Yamanashi Med. J. 5(2), 97~103, 1990

Original Article

Tumor Necrosis Factor Stimulates Acetyltransferase Activity of Human Neutrophils

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Abstract: Of the five cytokines tested (tumor necrosis factor, interleukin 1, colony-stimulating factor, interferon *alpha, beta,* and *gamma*), only tumor necrosis factor (TNF) showed a potent stimulatory effect on acetyltransferase activity of human neutrophils. Acetyltransferase is a key enzyme for the production of platelet-activating factor. This potentiating effect of TNF was time- and dose-dependent and was effectively blocked by TNF antiserum. Intracellular Ca^{++} elevation, protein kinase C activation, and arachidonic acid metabolites appeared not to be involved in the TNF-induced potentiation of the acetyltransferase activity in neutrophils.

Key words: tumor necrosis factor, acetyltransferase, platelet-activating factor, neutrophils, cytokines

INTRODUCTION

Neutrophils play a pivotal role in the termination of bacterial infections. In the absence of a large number of neutrophils in the circulating blood and tissues, recovery from bacterial infection is often difficult regardless of the quality of medical care. It has been well established that neutrophils from patients with bacterial infection show enhanced functions, including phagocytosis and oxygen radical production[1,2] while the precise mechanism for this potentiation remains to be elucidated.

Factors that potentiate neutrophil function during infection are multiple, including bacterial cell walls and cytokines. Beside these exogenous agents, neutrophils are known to produce endogeneous secondary messengers for potentiation. These include arachidonic acid metabolites such as leukotriene B_4, and platelet-activating factor (PAF)[3]. The major, and regulatory pathway for the biosynthesis of PAF in neutrophils is considered to be through acetylation of 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso PAF), catalyzed by lyso PAF: acetyl CoA acetyltransferase (AT)[4]. In a search for parameters that most accurately represent enhancement of neutrophil function in various inflammatory disorders, we found that AT activity of human neutrophils is most closely correlated with bacterial infection[5]. Multiple regression analysis of clinical data demonstrated that fever is one of the major determinants of elevated AT activity. Elevated levels of AT are not directly associated with enhanced production of PAF, since the precursor, lyso PAF, is absent in the resting cells. However, when neutrophils are in contact with other stimulators that are capable of activating phospholipase A_2 which produces lyso PAF, there occurs a massive production of PAF leading to the potentiated function of neutrophils.

Received January 17, 1990
Accepted March 22, 1990

Recent investigations have revealed that fever in bacterial infection is mostly attributable to the production of cytokines, especially interleukin 1 (IL-1) and TNF[6,7]. These cytokines are known to potentiate neutrophil functions, which favors termination of bacterial infection, and it may be that their effects are exerted on neutrophils by activating AT activity. In the present study, we evaluate the effects of various cytokines on AT activity of human neutrophils.

Experimental Procedures

Materials

Recombinant DNA-produced TNF was a generous gift from Asahi Chemical Industry Co. Ltd., Shizuoka, Japan (cytotoxic activity against tumor cell lines, 2.2×10^6 units/mg protein). This TNF preparation contained less than 50 pg endotoxin/mg TNF determined by Limulus amoebae test. Polyclonal antiserum against TNF was also provided by Asahi Chemical. Recombinant DNA-produced IL-1 was kindly provided by Dainippon Pharmaceutical Co. Ltd., Suita, Osaka, Japan (the LAF activity, 2×10^7 units/mg protein). Colony-stimulating factor (G-CSF) was obtained from Cosmo-Bio Co. Ltd., Tokyo, Japan (the specific activity, 2,200 CFU/μg). Interferon *alpha, beta,* and *gamma*, and AA861 were from (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). Aequorin and fura 2-AM were purchased from Wako Chemicals (Tokyo, Japan), and Dojin Chemicals (Kumamoto, Japan), respectively. H-7 was from Nippon Seikagaku Co. Ltd., Tokyo, Japan. Ethyleneglycol-bis (*b*-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA) and n-formyl-Methionyl-Leucyl-Phenylalanine (fMLP) were purchased from Sigma (St. Louis, MO.). PAF and lyso-PAF were obtained from Funakoshi Chemicals (Tokyo, Japan). [^{14}C] acetylcoenzyme A (50 mCi/mM) was purchased from Amersham International plc. (Bukinghamshire, England).

Preparation of human neutrophils

Blood was collected from healthy donors. Neutrophils were purified by dextran sedimentation and Ficoll-Hypaque density centrifugation. Contaminating red blood cells were lyzed by a hypotonic shock. The cells were suspended in Krebs-Ringer's phosphate buffer with 0.1% gelatin and 5 mM glucose (KRP solution) at the concentration indicated in each measurement. This cell suspension contained more than 95% of neutrophils, as checked on a Giemsa-stained smear. To neutrophils suspended in a KRP solution (10^7 cells/ml) an appropriate concentration of a stimulant or an equal volume of physiological saline was added, and the mixture was incubated at 37°C for the indicated period of time. At the end of incubation, the reaction was stopped by adding an excess of ice-cold KRP solution, and the cells were separated by centrifugation (200×g, 10 min) at 4°C.

Measurement of acetyltransferase activity

Acetyltransferase activity of the homogenate neutrophil preparations was measured according to the method of Alonso *et al.*[8] In brief, stimulated neutrophils in KRP solution were washed once and resuspended at a concentration of 10^7 cells/ml in ice-cold 0.1 M Tris-HCl buffer, pH 6.9. A cell homogenate was obtained by 10 sec of sonication with a Branson 200 sonifier equipped with a micro-tip at position 3 on ice. To this homogenate preparation was added 25 μM of lyso-PAF and 0.6 μCi of [^{14}C] acetylcoenzyme A, and 25 μM cold acetylcoenzyme A. The mixture was incubated at 37°C for 10 min and the reaction was stopped by the addition of 3 ml methanol: chloroform (2: 1, V/V). The chloroform layer containing lipids extracted from the homogenated preparations was evaporated under N_2 gas. The extracted lipids were subjected to silica-gel thin-layer chromatography, with chloroform: methanol: water (65: 25: 4, V/V) as solvent. The area corresponding to [^{14}C] PAF was scraped and the radioactivity was counted.

Measurement of intracellular Ca^{++}

Aequorin loading into neutrophils and measurement of intracellular Ca^{++} was performed essentially according to the method of Johnson *et al.*[9]. Fura 2 loading and measurement of intracellular Ca^{++} with dual wavelength spectrophotometry was performed as described elsewhere[10], with a Hitachi F-2000 spectrophotometer.

Results

Effects of various cytokines on the AT activity of neutrophils

The potentiating effects of IL-1, TNF, and CSF on neutrophil functions have been well documented, while that of interferons is more or less controversial[11–16]. Various doses of these cytokines sufficient to enhanced neutrophils functions were added to a neutrophil suspension, and the mixtures were incubated for 30 min at 37°C. After incubation, changes in AT activity were determined. As shown in Fig. 1, TNF showed a potent stimulatory effect on AT activity, whereas CSF had slight effect even at higher concentrations and with longer incubation. These findings indicate that the effect of TNF is qualitatively different from that of CSF. IL-1, and interferons at all the concentrations tested (up to 10^4 U/ml) had no effect on AT activity even after 2 hours of incubation. Therefore, the effect of TNF on AT activity was investigated in details.

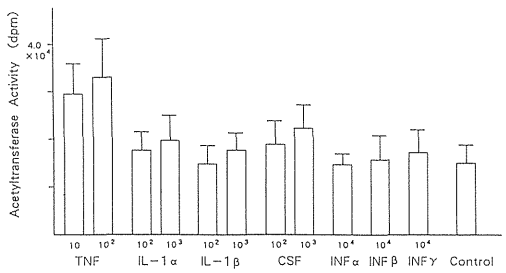

Fig. 1. Effects of various cytokines on the AT activity of neutrophils.
Neutrophils were incubated with various concentrations of cytokines at 37°C for 30 min. After incubation, the cells were washed and homogenated for the measurement of the AT activity. The column and bar represents the mean ±SD of three experiments. The number listed above each cytokine represents the concentration (U/ml).

TNF stimulated AT activity at concentrations as low as 1 U/ml, which is equivalent to those required for potentiation of neutrophil functions such as oxygen radical production (Fig. 2)[12]. The stimulatory effect of TNF on AT activity was effectively blocked by polyclonal anti-TNF antiserum, confirming that its effect is attributed to a specific property of TNF (Fig. 3). A time course study on the effect of TNF revealed that AT activity began to increase after one minute of incubation, and reached a plateau at 30 min (Fig. 4).

We then examined the mechanism by which TNF stimulates AT activity. Intracellular Ca^{++} elevation, diacylglycerol production with subsequent protein kinase C activation and leukotriene B_4 are known to play important roles in the signal transduction pathway in neutrophils. We, therefore, investigated whether these factors are involved in TNF-induced

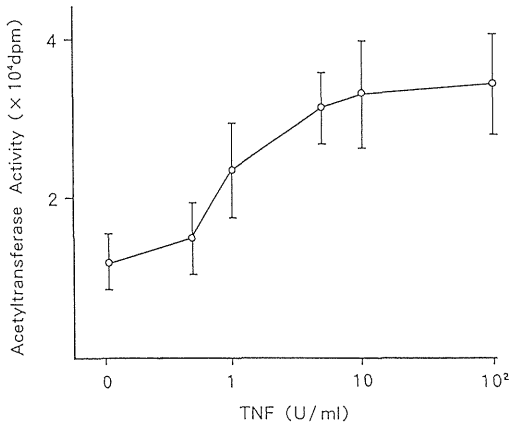

Fig. 2. Dose-dependency of TNF-induced AT activity. TNF at various concentrations was added to a neutrophil suspension, and incubated for 30 min at 37°C. After incubation, the AT activity of 10^7 neutrophils was determined as described for Figure 1. Open circles and bars represent the means ±SD of three experiments.

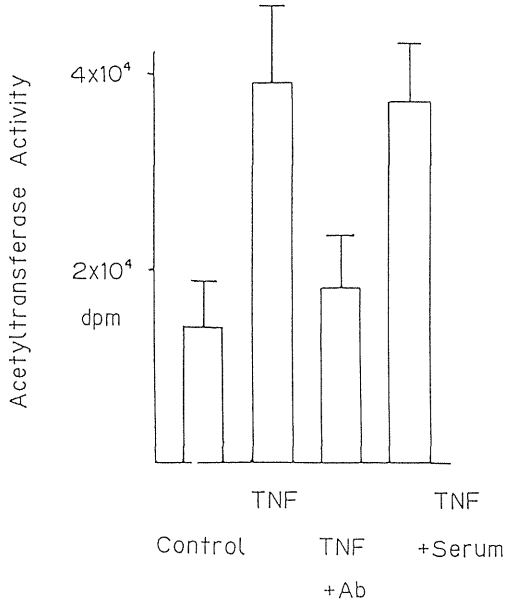

Fig. 3. Blocking effect of antiserum against TNF on AT activity.
Neutrophils were pretreated with antiserum against TNF (sufficient to block 100 U/ml TNF), normal serum or saline for 10 min. Then, TNF at the concentration of 10 U/ml was added to a neutrophil suspension, and the mixture incubated for another 30 min at 37°C. After incubation, AT activity was measured as described in Methods. Columns and bars represent the means ±SD of three experiments. "Control" represents the AT activity without TNF stimulation.

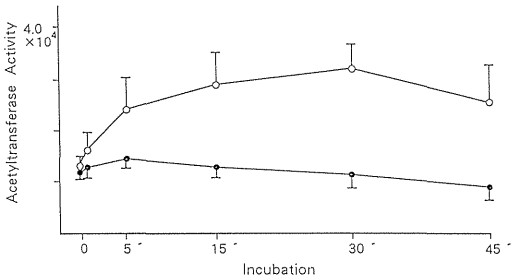

Fig. 4. Time course of TNF-induced AT activity. TNF at a concentration of 10 U/ml was added to a neutrophil suspension, and at various periods of time after the addition of TNF, the reaction was stopped to measure AT activity. Open circles and bars represent the means ±SD of three TNF-stimulated samples. Closed circles represent changes in AT activity of the controls.

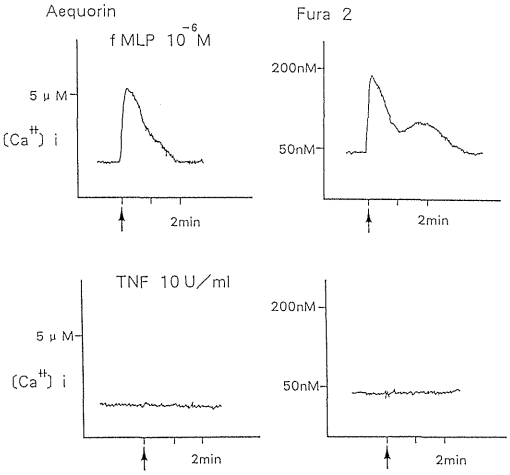

Fig. 5. Intracellular Ca^{++} elevation induced by fMLP and TNF. Intracellular Ca^{++} was measured by two different methods, aequorin, and fura 2 loading. The upper two panels show intracellular Ca^{++} changes induced by 10^{-6} fMLP. The lower panels represent changes induced by 10 U/ml TNF. Stimulators were added at the time indicated by arrows. The data are representative of three experiments.

potentiation of AT activity.

Intracellular Ca^{++} in neutrophils can be measured by two methods, the use of a Ca^{++} in neutrophils can be measured by two methods, the use of a Ca^{-+} in neutrophils can be measured by two methods, the use of a Ca^{++}-sensitive fluorescent dye, fura 2, and the use of a Ca^{++}-sensitive photoprotein, aequorin. The Ca^{++} pool detected by these two methods appear to differ from each other; aequorin may detect localized mobilization of Ca^{++}, while fura 2 demonstrates the average Ca^{++} concentration throughout the cytoplasm. As shown in Fig. 5, neither method detected intracellular Ca^{++} elevation induced by TNF at concentrations which stimulated the AT activity. Depletion of extracellular Ca^{++} with EGTA, which markedly diminishes Ca^{++}

influx into the cytoplasm, had no effect on TNF-induced AT activity (Fig. 6). In sharp contrast with TNF, fMLP, which is a synthetic analogue of bacterial cell walls, induced intracellular Ca^{++} elevation detectable by both methods, and fMLP-induced AT activity was markedly attenuated by Ca^{++} depletion. These findings suggested that intracellular Ca^{++} elevation plays a pivotal role in fMLP-induced potentiation of AT activity, while that of TNF has no requirement for intracellular Ca^{++} elevation.

The role of protein kinase C and leukotriene B_4 was evaluated with their specific inhibitors, H-7, and AA861, respectively[17,18]. Neither H-7 at 50 μM nor AA861 at 25 μM inhibited TNF-induced potentiation of AT activity, excluding the role of protein kinase C and leukotriene B_4.

Discussion

Among the 5 cytokines tested in the present study, only TNF showed a significant potentiating effect on AT activity of human neutrophils. This result was somewhat surprising, since AT activity was elevated in neutrophils of febrile patients, and we expected to find the potentiating effects of IL-1, which is known to be one of the major endogenous pyrogens[6,7].

This potentiating effect of TNF was independent of intracellular Ca^{++} elevation, arachidonic acid metabolites and protein kinase C activation, which play pivotal roles in a wide variety of cell systems. Although this effect is attributable to a specific property of TNF, which was blocked by anti-TNF antiserum, the mechanism by which TNF elevates AT activity remains to be elucidated.

It is also of great interest that of IL-1, CSF and TNF, all of which are potentiators of neutrophil functions[13,15,16] only TNF elevates AT activity. TNF, IL-1, and CSF stimulate neutrophil functions to almost the same extent, and it is suggested that these cytokines work in synergy to fight infection. While the mechanism by which these cytokines activate neutrophils remains largely unknown, it may be that the signal transduction pathway for TNF is different from those of IL-1 and CSF.

Clinical trials of TNF on cancer patients have revealed several side effects of TNF, though far from being severe. Among these,

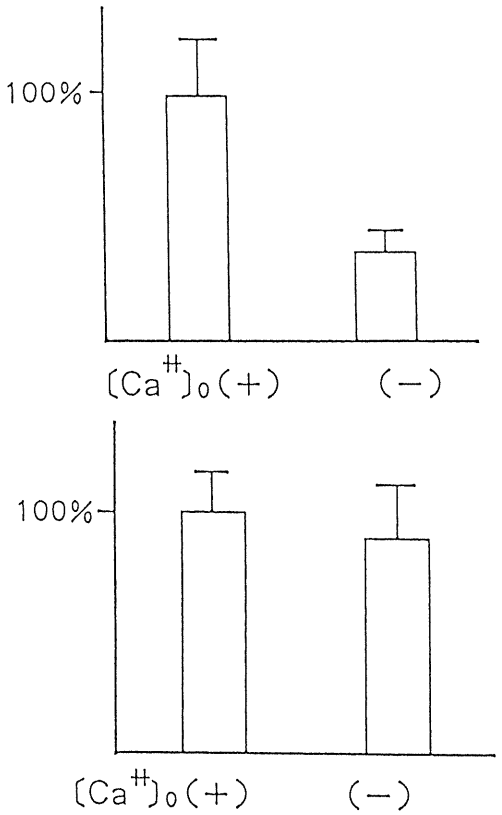

Fig. 6. Effect of depletion of extracellular Ca^{++} on AT activity induced by TNF and fMLP.
Neutrophils were either suspended in buffer containing 1 mM Ca^{++} ($[Ca^{++}]_0$ (+)) or in buffer containing 1 mM EGTA ($[Ca^{++}]_0$ (−)). fMLP (10^{-6}M) or TNF (10 U/ml) was added to the neutrophil suspension, and the mixture incubated for 30 min at 37°C. AT activity was determined as described for Figure 1. Upper panel, fMLP; lower panel, TNF. The data represent the means ±SD of three experiments (AT activity was expressed as percentage of that induced in the presence of extracellular Ca^{++}).

occasional hypotension has been noted after TNF administration. Although there is not yet an accepted TNF dose for cancer therapy, the effects of 10^5-5×10^6 U/m^2/day administration have been evaluated (19–21). With these administration doses, serum TNF concentrations sometimes reach the level of several U/ml, which, as shown in the present study, is sufficient to elevate neutrophil AT activity. Since PAF is alleged to be one of the agents causing hypotension in physiological states as well as in endotoxin shock[22], it may be that administration of TNF with consequent elevation of AT activity of peripheral neutrophils predisposes the enhanced production of PAF in patients suffering from bacterial infection.

We have observed that IL-1 administered intramuscularly prevented mice from succumbing to fatal bacterial infections[23] while TNF at all tested doses had no effect on the survival rates of severely infected mice (unpublished observations). Infected mice appeared to succumb earlier upon administration of TNF. Infected mice upon administration of TNF may have developed fatal hypotension caused by PAF before TNF potentiated neutrophils to eradicate infection. In this respect, a recent finding showed that patients suffering from meningits show higher mortality when TNF is detected in their sera[24]. In agreement with this concept, we have also demonstrated that TNF contaminated with substantial amounts of endotoxin induces severe side effects on rats, while pure TNF itself shows a qualitatively different profile[25]. These findings taken altogether suggest that anti-cancer therapy employing TNF should be directed only to patients with no overt bacterial infections.

References

1) Hill HR, Warwick WJ, Dettloff BS and Quie PG Neuteophil granulocytes function in patients with pulmonary infection. J Pediatr 1974; **84**: 55–58.
2) Ozaki Y, Ohashi T and Niwa Y Oxygen radical production by neutropils from patients with bacterial infection and rheumatoid arthritis. Inflammation 1986; **10**: 119–130.
3) Jaquemin C and Vargaftig, BB Drugs interfering with lipid mediators. *In*: Glynn LE, Houck JC, and Weissmann G, eds. The Pharmacology of Inflammation. Amsterdam: Elservier, 1985: 283–295.
4) Albert DH and Snyder F Biosynthesis of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor) from 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by rat alveolar macrophages. J Biol Chem 1983; **258**: 97–102.
5) Ozaki Y, Ohashi T and Niwa Y Evaluation of neutrophil acetyltransferase activity in patients with inflammatory disorders. Life Sciences 1986; **39**: 2343–2350.
6) Dinarello CA An update on human interleukin 1: From molecular biology to clinical relevance. J Clin Immunol 1985; **5**: 287–297.
7) Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA Jr, and O'Connor JV Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med 1986; 163: 1433–50.
8) Alonso F, Gil MG, Sanchez-Crespo M and Mato JM Activation of 1-alkyl-2-lyso-glycero-3-phosphocholine: acetyl-CoA transferase during phagocytosis in human polymorphonuclear leukocytes. J Biol Chem 1982; **257**: 3376–3378.
9) Johnson PC, Ware JA, Cliveden PB, Smith M, Dvorak AM and Salzman EW Measurement of ionized calcium in blood platelets with the photoprotein aequorin. J Biol Chem 1985; **260**: 2069–2076.
10) Ozaki Y and Kume S Functional responses of aequorinloaded human neutrophils. Comparison with fura 2-loaded cells. Biochim Biophys Acta 1988; **972**: 113–119.
11) Klebanoff SJ, Vada MA, Harlan JM, Sparks LH, Gamble JR, Agosti JM and Waltersdorph AM Stimulation of neutrophils by tumor necrosis factor. J Immunol 1986; **136**: 4220–.
12) Ozaki Y, Ohashi T, Niwa Y and Kume S Effect of recombinant DNA-produced tumor necrosis factor on various parameters of neutrophil function. Inflammation 1988; **12**: 297–309.
13) Smith RJ, Speziale SC and Bowman BJ Properties of interleukin 1 as a complete secrectagogue for human neutrophils. Biochem. Biophys. Res. Commun. 1985; **130**: 1233–1240.
14) Refaat Shalaby M, Aggarwal BB, Rinderknecht

E, Svedersky LP, Finkle BS, and Palladino MA Activation of human polymorphonuclear neutrophil functions by interferon-*gamma* and tumor necrosis factors. J Immunol 1985; **135**: 2069–2073.

15) Ozaki Y, Ohashi T and Kume S Potentiation of neutrophil function by recombinant DNA-produced interleukin 1 *a*. J Leukocyte Biol 1987; **42**: 621–627.

16) Fleischmann J, Golde DW, Weisbart RH and Gasson JC Granulocyte-macrophage colony-stimulating factor enhances phagocytosis of bacteria by human neutrophils. Blood 1986; **68**: 708–711.3

17) Kawamoto S and Hidaka H 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem Biophys Res Commun 1984; **125**: 258–264.

18) Ozaki Y, Ohashi T and Niwa Y A comparative study on the effects of inhibitors of the lipoxygenase pathway on neutrophil function. Biochem Pharmacol 1986; **35**: 3481–3488.

19) Selby P, Hobbs S, Viner C, Jackson E, Jones A, Newell D, Calvert AH, McElwain T, Fearon K, Humphreys J and Shiga T. Tumour necrosis factor in man: Clinical and biological observations. Br J Cancer 1987; **56**: 803–808.

20) Sherman ML, Spriggs DR, Arthur KA, Imamura K, Frei III E and Kufe DW Recombinant human tumor necrosis factor administered as a five-day continous infusion in cancer patients: Phase I toxicity and effects on lipid metabolism. J Clin Oncol 1988; **6**: 344–350.

21) Lenk H, Tanneberger S, Muller U, Ebert J and Shiga T Phase II clinical trial of high-dose recombinant human tumor necrosis factor. Cancer Chemother Pharmacol 1989; **24**: 391–392.

22) Terashita Z, Imura Y, Nishikawa K and Sumida S Is platelet activating factor (PAF) a mediator of endotoxin shock? Eur J Pharmacol 1985; **109**: 257–261.

23) Ozaki Y, Ohashi T, Minami K and Nakamura S Enhanced resistance induced by recombinant DNA-produced interleukin 1 alpha in experimentally infected mice. Infect Immun 1987; **55**: 1436–1440.

24) Waage A, Halstensen A and Espevik T Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet 1987; Feb. **14**, 355–357.

25) Ozaki Y, Oyama T and Kume S Exacerbation of toxic effect by endotoxin contamination of recombinant human tumor necrosis factor. Cancer Chemother Pharmacol 1989; **23**: 231–237.