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DT-5461, a New Synthetic Lipid A Analogue, Inhibits Lung and Liver Metastasis of Tumor in Mice

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We have investigated the antimetastatic effect of a new synthetic lipid A analogue, of low endotoxicity, DT-5461, against two highly metastatic tumor cell lines, L5178Y-ML25 T-lymphoma and B16-BL6 melanoma cells in mice. Four intermittent i.v. administrations of DT-5461 at intervals of 4 days resulted in a significant inhibition of liver metastasis caused by i.v. injection of L5178Y-ML25 cells and lung metastasis of B16-BL6 cells in the experimental metastasis models. Intraperitoneal and intranasal administrations as well as i.v. administration of DT-5461 were also effective in preventing lung metastasis of the melanoma cells. Multiple administrations of DT-5461 before the surgical excision of primary tumors significantly reduced the number of lung colonies of melanoma cells and primary tumor size. Similarly, this treatment modality after the surgical excision of primary tumors showed a greater reduction of lung tumor colonies as compared with lipopolysaccharide, a synthetic lipid A (No. 506) and its analogue as well as untreated control in the spontaneous lung metastasis model. Furthermore, the group that received DT-5461 after the inoculation of lymphoma or melanoma cells showed significantly enhanced survival rate compared with the untreated control. These results suggested that DT-5461 may be therapeutically useful for the inhibition of tumor metastasis.

Key words: Synthetic lipid A analogue — Liver metastasis — Lung metastasis — Melanoma — Lymphoma

Lipopolysaccharide (LPS), an endotoxin of Gram-negative bacteria which consists of a polysaccharide-protein complex, has been shown to possess various biological activities. Lipid A has also been established to be the active component responsible for the endotoxic properties of LPS.[1)] Recently the chemical structure of the lipid A moiety of several enterobacterial LPSs has been extensively investigated[2,3)] and it was found that *Escherichia coli* lipid A has a comparatively simple structure.[1,4)] The chemical synthesis of lipid A based on the structure of lipid A of *E. coli* type was carried out by Shiba's group[4,5)] and a synthetic lipid A (No. 506) has been shown to have biological activities, including endotoxic activities, identical with those of LPS.[6)] It is well known that synthetic lipid A, as well as natural lipid A, exhibits antitumor activity and enhancement of nonspecific protective activity against microbial infections. However, the use of LPS and synthetic lipid A can cause severe side effects, and should be avoided in the treatment of cancer metastasis and microbial infections. Therefore, the chemical synthesis and biological activities of synthetic lipid A analogues have been extensively studied,[7,8)] and there are several reports of attempts to develop synthetic lipid A analogues possessing considerable immunopharmacological activities without endotoxic activities.

Among the various synthetic lipid A analogues, the monosaccharide-type lipid A subunit analogues (GLA compounds) were shown to have low endotoxic activity and various immunomodulating activities.[9–14)] Several investigators have demonstrated that a lipid A subunit analogue, GLA-60, inhibited *in vivo* tumor growth and tumor metastasis in an experimental murine model.[15,16)] We have previously reported that the administration of recombinant interferon-γ followed by GLA-60 (rIFN-γ/GLA-60) could induce the endogenous tumor necrosis factor (TNF) in mice under a certain administration schedule, and this treatment modality caused significant inhibition of experimental and spontaneous lung metastasis of murine malignant melanoma as compared with either rIFN-γ or GLA-60 alone or the mixture.[17,18)]

Kusama *et al.*[7)] have reported that a new synthetic lipid A analogue, DT-5461, possesses much lower endotoxicity, such as lethal toxicity and pyrogenicity, than LPS or synthetic lipid A analogue No. 506 [approximately 1/10,000–1/100,000 in rabbits,[7)] 1/40 in normal mice or 1/32–1/128 in tumor-bearing mice (unpublished data)]. We describe here the effect of DT-5461 on the liver or lung metastasis of two metastatic murine tumors in syngeneic mice.

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MATERIALS AND METHODS

Animals Specific pathogen-free C57BL/6 and CDF_1 (BALB / c × DBA / 2) mice, 7 – 10 weeks old, were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. Mice were maintained in the Laboratory of Animal Experiment, the Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. All the mice used in this study were sex-matched.

Cells and cell cultures Lung metastatic B16-BL6 melanoma cells were kindly provided by Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX and maintained as monolayer cultures in Eagles's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, non-essential amino acids, and L-glutamine. Liver metastatic L5178Y-ML25 T-lymphoma cells, obtained from L5178Y parent cells by *in vivo* selection for invasion,[19] were maintained in RPMI-1640 supplemented with 7.5% FBS and L-glutamine.

Synthetic lipid A analogues and chemical reagents The chemical structures of a synthetic lipid A (No. 506) and its analogues (DT-5461 and GLA-60) used in this study are shown in Fig. 1. No. 506 (LA-15-PP), which was synthesized based on the defined structure of *E. coli*-type lipid A,[5] was kindly provided by Daiichi Seiyaku Co., Ltd., Tokyo. DT-5461, 1,3-dicarboxyisopropyl 2-deoxy-6-*O*-[2-deoxy-3-*O*-(*N*-dodecanoylglycyl)-4-*O*-phosphono-2-tetradecanoylamino-*β*-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-*α*-D-glucopyranoside, was chemically synthesized according to the method described previously[7] and was easily solubilized in 1 mg/ml meglumine-5% glucose solution before use. A lipid A subunit analogue, GLA-60, was chemically synthesized according to the method described previously.[10] The purity of DT-5461 was more than 97% by high-performance liquid chromatography (HPLC) analysis.[7] No. 506 and GLA-60 were easily solubilized in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) by adding trimethylamine (0.025%) before use. LPS from *E. coli* 0127:B8 was kindly provided by Daiichi Seiyaku Co., Ltd.

Assay for liver metastasis of lymphoma cells Four of five CDF_1 mice per group were given i.v. injection of 2 or 4×10^4 L5178Y-ML25 T-lymphoma cells and treated with synthetic lipid A analogues. Mice were killed 14 days after tumor inoculation and the weights of liver and spleen were recorded to evaluate tumor metastasis as previously described in detail.[19] The survival time of the animals given i.v. injection of tumor cells and treated with synthetic lipid A analogues was also determined by allowing the animals to live until they succumbed naturally from the tumor burden. Animals were autopsied at the same time of death to verify the presence of the tumor in the liver. The survival (%) was calculated as a function of time.

Assay for experimental and spontaneous lung metastasis of melanoma cells Experimental lung metastasis was assessed by means of tumor cell injection into the lateral tail vein of mice. Five C57BL/6 mice per group were given i.v. injection of 4×10^4 B16-BL6 melanoma cells. The treatment with synthetic lipid A analogues began

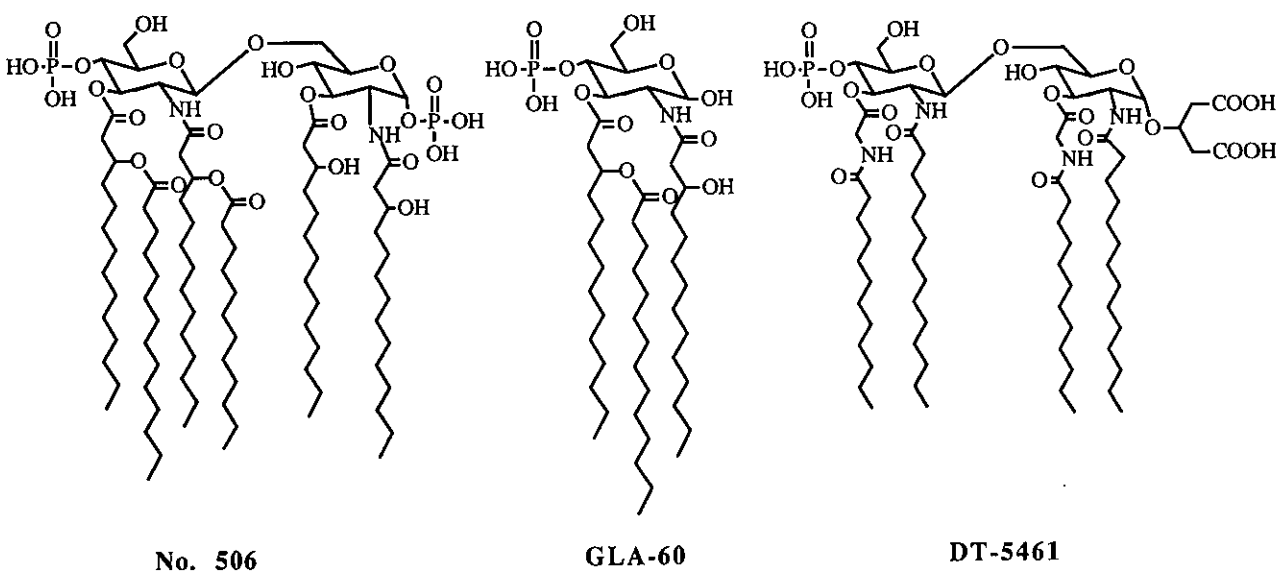

Fig. 1. Chemical structures of No. 506, GLA-60 and DT-5461.

one day after tumor inoculation and was carried out every 4 days for a total of 4 treatments. Mice were killed 14 days after tumor inoculation. The lungs were fixed in Bouin's solution and the lung tumor colonies were counted under a dissecting microscope. In spontaneous lung metastasis assay, eight C57BL/6 mice per group were injected s.c. with 5×10^5 B16-BL6 melanoma cells into the right hind footpad and subsequent primary tumors were surgically removed by amputation on day 21. In this experiment, the treatment with LPS, synthetic lipid A (No. 506) and lipid A analogues was carried out every 4 days for a total of 4 or 5 times before or after tumor excision. Mice were killed 35 days after tumor inoculation. The survival time of the animals given s.c. injection of tumor cells and treated with LPS or DT-5461 before surgical excision of primary tumors was determined by allowing the animals to live until they succumbed naturally from the tumor burden. Animals were autopsied at the time of death to verify the presence of the tumor in the liver. The survival (%) was calculated as a function of time.

Statistical analysis The statistical significance of differences between the groups was determined by Student's two-tailed *t* test or Mann-Whitney's U-test.

RESULTS

Effect of administration schedule of DT-5461 on tumor metastasis of two different types of metastatic tumors We first investigated the effect of multiple systemic administrations of DT-5461 on liver metastasis of L5178Y-ML25 lymphoma cells. Mice were given multiple i.v. injections of 40 μg DT-5461 after i.v. injection of 4×10^4 L5178Y-ML25 cells, and the weights of the liver and spleen were measured 14 days after tumor inoculation (Table I). When the tumor cells were injected i.v. into CDF_1 mice, liver and spleen weights of the mice were increased more than 4-fold, compared with those of normal mice. Four intermittent i.v. injections of DT-5461 at intervals of 2 or 4 days significantly inhibited liver and spleen metastasis, while 2 or 3 intermittent administrations of DT-5461 at 4-day intervals achieved less reduction of liver and spleen weights. In contrast, four consecutive administrations of DT-5461 showed only a slight inhibition of liver metastasis (not significant). These results indicated that the treatments with DT-5461 at intervals of 2 or 4 days were effective for the inhibition of liver metastasis of lymphoma cells. Therefore, the administration of DT-5461 at intervals of 4 days was carried out in the following experiments.

In the next set of experiments, we examined the dose-response relation of DT-5461 on tumor metastasis of two cell types of metastatic tumors such as L5178Y-ML25

Table I. Effect of Administration Schedule of DT-5461 on Liver Metastasis of L5178Y-ML25 Lymphoma Cells

Administered i.v. with;	Timing	Mean weight (g) ± SD	
		Liver	Spleen
Untreated	—	4.76±0.48	0.29±0.38
DT-5461	1, 2, 3, 4	3.52±0.80	0.26±0.03
	1, 3, 5, 7	1.87±0.46**	0.17±0.03*
	1, 5, 9, 13	1.76±0.53**	0.16±0.02**
	1, 5, 9	2.80±0.37*	0.22±0.04
	1, 5	2.87±0.61*	0.24±0.04
Normal	—	0.98±0.14	0.08±0.02

Four CDF_1 mice per group were inoculated i.v. with 4×10^4 L5178Y-ML25 lymphoma cells and administered i.v. with DT-5461 on the indicated days after tumor inoculation. Mice were killed 14 days after tumor inoculation. *: $P<0.01$, **: $P<0.001$ (compared with the untreated control by Student's two-tailed *t* test.)

Table II. Dose-Response Relation of DT-5461 Effect on Liver Metastasis Following i.v. Injection of L5178Y-ML25 Lymphoma Cells

Administered i.v. with;	Dose (μg)	Mean weight (g) ± SD	
		Liver	Spleen
Untreated (PBS)	—	3.81±0.42	0.29±0.03
LPS	40	1.31±0.34*	0.23±0.04
DT-5461	10	1.55±0.29*	0.14±0*
	40	1.19±0.14*	0.14±0.02*
	200	1.14±0.03*	0.15±0.02*
Normal	—	1.07±0	0.07±0

Five CDF_1 mice per group were inoculated i.v. with 2×10^4 L5178Y-ML25 lymphoma cells and administered i.v. with LPS or DT-5461 on days 1, 5, 9 and 13 after tumor inoculation. Mice were killed 14 days after tumor inoculation. *: $P<0.001$ (compared with the untreated control by Student's two-tailed *t* test.)

Table III. Effect of DT-5461 on Experimental Lung Metastasis of B16-BL6 Melanoma Cells

Administered i.v. with;	Dose (μg)	No. of lung metastases on day 14
		Mean ± SD (Range)
Untreated (PBS)	—	64.8±8.1 (52–73)
LPS	40	55.2±8.9 (44–66)
DT-5461	10	49.0±2.8 (47–51)*
	40	36.0±3.8 (32–41)**
	200	30.0±9.0 (18–40)**

Five C57BL/6 mice per group were inoculated i.v. with 4×10^4 B16-BL6 melanoma cells and administered i.v. with LPS or DT-5461 on days 1, 5, 9 and 13 after tumor inoculation. Mice were killed 14 days after tumor inoculation. *: $P<0.01$ **: $P<0.001$ (compared with the untreated control by Student's two-tailed *t* test.)

lymphoma and B16-BL6 melanoma. Table II showed that liver and spleen metastases of L5178Y-ML25 cells were dramatically inhibited by four intermittent treatments with DT-5461 at doses ranging from 10 to 200 μg per mouse. Administration of 40 μg of LPS caused a similar reduction of liver weight to that of 10 μg of DT-5461. Similarly, multiple administrations of DT-5461 significantly reduced the number of B16-BL6 melanoma colonies in lungs in a dose-dependent manner in the range from 10 to 200 μg (Table III). In contrast, treatment with 40 μg of LPS did not inhibit lung metastasis of melanoma cells.

Table IV. Effect of DT-5461 in Various Administration Routes on Experimental Lung Metastasis of B16-BL6 Melanoma Cells

Administration with;	Route	No. of lung metastases on day 14 Mean±SD (Range)
Untreated (PBS)	—	114±22 (80–135)
DT-5461	i.v.	66±55 (52–86)*
	i.p.	52±24 (21–78)*
	s.c.	88±41 (48–134)
	i.n.	67±14 (56–87)*

Six C57BL/6 mice per group were inoculated i.v. with 4×10^4 B16-BL6 melanoma cells, and administered with DT-5461 (40 μg) on days 1, 5, 9 and 13 after tumor inoculation. Mice were killed 14 days after tumor inoculation. *: $P<0.01$ (compared with the untreated control by Student's two-tailed t test).

Table V. Effect of DT-5461 on Spontaneous Lung Metastasis of B16-BL6 Melanoma Cells

Administered i.v. with;	Primary tumor size on day 21 (mean±SD)	No. of lung metastases on day 35 Mean±SD (Range)
Expt. I		
Untreated (PBS)	11.1±0.7	44±22 (31–56)
LPS	9.9±1.2*	16±14 (4–39)*
DT-5461	8.9±0.6***	15±10 (1–36)**
Expt. II		
Untreated (PBS)	(10.4±0.5)	54±27 (26–87)
LPS		24±19 (6–36)
No. 506		39±16 (15–48)
GLA-60		28±17 (8–60)
DT-5461		19±9 (1–26)**

Eight C57BL/6 mice per group were administered i.v. with 40 μg of LPS, DT-5461 or synthetic lipid A analogues on days 1, 5, 9, 13 and 17 (Expt. I) or on days 22, 26, 30 and 34 (Expt. II) after tumor inoculation. Primary tumors were surgically removed on day 21 and mice were killed 35 days after tumor inoculation. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ (compared with the untreated group by Student's two-tailed t test).

Effect of administration route of DT-5461 on experimental lung metastasis The above study demonstrated that multiple i.v. administrations of DT-5461 significantly inhibited liver and lung metastasis produced by i.v. injection of two different tumors. We next examined the effect of the administration route of DT-5461 on experimental lung metastasis produced by i.v. injection of B16-BL6 cells. Table IV shows that intraperitoneal (i.p.) or intranasal (i.n.) administration of DT-5461 resulted in significant reductions of lung tumor colonies, as did systemic administrations of DT-5461, whereas subcutaneous (s.c.) administration showed only a slight inhibitory effect.

Inhibition of spontaneous lung metastasis of melanoma by multiple treatments with DT-5461 We investigated

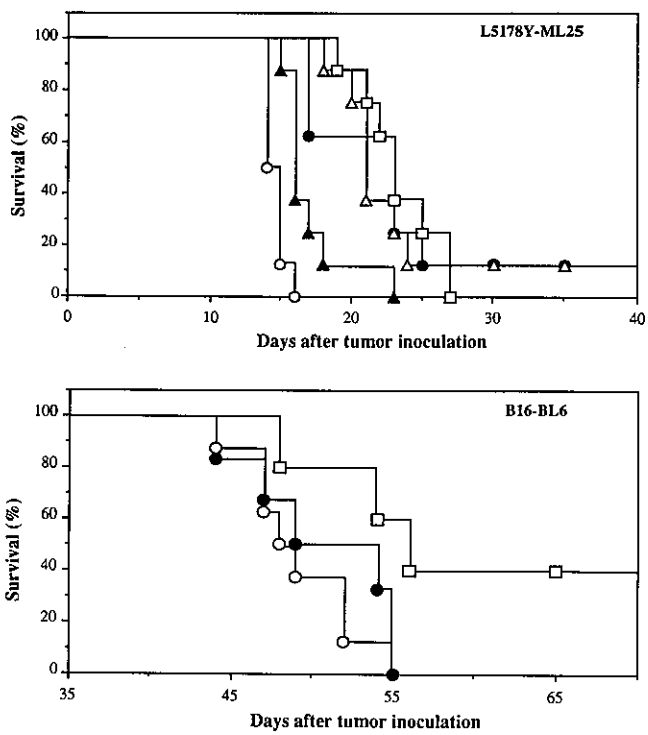

Fig. 2. Effect of i.v. administration of DT-5461 on survival of mice inoculated with L5178Y-ML25 lymphoma or B16-BL6 melanoma cells. Eight CDF 1 mice per group were inoculated i.v. with 4×10^4 L5178Y-ML25 lymphoma cells and administered i.v. with PBS (○), or 40 μg of LPS (●), No. 506 (△), GLA-60 (▲) or DT-5461 (□) on days 1, 5, 9 and 13 after tumor inoculation (upper panel). Eight C57BL/6 mice per group were inoculated s.c. with 5×10^5 B16-BL6 melanoma cells into right hind footpad and administered i.v. with PBS (○), or 40 μg of LPS (●) or DT-5461 (□) on days 1, 5, 9, 13 and 17 after tumor inoculation (lower panel). $P<0.01$, LPS-, No. 506-, GLA-60- or DT-5461-treated vs. untreated control (L5178Y-ML25); $P<0.05$, DT5461-treated vs. untreated control (B16-BL6) by Mann-Whitney's U-test.

whether or not multiple treatments with DT-5461 before or after surgical excision of primary tumors are able to inhibit primary tumor growth and lung metastasis caused by intrafootpad inoculation of B16-BL6 melanoma cells (Table V). Multiple administrations of DT-5461 as well as LPS before the excision of the primary tumor on day 21 significantly inhibited primary tumor growth at the time of amputation and lung metastasis of B16-BL6 melanoma (Expt. I in Table V). The administration of DT-5461 after amputation of the primary tumor significantly inhibited lung metastasis of B16-BL6 melanoma, whereas the administration of LPS, a synthetic lipid A (No. 506) or its subunit analogue (GLA-60) did not show any significant inhibitory effect (Expt. II in Table V). These results indicated that multiple treatments with DT-5461 before tumor amputation were effective for reduction of the primary tumor size and the number of tumor colonies in the lung. The treatment modality after primary tumor excision was also effective for the inhibition of lung metastasis.

Effect of DT-5461 on survival times of mice inoculated with lymphoma or melanoma cells We finally tested the prolongation of survival times of L5178Y-ML25- or B16-BL6-bearing mice by DT-5461. Multiple treatments with DT-5461, LPS, No. 506 or GLA-60 at a dose of 40 μg were performed on days 1, 5, 9 and 13 after i.v. inoculation of L5178Y-ML25 lymphoma cells (Fig. 2, upper). In this experiment, all untreated mice succumbed to the tumor burden within 16 days after tumor inoculation. The group that received DT-5461 as well as LPS and No. 506 showed significantly enhanced survival ($P<0.01$ by Mann-Whitney's U test) as compared with the GLA-60-treated or untreated control group. On the other hand, multiple i.v. administrations of DT-5461 on days 1, 5, 9, 13 and 17 after s.c. inoculation of B16-BL6 melanoma cells substantially prolonged the survival of mice inoculated with melanoma cells ($P<0.05$ by Mann-Whitney's U test) as compared with the untreated control (Fig. 2, lower).

DISCUSSION

The present study demonstrated that a new synthetic lipid A analogue of low endotoxicity,[7] DT-5461, exhibited therapeutic effects on liver and lung metastasis of L5178Y-ML25 lymphoma and B16-BL6 melanoma cells in mice. Multiple intermittent administrations of DT-5461 at intervals of 4 days significantly inhibited lung and liver metastasis of two different types of tumors in the experimental metastasis models (Tables I, II and III). In particular, Table I indicates that the administration schedule of DT-5461 is important for development of effective inhibition of tumor metastasis. The consecutive administrations of DT-5461 did not inhibit tumor metastasis. Our previous study has demonstrated that multiple treatments with rINF-γ/GLA-60 at intervals of 2 days caused the induction of endogenous TNF production and consequently the inhibition of lung metastasis of melanoma cells in mice, but consecutive treatments did not show any effect.[17,18] Tohgo *et al.* observed that consecutive administrations of DT-5461 failed to induce detectable TNF production in the serum, but administrations at intervals of 3 days or more amplified the production of endogenous TNF in the serum (manuscript submitted). Therefore, the antimetastatic activity of DT-5461 may be due to the effect of endogenous TNF produced by the administration schedule. The reason why the consecutive treatments of DT-5461 could provide no significant inhibition of liver metastasis is still unclear, but one possibility is a mechanism similar to that involved in the development of LPS tolerance resulting from depletion of intracellular TNF or down-regulation of TNF production by multiple LPS stimulations.[20] Further study will be need to establish the precise mechanism.

We also showed that not only i.v. administration of DT-5461 but also i.p. or i.n. administration of DT-5461 inhibited pulmonary metastasis of B16-BL6 cells (Table IV). These results indicate that DT-5461 was effective for the inhibition of lung metastasis by both systemic and local administration. Multiple administrations of DT-5461 at intervals of 4 days before or after the excision of primary tumors significantly reduced the number of lung tumor colonies and primary tumor size at the time of amputation, whereas multiple administrations of LPS or other synthetic lipid A analogues did not (Table V). These results indicated that DT-5461 has more potent antimetastatic activity against tumor growth and metastasis of B16-BL6 melanoma than LPS or other synthetic lipid A analogues. This treatment modality with DT-5461 as well as LPS or other synthetic lipid A analogues significantly prolonged the survival time of mice inoculated with lymphoma or melanoma cells (Fig. 2). Since DT-5461 is much less toxic than LPS and a synthetic lipid A (No. 506),[7] it should be useful for the treatment of cancer metastasis.

The mechanism of the inhibition of tumor metastasis by the administration of DT-5461 is not well understood. DT-5461 did not exhibit direct cytotoxicity against tumor cells, nor did it affect cell growth, i.e. incorporation of ^{3}H-thymidine in the cells (data not shown). This suggests that the antimetastatic effect of DT-5461 cannot simply be explained in terms of direct cytotoxicity toward tumor cells. Tohgo *et al.* have shown that the effectiveness of DT-5461 on tumor growth *in vivo* may be largely attributable to its ability to induce high levels of TNF in tumor tissue (manuscript submitted). Furthermore, we have previously reported that the treatment modality for induction of endogenous TNF by adminis-

tration of rINF-γ/GLA-60 inhibited murine lung metastasis of B16-BL6 melanoma.[18] Therefore, the inhibitory effect of DT-5461 on tumor metastasis may be associated with the stimulation of host immune defense mechanisms, including the activation of macrophages and NK cells. Further study will be needed to establish in detail the mechanism of the inhibitory effects.

In conclusion, the present study has demonstrated that a new synthetic lipid A analogue, DT-5461, inhibited liver and lung metastasis of different tumors and enhanced the survival rate. Although the mechanism of the regression of tumor metastasis by the administration of DT-5461 remains to be determined in detail, it may be potentially useful in the prevention of cancer metastasis.

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