest.* **43:** 574, 1986.

4. PATY, P. B., BANDA, M. J., AND HUNT, T. K. Activation of macrophages by l-lactic acid. *Surg. Forum* **39:** 27, 1988.
5. THAKRAL, K. K., GOODSON, W. H. J., AND HUNT, T. K. Stimulation of wound blood vessel growth by wound macrophages. *J. Surg. Res.* **26:** 430, 1979.
6. LEIBOVICH, S. J., AND ROSS, R. The role of macrophage in wound repair. *Amer. J. of Pathol.* **78:** 73, 1975.
7. POLVERINI, P. J., COTRAN, R. S., GIMBRONE, M. A., AND UNANUE, E. R. Activated macrophages induce vascular proliferation. *Nature (London)* **269:** 804, 1977.
8. ALEXANDER, P., ECCLES, S. A., AND GAUCI, C. L. L. The significance of macrophages in human and experimental tumors. *Ann. N.Y. Acad. Sci.* **276:** 124, 1976.
9. TALMADE, J. E., KEY, M., AND FIDLER, I. J. Macrophage content of metastatic and nonmetastatic rodent tumors. *J. Immunol.* **126:** 2245, 1981.
10. STOVROFF, M. C., *et al.* Cachectin activity in the serum of cachectic, tumor bearing rats. *Arch. Surg.* **124:** 94, 1989.
11. LEIBOVICH, S. J., POLERINI, P. J., SHEPARD, H. M., WISEMAN, D. M., SHIVELY, V., AND NUSEIR, N. Macrophage induced angiogenesis is mediated by tumor necrosis factor-α. *Nature (London)* **329:** 630, 1987.
12. FRAKER, D. L., STOVROFF, M. C., MERINO, M. J., AND NORTON, J. A. Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. *J. Exp. Med.* **168:** 95, 1988.
13. DAVIS, L. G., DIBNER, M. D., AND BATTEY, J. F. *Basic Methods in Molecular Biology.* New York: Elsevier Press, 1986.
14. LE, J., AND VILCEK, J. Biology of Disease: Tumor Necrosis Factor and Interleukin-1: Cytokines with multiple overlapping biological activities. *Lab. Invest.* **56:** 234, 1987.
15. MICHIE, H. R., KIRK, M. B., MANOGUE, K. R., SPRIGGS, D. R., REVHAUG, A., O'DWYER, S., DINARELLO, C. A., CERAMI, A., WOLFF, S. M., AND WILMORE, D. W. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* **318:** 1481, 1988.
16. TRACEY, K. J., BEUTLER, B., LOWRY, S. F., MERRYWEATHER, J., WOLPE, S., STEPHEN, W., MILSARK, I. W., HARIRI, R. J., FAHEY, T. J., ZENTELLA, A., ALBERT, J. D., SHIRES, G. T., AND CERAMI, A. Shock and tissue injury induced by recombinant human cachectin. *Science* **234:** 470, 1986.
17. BEUTLER, B., MILSARK, I. W., AND CERAMI, A. C. Passive immunization against Cachectin/Tumor Necrosis Factor protects mice from lethal effect of endotoxin. *Science* **229:** 869, 1985.
18. ADERKA, D., FISHER, S., LEVO, Y., HOLTMANN, H., HAHN, T., AND WALLACH, D. Cachectin/tumor necrosis factor production by cancer patients. *Lancet* November 23, 1985.
19. BALKWILL, F., BURKE, F., TALBOT, D., RAVERNEIR, J., OSBORNER, R., NAYLOR, S., DURBIN, H., AND FIERS, W. Evidence for tumor necrosis factor/cachectin production in cancer. *Lancet* November 28, 1987.
20. WAAGE, A., ESPEVIK, T., AND LAMVIK, J. Detection of tumor necrosis factor-like cytotoxicity in serum from patients with septicemia but not from untreated cancer patients. *Scand. J. Immunol.* **24:** 739, 1986.
21. LANGSTEIN, H. M., FRAKER, D. L., AND NORTON, J. A. Reversal of cancer cachexia by antibodies to interferon-γ but not cachectin/tumor necrosis factor. *Surg. Forum* **40:** 408, 1989.
22. SHERRY, B. A., GELIN, J., FONG, Y., MARANO, M., WEI, M., CERAMI, A., LOWRY, S. F., LUNDHOLM, K. G., AND MOLDAWER, L. L. Anticachectin/tumor necrosis factor-antibodies attenuate development of cachexia in tumor models. *FASEB J.* **3:** 1956, 1989.
23. SHERRY, B., AND CERAMI, A. Cachectic/tumor necrosis factor exerts endocrine, paracrine, and autocrine control of inflammatory responses. *J. Cell Biol.* **107:** 1269, 1988.