Neutralization of Microcystin Shock in Mice by Tumor Necrosis Factor Alpha Antiserum

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Microcystis aeruginosa is a common cyanobacterium in water blooms that appear widely in nutrient-rich, fresh, and brackish waters, and its toxic blooms cause the death of domestic animals. The administration of a crude toxic cell extract of *M. aeruginosa* K-139 to mice can produce tumor necrosis factor (TNF) and prompt severe physiological disturbances, especially liver damage, which can lead to death. The in vitro production of TNF-α by peritoneal macrophages was observed after stimulation with the cell extract or the purified toxin from K-139 cells. The expression of a TNF-α mRNA was also detected in spleen cells and peritoneal macrophages after stimulation with the cell extract. However, a previous injection of rabbit anti-murine TNF-α serum could prevent the liver damage to some extent and protect the mice from death. These findings indicate the involvement of TNF in microcystin shock.

Toxic water blooms have been found throughout the world, and wild and domestic animal deaths caused by the consumption of these toxic water blooms have been recorded (10). The toxicity of water blooms has been mostly associated with *Microcystis aeruginosa* (4), whereas cyclic heptapeptide toxins, such as microcystin and cyanoginosin, have been isolated from various *Microcystis* species (3, 4). The cells and the toxin(s) of *M. aeruginosa* are cytotoxic and cause extensive hemorrhaging in the livers of animals (8, 9, 11, 20). Acute and extensive centrilobular necrosis of the liver has occurred, with disruption of the architecture of the hepatic cord (4, 16). Falconer et al. (9) and Theiss and Carmichael (21) reported that death appears to be due to hemorrhagic shock caused by massive pooling of the blood in the liver following destruction of the sinusoids. However, the mode of action of microcystin in the liver is not yet clear.

M. aeruginosa K-139 was isolated as an axenic clone from water blooms in Lake Kasumigaura, Ibaraki, Japan (16, 19). The cells of this strain contained two different types of microcystin (a relatively large amount of 7-desmethyl microcystin-LR [7-DMLR] and a trace amount of an unknown toxin) (16). The 50% lethal dose of K-139 cells grown in CB medium (19) was 7.3 mg/kg per mouse, and acute toxic death occurred within 3 h when cells disrupted by freezing-thawing and sonication were intraperitoneally (i.p.) injected (13, 16). Furthermore, disrupted K-139 cells were capable of eliciting a soluble factor(s) such as interleukin-1 from macrophages (13). Interleukin-1 and tumor necrosis factor alpha (TNF-α) are thought to mediate endotoxin shock (1, 23). TNF-α and interleukin-1 are products of activated macrophages that were originally found in the sera of *Mycobacterium bovis* BCG-primed and bacterial lipopolysaccharide (LPS, endotoxin)-stimulated mice to mediate the antitumor effect (5) but were subsequently found to display a variety of other biological functions beyond tumor cytotoxicity (17). TNF-α mimics the toxic effects of endotoxin (6), and passive immunization with the TNF-α antiserum can protect mice from the lethal effect of endotoxin (2). Although the participation

of TNF in endotoxin shock seems to be obvious, its role in microcystin shock has not been examined yet. In the present study, we examined whether TNF is involved in microcystin shock.

Axenic K-139 cells were grown in CB medium at 30°C for 4 days (19). The grown cells were collected and washed by centrifugation. After three consecutive cycles of freezing-thawing, the cells were disrupted (model 185 Sonifier; Branson) at 50 W for 3 min. The disrupted cell suspension was centrifuged at 30,000 × *g* for 15 min. The resulting supernatant was filtered with a membrane filter (pore size, 0.45 μm; Acrodisc; Gelman Sciences Inc., Ann Arbor, Mich.) and lyophilized. One milligram of K-139 cells yielded 0.75 mg of extract containing protein, sugar, and microcystin (7-DMLR; molecular weight, 980) at 0.4913 mg, 0.0466 mg, and 6 μg, respectively. Microcystin (7-DMLR) was extracted from K-139 cells (16) and lyophilized. When used, the lyophilized cell extract and toxin were dissolved in saline. The anti-murine TNF-α serum was prepared from rabbits immunized with recombinant murine TNF-α (14).

BALB/c AnN female mice, 10 weeks of age, were injected i.p. with the cell extract (0.5 mg [dry weight] per mouse). Groups of mice were injected i.p. with TNF-α antiserum (10^5 neutralizing U/0.2 ml per mouse) 0.5 h before injection of the extract. After injection of the extract, blood was obtained from the mice by heart puncture over a 0.5- to 3-h period, and the TNF activity in the serum was determined as the cytolytic activity against murine L-929 cells (12) in comparison with the standard activity of recombinant murine TNF-α (provided by Suntory Co., Ltd., Institute for Biomedical Research, Osaka, Japan). TNF was found in the bloodstream of the BALB/c mice shortly after i.p. injection of the cell extract (Fig. 1). Maximum TNF activity was seen 0.5 h after injection, and the activity decreased quickly thereafter. However, TNF activity was completely blocked when rabbit anti-murine TNF-α serum was injected before injection of the extract.

Next, we examined the production of TNF by macrophages after stimulation with the extract or microcystin. Peritoneal exudate cells were obtained by washing the peritoneal cavities with 5 ml of RPMI 1640 medium supple-
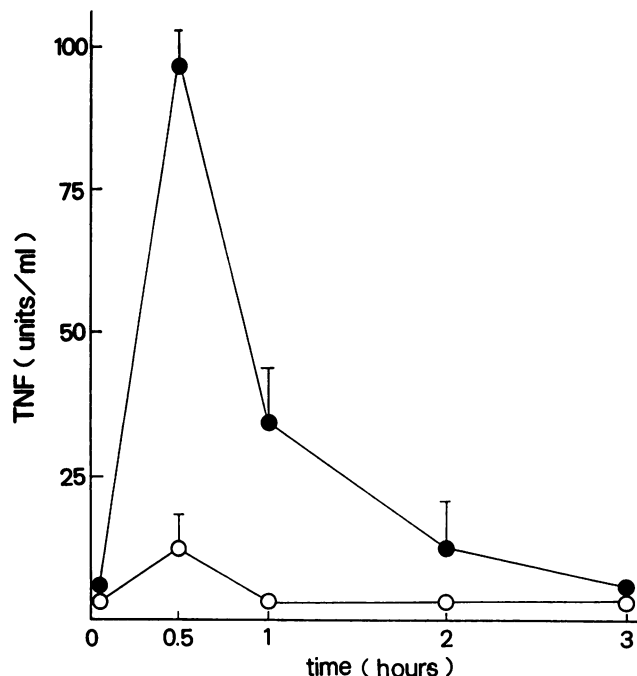
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FIG. 1. Kinetics of TNF activity in serum obtained from mice injected with the crude cell extract of *M. aeruginosa* K-139. Symbols: ●, injected i.p. with the extract (0.5 mg/mouse); ○, injected i.p. with the extract (0.5 mg) plus TNF-α antiserum (10^5 neutralizing U/0.2 ml per mouse). Each point represents the mean TNF titers for five mice and the standard deviations.

TABLE 1. Production of TNF by peritoneal macrophages stimulated with *M. aeruginosa* K-139 microcystin (7-DMLR)

Microcystin dose (μg/ml)	TNF activity (U/ml) at:		
	1 h	3 h	6 h
500	NT[a]	32	NT
100	6	9	7
20	<2	<2	<2
0	<2	<2	<2

[a] NT, Not tested.

(Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. Nonadherent cells were washed from the dishes. The remaining, adherent cells (macrophages) were cultured in RPMI 1640 medium supplemented with 1% fetal calf serum and various doses of the extract or microcystin in a humidified 5% CO_2-95% air chamber. Supernatants of the cultures were removed at various times, and TNF activities were assessed with L-929 cell cultures. The cell extract or microcystin itself, at the dose used, did not destroy L-929 cells.

Cultured murine peritoneal exudate macrophages produced TNF in the presence of the extract or microcystin in a dose-dependent manner (Fig. 2 and Table 1). Maximum activity was observed 3 h after stimulation with the extract, and the activity diminished thereafter. The extract seemed to be more effective in inducing TNF production by macrophages than did microcystin. However, a cell extract (50 to 500 μg) from nontoxic *M. aeruginosa* K-17 could hardly induce TNF production in the macrophage cultures. These results suggest that microcystin can induce TNF production but that a cell extract presumably enhances the production. We are now investigating the substance that enhances TNF production induced by microcystin. We do not know the exact reasons why the culture macrophages were retarded in reaching the maximum (Fig. 2) when compared with the in
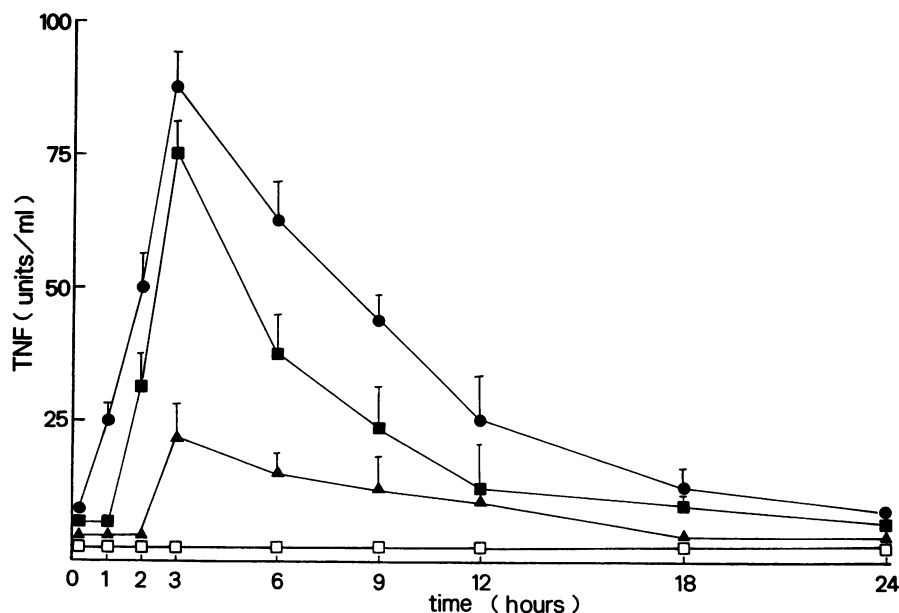
mented with 1% fetal calf serum from BALB/c mice that had been injected i.p. with 2 ml of thioglycolate broth 4 days earlier. The peritoneal exudate cells (10^6 cells per ml) were poured into a Multiplate with 24 wells (1 ml per well)

FIG. 2. Production of TNF by peritoneal macrophages stimulated in vitro with K-139 cell extract at 50 μg/ml (□), 100 μg/ml (▲), 250 μg/ml (■), and 500 μg/ml (●).
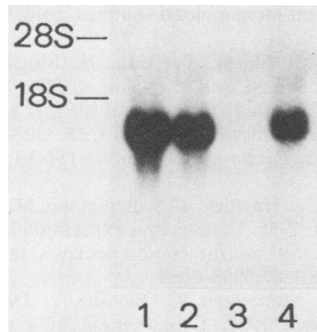
FIG. 3. Northern blot analysis of TNF-α mRNA induction by K-139 cell extract. Lanes: 1 to 3, cultured macrophages tested with 2 μg of LPS per ml of culture (positive control) (lane 1), 200 μg of K-139 cell extract per ml of culture (lane 2), and medium alone (control) (lane 3); 4, spleen cells tested with 0.5 mg of K-139 cell extract injected i.p. per mouse.

TABLE 2. Protective effect of TNF-α antiserum against
M. aeruginosa K-139 cells in mice

Injection (per mouse)[a]	Survival rate (%)[b]
K-139 cell extract (0.6 mg)	10
TNF-α antiserum (10^4 U)	100
TNF-α antiserum (10^5 U)	100
K-139 cell extract (0.6 mg) + TNF-α antiserum (10^3 U)	10
K-139 cell extract (0.6 mg) + TNF-α antiserum (10^4 U)	40 (Not significant)
K-139 cell extract (0.6 mg) + TNF-α antiserum (10^5 U)	70 ($P < 0.01$)
K-139 cell extract (0.6 mg) + TNF-α antiserum (10^6 U)	90 ($P < 0.005$)

[a] Mice (10 per group) were injected i.p. with K-139 cell extract (0.6 mg [wet weight] per mouse) and/or rabbit anti-murine TNF-α serum (10^3 to 10^6 neutralizing U/0.2 ml per mouse).

[b] The survival rate was scored on the fifth day after the injection. The statistical significance between the group receiving K-139 cell extract and the groups receiving K-139 cell extract plus antiserum was determined with the χ^2 test.

vivo results (Fig. 1), but they may involve the superior production and destruction of TNF in vivo rather than in vitro.

Stimulation of the production of TNF by the K-139 cell extract was also confirmed by the expression in macrophages and spleen cells of TNF-α mRNA. Adherent peritoneal macrophages (10^6 cells per ml of culture) were cultured in the presence or absence of the extract for 3 h. As a positive control, the macrophages were cultured with LPS for 3 h. To examine the expression of TNF-α mRNA in vivo, we injected mice with the extract and obtained spleen cells 0.5 h later. Total cellular RNA was isolated from the cultured macrophages or the spleen cells by the guanidine isothiocyanate-cesium chloride method (7). The RNA samples (20 μg) were denatured with formamide-formaldehyde, subjected to electrophoresis in 0.1% agarose, and transferred to nylon membranes (Hybond-N$^+$; Amersham, Tokyo, Japan). TNF-α mRNA was detected by Northern (RNA) blot hybridization (22) as follows. After 1 h of prehybridization at 65°C, mRNA was hybridized at 65°C with a ^{32}P-labeled cDNA murine TNF probe. The 733-bp cDNA probe encoding mature TNF was cloned by Suntory Co., Ltd., and its nucleotide sequence was found completely identical to that described by Pennica et al. (18). After hybridization, the membranes were washed twice with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at room temperature and washed twice with 0.2× SSC–0.1% sodium dodecyl sulfate at 45°C. The membranes were exposed to X-ray film with an intensifying screen at −80°C. TNF-α mRNA was clearly expressed in in vitro cell extract-stimulated macrophages and in vivo cell extract-stimulated spleen cells (Fig. 3).

If the TNF-α produced by macrophages in mice after injection of the K-139 cell extract plays some important role in acute toxic death, death should be able to be prevented by an earlier administration of TNF-α antiserum. To test this idea, we injected BALB/c mice i.p. with the extract and/or anti-murine TNF-α serum. An earlier injection of mice with 10^5 or 10^6 U of TNF-α antiserum caused a significant increase in the survival rate, while the majority of mice that did not receive TNF-α antiserum died after the injection of the extract (Table 2). In addition, when the livers of the mice that had been pretreated or not pretreated with TNF-α antiserum and injected with the extract were removed and examined histologically, there was apparently less liver

damage in the TNF-α antiserum-pretreated mice than in the untreated mice (data not shown). These findings provide evidence that the TNF-α that is synthesized de novo by macrophages stimulated with toxic M. aeruginosa participates in leading to severe liver damage and toxic death in mice. Contamination with endotoxin or bacterial LPS in the K-139 cell sample used in these experiments was ruled out, since the toxicolor test (15), an improved Limulus lysate test, revealed no LPS activity and the toxic effect was seen in LPS-nonresponsive C3H/HeJ mice as well as LPS-responsive BALB/c mice (13). Our results, however, do not rule out other causes, such as a direct toxicity of microcystin for liver cells and the participation of other endogenous mediators, such as interleukin-1, produced by microcystin.

The present study demonstrated that the macrophage-derived mediator TNF is involved in microcystin shock in this animal model and that prophylactic administration of TNF antibodies in mice protects against lethal doses of microcystin.

REFERENCES

1. **Bertini, R., M. Bianchi, and P. Ghezzi.** 1988. Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. J. Exp. Med. **167:**1708–1712.
2. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229:**869–871.
3. **Botes, D. P., A. A. Tuinman, P. L. Wessels, C. C. Viljoen, H. Kruger, D. H. Williams, S. Santikarn, R. J. Smith, and S. J. Hammond.** 1984. The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium Microcystis aeruginosa. J. Chem. Soc. Perkin Trans. I **1984:**2311–2318.
4. **Carmichael, W. W.** 1988. Toxins of freshwater algae, p. 121–147. In A. Tu (ed.), Handbook of natural toxins: marine toxins and venoms, vol. 3. Marcel Dekker, Inc., New York.
5. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72:**3666–3670.
6. **Cerami, A., and B. Beutler.** 1888. The role of cachectin/TNF in endotoxin shock and cachexia. Immunol. Today **9:**28–31.

7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.

8. **Codd, G. A., and G. K. Poon.** 1988. Cyanobacterial toxins, p. 283–296. *In* L. J. Rogers and J. R. Gallon (ed.), Biochemistry of the algae and cyanobacteria. Clarendon, Oxford.

9. **Falconer, I. R., A. R. B. Jackson, J. Langley, and M. T. Runnegar.** 1981. Liver pathology in mice in poisoning by the blue-green alga *Microcystis aeruginosa.* Aust. J. Biol. Sci. **34:**179–187.

10. **Gorham, P. R., and W. W. Carmichael.** 1988. Hazards of freshwater blue-green algae (cyanobacteria), p. 403–431. *In* A. A. Limbi and J. R. Waaland (ed.), Algae and human affairs. Cambridge University Press, Cambridge.

11. **Hughes, E. O., P. R. Gorham, and A. Zehnder.** 1958. Toxicity of a unialgal culture of *Microcystis aeruginosa.* Can. J. Microbiol. **4:**225–236.

12. **Nakane, A., T. Minagawa, and K. Kato.** 1988. Endogenous tumor necrosis factor (cachectin) is essential to host resistance against *Listeria monocytogenes* infection. Infect. Immun. **56:** 2563–2569.

13. **Nakano, M., Y. Nakano, T. Saito-Taki, N. Mori, M. Kojima, A. Ohtake, and M. Shirai.** 1989. Toxicity of *Microcystis aeruginosa* K-139 strain. Microbiol. Immunol. **33:**787–792.

14. **Nakano, Y., K. Onozuka, Y. Terada, H. Shinomiya, and M. Nakano.** 1990. Protective effect of recombinant tumor necrosis factor-α in murine salmonellosis. J. Immunol. **144:**1935–1941.

15. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, and T. Kawai.** 1986. Endotoxin-inactivating activity in normal and pathological human blood samples. Infect. Immun. **53:**294–297.

16. **Ohtake, A., M. Shirai, T. Aida, N. Mori, K. Harada, K. Matsuura, M. Suzuki, and M. Nakano.** 1989. Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. Appl. Environ. Microbiol. **55:**3202–3207.

17. **Old, L. J.** 1985. Tumor necrosis factor (TNF). Science **230:**630–632.

18. **Pennica, D., J. S. Hayflick, T. S. Bringman, M. A. Palladino, and D. V. Goeddel.** 1985. Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor. Proc. Natl. Acad. Sci. USA **82:**6060–6064.

19. **Shirai, M., K. Matumaru, A. Ohotake, Y. Takamura, T. Aida, and M. Nakano.** 1989. Development of a solid medium for growth and isolation of axenic *Microcystis* strains (cyanobacteria). Appl. Environ. Microbiol. **55:**2569–2571.

20. **Shirai, M., Y. Takamura, H. Sakuma, M. Kojima, and M. Nakano.** 1986. Toxicity and delayed type hypersensitivity caused by *Microcystis* blooms from Lake Kasumigaura. Microbiol. Immunol. **30:**731–735.

21. **Theiss, W. W., and W. W. Carmichael.** 1986. Physiological effect of a peptide toxin produced by the fresh water cyanobacteria (blue-green algae) *Microcystis aeruginosa* strain 7820, p. 353–364. *In* P. S. Steyn and R. Vleggaar (ed.), Mycotoxins and phycotoxins. Elsevier Science Publishers B.V., Amsterdam.

22. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:**5201–5205.

23. **Waage, A., and T. Espevik.** 1988. Interleukin 1 potentiates the lethal effect of tumor necrosis factor α/cachectin in mice. J. Exp. Med. **167:**1987–1992.