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Synergistic Antitumor Effects of Natural Human Tumor Necrosis Factor and Mouse Interferon Beta and Gamma

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ABSTRACT

Referring to synergistic antitumor effects of natural human tumor necrosis factor (n-TNF) derived from human acute lymphoblastic leukemia BALL-1 cell as well as mouse interferon beta (mIFNbeta) and mouse interferon gamma (mIFNgamma), a series of the study was made using Lewis lung carcinoma grafted on BDF1 mice. With a combination dose of n-TNF (1×10^2 U/kg/day) and mIFNbeta (1×10^2 IU/kg/day) as well as that of n-TNF (1×10^2 U/kg/day) and mIFNgamma (1×10^2 IU/kg/day), a significant enhancement of antitumor effect was observed. Furthermore, with a triple combination dose of n-TNF (1×10^2 U/kg/day), mIFNbeta (1×10^2 IU/kg/day) and mIFNgamma (1×10^2 IU/kg/day), too, a strong synergistic effect was noted. The concentration of n-TNF required for concomitant use with mIFNbeta and mIFNgamma was 1 over 5×10^3 of that required for single dose of n-TNF, to obtain the same level of effect.

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 recombinant type 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 hamster method are treated with HVJ[7].

Our previous studies demonstrated that the antitumor effect of the n-TNF was noted to be dose dependent, and that the synergistic antitumor effect was found when n-TNF was administered in combination with conventional anticancer drugs[8,9]. In this study, the synergistic antitumor effects of n-TNF as well as mouse interferon beta (mIFNbeta) and mouse interferon gamma (mIFNgamma) were investigated with the in vivo experimental systems.

MATERIALS AND METHOD

n-TNF

Natural human TNF was supplied by Hayashibara Biochemical Laboratories, Inc.,Okayama, and used. n-TNF is a protein consisting 161 amino acids with molecular weight of 17,000 and isoelectric point of 5.2—6.2, being stable at 56°C in 30 min[7]. Its cytotoxicity was determined by the high sensitive and rapid assay of Eifel et al[5] for lymphotoxin, using mouse L929 cells as the target cells. One unit of activity is designated as the reciprocal of the dilution that effects cytopathic effect (CPE) in 50% of the target cells.

mIFNbeta

mIFNbeta was supplied by Hayashibara Biochemical Laboratories, Inc., Okayama, and used. This mIFNbeta is a natural type containing 80 to 90% of interferon beta and 10 to 20% of interferon alpha.

mIFNgamma

mIFNgamma was supplied by Shionogi Pharmaceutical Co., Ltd., Tokyo, and used. It was the recombinant mIFNgamma.

Experimental Animals

Female BD (C57BL/6 × DBA/2)F 1 mice at 8 weeks of age weighting about 25 g 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 with Hanks' 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 tumors

To the left foot pad of BDF1 mice 1×10^6 3LL cells were inoculated, and these tumors 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 each group. The metastatic pulmonary tumors in mice were evaluated on the 21st day from the inoculation. the evaluation was carried out by Wexler's method[21]. 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, then the number of metastatic tumors was counted.

Administration of n-TNF, mIFNbeta and mIFNgamma

Saline (control), n-TNF, mIFNbeta and mIFNgamma were administered into the tail veins of mice, singly or in combination, since the next day of the amputation, daily for 10 days (Fig. 1).

Weight of the mouse's Body and Spleen

The animals were weighted immediately before sacrifice on the 21st day from the inoculation. Furthermore, the spleen weight was measured upon excitation after sacrifice.

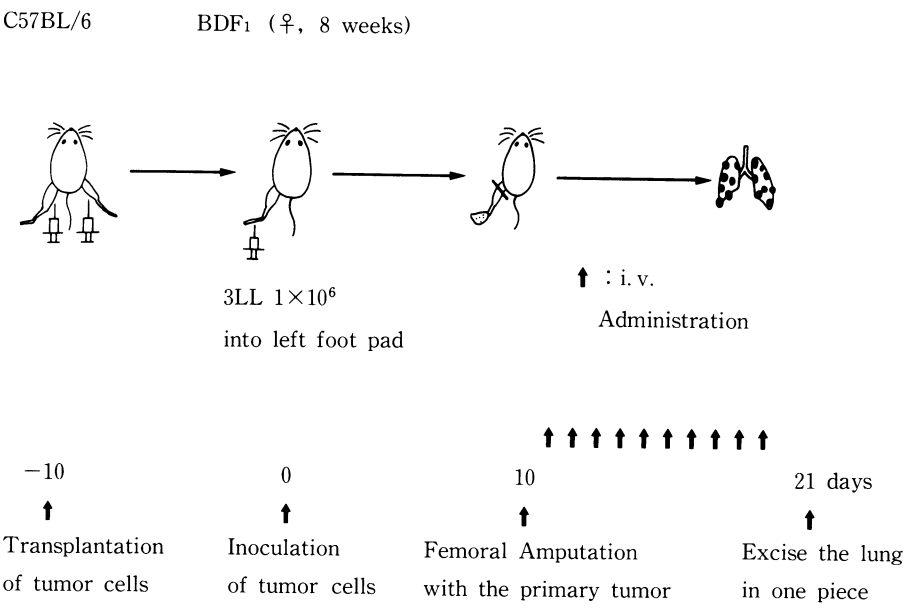

Fig. 1. Method of Experiments

Table 1. The synergistic effects of n-TNF and mIFNbeta

	n	Inhibition rate(%)[a] of pulmonary tumors	No. of pulmonary tumors (mean ± SD)	Range	Incidence	P(t-test)
Saline	6	0	49.0 ± 17.0	25—72	6/6	—
n-TNF 10^4U/kg/day	6	57.8	20.7 ± 14.1	4—37	6/6	0.02
mIFNβ 10^4IU/kg/day	6	87.1	6.3 ± 7.5	1—21	6/6	0.001
n-TNF 10^4+mIFNβ 10^4	6	85.7	7.0 ± 6.2	0—16	5/6	0.001
n-TNF 10^3U/kg/day	6	39.2	29.8 ± 20.6[b]	9—60	6/6	N.S.
mIFNβ 10^3IU/kg/day	6	79.6	10.0 ± 8.5	3—24	6/6	0.001
n-TNF 10^3+mIFNβ 10^3	6	81.2	9.2 ± 5.8[c]	3—18	6/6	0.001
n-TNF 10^2U/kg/day	6	39.8	29.5 ± 17.8[d]	12—58	6/6	N.S.
mIFNβ 10^2IU/kg/day	6	45.9	26.5 ± 14.1[e]	12—50	6/6	0.05
n-TNF 10^2-mIFNβ 10^2	6	80.0	9.8 ± 9.3[f]	3—28	6/6	0.001

N.S.: not significant

a) Inhibition rate(%)= $\left(1 - \frac{\text{mean No. of pulmonary tumors of each group}}{\text{mean No. of pulmonary tumors of control group}}\right) \times 100$

b) vs c); $p<0.1$, d) vs f); $p<0.05$, e) vs f); $p<0.05$

Table 2. The synergistic effects of n-TNF and mIFNgamma

	n	Inhibition rate(%)[a] of pulmonary tumors	No. of pulmonary tumors (mean ± SD)	Range	Incidence	P(t-test)
Saline	6	0	37.5 ± 13.5	25—62	6/6	——
n-TNF 10^4U/kg/day	5	74.1	9.4 ± 3.0[b]	5—12	5/5	0.01
mIFNγ 10^4IU/kg/day	5	89.3	4.0 ± 3.5	1—10	5/5	0.001
n-TNF 10^4+mIFNγ 10^4	5	90.4	3.6 ± 2.9[c]	0— 7	4/5	0.001
n-TNF 10^3U/kg/day	5	49.3	19.0 ± 12.6[d]	5—35	5/5	0.05
mIFNγ 10^3IU/kg/day	5	85.1	5.6 ± 1.5	4— 8	5/5	0.001
n-TNF 10^3-mIFNγ 10^3	5	90.9	3.4 ± 2.5[e]	0— 7	4/5	0.001
n-TNF 10^2U/kg/day	5	27.5	27.2 ± 8.3[f]	18—40	5/5	N.S.
mIFNγ 10^2IU/kg/day	5	53.1	17.6 ± 7.5[g]	10—30	5/5	0.05
n-TNF 10^2+mIFNγ 10^2	5	87.7	4.6 ± 3.6[h]	0—10	4/5	0.001

N.S.: not significant

a) Inhibition rate(%)= $\left(1 - \frac{\text{mean No. of pulmonary tumors of each group}}{\text{mean No. of pulmonary tumors of control group}}\right) \times 100$

b) vs c); $p<0.05$, d) vs e); $p<0.1$, f) vs h); $p<0.001$, g) vs h); $p<0.01$

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

RESULTS

The effects of n-TNF and mIFNbeta, singly or in combination, on metastatic pulmonary tumors are shown in Table 1. n-TNF showed a significant antitumor effect ($p<0.02$) at 1×10^4 U/kg/day. Antitumor effects of n-TNF at 1×10^2 U/kg/day and 1×10^3 U/kg/day were not significant. mIFNbeta showed at 1×10^2 IU/kg/day or over a significant antitumor effect ($p<0.05$) and at 1×10^3 IU/kg/day or over a strong antitumor effect ($p<0.001$). When n-TNF and mIFNbeta were dosed combinedly at 1×10^2 U/kg/day and 1×10^2 IU/kg/day respectively, a significant enhancement of antitumor effect was obtained. Making a comparison between the combination dose group and the single dose group, a statistically significant enhancement ($p<0.05$, respectively) was observed on each of them.

The effect of n-TNF and mIFNgamma, singly or in combination, on metastatic pulmonary tumors are shown in Table 2. In this experiment with n-TNF, the antitumor effect was significant

Table 3. The synergistic effects of n-TNF, mIFNbeta and mIFNgamma

	n	Inhibition rate(%)[a] of pulmonary tumors	No. of pulmonary tumors (mean ± SD)	Range	Incidence	P(t-test)
Saline	6	0	37.5 ± 13.5	25~62	6/6	——
10^4 [a)]	5	97.8	0.8 ± 1.3	0~3	2/5	0.001
10^3 [b)]	5	96.3	1.4 ± 1.3	0~3	3/5	0.001
10^2 [c)]	5	94.1	2.2 ± 1.8	0~9	4/5	0.001

a) n-TNF 10^4U/kg/day + mIFNβ 10^4IU/kg/day + mIFNγ 10^4IU/kg/day
b) n-TNF 10^3U/kg/day + mIFNβ 10^3IU/kg/day + mIFNγ 10^3IU/kg/day
c) n-TNF 10^2U/kg/day + mIFNβ 10^2IU/kg/day + mIFNγ 10^2IU/kg/day
d) Inhibition rate(%) $= \left(1 - \frac{\text{mean No. of pulmonary tumors of each group}}{\text{mean No. of pulmonary tumors of control group}}\right) \times 100$

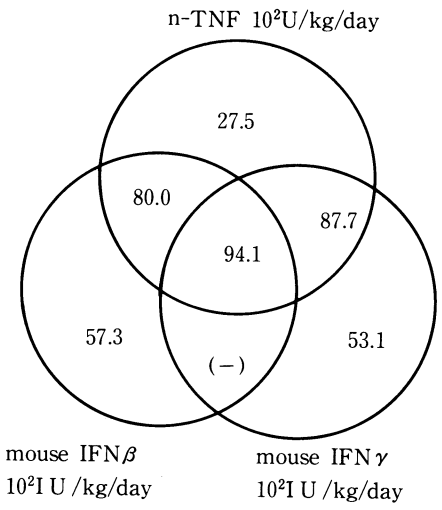

Fig. 2. The synergistic effects of n-TNF, mIFNbeta and mIFNgamma in 1 × 10^2 U/kg/day dose group

($p<0.05$) at 1 × 10^3 U/kg/day or over, but not significant at 1 × 10^2 U/kg/day. Antitumor effect of mIFNgamma was significant ($p<0.05$) at 1 × 10^2 IU/kg/day or over, and strongly significant ($p<0.001$) at 1 × 10^3 IU/kg/day or over. When n-TNF and mIFNgamma were dosed in combination, at 1 × 10^2 U/kg/day and 1 × 10^2 IU/kg/day respectively, a significant enhancement of antitumor effect was obtained. In comparison of the combination dose group with the single dose group of n-TNF and that of mIFNgamma respectively, a statistically significant enhancement ($p<0.001$, $p<0.05$, respectively) was observed on each of them. When the antitumor effect of mIFNgamma was compared with that of mIFNbeta, an equally leveled effect was noted at the same concentration showing no statistical difference. No statistical difference was observed either, when the combination dose group of n-TNF plus mIFNbeta was compared with that of n-TNF plus mIFNgamma, as to their antitumor effects.

Next, a triple combination dose of n-TNF, mIFNbeta and mIFNgamma was studied. The effects of n-TNF, mIFNbeta and mIFNgamma in combination on metastatic pulmonary tumors are shown in Table 3. In the triple combination dose group, a strong enhancement of antitumor effect ($p<0.001$, respectively) was found. The effect of the triple combination dose group at 1 × 10^2 U/kg/day was also compared with the single dose or the combination dose groups (Fig. 2). The effect of the triple combination dose group was significantly ($p<0.05$) stronger than that of the single dose group. However the triple combination dose group revealed no statistically significant enhancement of the effect as the double combination dose group.

Table 4 and 5 show the weights of the body and spleen of mice. No change was observed on the body weight and spleen weight of mice in either group of the single of combination dose of n-TNF, mIFNbeta and mIFNgamma. In the triple combination group, too, weights of the body and spleen of mice showed no change at all (data not shown).

DISCUSSION

TNF was reported by Carswell et al[3] as a macrophage-derived polypeptide, which causes hemorrhagic necrosis of transplanted tumors.

Table 4. Mouse body weight and spleen weight in n-TNF group and mIFNbeta group

	n	Body Weight(g) (mean ± SD)	P(t-test)	Spleen Weight(g) (mean ± SD)	P(t-test)
Saline	6	26.2 ± 0.8	—	0.21 ± 0.05	—
n-TNF 10^4U/kg/day	6	24.3 ± 2.7	N.S.	0.19 ± 0.03	N.S.
mIFNβ 10^4IU/kg/day	6	25.0 ± 1.1	N.S.	0.18 ± 0.04	N.S.
n-TNF 10^4+mIFNβ 10^4	6	23.5 ± 2.9	N.S.	0.18 ± 0.02	N.S.
n-TNF 10^3U/kg/day	6	24.0 ± 2.6	N.S.	0.18 ± 0.03	N.S.
mIFNβ 10^3IU/kg/day	6	25.0 ± 1.7	N.S.	0.18 ± 0.03	N.S.
n-TNF 10^3+mIFNβ 10^3	6	23.8 ± 3.5	N.S.	0.19 ± 0.05	N.S.
n-TNF 10^2U/kg/day	6	24.6 ± 1.4	N.S.	0.19 ± 0.06	N.S.
mIFNβ 10^2IU/kg/day	6	25.7 ± 1.6	N.S.	0.20 ± 0.02	N.S.
n-TNF 10^2+mIFNβ 10^2	6	24.8 ± 1.9	N.S.	0.19 ± 0.05	N.S.

N.S.: not significant

Table 5. Mouse body weight and spleen weight in n-TNF group and mIFNgamma group

	n	Body Weight(g) (mean ± SD)	P(t-test)	Spleen Weight(g) (mean ± SD)	P(t-test)
Saline	6	19.8 ± 1.5	—	0.27 ± 0.06	—
n-TNF 10^4U/kg/day	5	19.0 ± 1.0	N.S.	0.23 ± 0.06	N.S.
mIFNγ 10^4IU/kg/day	5	19.2 ± 1.1	N.S.	0.27 ± 0.05	N.S.
n-TNF 10^4+mIFNγ 10^4	5	18.4 ± 1.5	N.S.	0.29 ± 0.04	N.S.
n-TNF 10^3U/kg/day	5	18.8 ± 1.6	N.S.	0.24 ± 0.05	N.S.
mIFNγ 10^3IU/kg/day	5	18.8 ± 0.8	N.S.	0.22 ± 0.07	N.S.
n-TNF 10^3+mIFNγ 10^3	5	19.0 ± 1.4	N.S.	0.24 ± 0.04	N.S.
n-TNF 10^2U/kg/day	5	19.6 ± 0.5	N.S.	0.23 ± 0.03	N.S.
mIFNγ 10^2IU/kg/day	5	19.2 ± 0.8	N.S.	0.21 ± 0.04	N.S.
n-TNF 10^2+mIFNγ 10^2	5	19.6 ± 1.1	N.S.	0.22 ± 0.02	N.S.

N.S.: not significant

After that Williamson et al[22] clarified that TNF is produced from the B cell line, too. Furthermore, Pennica et al[13] and Shirai et al[17] succeeded in production of human recombinant TNF, and clarified its amino acid sequence. TNF used by the authors is a natural type human TNF obtained from BALL-1 mass-produced by the Hayashibara-Hamster's method[7] and processed by HVJ. The authors' previous studies[8] demonstrated that this n-TNF showed strong dose dependent antitumor effects, and that the daily administration by the route of i.v., i.m. and i.t. of this n-TNF was effective. In our experiments, n-TNF showed constantly antitumor effects at 1×10^4 U/kg/day or over ($p<0.05$). And at 5×10^5 U/kg/day or over, strong antitumor effects ($p<0.001$) were noted in the previous study of the authors[8].

Recently an enhancement of antitumor effect is expected by a combined use of TNF and interferons. The synergistic effects has been reported by Williamson et al[22] and Naomoto et al[11] upon use of human TNF and human interferon alpha in combination. Furthermore, a combination of TNF and interferon gamma has also been reported[1,4,12,15,16,18,19,22]. They are referred to the in vitro experiments, but the authors made it in vivo. Thus, mouse interferon was used for interferon beta and interferon gamma, because the species specificity is not shown by TNF but interferons[10]. It has been reported that mouse interferon has the antitumor effects[23,24]. In this study of the authors, mIFNbeta and mIFNgamma showed the significant antitumor effect with single dose respectively, too. The significant enhancement of antitumor effect was also indicated by use of n-TNF and mIFNbeta in combination. Specially the strong antitumor ef-

fect ($p<0.001$) was obtained upon combined use of mIFNbeta at 1×10^2 IU/kg/day and n-TNF at 1×10^2 U/kg/day, although the effect of n-TNF was not significant in its single use. In combination of n-TNF with mIFNgamma, too, the same significant enhancement of antitumor effect was indicated. In combination with mIFNgamma, too, a strong antitumor effect ($p<0.001$) was obtained at 1×10^2 IU/kg/day with n-TNF at 1×10^2 U/kg/day, that was not significantly effective in single dose. Antitumor effect of mIFNbeta and mIFNgamma in single dose respectively was nearly on the same level at the same concentration, showing no statistical difference. Furthermore, when the synergistic effect of n-TNF and mIFNbeta was compared with that of n-TNF and mIFNgamma, an equal enhancement of antitumor effect was obtained at the same concentration, showing no statistical difference. In addition when the triple combination dose of n-TNF, mIFNbeta and mIFNgamma was studied, the strongest antitumor effect was noted at inhibition rate of 94.1% to 97.8% ($p<0.001$). However, no significant enhancement was observed when antitumor effect of the triple combination dose group was compared with the combination dose group of n-TNF and mIFNbeta only or mIFNgamma only.

In the recent studies on mechanism of cytotoxic effect with TNF, it has been reported that due to the presence of TNF receptor in tumor cells, cytotoxic effect is revealed when such TNF meets the TNF receptor[1,2,6,14]. Baglioni et al[2] report in their study on TNF receptors of L929 cells that a KD of 2×10^{-10}M and 6000 receptors/cell were calculated, while Hass et al[6] in their study using L929 cells report that an apparent KD of 6.7×10^{-11}M on a capacity of 3200 binding sites/cell was calculated. According to the report of Aggarwel et al[1] in their study on the combined dose of TNF and IFNgamma, preincubation of cells with IFNgamma increases the total number of TNF receptors two to threefolds without any significant change in the affinity constant. Tsujimoto et al[19] state that TNF receptors of L929 cells increase by murine-IFNgamma, and report that a maximal increase of TNF binding was seen after 6 to 12 hours of incubation with IFN. Furthermore, Tsujimoto et al. have studied on IFNalpha and IFNbeta, too, and state that IFNalpha and IFNbeta also produce the increased TNF binding, with the lower efficacy than that of IFNgamma though. In addition, Tsujimoto et al[20] and Ruggiers et al[15] report as stated by Aggarwel et al[1] that IFNgamma dose not change the binding affinity of TNF receptor. In the authors' *in vivo* experiment, too, the synergistic effect of mIFNbeta and n-TNF as well as mIFNgamma and n-TNF are apparent, both of them being on the same level.

In this series of the study, either of the n-TNF, mIFNbeta and mIFNgamma groups showed no change in weights of the body and spleen of mice, which assured the safety of every drug. In the experiment using mice, the safety of n-TNF was suggested to be assured by increasing up to 5×10^6 U/kg/day[8] but in the clinical use some unknown side effect might occur. According it is better to obtain the maximum effect with the minimum dose, which requires a combination dose with other drugs. In this study a strong antitumor effect equivalent to a single dose of n-TNF at 5×10^5 U/kg/day[8] was obtainable by use of IFN in combination at 1×10^2 U/kg/day, which is 1 over 5×10^3 only, well serving the purpose. In future upon further studies on the ratio on n-TNF and IFN in combination dose, it is expected to obtain the maximum antitumor effect with the minimum side effect.

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