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Role of first stimulating agents in the production of tumor necrosis factor

Katsuyuki Haranaka[1], Nobuko Satomi[1], Akiko Sakurai[1], and Ruriko Haranaka[2]

[1] Department of Internal Medicine, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan
[2] Department of Biochemistry, Nihon University School of Medicine, 30-1, Ooyaguchikamimachi, Itabashi-ku, Tokyo 173, Japan

Summary. *The conditions and kinetics of tumor necrosis factor (TNF) production were examined. For TNF production, dual stimulation is necessary. Priming agents such as BCG, Corynebacterium parvum, and zymosan, which can stimulate the reticuloendothelial system (RES), are good substances for TNF production with the aid of lipopolysaccharide. Wide differences are observed in TNF producibility among different priming agents. The producibility of TNF depends on the degree of stimulation of the RES by the priming agents. Those priming agents, e.g., Propionibacterium acnes and Corynebacterium anaerobium, that are able to induce substantial RES hyperplasia are also able to induce high levels of TNF activity. Following administration of large doses of BCG or zymosan, mice were found to produce TNF activity. However, PPD, OK 432, PSK, and Choreito were unable to induce TNF activity.*

Introduction

The antitumor effects of bacterial cells and their products have been known for over 100 years, since the work of Bruns and Coley [16]. Parenteral administration of bacterial products such as lipopolysaccharide (LPS) to experimental animals or human patients can produce a severe hemorrhagic reaction within tumors. If administered in effective amounts, LPS causes circulatory collapse and then death [1]. When tumor-bearing animals are treated with other immunostimulatory agents, such as BCG or *Corynebacterium parvum (C. parvum)*, significant regression rates can be obtained [14, 15].

Carswell et al. [2] demonstrated a necrotizing factor in the serum of BCG-primed mice challenged with endotoxin. This factor, termed tumor necrosis factor (TNF), causes hemorrhagic necrosis of tumors in vivo and displays cytotoxicity against cancer cells in vitro [4, 5, 8, 12]. TNF is presumably produced by macrophages [9, 20] and can be purified from the sera of presensitized animals with endotoxin shock [13]. TNF seems to have unique characteristics, e.g., absence of species specificity [11] and the ability to discriminate between normal and certain tumor cells in vitro [4, 16].

It has been reported that priming agents that stimulate the reticuloendothelial system (RES) are helpful in the production of TNF [2]. The best eliciting agent of TNF production is LPS, especially its lipid A portion [7]. It is commonly accepted that dual stimulation is necessary for the production of TNF. In the present study the conditions and kinetics of TNF production were examined and wide differences were noted among various priming agents in the amounts of TNF it was possible to produce.

Offprint requests to: K. Haranaka

Materials and methods

Animals. DDY strain mice (Shizudokyo, Shizuoka, Japan) were used for the production of TNF due to the good TNF production noted in this strain in the past [6].

Vaccine and chemicals. The following materials were used: *C. parvum* (Wellcome Reagents Limited, Beckenham, England); *Propionibacterium acnes (P. acnes)* IID 912 [ATCC 11827], *P. granulosum* IID 902 and *Listeria monocytogenes (L. monocytogenes)* IID 573 [NIAH L8-12028] (provided by our Institute); *C. anaerobium* strains 575 and 585, and *C. parvum* ATCC 11829 (from Nihon University); *P. acnes* ATCC 11828 and ATCC 6919, and *P. granulosum* ATCC 25564 (from Gifu University); BCG vaccine (from B.C.G. Co. Ltd, Tokyo, Japan); PPD from *Mycobacterium tuberculosis* $H_{37}Ra$ strain (Mitsui Chemical Industry, Tokyo, Japan); OK 432 (Picibanil) (*Streptococcus pyogenes* Group A type 3; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan); PSK (Krestin) (*Coriolus versicolor* Qu'el CM-101 strain; Kureha Chemical Industry, Tokyo, Japan); zymosan A *(Saccharomyces cerevisiae;* Sigma Chemical Co., Mo., USA); Chinese medicine (Choreito) (Tsumura Juntendo Inc., Tokyo, Japan), and LPS of *Escherichia coli* 0111: B4 *w* (Difco Lab., Mich., USA).

Schedule of TNF production. Doses of 1, 2, or 4 mg/mouse of formalin-killed bacteria were administered IP 9 days prior to injection of LPS 10 μg IV. Blood was collected 2 h after the administration of LPS. Each group consisted of five mice. The spleen and liver weights were determined as convenient markers of RES stimulation. TNF activity was expressed as the mean dilution factor which revealed 50% killing activity of L(S) cells (mouse fibroblast cells).

BCG 0.05, 0.1, 0.2, 1.0, or 5 mg/mouse (3.11×10^8 cells/0.5 mg), PPD 125 or 250 μg/mouse, OK 432 0.5 or 1 KE/mouse, or zymosan A 5, 10, or 20 mg/mouse was injected IP. Ten 0.5-KE doses of OK 432 were given by SC injection. PSK 5 or 10 mg/mouse/day for 2 weeks and Choreito 3, 6 or 130 mg/mouse/day for 2 weeks were administered PO.

TNF assay in vitro. Serially diluted TNF test samples were incubated with 2×10^5/ml of L(S) cells in a 96-well microplate

Table 1. TNF activity and spleen and liver weights induced by various kinds of priming agents

Stimulants	Dose (mg)	Route	Spleen weight[a] (mg)	Liver weight[a] (mg)	TNF activity	
					L assay (DU)[b]	Meth A[c]
P. acnes	1	IP	315.2 ± 54.3	1,673.2 ± 165.6	18,249.5 ± 10,899.4	+++
IID 912	2	IP	381.9 ± 55.0	2,201.7 ± 176.8	55,726.6 ± 11,549.3	+++
ATCC 11827	4	IP	561.8 ± 135.3	2,594.5 ± 356.3	64,012.5 ± 4,641.5	+++
C. parvum	1	IP	321.4 ± 89.1	2,105.2 ± 250.6	6,387.4 ± 2,513.4	++
Wellcome	2	IP	569.9 ± 96.9	2,547.4 ± 773.0	20,477.9 ± 3,944.4	+++
ATCC 11829	4	IP	666.3 ± 91.3	3,128.7 ± 213.9	19,819.5 ± 10,411.1	+++
P. acnes	1	IP	298.8 ± 94.7	1,915.9 ± 315.6	7,133.0 ± 1,619.1	++
Nihon Univ. ATCC 11829	2	IP	546.7 ± 120.1	2,482.7 ± 251.4	25,341.7 ± 1,215.1	+++
P. acnes	1	IP	364.5 ± 81.7	2,247.2 ± 206.2	33,599.6 ± 11,437.6	+++
Gifu Univ ATCC 11828	2	IP	468.1 ± 126.0	2,376.2 ± 174.2	36,242.7 ± 14,659.1	+++
P. acnes	1	IP	373.2 ± 223.3	1,657.9 ± 318.5	164.8 ± 240.6	–
Gifu Univ. ATCC 6919	2	IP	579.1 ± 107.9	1,628.6 ± 536.7	12,100.0 ± 6,447.9	+++
C. anaerobium	1	IP	412.5 ± 105.6	1,615.6 ± 413.2	16,812.5 ± 2,015.7	+++
Nihon Univ. strain 575	2	IP	587.7 ± 124.1	1,958.1 ± 381.7	27,182.4 ± 1,050.1	+++
C. anaerobium	1	IP	342.1 ± 49.5	1,719.2 ± 210.1	10,251.6 ± 4,136.5	++
Nihon Univ. strain 578	2	IP	498.7 ± 147.0	2,056.1 ± 181.9	24,982.1 ± 2,175.7	+++
P. granulosum	1	IP	240.4 ± 42.5	1,518.9 ± 115.1	804.6 ± 1,023.4	–
IID 902	2	IP	318.7 ± 105.6	1,641.8 ± 205.1	1,259.1 ± 895.1	–
P. granulosum	1	IP	199.2 ± 28.6	1,449.7 ± 116.5	4,060.6 ± 3,893.4	+
ATCC 25564	–	IP	211.2 ± 32.8	1,530.4 ± 120.0	6,509.0 ± 2,879.2	++
L. monocytogenes	1	IP	201.1 ± 45.3	1,542.8 ± 145.1	23.9 ± 58.5	–
IID 573	2	IP	239.4 ± 39.7	1,741.1 ± 189.9	159.7 ± 41.5	–
(−)	(−)		117.5 ± 29.2	1,325.0 ± 65.7	–	–

[a] Spleen and liver weights on 9th day. Mean ± SD ($n = 5$)
[b] In vitro cytotoxicity test against L(S) cells (DU, dilution units)
[c] In vivo test against Meth A sarcoma. The degree of tumor necrosis was assessed 24 h later

Table 2. TNF activity and spleen and liver weights induced by various kinds of priming agents

Stimulants	Dose (mg)	Route	Spleen weight[a] (mg)	Liver weight[a] (mg)	TNF activity	
					L assay (DU)[b]	Meth A[c]
BCG	0.05	IP	138.2 ± 16.1	1,309.1 ± 117.9	–	–
	0.1	IP	132.4 ± 25.7	1,414.8 ± 107.3	–	–
	0.2	IP	180.3 ± 17.6	1,317.7 ± 111.0	–	–
	1	IP	201.5 ± 13.8	1,317.2 ± 40.0	–	–
	5	IP	358.3 ± 79.4	1,778.5 ± 335.6	6,387.1 ± 6,886.7	+
PPD	0.125	IP	134.7 ± 34.4	1,273.3 ± 102.3	–	–
	0.25	IP	129.4 ± 11.5	1,243.6 ± 39.5	–	–
OK 432	0.5 KE	IP	102.7 ± 20.5	1,254.6 ± 38.1	–	–
	1 KE	IP	110.5 ± 19.8	1,263.7 ± 95.1	–	–
	0.5 KE × 10 T	SC	238.7 ± 53.0	1,322.5 ± 192.5	18.4 ± 7.2	–
PSK	5 × 14 T	PO	170.9 ± 29.3	1,339.6 ± 97.6	–	–
	10 × 14 T	PO	185.3 ± 11.8	1,312.4 ± 127.0	–	–
Zymosan	5	IP	207.2 ± 38.3	1,243.8 ± 197.2	–	–
	10	IP	280.3 ± 18.1	1,424.7 ± 127.0	3,467.8 ± 2,497.3	+
	20	IP	339.0 ± 75.2	1,472.4 ± 171.0	2,578.4 ± 1,174.4	+
Chinese medicine	3 × 14 T	PO	191.9 ± 16.8	1,375.2 ± 89.3	–	–
(Choreito)	6 × 14 T	PO	211.2 ± 28.3	1,584.5 ± 95.2	–	–
	130 × 14 T	PO	197.4 ± 10.9	1,461.7 ± 24.0	–	–
(−)	(−)		117.5 ± 29.2	1,325.0 ± 65.7	–	–

[a] Spleen and liver weights on 9th day or 14th day (PO) Mean ± SD ($n = 5$)
[b] In vitro cytotoxicity test against L(S) cells (DU, dilution units)
[c] In vivo test against Meth A sarcoma. The degree of tumor necrosis was assessed 24 h later

for 48 h in 5% CO_2 in air at 37° C. The dilution of TNF sample having 50% cytotoxicity was assessed by the dye exclusion method. To exclude interferon activity we also used L(R) cells, which are resistant to TNF but sensitive to interferon.

TNF assay in vivo. TNF activity was also bioassayed in Balb/c mice bearing 7-day SC transplants of methylcholenthrene-induced fibrosarcoma (Meth A) averaging 7–8 mm in diameter. The tumor necrosis developing at 24 h after IV injection of 0.5 ml mixed sera (each group consisted of 5 mice) was examined to assess the TNF activity.

Results

The formalin-killed bacteria, BCG, and zymosan all induced hepatosplenomegaly. The TNF activity paralleled the degree of hepatosplenomegaly. The coefficient of correlation between TNF activity and spleen weight was 0.8554, and that between TNF activity and liver weight was 0.7450. As shown in Table 1, *P. acnes* IID 912 was found to be the best priming agent, followed by *P. acnes* ATCC 11828 and ATCC 11829. *P. granulosum* was fourth. As shown in Table 2, BCG and zymosan were also able to prime mice for TNF activity, but their ability was far inferior to that of the anaerobic *Corynebacterium* group. If a large dose of BCG or zymosan was administered the mice produced TNF activity. PPD, OK 432, PSK, and Choreito induced only slight splenomegaly and TNF activity was not observed after these preparations.

Discussion

Green et al. [3] reported that mice primed with *C. parvum* strain CN 6134 could produce high TNF activity, whereas *C. parvum* strain CN 5888 did not prime the mice for TNF production. On the basis of our experiments it was considered that important points for TNF production were the kind of bacterial strain and the dose of vaccine. In the *Corynebacterium* group *P. acnes* is the best priming agent, and *C. anaerobium, P. granulosum* and *L. monocytogenes* are inferior to *P. acnes*.

BCG and zymosan are known to be good priming agents [2]. However, in the present study, the amounts of TNF produced with BCG or zymosan were very low compared with other reports (Table 2). A standard assay system has not yet been established and awaits international agreement.

OK 432 is reported to be a cytotoxic agent and a host defense stimulator. In vitro contact of Yoshida sarcoma or rat ascites hepatoma cells with OK 432 revealed a direct cytocidal action, and in in vivo studies OK 432 demonstrated a host-mediated antitumor activity [10, 18, 19]. In the present study, OK 432 was chosen for TNF production to examine whether TNF was involved in this phenomenon. OK 432-primed mice produced cytotoxic substances that were cytotoxic not only against L(S) cells but also against L(R) cells, even if the cytotoxicity was very low. L(R) cells were chosen as a TNF-resistant and interferon-sensitive cell line. Saito et al. reported that OK 432 was an interferon inducer [17], and that interferon-γ induced by OK 432 had antitumor effects on Meth A sarcoma [18].

Morphologically speaking, spleen and liver from bacteria-stimulated mice revealed infiltration of mononuclear cells, but those from OK 432-stimulated mice revealed polymorphonuclear cell infiltration. The OK 432-stimulated mice had slight lymphoadenopathy, but a lower degree of hepatosplenomegaly. Single injections of OK 432 induced neither hepatosplenomegaly nor TNF activity, while only a low degree of splenomegaly and no TNF production were observed after several injections.

PSK has been found to display antitumor activity against several experimental tumors, including sarcoma 180, hepatoma AH-13, 3-methylcholanthrene-induced fibrosarcoma, and squamous cell carcinoma [21, 22]. It is speculated that the activity was exerted through a host-mediated immunomechanism.

Choreito is one of the prescriptions of traditional Chinese medicine and contains Polyporus and Hoelen, which are known to be RES stimulants. This preparation was administered PO. Slight hepatosplenomegaly and no TNF production were observed in mice treated with it.

Our previous study indicated that the production of TNF was located in activated macrophages [20]; that is, following injection of a large dose of carrageenan before *P. acnes* treatment TNF production was completely blocked. Macrophage-enriched peritoneal exudate cells (PEC) taken from mice infected with *P. acnes* revealed TNF activity in the supernatant after stimulation with LPS.

On the basis of our findings, it is considered that for the production of TNF, *P. acnes* is the best stimulant of the RES and the amount of TNF that can be produced is dependent on the degree of RES stimulation by priming agents. As mentioned by us in other reports, there are wide differences in TNF-productive activity among different strains in mice. The sensitivity of animals to LPS is considered to represent another important factor their ability to produce TNF [6].

In conclusion, it can be said that priming agents that can induce a high degree of RES hyperplasia are also able to induce high levels of TNF activity.

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