Brief Definitive Report

SPONTANEOUS PRODUCTION OF TUMOR NECROSIS FACTOR α BY KUPFFER CELLS OF MRL/lpr MICE

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Tumor necrosis factor α (TNF-α), a secretory product of activated macrophages (1), has been shown to have multiple inflammatory and immunoregulatory properties, including the induction of release of IL-1 by monocytes and endothelial cells (2–3), alteration of antigenic expression on (4) and direct toxicity to (5) vascular endothelium, activation of NK cells (6), and stimulation of T cell proliferation and production of IFN-γ (7). In the MRL/lpr mouse strain, a murine model of autoimmunity and vasculitis (reviewed in reference 8), endogenous activation of macrophages (9–10) and hepatic NK cells (11) has been described. We report here that freshly isolated, unstimulated Kupffer cells (KC) from MRL/lpr female mice spontaneously produce high levels of TNF-α in short-term culture.

Materials and Methods

Mice. C3H/HeN, C3H/HeJ, BALB/c DBA/2J, (NZB × NZW)F_1, C57BL/6, MRL/MpJ-*lpr/lpr* (MRL-lpr), and MRL/MpJ-+/+ (MRL-+/+) female mice 3–40 wk old were obtained from colonies from The Jackson Laboratories, Bar Harbor, ME. The mice were housed in barrier facilities with sterile caging, bedding, water, and food. They were screened routinely for pathogens.

Reagents and Cells. Collagenase IV, Metrizamide, and LPS were purchased from Sigma Chemical Co., St. Louis, MO. Mouse IFN-γ was purchased from Lee Biomolecular Research Laboratories, Inc., San Diego, CA. MTT (3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyl tetrazolium bromide) was purchased from Molecular Devices, Palo Alto, CA. *Corynebacterium parvum* was purchased from Burroughs Wellcome Co., Research Triangle Park, NC. Rabbit antisera to recombinant mouse TNF-α was kindly provided by Genentech, Inc., South San Francisco, CA.

Kupffer Cell Isolation. Under sterile conditions, hepatic nonparenchymal cells (NPC) were purified as described previously (11) by collagenase perfusion of the portal vein and Metrizamide density centrifugation. Total recovery ranged from 0.2 to 1.2 × 10^7 NPC per liver. Contamination by hepatocytes, as determined by counting 200 cells from the preparation, was <1%. After washing twice, the NPC were resuspended at a concentration of 10^6 cells/ml in complete media containing RPMI 1640 plus 5% FCS, penicillin, and streptomycin, and were adhered to flat-bottomed 24-well microtiter plates (Costar, Cambridge, MA) for 90 min at 37°C (5% CO_2, 100% humidity). The supernatant was removed by vigorous pipetting. 35–40% of NPC were adherent and >90% of the adherent cells were nonspecific esterase-positive in all mouse strains. 1 ml of fresh complete media was then added to each well. KC were cultured for 24 h in complete media. The supernatants were then harvested, centrifuged at 400 *g* to remove cells, and stored at 4°C before being assayed for TNF.

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In selected wells, 0.01–1.0 μg of LPS (in 10 μl) was added after adherence. After 2 h the supernatants were removed by pipetting; the adherent cells were washed twice and cultured for 24 h in complete media. The supernatants were then harvested as above.

Assay for Endotoxin. Media and perfusates were assayed for the presence of endotoxin by the Limulus amebocyte lysate agglutination (No. 210-A; Sigma Chemical Co.) (12).

Assay for Tumor Necrosis Factor. The UV-induced 1591-RE tumor cell line has been shown to be highly sensitive to the cytotoxic activity of TNF-α and TNF-β and resistant to IL-1 in vitro (13). A clone of this cell line, 1591-RE3.5, was used in these experiments. A standard MTT assay described by Mosmann (14) was used to quantify TNF in cultured supernates. Briefly, supernatants from KC in 100 μl RPMI 1640 plus 5% FCS, penicillin, and streptomycin were serially diluted in duplicate in 96-well microtiter trays (Dynatech Laboratories, Inc., Alexandria, VA) and mixed with 5×10^3 1591 RE3.5 tumor cells in 100 μl of medium. After a 72-h incubation at 37°C in a 5% CO_2 humidified incubator, 100 μl of the culture media was removed and 20 μl of MTT (5 mg/ml in PBS) were added per well and incubated an additional 4 h. The formazan crystals were dissolved by overnight incubation with 100 μl of a 10% SDS–0.01 N HCl solution per well. Lysis was quantified on a microelisa reader (model MR1000; Dynatech Laboratories, Inc.) using a 690-nm reference and a 570-nm test filter. Optical density values were converted to percent cytotoxicity by comparing LD_{50} values from untreated controls using the following formula: Percent cytotoxicity = 100 × [1−(OD test/OD control)]. The LD_{50} of 1591-RE3.5 cell line is 0.158 ± 0.01 U/ml using a recombinant human TNF-α (sp act 5×10^7 U/mg; Genentech, Inc.) as a reference source of TNF. Picomolar values were based upon the average molecular weight of TNF-α (17,000 mol wt).

Results and Discussion

Using a cytotoxicity assay against a TNF-α- and TNF-β–sensitive, IL-1 resistant cell line, we have found that freshly isolated, unstimulated KC from MRL/lpr female mice produce high levels of TNF in 24-h culture. TNF production was detected in KC cultures from MRL/lpr mice > 6 wk old and increased with age of the mice (Fig. 1). Moreover, TNF production was also observed in splenic macrophages from MRL/lpr mice >12 wk (data not shown). Pretreatment of the KC culture supernatants with an antiserum made against recombinant TNF eliminated cytotoxicity to the 1591 RE 3.5 cell line, suggesting that the adherent hepatic nonparenchymal cells are producing TNF-α and not lymphotoxin (Table I). Although *C.parvum* in vivo (1.4 mg i.v. injection) could induce TNF production in KC in the nonautoimmune C3H/HeN, BALB/c, DBA/2, and C57B16 strains, the only other strain in which spontaneous TNF production by KC was consistently observed was MRL/++ mice >10 mo old.

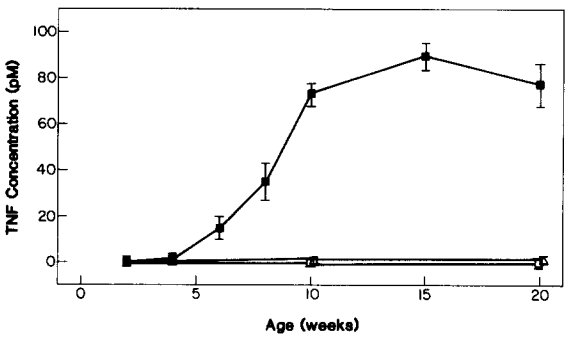

FIGURE 1. Tumor necrosis factor production by Kupffer cells. 10^6 hepatic NPC from female MRL/lpr (■), MRL/++ (□), and NZB × NZW)F_1 (Δ) mice of different ages were adhered to flat-bottomed 24-well microtiter plates. The nonadherent cells were removed by vigorous pipetting, and 1 ml of RPMI 1640 + 5% FCS was added to the well. After a 24-h incubation, the supernatant was assayed for TNF activity by cytotoxicity to 1591 RE 3.5, a TNF-sensitive, IL-1-resistant cell line. The LD_{50} is 0.158 ± 0.01 U/ml. Picomolar values are based upon the sp act of 5×10^7 U/mg and molecular weight of 17,000.

TABLE I

Cytotoxic Activity to 1591 RE 3.5 Tumor Cells of Cultured Supernatants from MRL/lpr or Old MRL-+/+ Kupffer Cells Is Neutralized by an Antibody to TNF-α

Kupffer cell culture from mouse strain	Age	Percent inhibition of cytotoxicity by anti-TNF-α antibody at a concentration (μl/well) of: 2.5	5
	wk		
MRL/lpr	6	81	90
MRL/lpr	10	90	97
MRL-+/+	40	84	89

Rabbit antiserum to recombinant murine TNF-α (4×10^5 neutralizing units/ml) was preincubated with culture supernatants for 16 h at 37°C before the addition of 1591RE-3.5 targets. After an additional 72 h the assay was harvested as described in Materials and Methods. Cultured supernatants were tested at a final dilution of 1:2 in the culture well (100 μl target cells, 100 μl culture supernatant). Without the addition of antibody, the specific cytotoxicity of KC supernatants from 6-wk-old MRL/lpr was 97%, 10-wk-old MRL/lpr was 97%, and 40-wk-old MRL-+/+ was 95%.

TNF can be induced by sublethal injection of endotoxin. However, it is unlikely that contamination of endotoxin is the cause of the spontaneous production of TNF-α by KC in the MRL/lpr strain. Buffers, media, and perfusates were routinely tested for the presence of LPS by the Limulus amebocyte lysate assay (12). Less than 0.1 ng/ml was detected. Moreover, macrophages from at least one other mouse strain were usually isolated on the same day as MRL/lpr mice under identical conditions with the same perfusate preparations (Fig. 2). The possibility of enhanced LPS sensitivity of MRL/lpr KC to produce TNF has also been excluded. Indeed, we have observed unresponsiveness to LPS in KC from older MRL/lpr mice. KC were incubated for 2 h with increasing concentrations of LPS (0.05–0.5 μg/ml). In contrast to KC from nonautoimmune strains, TNF production *decreased* after incubation with LPS in KC from MRL/lpr mice >10 wk old (Fig. 3). In support of this latter finding

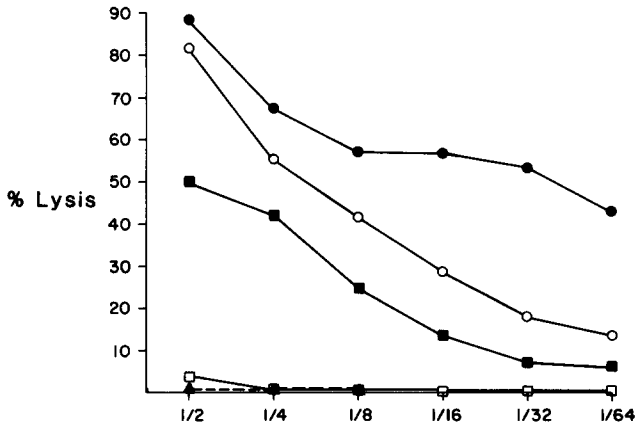

FIGURE 2. Tumor necrosis factor by Kupffer cells: cytotoxicity to 1591 RE 3.5 tumor cells. Single experiment in which 5×10^3 tumor cells were incubated for 72 h with increasing dilutions of Kupffer cell supernatants from 16-wk-old (●), 12-wk-old (○), and 10-wk-old (■) MRL/lpr; 16-wk-old C3H/HeN (▲); and 30-wk-old (NZB × NZW)F_1 (□) female mice.

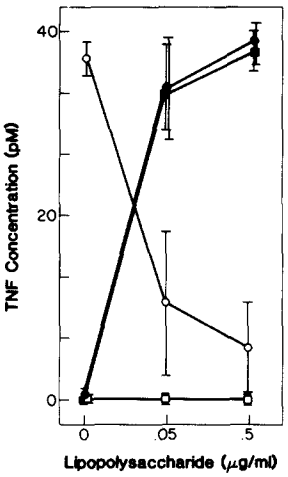

FIGURE 3. In vitro effects of LPS on the TNF production by Kupffer cells. Kupffer cells from different mouse strains were incubated with 0.05-05 μg/ml of LPS. After 2 h the wells were washed, and 1 ml of RPMI 1640 + 5% FCS was added to the wells. After a 24-h incubation, the supernatants were assayed for TNF activity. (○) 12-wk-old MRL/lpr; (●) 12-wk-old MRL/++; (■) 10-wk-old C3H/HeN; (□) 13-wk-old C3H/HeJ female mice.

is the recent report of defective induction of TNF by LPS in peritoneal exudate cells from another autoimmune mouse strain, (NZB × NZW)F_1 (15).

Spontaneous TNF production by Kupffer cells of MRL/lpr mice may only represent an epiphenomenon related to endogenous macrophage activation (9, 10, 16). However, in addition to its metabolic, antitumor and antiviral properties (reviewed in reference 17), TNF has pleiotropic immunoregulatory effects, including activation of NK cells (6) and stimulation of activated T cells to proliferate and produce IFN-γ (7). Recently, Ghiara et al. (18) have demonstrated that TNF-α can enhance the immune response in vivo at doses far lower than required for antitumor activity. Moreover, TNF has been shown to have profound in vitro effects on the vascular endothelium which could initiate a local inflammatory response. Enhancement of the expression of HLA antigens (19), inhibition of the expression of thrombomodulin (20), and stimulation of the production of IL-1 (3) and procoagulant activity (21) have been reported. TNF also stimulates neutrophils both to adhere to endothelium (22) and, when adherent, to release hydrogen peroxide (23). Therefore, endogenous production of TNF in the liver may have a role in the pathogenesis of both the multiple immunoregulatory abnormalities (8) and the necrotizing vasculitis of medium-sized arteries (25) in the MRL/lpr mouse.

Summary

We report that freshly isolated, unstimulated Kupffer cells (KC) from MRL/lpr female mice in short-term culture spontaneously produce high levels of TNF-α. TNF production was first detected in KC cultures at age 6 wk and increased with the age of the mice. Moreover, the levels of spontaneous TNF production by KC directly correlated with the age of the MRL/lpr mice. Although TNF production by KC could be induced with *C. parvum* in vivo or LPS in vitro in all nonautoimmune C3H/HeN, BALB/c, DBA/2, C57B16 mice, the only other strain in which spontaneous TNF production by KC was observed was MRL/++ mice >10 mo old.

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References

1. Mannel, D. N., R. N. Moore, and S. E. Mergenhagen. 1980. Macrophages as a source of tumoricidal activity (tumor-necrotizing factor). *Infect. Immun.* 30:523.
2. Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, and J. V. O'Connor. 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med.* 163:1433.
3. Nawroth, P. P., I. Bank, D. Handley, J. Cassimeris, L. Chess, and D. Stern. 1986. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J. Exp. Med.* 163:1363.
4. Collins, T., L. A. Lapierre, W. Fiers, J. L. Strominger, and J. S. Prober. 1986. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A, B antigens in vascular endothelial cells and dermal fibroblasts in vitro. *Proc. Natl. Acad. Sci. USA.* 83:446.
5. Sato, N., T. Goto, and K. Haranaki. 1986. Actions of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition, and cytotoxicity. *J. Natl. Cancer Inst.* 76:1113.
6. Ostensen, M. E., D. L. Thiele, and P. E. Lipsky. 1987. Tumor necrosis factor enhances cytolytic activity of human natural killer cells. *J. Immunol.* 138:4185.
7. Scheurich, P., B. Thoma, U. Ucer, and K. Pfizenmaier. 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α induction of TNF receptors on human T cells and TNF-mediated enhancement of T cell responses. *J. Immunol.* 138:1786.
8. Theofilopoulos, A. N., and F. J. Dixon. 1985. Murine models of systemic lupus erythematosus. *Adv. Immunol.* 37:269.
9. Kelley, V. E., and J. B. Roths. 1982. Increase in macrophage Ia expression in autoimmune mice: role of the lpr gene. *J. Immunol.* 129:923.
10. Magilavy, D. B., T. R. Hundley, A. D. Steinberg, and I. M. Katona. 1987. Hepatic reticuloendothelial system activation in autoimmune mice. *Clin. Immunol. Immunopathol.* 42:386.
11. Magilavy, D. B., A. D. Steinberg, and S. L. Latta. 1987. High hepatic natural killer cell activity in murine lupus. *J. Exp. Med.* 166:271.
12. Levin, J., P. A. Tomasulo, and R. S. Oser. 1970. Detection of endotoxin in human blood and demonstration of an inhibitor. *J. Lab. Clin. Med.* 75:903.
13. Urban, J. L., H. M. Shephard, J. L. Rothstein, B. J. Sugarman, and H. Schreiber. 1986. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc. Natl. Acad. Sci. USA.* 83:5233.
14. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55.
15. Jacob, C. O., and H. O. McDevitt. 1988. Tumour necrosis factor-α in murine autoimmune lupus nephritis. *Nature (Lond.).* 331:356.
16. Dang-Vu, A. P., D. S. Pisetsky, and J. B. Weinberg. 1987. Functional alterations of macrophages in autoimmune MRL-lpr/lpr mice. *J. Immunol.* 138:1757.
17. Beutler, B., and A. Cerami. 1987. Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med.* 316:379.
18. Ghiara, P., D. Boraschi, L. Nencioni, P. Ghezzi, and A. Tagliabue. 1987. Enhancement

of in vivo immune response by tumor necrosis factor. *J. Immunol.* 139:3676.

19. Collins, T., L. A. Lapierre, W. Fiers, J. L. Strominger, and J. S. Pober. 1986. Recombinant human tumor necrosis factor increases in RNA levels and surface expression of HLA-A, B antigens in vascular endothelial cells and dermal fibroblasts in vitro. *Proc. Natl. Acad. Sci. USA.* 83:446.
20. Nawroth, P. P., and D. M. Stern. 1986. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* 163:740.
21. Bevilacqua, M. P., J. S. Pober, G. R. Majeau, W. Fiers, R. S. Cotran, and M. A. Gimbrone. 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with interleukin 1. *Proc. Natl. Acad. Sci. USA.* 83:4533.
22. Gamble, J. R., J. M. Harlan, S. J. Klebenoff, and M. A. Vadas. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA.* 82:8667.
23. Nathan, C. F. 1987. Neutrophil activation on biological surfaces. *J. Clin. Invest.* 80:1550.
24. Andrews, B. S., R. O. Eisenberg, A. N. Theofilopoulos, S. Izui, C. B. Wilson, P. J. McConahey, E. D. Murphy, J. B. Roths, and F. J. Dixon. 1978. Spontaneous murine lupus-like syndromes. *J. Exp. Med.* 148:1198.