Jpn. J. Cancer Res. (Gann), 79, 626–631 ; May, 1988

Tumor Necrosis Factor-inducing Activities of Lipid A Preparations from *Pseudomonas diminuta* and *Pseudomonas vesicularis*

Satoru ARATA,*1 Kazuyasu NAKAYA,*2 Hisayoshi FURUHASHI,*2 Yasuharu NAKAMURA,*2 Tohru HIRAYAMA,*1 Jun-ichi MASHIMO*1 and Nobuhiko KASAI*1
*1*Department of Microbial Chemistry and* *2*Department of Biological Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142*

Tumor necrosis factor (TNF)-inducing activities of lipid A preparations from *P. diminuta* and *P. vesicularis*, which contain mainly 2 mol of 2,3-diamino-2,3-dideoxy-D-glucose and 1 mol of nonglycosidic phosphate as the backbone component and have partly different fatty acid compositions, were examined. TNF was induced by injecting various lipid A fractions into mice that had previously been sensitized with *Mycobacterium bovis* BCG vaccine. A major component of lipid A of both strains, referred to as A3 fraction, exhibited stronger TNF-inducing activity than A2 fraction having incomplete acyl residues. The removal of ester-linked fatty acyl groups by mild hydrazinolysis of the *P. diminuta* lipid A results in a marked decrease of the activity. These results suggest that the structure of the hydrophobic part, including the amide-linked acyloxyacyl group(s), of the lipid A molecule play an important role in inducing TNF in the sera of mice.

Key words: Tumor necrosis factor — Lipid A — Lipopolysaccharide

TNF, first found in the sera of animals previously injected with BCG and subsequently with endotoxin, is known to cause necrosis in transplanted tumor cells *in vivo*[1] and to be cytotoxic to various tumor cells *in vitro*.[2–4] Recently, multiple cellular functions of TNF have been reported, including stimulation of growth of normal diploid skin and lung fibroblasts,[4] induction of differentiation in myeloid leukemia cells,[5–8] modulation of cell surface antigen expression in various cells,[9–11] and modulation and mediation of monocyte cytotoxicity induced by cytokines[12] and by LPS.[13] Therefore, regulation of the production of TNF, which is now known to be produced by endotoxin-stimulated macrophages,[14] is important in various cellular functions.

In the previous study,[15] we found that *Pseudomonas diminuta* LPS contains a novel endotoxic lipid A structure which is composed mainly of 2,3-diamino-2,3-dideoxy-D-glucose, nonglycosidic phosphate and fatty acids in the molar ratio of 2:1:5–6. It would be interesting to see whether or not such unusual lipid A preparations have TNF-inducing activity.

Abbreviations: TNF, tumor necrosis factor; LPS, lipopolysaccharide.

In the present report, we describe the TNF-inducing activity of lipid A preparations from *P. diminuta* and *P. vesicularis* LPS.

Materials and Methods

Materials BCG was purchased from Japan BCG Ind., Tokyo. Recombinant human TNF was kindly provided by Fujisawa Phamaceutical Co., Ltd. RPMI 1640 medium was from GIBCO.

LPS and Lipid A Preparations LPS and free lipid A were prepared from *E. coli* F515, *P. diminuta* JCM 2788 and *P. vesicularis* JCM 1477 as previously described.[15,16] Purified lipid A components, A3 and A2 fractions were isolated from free lipid A of *P. diminuta* and *P. vesicularis* by preparative thin-layer chromatography (TLC) as previously described.[15] To obtain partially deacylated lipid A containing only amide-linked fatty acids, free lipid A of *P. diminuta* was treated with anhydrous hydrazine at 60° for 1 hr[17] and purified by silica gel chromatography.[18] The synthetic *E. coli*-type lipid A, compound 506 was kindly provided by Daiichi Pure Chemicals Co., Ltd. The chemical composition of purified lipids was determined by the methods described previously.[15]

Assay for TNF-inducing Activity Serum containing TNF was prepared by the method of Carswell *et al.*[1] Briefly, female mice (ddy) were injected intravenously with 1 mg of BCG. After 2 weeks, 20 μg of LPS or lipid A was injected in-

travenously, and the mice were bled 2 hr later. The activity of TNF was assayed as described by Ruff and Gifford.[19] L929 cells were seeded at a density of 5×10^3 cells per well in 96-well tissue culture plates in RPMI 1640 medium containing 10% FBS. Serum dilutions were incubated with L929 cells for 48 hr at 37° in a 5% CO_2 atmosphere. The number of cells remaining was then quantitated. After the incubation, the medium was removed and RPMI 1640 medium containing 10% FBS and 0.25 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol) was added. After incubation for a further 4 hr, the cells were treated with 0.1% trypsin in Hanks' solution, and harvested by filtration on glass fiber filters (Whatman GF/C). The filters were placed in a scintillation vial and dried with an infrared lamp, then the radioactivity was determined in Triton-toluene scintillator with an ALOKA LSC1000 liquid scintillation counter. The specific activity of recombinant human TNF assayed as described above was calculated to be 1.18×10^8 U/mg (average of four determinations), which is very close to the value of 10^8 U/mg reported by Aggawal *et al.*[20]

Results

Proposed Chemical Structure of Purified Lipids The thin-layer chromatograms and the chemical compositions of the purified lipids are shown in Fig. 1 and Table I. Free lipid A preparations from *P. diminuta* and *P. vesicularis* had 2 mol of 2,3-diamino-2,3-dideoxy-D-glucose and 1 mol of nonglycosidic phosphate as the backbone components. All purified lipid A fractions have the same backbone, and the only structural difference among these preparations was considered to be in their fatty acid compositions. The A3 fraction of *P. diminuta* lipid A had about 4 mol of amide-linked 3-hydroxy fatty acids (3-OH-12:0, 3-OH-13:0 and 3-OH-14:0), in which the 3-hydroxyl groups were partially substituted by 1–2 mol of nonhydroxy fatty acids (14:0 and 14:1) and 3-hydroxydodecanoic acid. The A2 fraction of *P. diminuta* lipid A contained fewer fatty acyl groups than did the A3 fraction. A similar composition was also seen in *P. vesicularis* lipid A fractions, except that neither tetradecenoic acid or 3-hydroxytridecanoic acid was detected in the latter preparations. The hydrazinolyzed lipid A (N_2H_4-PdA) had no ester-linked fatty acids but retained almost all amide-linked fatty acids. Thus, it has been proposed that the main component of both

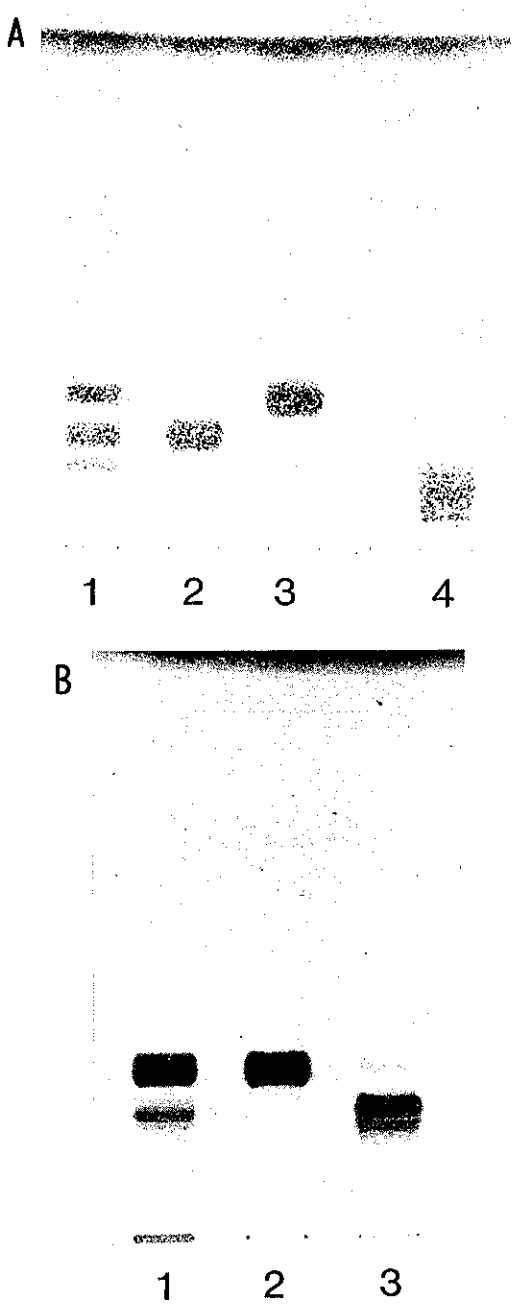

Fig. 1. A. Thin-layer chromatogram of purified lipid A preparations from *P. diminuta*. The lipids were applied to a silica gel plate, developed with chloroform-methanol-water-0.02*N* EDTA (100:50:7:4), and the chromatogram was charred with sulfuric acid. 1, Free lipid A; 2, A2 fraction; 3, A3 fraction; 4, hydrazinolyzed lipid A (N_2H_4-PdA). B. Thin-layer chromatogram of purified lipid A preparations from *P. vesicularis*. 1, Free lipid A; 2, A3 fraction; 3, A2 fraction.

Table I. Chemical Composition of Purified Lipid A Preparations

Preparations	Fatty acid						Phosphorus
	14:0	14:1	3-OH-12:0	3-OH-13:0	3-OH-14:0	Unidentified	
P. diminuta							
A3	0.15 (0.3)	0.08 (0.1)	1.76 (0.3)	0.33 (0.6)	0.49 (0.9)	0.28 (0.5)	0.58 (1.0)
A2	0.09 (0.2)	0.06 (0.1)	1.40 (2.9)	0.25 (0.5)	0.23 (0.5)	0.06 (0.1)	0.49 (1.0)
N_2H_4-PdA	ND	ND	0.93 (1.4)	0.29 (0.5)	0.40 (0.6)	0.22 (0.3)	0.65 (1.0)
P. vesicuralis							
A3	0.44 (1.0)	ND	1.38 (3.0)	ND	0.63 (1.4)	0.15 (0.3)	0.46 (1.0)
A2	0.40 (0.8)	ND	1.56 (3.0)	ND	0.57 (1.1)	0.07 (0.1)	0.52 (1.0)

Analytical values represent μmol/mg, and the figures in parentheses represent the molar ratio to phosphorus. Abbreviations: 14:0, tetradecanoic acid; 14:1, tetradecenoic acid; 3-OH-12:0, 3-hydroxydodecanoic acid; 3-OH-13:0, 3-hydroxytridecanoic acid; 3-OH-14:0, 3-hydroxytetradecanoic acid; ND, not detected.

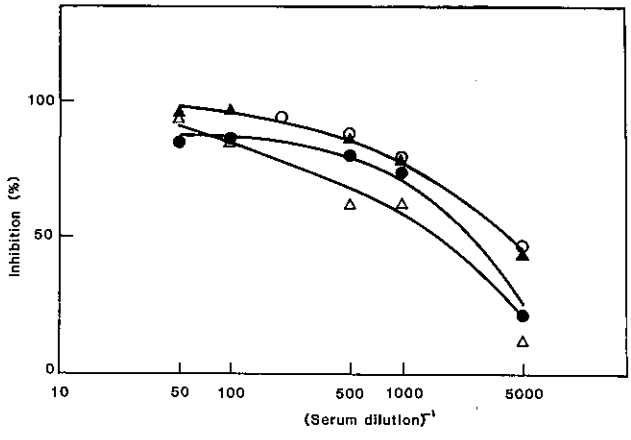

Fig. 2. TNF-inducing activity of LPS and lipid A preparations. Sera were obtained from mice treated first with 1 mg of BCG and subsequently with 20 μg of *P. diminuta* LPS (○), synthetic lipid A (506, △), *P. diminuta* lipid A (●), or *E. coli* lipid A (▲) as described in "Materials and Methods." The TNF activities were determined by measuring the incorporation of [^{3}H]thymidine into L929 cells.

P. diminuta and *P. vesicularis* lipid A has a similar structure containing diaminosugar disaccharide phosphomonoester, which probably carries penta- or hexa-acyl groups including acyloxyacyl residues.[15]

TNF-inducing Activity Figure 2 shows typical curves of TNF-inducing activities of lipid A preparations from *P. diminuta* and *E. coli* F515 and of LPS from *P. diminuta* as compared with that of synthetic *E. coli*-type lipid A (506). As seen in this figure, *P. diminuta* LPS was able to induce TNF activity in mice, as did synthetic lipid A. Moreover, the TNF-inducing activity of lipid A from *P. diminuta* was very similar to that of *P. diminuta* LPS as well as *E. coli* lipid A. From the serum concentration that caused 50% cytotoxicity against L929 cells, TNF activities of sera treated with the *P. diminuta* and *E. coli* lipid A were calculated to be 3,100 and 4,100, respectively.

To examine the relation of the fatty acyl moiety of *P. diminuta* lipid A to TNF-inducing activity, the activities of three preparations including A3, A2 fractions and hydrazinolyzed lipid A (N_2H_4-PdA) were ex-

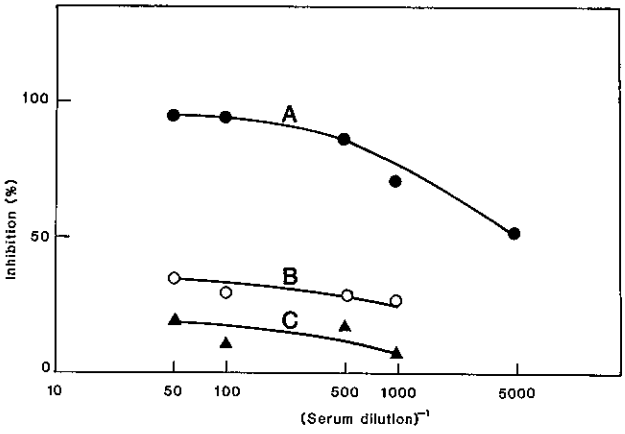

Fig. 3. TNF-inducing activity of three fractions derived from *P. diminuta* lipid A, A3 fraction (curve A, ●), A2 fraction (curve B, ○), and the hydrazinolyzed lipid A, N_2H_4-PdA (curve C, ▲). Induction of TNF by these preparations in mice and measurement of the TNF activities were conducted as described in Fig. 2.

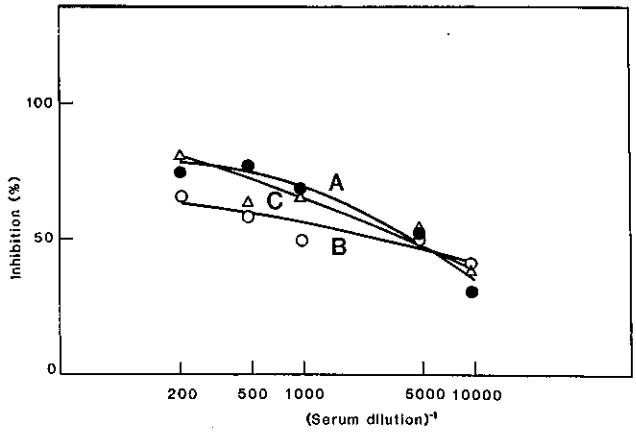

Fig. 4. TNF-inducing activity of *P. vesicularis* lipid A preparations, A3 fraction (curve A, ●), A2 fraction (curve B, ○), and free lipid A (curve C, △). Induction of TNF by these preparations in mice and measurement of the TNF activities were conducted as described in the legend to Fig. 2.

amined. The results (Fig. 3) showed that A3 fraction of *P. diminuta* lipid A had a strong TNF-inducing activity (curve A in Fig. 3). By contrast, A2 fraction exhibited a very weak activity, from which the serum concentration causing 50% inhibition of L929 cells could not be determined (curve B in Fig. 3). The hydrazinolyzed lipid A (N_2H_4-PdA) was hardly active (curve C in the same figure).

TNF-inducing activities of *P. vesicularis* lipid A preparations are shown in Fig. 4. Both lipid A and A3 fraction had strong TNF-inducing activities as shown by curves C and A in Fig. 4. As shown by curve B in the same figure, the A2 fraction expressed a slightly weaker activity as compared to A3 fraction.

Table II summarizes the TNF-inducing activities of various lipid A preparations as well as *P. diminuta* LPS, with standard deviations. As described above, the A3 fractions of *P. diminuta* and *P. vesicularis* lipid A both had significantly stronger TNF-inducing activity than synthetic *E. coli*-type lipid A (506). The A2 fraction derived from lipid A of both strains had clearly weaker TNF-inducing activity as compared to the A3 fraction. In the case of the A2 fraction of *P. diminuta* lipid A, the TNF-inducing activity was so weak that its activity could not be estimated. We repeated the same experiments as in Table II four times and confirmed the reproducibility of these results.

Table II. TNF-inducing Activity of Lipid A Preparations

Preparation		TNF-inducing activity (mean ± SD)
P. diminuta	LPS	5563 ± 2727
	Lipid A	3136 ± 1247
	A3	5655 ± 926
	A2	ND
	N_2H_4-PdA	ND
P. vesicularis	Lipid A	2760 ± 1391
	A3	4200 ± 1883
	A2	2550 ± 650
E. coli F515	Lipid A	4093 ± 564
Synthetic lipid A (506)		1726 ± 629

TNF activity is given as the reciprocal of the final dilution of test serum that results in 50% inhibition of [^{3}H]-thymidine incorporation into L929 cells. Values shown are averages ± SD determined for individual serum samples obtained from three to five mice. ND: not determined; the TNF-inducing activity of these fractions was so weak that the serum concentration causing 50% inhibition of L929 cells could not be estimated.

Discussion

The present study demonstrated that free lipid A of *P. diminuta* and that of *P. vesicularis* express TNF-inducing activity in mice; in both cases the major component (A3) had the strongest activity, while the activity of the A2 fraction was significantly weaker. The activity of *P. diminuta* lipid A fraction which had been subjected to hydrazinolysis was much lower than that of the A2 fraction. The main structural difference between A3 and A2 fractions of both strains has been found in their fatty acyl groups: A2 fraction has an incomplete fatty acyl moiety, which lacks partially amide-linked fatty acids, as compared with A3 fraction. The hydrazinolyzed lipid A (N_2H_4-PdA) contains no acyloxyacyl group. These results suggest that the structure of the hydrophobic part, including amide-linked acyloxyacyl groups, of the lipid A molecule plays an important role in inducing TNF in the sera of mice.

Similar relations between structure and function of purified lipids of *P. diminuta* and *P. vesicularis* have been observed in other assays of biological activities. For example, the local Shwartzman activity of A3 fraction of both strains was about ten-fold greater than that of A2 fraction (unpublished results). The results of lipid A epitope analysis by means of the ELISA-inhibition test[21] with conventional and monoclonal antibodies also showed that both *P. diminuta* and *P. vesicularis* lipid A have closely related, if not identical, epitopes, but the antigenic reactivity significantly decreases on partial deacylation, indicating the important contribution of acyl groups including acyloxyacyl residues (unpublished results).

Since endotoxic reactions are known to be mediated by many cytokines including TNF, interleukin 1 and colony stimulating factor, it is likely that the *P. diminuta* lipid A and *P. vesicularis* lipid A may induce other cytokines as well as TNF. This possibility is under examination.

(Received Jan. 21, 1988/Accepted March 16, 1988)

References

1) Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamsons, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA*, **72**, 3666–3670 (1975).
2) Helson, L., Green, S., Carswell, E. and Old, L. J. Effect of tumor necrosis factor on cultured human melanoma cells. *Nature*, **258**, 731–732 (1975).
3) Wang, A. M., Creasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., Van Arsdell, J. N., Yamamoto, R. and Mark, D. F. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science*, **228**, 149–154 (1985).
4) Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A. and Shepard, H. M. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. *Science*, **230**, 943–945 (1985).
5) Nakaya, K., Nakajo, S., Shimizu, T., Hayashi, Y. and Nakamura, Y. Tumor necrosis factor is partly responsible for the differentiation of mouse myeloid leukemia cells. *Biochem. Int.*, **15**, 1213–1219 (1987).
6) Takeda, K., Iwamoto, S., Sugimoto, H., Takuma, T., Kawatani, N., Noda, M., Masaki, A., Morise, H., Arimura, H. and Konno, K. Identity of differentiation inducing factor and tumour necrosis factor. *Nature*, **323**, 338–340 (1986).

7) Trinchieri, G., Kobayashi, M., Rosen, M., Loudon, R., Murphy, M. and Perussia, B. Tumor necrosis factor and lymphotoxin induce differentiation of human myeloid cell lines in synergy with immune interferon. *J. Exp. Med.*, **164,** 1206–1225 (1986).
8) Tamatani, T., Urawa, H., Hashimoto, T. and Onozaki, K. Tumor necrosis factor as an interleukin 1-dependent differentiation inducing factor (D-factor) for mouse myeloid leukemic cells. *Biochem. Biophys. Res. Commun.*, **143,** 390–397 (1987).
9) Gamble, J. R., Harlan, J. M., Klebanoff, S. J. and Vadas, M. A. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA*, **82,** 8667–8671 (1985).
10) Pober, J. S., Bevilacqua, M. P., Mendrich, D. L., Lapirre, L. A., Fiers, W. and Gimbrone, M. A. Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J. Immunol.*, **136,** 1680–1687 (1986).
11) Collins, T., Lapierre, L. A., Fiers, W., Strominger, J. L. and Pober, J. S. 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,** 446–450 (1986).
12) Philip, R. and Epstein, L. B. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. *Nature*, **323,** 86–89 (1986).
13) Chen, A. R., McKinnon, K. P. and Koren, H. S. Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin D-treated WEHI-164 target cells via increased secretion of a monokine similar to tumor necrosis factor. *J. Immunol.*, **135,** 3978–3987 (1985).
14) Kelker, H. C., Oppenheim, J. D., Stone-Wolff, D., Herriken-Destefano, D., Aggarwal, B. B., Stevenson, H. C. and Vilcek, J. Characterization of human tumor necrosis factor produced by peripheral blood monocytes and its separation from lymphotoxin. *Int. J. Cancer*, **36,** 69–73 (1985).
15) Kasai, N., Arata, S., Mashimo, J., Akiyama, Y., Tanaka, C., Egawa, K. and Tanaka, S. *Pseudomonas diminuta* LPS with a new endotoxic lipid A structure. *Biochem. Biophys. Res. Commun.*, **142,** 972–978 (1987).
16) Kasai, N., Arata, S., Mashimo, J., Okuda, K., Aihara, Y., Kotani, S., Takada, H., Shiba, T. and Kusumoto, S. *In vitro* antigenic reactivity of synthetic lipid A analogues as determined by monoclonal and conventional antibodieds. *Biochem. Biophys. Res. Commun.*, **128,** 607–612 (1985).
17) Wollenweber, H. W., Broady, K. W., Lüderitz, O. and Rietschel, E. T. The chemical structure of lipid A: demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide. *Eur. J. Biochem.*, **124,** 191–198 (1982).
18) Kasai, N. and Yamano, A. Studies on the lipids of endotoxins: thin-layer chromatography of lipid fractions. *Jpn. J. Exp. Med.*, **34,** 329–344 (1964).
19) Ruff, M. R. and Gifford, G. E. Purification and physicochemical characterization of rabbit tumor necrosis factor. *J. Immunol.*, **125,** 1671–1677 (1980).
20) Aggarwal, B. B., Kohr, W. J., Hass, P. E., Moffat, B., Spencer, S. A., Henzel, W. J., Bringman, T. S., Nedwin, G. E., Goeddel, D. V. and Harkins, R. N. Human tumor necrosis factor: production, purification, and characterization. *J. Biol. Chem.*, **260,** 2345–2354 (1985).
21) Kasai, N., Arata, S., Mashimo, J., Okuda, K., Aihara, Y., Kotani, S., Takada, H., Shiba, T., Kusumoto, S., Imoto, M., Yoshimura, H. and Shimamoto, T. Synthetic *Salmonella*-type lipid A antigen with high serological specificity. *Infect. Immun.*, **51,** 43–48 (1986).