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Antitumor Effects of Natural-Human TNF on BDF1 Mice Bearing Lewis Lung Carcinoma

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ABSTRACT

Natural-human tumor necrosis factor (n-TNF) was obtained by isolating and refining lymphokines which were extracted from human acute lymphoblastic leukemia BALL-1 cells. Antitumor effects of this n-TNF were studied by using Lewis lung carcinoma (3LL) which was transplanted on BDF1 mice.

n-TNF showed inhibitory effects of the proliferation of metastatic tumors dose-dependently through i.v. injection daily for 10 days. And the study of the dose schedule of the administration and the route of the administration showed that routes of i.v., i.m. and i.t. injections were effective respectively through daily administration. Histological study showed effects which were ranked Grade IIb (and partially III) of Shimosato and Ohboshi's histological criteria.

Tumor necrosis factor (TNF) is remarked because of its strong antitumor effects, and many investigations have been reported. TNFs which were reported in many papers are recombinate type TNF (r-TNF). However, recently, it became possible to obtain a large amount of natural type TNF (n-TNF), when BALL-1 cells (human acute lymphoblastic leukemia cell line) which are mass-produced by the Hayashibara hamster method are treated with HVJ[10].

In the present report, antitumor effects of the n-TNF were discussed regarding the dose schedule of the administration, the route of the administration and histological antitumor effects by using Lewis lung carcinoma (3LL) of mice.

MATERIALS AND METHOD

n-TNF

Natural-human TNF was supplied by Hayashibara biochemical Laboratories, Inc., Okayama, and used. n-TNF, with the molecular weight of 17,000, is a protein which is stable at the isoelectric point from 5.2 to 6.2 at 52°C for 30 min, which consists of 161 amino acids[10]. It's cytotoxicity was determined by the high sensitive and rapid assay of Eifel et al[5] for lymphotoxin, using mice L929 cell as the reciprocal of the dilution that effects cytopathic effect (CPE) in 50% of the target cells.

Experimental animal

Eight-weeks old female BD (C57BL/6 × DBA/2) F1 mice, about 25g each, were purchases from Shizuoka Laboratory Animal Center, Shizuoka, Japan, and used in the experiments.

Tumor

Lewis lung carcinoma (3LL) cells successively maintained subcutaneously in C57BL/6 mice by the First Department of Surgery, Okayama University medical School were used. For the experiment, the tumor was excised aseptically on the 10th day from the successive transplantation, minced, washed three times in Hanks

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solution, treated with 0.25% trypsin (Difco Co., USA) at 37°C for 15 min, washed twice with Eagle's essential medium supplemented with 10% FCS, filtered through #80 and #150 wire meshes and prepared into single cells.

Experimental design of mice pulmonary metastatic tumor

BDF1 mice were inoculated to their left foot pad 1×10^6 3LL cells, and these tumor were regarded as the primary tumor. The primary tumor was removed by femoral amputation under ether anesthesia on the 10th day from the inoculation. After the amputation, mice were mixed up and divided into the each group. The mice pulmonary metastatic tumors were evaluated on the 21th day from the inoculation. The evaluation was carried out by the Wexler's method[17]. In brief, their lungs were excised in one piece, dyed with India ink, washed for 5 min with flowing water, bleached and fixed in Fekete's solution for 24 hr and counted the number of the metastatic tumors.

Study of the dose dependent antitumor effect

The mice were administered into their tail veins saline, 1×10^2 u/kg/day, 5×10^2 u/kg/day, 1×10^3 u/kg/day, 5×10^3 u/kg/day, 1×10^4 u/kg/day, 5×10^4 u/kg/day, 1×10^5 u/kg/kay, 5×10^5 u/kg/day, 1×10^6 u/kg/day and 5×10^6 u/kg/day of n-TNF since the next day of the amputation daily for 10 days.

Study of the dose schedule of the administration

The mice were divided into seven groups. Saline and n-TNF were administered into the tail vein according to each protocol. In group 1, 0.2 ml saline was administered daily for 10 days since the first day after the amputation of the primary tumor. In group 2 and 3, 5×10^5 u/kg n-TNF was administered once on the amputated day (group 2) and the first day after the amputation of the primary tumor (group 3). In group 4 and 5, 1×10^5 u/kg/day n-TNF was administered every other day since the amputated day (group 4) and the first day after the amputation of the primary tumor (group 5). In group 6 and 7, 5×10^4 u/kg/day n-TNF was administered daily for 10 days since the amputated day (group 6) and the first day after the amputation of the primary tumor (group 7).

Study of the route of the administration

The mice were divided into four groups. n-TNF was administered 5×10^4 u/kg/day daily for 10 days by intravenous (i.v.), intraperitoneal (i.p.), intramuscular (i.m.), subcutaneous (s.c.) and intratumorous (i.t.) routes. In i.t. group, BDF1 mice were inoculated subcutaneously 1×10^6 3LL cells on the back. The tumor size was measured on the 10th day from the inoculation, and this tumor size was regarded as the preadministration size. Saline and n-TNF 5×10^4 u/kg/day were administered intratumorously (i.t.) daily for 10 days since the 11th day from the inoculation. The tumor size was measured on the 21th day from the inoculation and this tumor size was regarded as the postadministration size. The longest diameter (a mm) and the shortest diameter (b mm) were calculated with the following equation and the presumptive tumor weight was determined.

$$\text{Tumor weight (mg)} = \sqrt{a \times b^2}$$

Histological examination

The metastatic pulmonary tumors in study of the dose dependent antitumor effect were examined histologically. After fixed with the 10% formarin solution, the pulmonary tumor was sliced and dyed with hemoxylin and eosin solution. The specimen was evaluated with the Shimosato and Ohboshi's histological criteria for evaluation of therapeutic effects[15].

Statistical analysis was carried out by Student's t-test.

RESULTS

The dose dependent antitumor effect of n-TNF

Table 1 shows results of study of the dose dependent antitumor effect of n-TNF. n-TNF showed the statistically significant antitumor effect over 5×10^2 u/kg/day concentration and the antitumor effect increased dose dependently. Especially, over 5×10^5 u/kg/day, great inhibition of metastatic pulmonary tumor was found (82.4% — 92.2%, $p<0.001$).

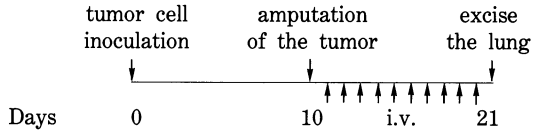
The dose schedule of the administration

In group 6 and 7, the daily administration groups, the statistically significant antitumor effect was shown ($p<0.1$, $p<0.05$). The statistical difference between group 6 and group 7 was not found (Table 2). When the total dose of n-TNF was fixed, the daily administration of divided dose was significantly effective.

The route of the administration

The route of the administration was studies.

Table 1. Study of the dose dependent antitumor effect

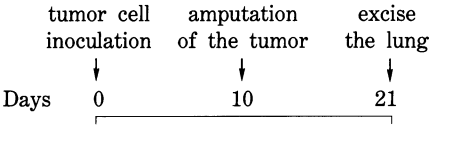


		n	Pulmonary tumor inhibition rate(%) a)	Range of No. of pulmonary tumor (mean ± SD)	Incidence	P(t-test)
	Saline	6	0	25-72(49.0 ± 17.0)	6/6	—
n-TNF	1×10^2 u/kg/day	5	25.0	12-30(29.4 ± 7.7)	5/5	not significant
	5×10^2	5	69.1	3-23(12.0 ± 8.1)	5/5	0.05
	1×10^3	5	70.4	3-23(11.6 ± 10.0)	5/5	0.05
	5×10^3	5	58.7	2-30(16.2 ± 12.6)	5/5	0.05
	1×10^4	5	53.6	8-28(18.2 ± 9.1)	5/5	0.05
	5×10^4	5	49.5	10-29(19.8 ± 7.5)	5/5	0.05
	1×10^5	5	73.9	3-30(12.8 + 11.2)	5/5	0.01
	5×10^5	5	82.4	1-17(8.6 ± 5.8)	5/5	0.001
	1×10^6	5	91.0	2- 8(4.4 ± 2.2)	5/5	0.001
	5×10^6	5	92.2	1- 6(3.8 ± 2.2)	5/5	0.001

a) pulmonary tumor inhibition rate

$$= \left(1 - \frac{\text{mean numbers of pulmonary metastatic tumors in n-TNF group}}{\text{mean numbers of pulmonary metastatic tumors in control group}} \right) \times 100\%$$

Table 2. Study of the dose schedule of the administration

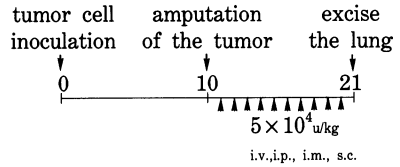


	n	Pulmonary Metastasis: No. of pulmonary tumor (mean ± SD)	Range	Incidence	P(t-test)
1. Saline (0.2ml × 10)	7	65.3 ± 30.6	36-130	7/7	—
2. n-TNF(5×10^5u/kg × 1)	7	56.3 ± 26.4	32-110	7/7	N.S.
3. n-TNF(5×10^5u/kg × 1)	6	48.4 ± 44.7	6-130	6/6	N.S.
4. n-TNF(1×10^5u/kg × 5)	7	55.0 ± 35.9	7-100	7/7	N.S.
5. n-TNF(1×10^5u/kg × 5)	7	41.3 ± 26.7	9- 81	7/7	N.S.
6. n-TNF(5×10^4u/kg × 10)	6	33.3 + 23.0	3- 70	6/6	0.1
7. n-TNF(5×10^4u/kg × 10)	6	30.5 ± 22.9	3- 68	6/6	0.05

N.S.: not significant

The mice were administered saline and n-TNF according to each protocol. The mice pulmonary metastatic tumors were evaluated on the 21th day from the inoculation by the Wexler's method.

Table 3. Study of the route of the administration (i.v., i.p., i.m., s.c.)

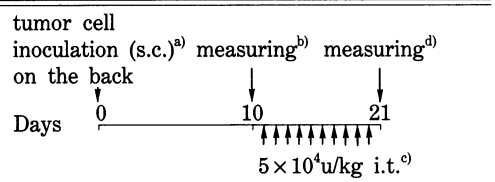


Route	Drug	n	Pulmonary Metastasis: No. of pulmonary tumor (mean ± SD)	Range	Incidence	P(t-test)	
i.v.	Saline	7	53.1 ± 20.1	28-96	7/7	—	—
	n-TNF	7	18.6 ± 11.9	4.32	7/7	0.01	
i.p.	Saline	5	36.0 ± 12.9	21-53	5/5	—	N.S.
	n-TNF	5	20.2 ± 18.0	3-50	5/5	N.S.	
i.m.	Saline	7	53.1 ± 23.7	21-56	7/7	—	N.S.
	n-TNF	5	18.4 ± 13.0	5-36	5/5	0.05	
s.c.	Saline	6	44.7 ± 32.4	20-99	6/6	—	N.S.
	n-TNF	6	24.2 ± 11.1	8-38	6/6	N.S.	

N.S.: not significant

n-TNF was administered 5×10^4 u/kg/day daily for 10 days by i.v., i.p., i.m. and s.c. routes. By the i.v. and i.m. routes, the antitumor effects were statistically found ($p<0.01$, $p<0.05$).

Table 4. Study of the intratumorously administration (i.t.)



	Tumor Weight(mg)e): preadministration	P(t-test)
Saline	30.9 ± 11.6 (n=9)	—
n-TNF	29.0 ± 13.2 (n=9)	N.S.
	postadministration	P(t-test)
Saline	99.7 ± 25.0 (n=9)	—
n-TNF	62.6 ± 25.1 (n=7)	0.05

N.S.: not significant

a) BDF1 mice were inoculated subcutaneously on the back 1×10^6 3LL cells. b) The tumor size was measured on the 10th day from the inoculation, and this size was regarded as the preadministration size. c) Saline and n-TNF 5×10^4 u/kg/day were administered intratumorously (i.t.) daily for 10 days since the 11th day from the inoculation. d) On the 21th day, the tumor size was measured and this size was regarded as the postadministration size. e) The presumptive tumor weight (mg) was determined by calculating with the following equation:

$$\text{Tumor weight (mg)} = \sqrt{a \times b^2}$$

a;the longest diameter (mm), b; the shortest diameter (mm)

Administering 5×10^4 u/kg/day n-TNF daily for 10 days, the statistically significant antitumor effect was shown by the route of i.v. ($p<0.01$), i.m. ($p<0.05$) and i.t. ($p<0.05$) (Table 3, 4).

Histological examination

The metastatic pulmonary tumors in the study of the dose dependent antitumor effect of n-TNF were examined histologically and evaluated with the Shimosato and Ohboshi's histological criteria[15]. Fig. 1 shows the pulmonary tumors specimen in control group (left) and n-TNF 5×10^6 u/kg/day group (right). IN n-TNF group, severe destruction of tumor structures was shown. Nuclear swelling and destruction, loosing the cell-cell interaction and cell lytic change were found and evaluated as the Grade IIb of the Shimosato and Ohboshi's histological criteria. Fig. 2 shows the specimen from n-TNF 1×10^3 u/kg/day group. Nuclear swelling and cell lytic change were similarly found and, especially, many cells (14 cells in this field) at the premetaphase of the cell cycle were shown. Nevertheless, a statistically significant antitumor effect was not found in n-TNF 1×10^2 u/kg/day group by the count of the number of metastatic pulmonary tumors, the significant cell damage was shown by the histological examina-

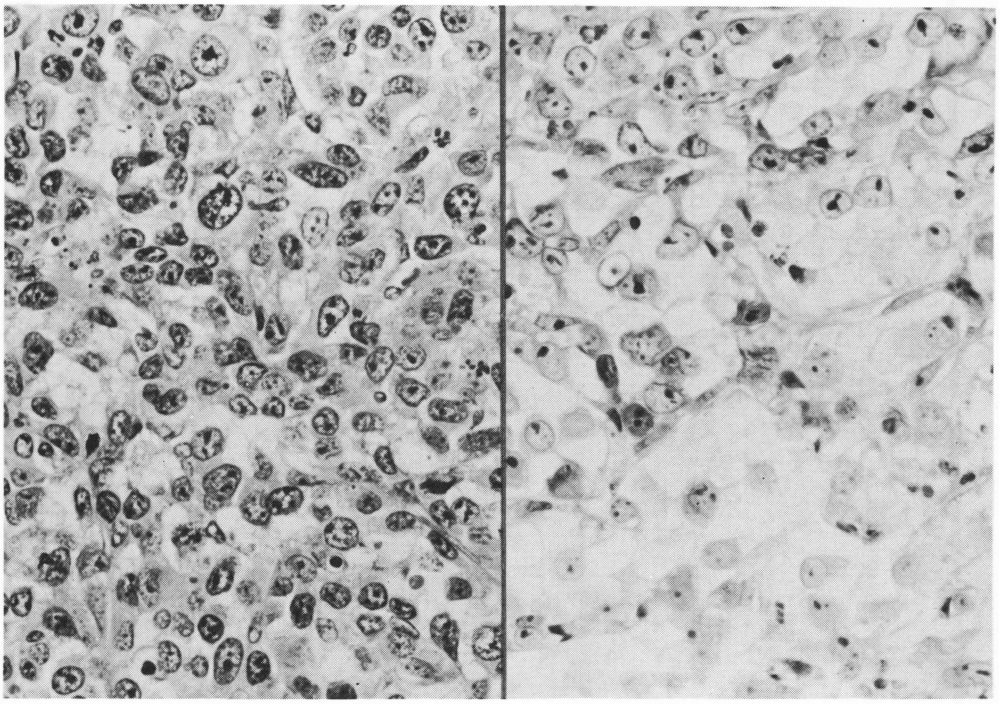


Fig. 1. Histological examination of the pulmonary metastatic tumors (1)
H-E stain, × 80

left) control group; Filling up with the fresh tumor cells. N/C ratio is high and some cells at the mitotic phase are visible. Right) n-TNF 5 × 10^6 u/kg/day group; Severe destruction of tumor structure is shown. This specimen can be evaluated as the Grade IIb of the Shimosato and Ohboshi's histological criteria. The lymphocyte infiltration is similar degree to that in control group and the hemorrhagic necrosis is never found.

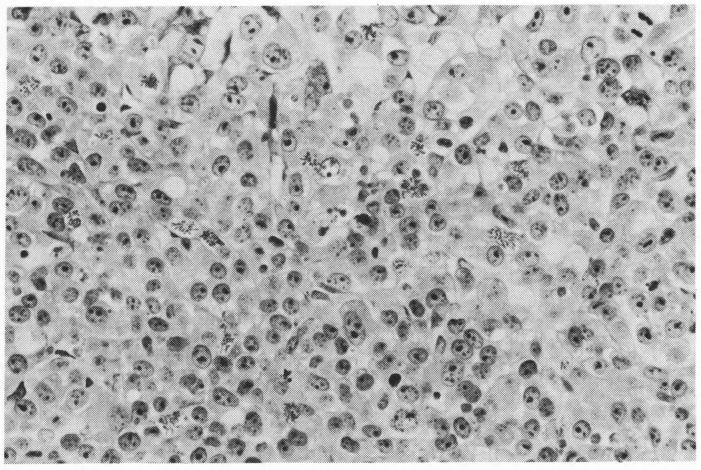


Fig. 2. Histological examination of the pulmonary metastatic tumors (2)
H-E stain, × 80

n-TNF 1 × 10^3 u/kg/day group; Nuclear swelling and cell lytic change are found and evaluated as the grade IIa of the Shimosato and Ohboshi's histological criteria. In this specimen, especially, many cells (14 cells in this field) at the premetaphase of the cell cycle are shown. The lymphocyte infiltration and the hemorrhagic necrosis are not found.

tion. In this study, the lymphocyte infiltration in n-TNF groups was similar degree to that in the control group and the hemorrhagic necrosis was never found.

DISCUSSION

Tumor necrosis factor (TNF) reported by Carswell et al[2] and Currie et al[3] in 1975 is especially remarked because of its strong antitumor effects, and many investigations have been continued. In this paper, we discussed the antitumor effect of natural type human TNF (n-TNF).

This n-TNF is one of the fractions which was isolated and refined through the monoclonal antibody-Sepharose column from crude lymphokines which were obtained by treating BALL-1 cells, one kind of B cells, with HVJ by the "Hamster method" of Hayashibara Biochemical Laboratories, Inco., Okayama[10]. By using the "Hamster method", we were able to obtain a large amount of n-TNF, and thus *in vivo* experiment in the present report became possible. This n-TNF was lately found to be a PAS stain positive protein which consists of 161 amino acids through the identification of the amino acids sequence. This is very similar to the amino acids sequence which was reported by Pennica et al[13] and Shirai et al[16], and thus it was considered to be alpha type natural-human TNF. TNF is generally thought to be derived from macrophage[2,3,6,12], but there is a report which says it is also produced from other lymphocytes than macrophage[18]. The n-TNF which was discussed in the present report was produced from BALL-1 cells which are one of B cells.

In our study, natural TNF showed antitumor effects on 3LL which is a tumor derived from mice, and effects were enforced dose dependently. Helson et al[8] showed, conversely to our study, antitumor effects of mice TNF on the human tumor cell line, and thus reported, as we did, that antitumor effects of TNF had no species specificity. In addition, Helson et al[9] reported that mice TNF had dose dependent effects and Ruff et al[14] reported that TNF was effective on *in vitro* L929 cells dose dependently, both of which agreed to our result.

Next, the dose schedule of the administration of n-TNF was discussed. When the total amount of the administration was fixed, the daily administration of divided doses was effective. It is considered from this result that TNF is more effective when it is administered continuously even if the amount is small. Ruff et al[14] reported that effects of TNF on *in vitro* L929 cells were time dependent, and thus their thought agreed to our thought.

Incidetally, the route of the administration is also important in using antitumor agents. Haranaka et al[7] reported that i.v. injection was more effective when i.v. injection was compared with i.t. injection in the experiment in which mice TNF was used. In our study, effects were observed in route of i.v., i.m. and i.t. through the daily administration. Intravenous injection was a little more effective than i.t. injection also in our study as Haranaka et al[7] reported. As for i.p. injection which was not effective in our study, it was anticipated that effects would not be expected because tumors which were set as objects were in lungs. Effects of i.p. injection will be needed to be discussed by using the model of the cancer cell line in the ascites, considering the case of peritoneal dissemination.

Histological examination was conducted lastly. Metastatic pulmonary tumors were discussed as specimens. The 5×10^6 u/kg/day dose group in which the largest effect was observed were ranked Grade IIb of the Shimosato and Ohboshi's histological criteria. And there were also observations of partially ranked Grade III. As the effects of Grade IIb or more than Grade IIb shows that the drug may well be judged to be effective as an antitumor drug, the effect of n-TNF was observed also histologically. Moreover, the significant effects were observed in the histological examination as to the 1×10^2 u/kg/day dose group in which no significant effects were observed in study of the pulmonary metastatic tumors, and thus n-TNF is considered to affect tumor cells though the amount is small. In our histological examination, lymphocyte infiltration was of a similar degree to that of the control group. And the hemorrhagic necrosis was not observed. This deviates from classic definitions of TNF[2,3]. The reason was unclear, but the amount of dosage of n-TNF in this study might be a little too small. The further investigation is needed for the future. n-TNF showed no change in the NK cell activity (data not shown), and thus it was considered that n-TNF did not affect tumor cells through the aid of the

effector but affected them directly. Also, many cells at the premetaphase of the cell cycle were observed than usual observation as to the 1×10^3 u/kg/day dose group. It suggests that n-TNF affects tumor cells directly and in association with the cell cycle. Kull et al[11] discussed the mechanism of action of TNF and reported, as we did, that TNF inhibited RNA and protein synthesis of cells. Also, Darzynkiewicz et al[4] discussed the effect of the TNF on the cell cycle through *in vitro*, and reported that the immediate effect of TNF added to cultures of L-cells was cytostasis, manifested as cell arrest in G2. They also reported that many tumor cells died between G1 phase and S phase.

Nevertheless the identity of TNF and the cachectin was reported[1], no changes were seen as to mice body weight and splenic weight throughout all our present experiments (data not shown). Although more investigations for the future is needed, n-TNF which is easily mass-produced can be looked forward to as a new antitumor agent which can be used safely in clinical use.

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