Cancer Immunol Immunother (1985) 20: 6-10

Cancer Immunology Immunotherapy

© Springer-Verlag 1985

Tumour necrosis factor and the lysosomal enzymes of macrophages or macrophage-like cell line

Akiko Sakurai, Nobuko Satomi, and Katsuyuki Haranaka

Department of Internal Medicine, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108 Japan

Summary. The relationship between tumour necrosis factor (TNF) and macrophages or macrophage-like cell line, especially the lysosomal enzymes was investigated. The serum lysosomal enzymes and LDH activities were increased in proportion to the TNF production even in different strains of mice. Lysosomal enzymes and TNF activity were released into the supernatant of the culture medium of macrophage-enriched peritoneal exudate cells (PEC) or spleen cells derived from *Propionibacterium acnes*-primed mice after addition of lipopolysaccharide (LPS). After passage through a Sephadex G-10 column, TNF activity could not be detected in the supernatant of these spleen cells after addition of LPS. Also TNF activity could not be detected in the supernatant following destruction of PEC. These results suggest that TNF producibility is strongly related to the degree of activation of macrophages, especially the lysosomal enzymes. The murine macrophage-like cell line, J 774, also released TNF activity and lysosomal enzymes after addition of LPS.

Introduction

Tumour necrosis factor (TNF) was discovered in 1975 as an active component of anti-tumour molecules in the serum of BCG-primed mice challenged with endotoxin [2]. The anti-tumour activity of TNF has been thoroughly investigated [4, 5, 8, 9, 13, 14]. Large differences in TNF productive ability have been observed among various rodents as reported by us [6]. Nevertheless, all animals receiving priming agents revealed hyperplasia of the reticuloendothelial system, and the TNF producibility has been found to be extremely different. The sensitivity of the animals to lipopolysaccharide (LPS) is considered to be the important factor in their TNF productive ability.

In our previous report, it was strongly suggested that the production site of TNF is activated macrophages [15]. That is, TNF production was completely blocked following the injection of a large dose of carrageenan, administered before receiving priming agents. Administering trypan blue or steroids which are known as lysosomal enzymes' inhibitor or membrane stabilizers before the LPS injection blocked TNF production. Therefore we speculated that a close relation existed between lysosomal enzymes and TNF production.

This report examines the participation of macrophages in the production of TNF in relation to the lysosomal enzymes in vivo employing different strains of mice and in vitro employing activated macrophages derived from *Propionibacterium acnes (P. acnes)*-primed mice. Furthermore, the production of TNF in relation to lysosomal enzymes in vitro employing the murine macrophage-like cell line, J 774, was examined.

Offprint requests to: A Sakurai

Materials and methods

Animals. DDY strain mice were purchased from Shizudokyo (Shizuoka, Japan), C3H/HeNCrj strain mice from Charles River Japan (Kanagawa, Japan) and C3H/HeJms, DDD and C57Bl/6J strain mice were provided by the Animal Facilities of our Institute.

Macrophages. The peritoneal exudate cells (PEC) or spleen cells were harvested from *P. acnes*-primed mice or control mice. Adherent cells were used for macrophages. The J 774, murine macrophage-like cell line, was also used.

Schedule of TNF production. The time course of TNF production was investigated using DDY strain mice. Formalin-killed *P. acnes* (1 mg/mouse) was injected i.p. as a priming agent, then 9 days later 10 µg/mouse of LPS of *Escherichia coli* 0111:B4*w* (Difco Lab., Mich. USA) was injected i.v. Blood was collected from the post-orbital venous plexus using a micropipette 2 h after the LPS administration.

In vitro TNF assay. Serially diluted test samples and 2×10^5 L(S) cells, mouse fibroblasts which are sensitive to TNF, were mixed and incubated for 48 h at 37 °C in 5% CO_2 in air. After removal from the medium, cells were fixed with methanol and stained with 0.05% methylene blue. The dye extract was measured using a spectrophotometer and the 50% cytotoxicity was assessed. L(R) cells, resistant to TNF, were incubated as above in order to exclude other contaminating biologically active substances.

Enzyme assay. The lysosomal enzymes, acid phosphatase (AcP) and β-glucuronidase (β-Gase), and lactate dehydrogenase (LDH) of the serum or medium were measured. Acid phosphatase (EC 3.1.3.2.) was determined by the method of Kind and King using disodium phenyl phosphate as substrate [11]. β-Glucuronidase (EC 3.2.1.31) was determined by the method of Kato using p-nitrophenyl-β-D-glucuronide as substrate [10], and the activity was expressed in µmole/ml per h. For estimating the activity in

the culture medium, the incubation time was prolonged to 18 h. Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Bergmeyer and Bernt [1]. Enzyme activities were always assayed immediately.

Time course of TNF activity and enzyme activities. Prior to the administration of *P. acnes* or on the 5th, 9th, 14th and 21st day after administration, mice were injected with 10 µg/mouse of LPS and blood was collected 2 h later. On the 9th day, at 30, 60, 90 and 120 min after LPS administration, blood was collected. The serum was separated and the TNF or enzyme activities were measured.

Strain difference. DDD, C57Bl/6J, C3H/HeNCrj or C3H/HeJms mice were given 100 µg/mouse of LPS, and blood was collected 2 h later. The spleen weights were monitored in all mice, and TNF or enzyme activities were measured individually. One group of each different strain consisted of five or more mice.

Measurement of TNF or enzyme activities released from macrophages or J 774. The adherent cells of the PEC or spleen cells from *P. acnes*-treated mice were incubated with 10 µg/ml of LPS. Whole spleen cells or Sephadex G-10 eluted cells were also examined. Release of cytoplasmic LDH was used as an indicator of cell death. The TNF activity following cell destruction with Triton X-100 was also estimated. Also J 774 cells were incubated with 10 µg/ml of LPS.

Results

Time course of TNF activity and enzyme activity

The TNF activity was highest 9 days after administration of *P. acnes,* with subsequent addition of LPS. The AcP, β-Gase and LDH activities were also highest at 9 days (Fig. 1). The TNF production and serum enzyme levels recorded at each time interval are shown in Fig. 2, TNF activity could be detected in the sera at 60 min after the administration of LPS. The serum enzyme levels were also increased in proportion to the increment in TNF activity.

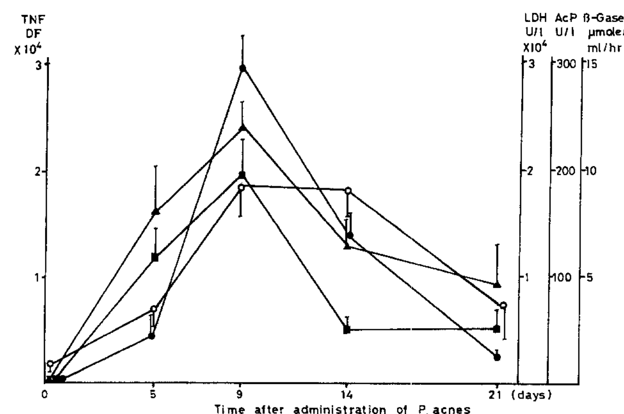

Fig. 1. Time course of TNF activity and enzyme activities after administration of *P. acnes. P. acnes* (1 mg/mouse) was injected i.p. as priming agent. Several days after the administration, LPS (10 µg/mouse) was injected i.v. and 2 h later blood was collected. The TNF activity (●) and Acp (○), β-Gase (▲) and LDH (■) were then measured. DF: Dilution factor giving 50% L cell cytotoxicity. ($n=5$, Mean ± SD)

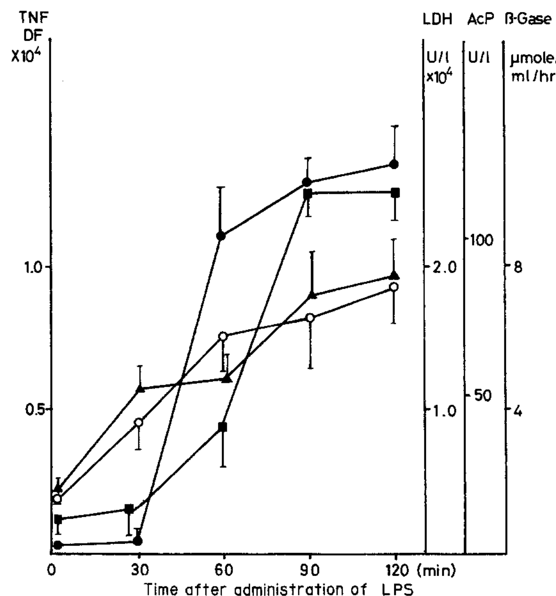

Fig. 2. Time course of TNF activity and enzyme activities after administration of LPS. The TNF activity (●) and enzyme activities (AcP (○), β-Gase (▲) and LDH (■)) at each interval after administration of LPS were measured. ($n=5$, Mean ± SD)

TNF activity, enzyme activities and spleen weight in different strains of mice

There was a good positive correlation between the TNF activity and serum AcP levels in the different strains of mice (Fig. 3 a). There was also a good positive correlation between the TNF activity and β-Gase (Fig. 3 b) or LDH (Fig. 3 c). Hepatosplenomegaly and TNF activity were parallel among mice of the same strain. However, there was no correlation between the TNF activity and spleen weight among different strains of mice (Fig. 3 d).

TNF production and enzyme activities in vitro

TNF activity could be detected in the supernatant of PEC from *P. acnes*-primed mice after a 1-h incubation with LPS. After a 1-h incubation, the AcP, β-Gase and LDH activities were also increased. The release of TNF or these enzymes was reduced 3 h after the addition of LPS (Fig. 4). Following mechanical destruction of the PEC, TNF activity could not be detected, but the LDH activity was remarkably increased (Table 1). Also TNF activity could be detected in the supernatant of whole spleen cells or adherent spleen cells from *P. acnes*-primed mice after a 1-h incubation with LPS. In contrast, Sephadex G-10 eluted spleen cells did not release TNF activity into the supernatant (Table 1). Using macrophage-like cell line, TNF activity could be detected after 3 days incubation with LPS. The release of these enzyme activities was also increased by addition of LPS (Fig. 5).

Discussion

The production of TNF has been speculated to be within activated macrophages, and to be closely related to lysosomes [15]. Priming agents, such as BCG, *P. acnes* or zymosan, have been shown to act as stimulants of the reticuloendothelial system [2]. These agents induce hyperplasia of macrophages, and an easy sign of this is the degree of hep-

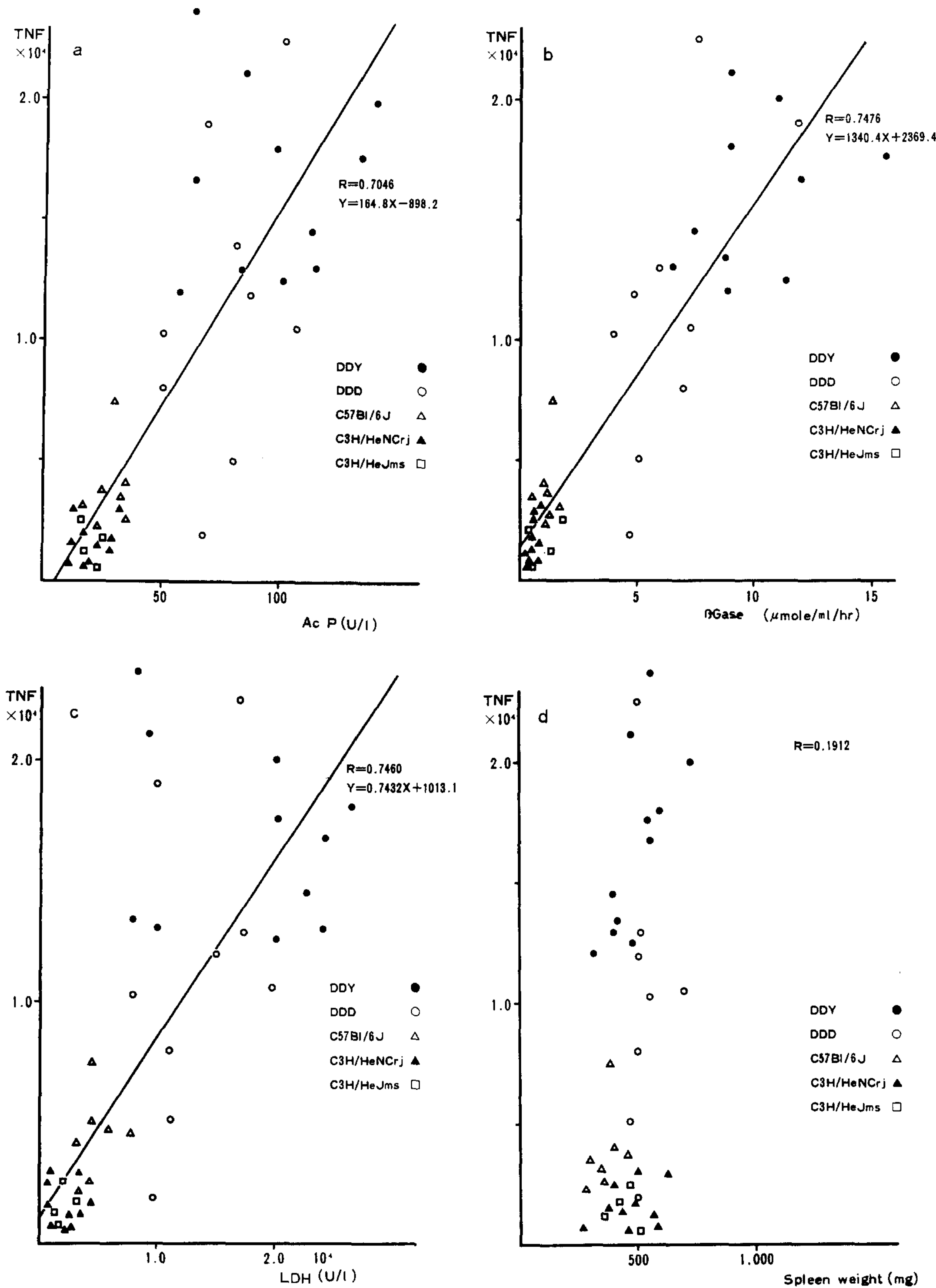

Fig. 3. Correlation between TNF activity and enzyme activities or spleen weight in different strains of mice. Mice received 1 mg/mouse of *P. acnes*, and 9 days later LPS was injected i.v. (DDY: 10 μg/mouse, other strains of mice: 100 μg/mouse). The TNF activity, enzyme activities and the spleen weights were measured individually. (a) TNF *vs.* AcP (b) TNF *vs.* β-Gase, (c) TNF *vs.* LDH, (d) TNF *vs.* spleen weight

atosplenomegaly [17]. In contrast, the administration of carrageenan, which has been reported to be cytotoxic for macrophages [16], before the injection of priming agents completely blocked the production of TNF [15]. The TNF producibility is also influenced by the kinds of stimulants [3], so *P. acnes* was selected as the priming agent due to its good TNF producibility [7].

Nine days after *P. acnes* administration, most of the contents of the PEC were mononuclear cells, and TNF was produced from these PEC by the addition of LPS to the medium in vitro (Fig. 4). Morphological changes are caused by the addition of LPS, namely that the cytoplasm gradually filled with phase-lucid vacuoles and was paralleled by the disappearance of the granules. Since these results clearly indicated the importance of lysosomal enzymes, the activities of lysosomal enzymes were measured in relation to the TNF production. Increment of AcP or β-Gase was found to parallel the levels of TNF production

Table 1. TNF production and enzyme activities *in vitro*

Treatmant	TNF activity (DF)	AcP (U/1)	LDH (U/1)
PEC (no treatment)	0	0.36 ± 0.09	79.3 ± 13.3
PEC with 10 μg/ml of LPS	1607.9 ± 136.6	2.17 ± 0.06	281.4 ± 9.9
PEC (Triton X)	0	30.33 ± 1.80	3363.6 ± 379.2
Whole spleen cells (WSC)	0	0.03 ± 0.01	50.0↓
WSC with 10 μg/ml of LPS	4.8 ± 0.4	0.68 ± 0.03	50.0↓
Sephadex G-10 eluted spleen cells (G10SC)	0	0.02↓	50.0↓
G10SC with 10 μg/ml of LPS	0	0.02↓	50.0↓
Adherent spleen cells (ASC)	0	0.13 ± 0.05	68.1 ± 16.0
ASC with 10 μg/ml of LPS	482.6 ± 59.8	1.94 ± 0.09	204.1 ± 18.9

Cells were incubated with and without LPS. Cell destruction was carried out using Triton X-100. TNF activity and enzyme activity were expressed (1×10^6 cells). (WSC: whole spleen cells, G10SC: Sephadex G-10 eluted spleen cells, ASC: adherent spleen cells.)

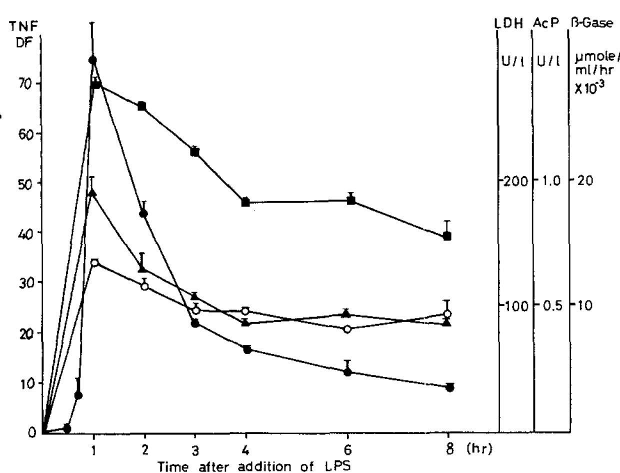

Fig. 4. TNF production and enzyme activities *in vitro*. PEC derived from *P. acnes*-primed mice were incubated with 10 μg/ml of LPS. The TNF activity (●) and enzyme activities (AcP (○), β-Gase (▲) and LDH (■) in the medium were measured. (n=4, Mean ± SD)

in vivo and in vitro. Sera with high TNF levels always contained a high level of AcP or β-Gase. The LDH level also paralleled the production of TNF. However, the LDH demonstrated cell destruction that was not specific to the lysosomal enzymes of macrophages (Table 1).

Large differences in producibility of TNF exist among different strains of mice [6]. The TNF producibility paralleled the degree of stimulation of the reticuloendothelial system and release of lysosomal enzymes in DDY, DDD and C57Bl/6J mice. However, in C3H/HeNCrj or C3H/HeJms mice, the TNF production was poor even when a sufficient dose of LPS was injected (Fig. 3). It was known that administered LPS is soon absorbed by the liver and spleen and selective lysis of Kupffer cells and splenic macrophages was observed shortly after LPS injection. Sensitivity of mice to LPS was increased after vaccination with BCG [18]. These phenomena resembled those of in vitro experiments.

Männel et al. reported that macrophage-enriched PEC from mice infected with BCG, macrophage-like tumour cells (PU 5-1, 8) and peritoneal macrophages propagated in vitro with macrophage growth factor, released tumouricidal activity into the culture medium within 2 or 3 h after stimulation with LPS. They estimated the molecular weight of this factor to be 50 K to 60 K by gel filtration [12]. Our experiments revealed that the molecular weight of TNF produced in vitro from PEC derived from *P. acnes*-primed

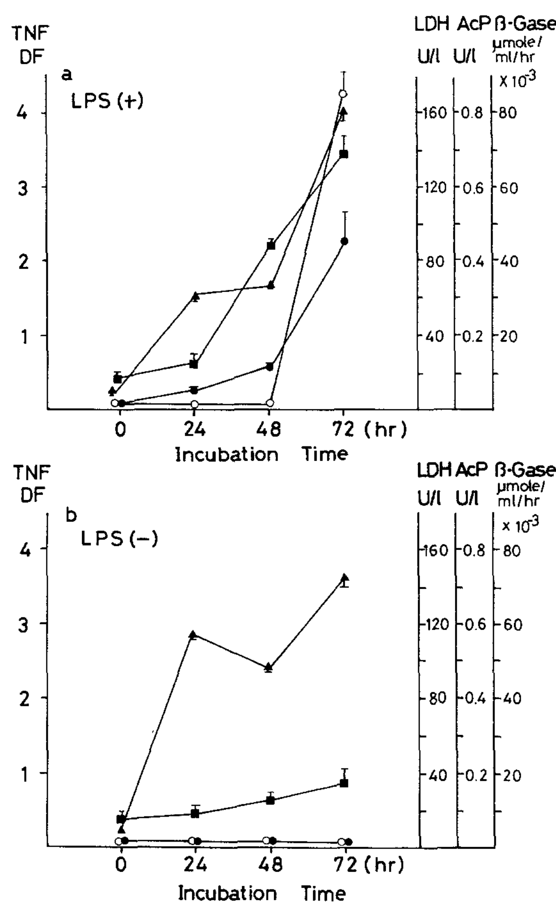

Fig. 5. TNF production and enzyme activities from the macrophage-like cell line, J 774, *in vitro*. J 774 cells were incubated with 10 μg/ml of LPS (a). The TNF activity (●) and enzyme activities (AcP (○), β-Gase (▲) and LDH (■)) were measured every day. The medium of J 774 cells without LPS were also measured (b).
TNF activity: with LPS *vs* without LPS, $P < 0.0001$
AcP activity: with LPS *vs* without LPS, $P < 0.0001$
LDH activity: with LPS *vs* without LPS, $P < 0.001$
β-Gase activity: with LPS *vs* without LPS, $P > 0.1$
(n=4, Mean ± SD)

mice was 39 K to 40 K as estimated by gel filtration, which was the same as that produced in mice after administration of LPS [4]. We succeeded in producing TNF from macrophage-like tumour cells (J 774) under conditions of priming stimulation. The TNF activity was released 6 to 8 h after stimulation with LPS. The molecular weight of TNF from tumour cell lines was found to be the same as above. The necrotizing activity against transplanted Meth A sarcoma was the same as that produced in mice (unpublished work). Enzyme activities could be detected in TNF from tumour cell lines without priming stimulation even when the TNF was low (Fig. 5).

Crude TNF samples always contain high lysosomal enzyme activities. However, in purified TNF samples, no such enzyme activities could be detected (data not included here).

In conclusion, it can be said that TNF producibility is closely related to the degree of activation of macrophages, especially the lysosomal enzymes. Also, TNF could be produced from macrophage-like tumour cells (J 774) after the addition of LPS, and its activity and molecular weight were the same as that produced in blood.

Acknowledgements. This work was supported in part by a Grant-in-Aid from the Ministry of Education of Japan. The authors gratefully acknowledge the helpful advice of Dr. L. J. Old, Sloan-Kettering Institute for Cancer Research, New York, USA, and Prof. S. Miwa, Institute of Medical Science, University of Tokyo.

References

1. Bergmeyer HU, Bernt Z (1974) Lactate dehydrogenase. In: Methods of Enzymatic Analysis. Academic Press, New York, Vol. 2, P. 574
2. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72: 3666
3. Cummins CS, Stimpson SA, Tuttle RL, Weck CJ (1981) Observation on the nature of the determinants in anaerobic Coryneforms which produce splenomegaly. J Reticuloendothel Soc 29: 1
4. Haranaka K, Satomi N (1981) Cytotoxic activity of tumor necrosis factor (TNF) on human cancer cells *in vitro.* Jpn J Exp Med 51: 191
5. Haranaka K, Satomi N, Sakurai A (1984) Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. Int J Cancer 34: 263
6. Haranaka K, Satomi N, Sakurai A (1984) Differences in tumor necrosis factor productive ability among rodents. Br J Cancer 50: 471
7. Haranaka K, Satomi N, Sakurai A, Haranaka R (1984) Role of first stimulating agents in the production of tumor necrosis factor. Cancer Immunol Immunother 18: 87
8. Helson L, Green S, Carswell EA, Old LJ (1975) Effect of tumor necrosis factor on cultured human melanoma cells. Nature 258: 731
9. Helson L, Helson C, Green S (1979) Effects of murine tumor necrosis factor on heterotransplanted human tumors. Exp Cell Biol 47: 53
10. Kato K, Yoshida K, Tsukamoto H, Nobunaga M, Masuya T, Sawada T (1960) β-D-Glucopyranosiduronic acid and its utilization as a substrate for the assay of β-glucuronidase activity. Chem Pharm Bull 8: 239
11. Kind PRN, King EJ (1954) Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. J Clin Path 7: 322
12. Männel N, Moore RN, Mergenhagen SE (1980) Macrophages as a source of tumoricidal activity (tumor necrotizing factor). Infect Immun 30: 523
13. Matthews N, Watkins JF (1978) Tumor-necrosis factor from the rabbit. I. Mode of action, specificity and physicochemical properties. Br J Cancer 38: 302
14. Old LJ (1976) Tumor necrosis factor. Clin Bull 6: 118
15. Satomi N, Haranaka K, Kunii O (1981) Research on the produciton site of tumor necrosis factor. Jpn J Exp Med 51: 317
16. Sawiki JE, Catanzaro RJ (1975) Selective macrophage cytotoxicity of carrageenan *in vivo.* Int Arch Allergy Appl Immun 49: 709
17. Schnyder J, Boggiolini M (1978) Secretion of lysosomal hydrolases by stimulated and nonstimulated macrophages. J Exp Med 148: 435
18. Suter E, Ullman GE, Hoffman RG (1958) Sensitivity of mice to endotoxin after vaccination with B.C.G. (Bacillus Calmette-Güerin) Proc Soc Exp Biol Med 99: 167

Received February 6, 1985/Accepted March 12, 1985