Antitumor Activity of Activated Lymphocytes and Macrophages by Liposome-borne Tumor-specific Transplantation Antigens on Postsurgical Tumor Recurrence in Murine

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ABSTRACT

Liposome-borne tumor-specific transplantation antigens (TSTA) potentiated the antitumor activity of cytotoxic lymphocytes and macrophages (Mϕ) much more efficiently than empty liposomes in murine. Mϕ obtained from peritoneum and lung as well as cytotoxic T-lymphocytes (CTL) such as lymphokine-activated killer (LAK) cells and tumor infiltrating lymphocytes (TIL) showed higher inhibitory activity on metastatic tumor cell growth in lung. Among the effector lymphocytes *in vivo*, TIL showed desiable antitumor activity by way of intravenous injection, while peritoneal Mϕ showed high cytotoxicity by intraperitoneal injection and intraveneously alveolar Mϕ also showed high cytotoxic activity. These results suggest that the suitable administrations of liposome-borne TSTA may be useful in potentiating the tumoricidal effect of effector cells *in vivo*, especially TIL, CTL, and Mϕ, and possibly may aid in overcoming tumor metastases.

Key words: Liposome, Tumor-specific transplantation antigens, Macrophages, Tumor infiltrating lymphocytes, Crude butanol extracts

INTRODUCTION

A large amount of evidence has been accumulated which points to cytotoxic

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[2] The abbreviations used are: TSTA, tumor-specific transplantation antigens; CBE, crude 1-butanol extracts; MCA, 3-methylcholanthrene; TIL, tumor infiltrating lymphocytes; Mϕ, macrophages; CTL, cytotoxic T-lymphocytes; LAK, lymphokine-activated killer; PC, phosphatidylcholine; PG, phosphatidylglycerol; Chol, cholesterol; rIL-2, recombinant interleukin-2.

lymphocyte activation as of great importance in host defense against primary and metastatic tumors (1, 15, 17, 18, 23). Inactive lymphocytes can be activated to manifest antitumor activity by a variety of immunomodulator agents, such as lymphokines designated as interleukin-2, interferons, and natural mitogeneic substances such as PHA and PWM. Besides these, TSTA[2] modulate host defense mechanisms through activation of natural killer (NK) cells and induction of antitumor T cell immunity. Recently, we have demonstrated that butanol extracted TSTA showed stimulated CTL and potentiated antitumor activity *in vivo* (19). Thus, TSTA seems to be a promining potentiator of host resistance against tumors; however, several major obstacles, such as its weak imunogeneity and specificity, remain to be overcome if it is to be used successfully for immunotherapy. In an attempt to tackle these problems, we have used liposomes as a vehicle for TSTA; this did result in efficient tumoricidal activation of CTL, which ingested liposomes *in vitro* and *in vivo* in murine. Liposome is an artificial membrane with extensive industrial, pharmaceutical, and medical uses, and interestingly has shown potent opsonic ability for macrophage- phagocytosis (26). Therefore, we have prepared liposomes and explored the applicability of the immunomodulatory agent as a carrier for delivering immunopotentiators to cytotoxic lymphocytes. Liposomes are capable of holding various drugs including immunopotentiators, and are stable physically as well as chemically under physiological conditions. In the last year, we also have reported that liposome-borne TSTA was far more efficient in enhancing the inhibitory activity of CTL for tumor growth than empty liposome *in vivo* (22).

The present study focused on the potentiation of the inhibition by macrophages (Mϕ) of *in vivo* tumor growth by liposome-borne TSTA. Incorporation of TSTA in the liposomes allowed not only effector lymphocytes but resident Mϕ to be effectively activated and prevent the host from tumor metastases *in vivo*.

MATERIAL AND METHODS

1. *Mouse and tumor cells*

Mouse fibrosarcoma MCA-F cell line was established in female C3H/HeJ mice with MCA (13) and used after seven *in vivo* generations. The minimum tumorigenic dose of this antigenically distinct fibrosarcoma cell was 10^2. Tumors were maintained by serial s. c. passage in 4 to 6-week-old specific pathogen-free female C3H/HeN (MTV^-) mice (Charles River, Kingston, NY) as previously reported (6). 5×10^5 of MCA-F tumor cells were injected into the right hind footpad (i. f. p.) of mice. When the growing subcutaneous tumors reached an average diameter of 1.0 cm, the tumor-bearing leg, including the popliteal lymphonode, was resected at midfemur. Three weeks after the resections, spon-

taneous metastatic lung colonies of 1-2 mm diameter were aseptically removed. From the same mouse, 4 colonies were isolated from each individual lung nodule and single cell suspensions were prepared, as described previously (18, 23). The spontaneous lung metastatic cell lines, designated as MCA-F-M1 through MCA-F-M4, were cloned twice *in vivo* by the limiting dilution technique as reported previously (17). Cultures were incubated in 5% CO_2 at 37℃. Two weeks later, the cultures showing predominant cell growth were harvested by brief incubation in 0.05% trypsin with 0.02% EDTA and a second single cell cloning was done for each clone. By contineous culturing in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine, each clone was propagated sufficiently *in vivo* before use.

2. *Butanol treatment and extraction of TSTA*

Tumor cell extracts were prepared as described previously (6, 8, 9). In brief, cultured cells were harvested from the flask by brief incubation in 0.05% trypsin-0.02% EDTA. Collected cells were washed 3 times with Hank's balanced salt solution (HBSS) prior to extraction with 2.5% (V/V) 1-butanol in Dulbecco's phosphate-buffered saline (PBS). The viability of the extracted cells was routinely determined by trypan blue dye exclusion test and was almost always over 96%. The yield of protein which was obtained by butanol treatment of the cultured tumor cells was 10 μg/10^6 cells. The antigen dose was based on the protein concentration in the CBE indicated by the Pierce protein assay (Pierce Chemical Co., Rockford, IL) with ovalbumin as a standard.

3. *Preparation of liposome*

For experimental immunoassays, butanol dialysis vesicles were prepared from 2.1 mg CBE and 60 μmol lipids as described previously (7, 22). The lipid composition for negatively charged liposomes was a 7:2:1 molar ratio of PC: PG: Chol. Lipids were combined into 9:1 (V/V) chloroform: methanol, rotoevaporated to dryness, and resuspended in 0.42 m*l* butanol. The butanol-lipid fraction was admixed with 6 m*l* CBE (2.1 mg), vortexed vigorously to obtain an opalescent suspension, and dialyzed overnight at 4℃ against 2,000 m*l* of PBS. The resulting liposomes were washed twice in adequate PBS by centrifugation at 24,900×g for 10 min at 4℃. The first supernatant was saved for determination of the protein incorporated into liposomes. As empty liposomes, 2.1 mg ovalbumin in 6 m*l* PBS was used instead of CBE. CBE protein incorporation into liposomes was around 40%.

4. *Collection of peritoneal and alveolar Mϕ*

Peritoneal exudate Mϕ (PEM) were collected by peritoneal lavage from C3H/HeN mice given i. p. or i. v. injections of 1.0 m*l* of each immunogen twice

weekly for 2 weeks, and alveolar Mϕ (AM), by tracheal lavage as well (16). The PEN and AM were centrifuged at 250×g for 10 min and resuspended in serum-free medium at a concentration of 5×10^5 cells/m*l*, and 0.2 m*l* of the cell suspension was plated into 96-well round-bottom tissue culture dishes (Corning 25850, Corning Glass Works, Corning, NY). After a 60-min incubation, the wells were rinsed with medium to remove nonadherent cells and the culture was re-fed with medium containing 10% FBS. The adherent cells were assessed for Mϕ by Giemsa staining (purity more than 98%).

5. *Preparation of CTL and TIL*

For the preparation of CTL, non-adherent lymphocytes were harvested from the spleen of C3H/HeN mice after immunization by CBE and liposome-borne TSTA twice weekly i. v. or i. p. for 2 weeks. Three days after the last injection, CTL and TIL were obtained from the metastatic tumor by passing over Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). After washing 3 times with HBSS, these lymphocytes were collected by passing the solution through a nylon wool column twice; they showed approximately 98% Thy-1 (monoclonal antibody reacted with T-lymphocyte) positive by indirect immunofluorescence. Monoclonal antibody L3T4, which reacted with cytotoxic/suppressor cells, showed 76% and 84% positive against CTL and TIL, respectively. Collected lymphocytes were cultured in RPMI-1640 medium containing 10% FBS with 1,000 Units/m*l* of rIL-2 and 2×10^{-5} Mol/m*l* of 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) for 7 days. Before the experiments, the number of lymphocytes were adjusted to an E/T ratio of 100. rIL-2 produced by recombinant DNA technology was provided by Biogen Research Corp., MA.

6. *Experimental protocol*

In order to examine the ability of liposome-borne TSTA to engender the antitumor activity *in vivo*, the immunoprotection assay was used as reported previously (7, 14, 22). Briefly, mice were inoculated into the right flank with 1×10^6 MCA-F-M4 tumor cells on day −14. Then, the growing tumors were resected completely (on day 0), and from these CBE were extracted and incorporated into liposomes. On day +7, CBE and liposome-borne CBE (15 μg) were injected s. c. into the abdomen. Tumor cells were cultured as the challenge tumor cells and injected s. c. into the left flank with 5×10^4 cells and the diameters of the resulting tumors were measured. Tumor recurrence was evaluated by the same procedures as reported previously (20, 22) to examine the efficacy of immunotherapy with liposome-borne TSTA against the spontaneous lung metastases. In the present study, 5×10^5 MCA-F-M4 viable cells were injected into the right hind footpad of the mice. The growing tumors were resected when the tumor reached 8-10 mm diameter without any necrosis or bleeding (day 0), and

the tumor-bearing legs including the popliteal lymphonode were resected at midfemur. Subsequently, CBE were extracted and incorporated into liposomes. On day +7, CBE and liposome-borne CBE were injected s. c. into the mice. Usually, 3 weeks after the tumor resections, spontaneous metastatic lung colonies of 1-2 mm diameter were observed. As the immunotherapy after the tumor resection, mice were given CBE (15 μg) or liposome-borne CBE injections intraperitoneally (i. p.) or intravenously (i. v.; via teil vein) twice a week for 4 weeks and the number of spontaneous lung metastatic colonies were counted (Fig. 1). Significant differences in mean tumor diameter of the control and therapy group were determined using the Student-Newman-Keuls multiple comparison test. The tumor growth rate (mm/day) was obtained at a certain interval, and the tumor growth ratio was calculated as: tumor growth rate of treatment group/tumor growth rate of control group. The fifty percent survival time (ST_{50}) was determined by the number of mice surviving in each group. The percent survival was converted to probits and plotted against the $\log_{10}$ of the days after tumor resection. The intersection of the probit value at 5.0 with the regressed survival line yielded the ST_{50} as described previously (14). The ST_{50} values of treated and control group were compared by Chi-square test.

7. *Cytotoxicity assay*

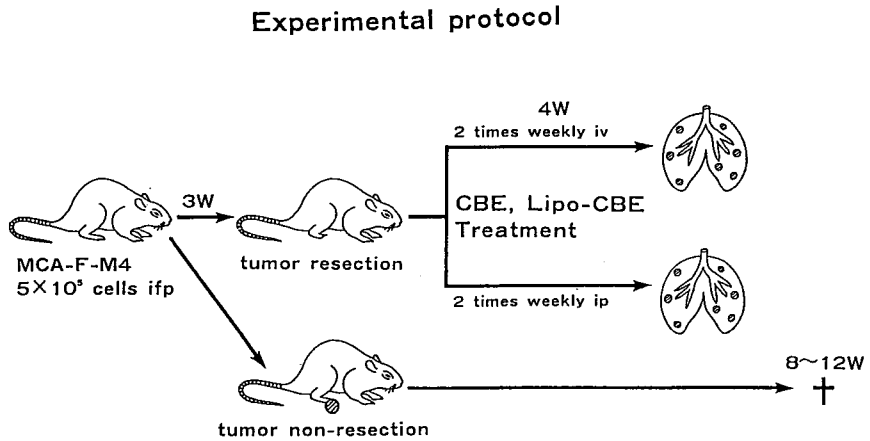

Fig. 1 MCA-F cells were injected into the right hind footpad of female C3H/HeJ mice when the tumors reached the average diameter of 1.0 cm, and the tumor-bearing legs, including the popliteal lymph nodes, were amputated at midfemur. The mice were then given CBE or Lipo-CBE treatments twice weekly for 4 weeks and the number of spontaneous lung metastatic colonies were counted from week 7.

The ^{51}Cr release assay for the determination of cytotoxic activity of CTL and TIL was carried out as described previously (17, 19, 21). In brief, target tumor cells were labeled by 100 μCi of ^{51}Cr Sodium chromate (New England Nuclear, Boston, MA) and incubated for 3 h at 37℃. The cells were washed 5 times with PBS, and 1×10^4 MCA-F-M4 target cells in 0.1 m*l* MEM were seeded into each U-bottomed microtiter plate (Corstar 3799, Cambridge, MA). Next, the predetermined dose of 0.1 m*l* of the effector lymphocyte-suspension was added, and the plates were centrifuged at 200×g for 5 min. After 6 h incubation at 37℃ in 5% CO_2 incubator, 0.1 m*l* of culture supernatant was harvested from each plate and the radioactivity was counted with a liquid gamma scintillation counter. The percent of lysis was calculated as follows: % cytotoxicity= (experimental release—spontaneous release)/(maximal release—spontaneous release)×100. To determine the maximal release, 0.1 m*l* of 0.1 N hydrochloric acid was added to the appropriate wells. Spontaneous release was obtained by incubation of target cells with medium alone, indicating usually below 15% in the all experiments. All determinations were carried out in triplicate, and the data were represented as the mean ± SE. On the other hand, Mϕ mediated cytotoxicity was assessed by a radioactive release assay, as previously reported by others (4, 5). MCA-F-M4 target cells (1×10^4) were incubated in the appropriate medium which contained [^{125}I] iododeoxyuridine (0.3 μCi/m*l*, specific activity, greater than 2,000 Ci/mmol; New England Nuclear, Boston, MA) for 24 h. The cells were then washed twice in HBSS to remove unbound radiolabel, harvested by a 1 min trypsinization, then resuspended in medium. The labeled cells were plated into culture wells to obtain an initial Mϕ : target cell ratio of 10 : 1. Radiolabeled target cells were plated alone as a control group. After 24 h the cultures were washed to remove the nonadherent target cells, re-fed with fresh medium, and then cultured for an additional 2 days at 37℃. At 72 h after the plating, the cultures were washed twice with HBSS, and the remaining, presumably adherent, viable target cells were lysed with 0.1 m*l* of 0.1 N NaOH. The lysate was absorbed on cotton swabs and monitored for radioactivity in a gamma counter. The percentage of generated Mϕ cytotoxicity was calculated as follows: % of specific cytotoxicity mediated by activated Mϕ=(release of target cells with control Mϕ—release of target cells with activated Mϕ)/release of target cells with control Mϕ×100. The statistical significance of differences between the control group and the therapy group were determined by Student's two-trailed t-test.

RESULTS

1. *Antitumor activity of liposome-borne TSTA in the tumor-bearing mice*

The antitumor efficacy of liposome-borne TSTA immunotherapy against challenge tumor cells was studied. A group of 6 mice were inoculated into the right flank with 1×10^6 MCA-F-M4 cells on day−14. The growing tumors were resected on day 0. On day+7, TSTA (15 μg of CBE) and liposome-borne TSTA were injected s. c. into the abdomen. Simultaneously, tumor cells were injected s. c. into the left flank of mice with 5×10^4 cells and mean tumor diameters were measured. On day+30, though the inoculation by PBS and empty liposome showed no effect on tumor growth, TSTA and liposome-borne TSTA therapy showed significant antitumor effects ($P<0.001$). Liposome-borne TSTA showed more remarkable antitumor activity than that of TSTA alone, ST_{50} in the liposome-borne TSTA treated mice showed the longest group survival rate (58.3 day; Table 1) and comprised the group of the longest-lived (Fig. 2) of the 4 therapy groups. The results clearly suggested that liposome- borne TSTA was effective to engender antitumor immune response *in vivo*.

2. *Immunoprotective activity of liposome-borne TSTA against spontaneons lung metastasis*

On the protocol described in *materials and methods*, liposome-borne CBE showed marked suppression of lung metastasis (91% tumor growth inhibition versus the non-resection group as a cotrol; $P<0.001$) on 10 weeks sutseguent to

Table 1. *Immunoprotective activity of liposome-borne CBE (TSTA).*

Therapy group	Mean tumor diameter[d] (mm ± SE)	P[e]	ST_{50}[f]	P[g]
PBS	24.2±1.6	— <0.001	36.2	—
CBE[a]	18.4±1.3	<0.001<0.001	46.4	<0.05
Lipo-CBE[b]	12.2±0.8	<0.001< —	58.3	<0.001
Empty-Lipo[c]	25.4±2.1	NS <0.001	38.3	NS

Groups of 6 mice were inoculated into the right flank with 1×10^6MCA-F-M4 tumor cells on day-14. The growing tumors were resected on day 0. Subsequently, CBE were extracted and incorporated into liposomes. On day +7, CBE and Liposome-borne CBE were injected sc. Tumor cells were cultured as challenge tumor cells and injected sc into the left flank with 5×10^4 cells and measured for mean tumor diameters.

[a] Immunized with 15 μg of CBE.

[b] Incorporated 15 μg of CBE protein in negatively charged liposomes.

[c] Instead of CBE, 15 μg ovalbumin was used.

[d] Measured on day +30.

[e] The significance was assessed by the Student-Newman-Keuls test, compared with either PBS control in the left column or Lipo-CBE group in the right column. NS is not significant.

[f] ST_{50}, the 50% survival time in days.

[g] The significance was assessed by Chi-square test, compared to PBS control group.

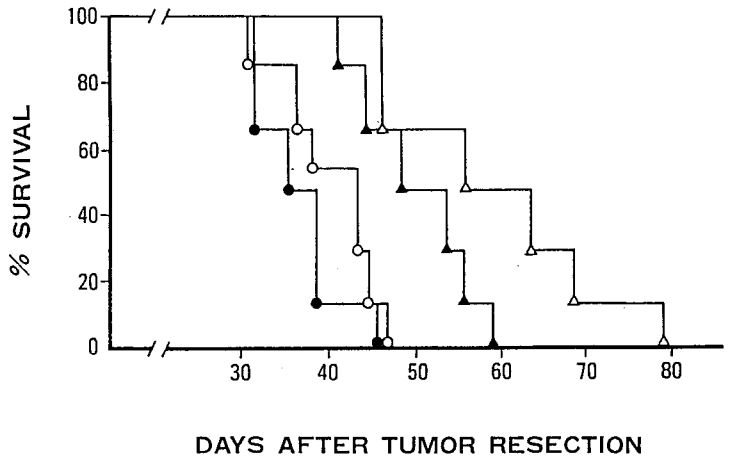

Fig. 2 Percent survivals of treated mice: Groups of 6 mice were treated with PBS (●), Empty liposomes (○), CBE (▲) and Liposome-borne CBE (△) in the same protocol as described above and observed till their termination.

i. v. injection. TSTA alone was also effective in antitumor activity but did not result in such long survival rate as liposome-borne TSTA. Comparing i. v. and i. p. inoculation, i. v. therapy showed higher effectiveness against lung metastasis (Table 2).

3. *Cytotoxic activity of Mϕ and effector lymphocytes*

Examinations of Mϕ mediated cytotoxicity showed higher cytotoxicity by i. v. injection of CBE alone and liposome-borne TSTA for AM, while, in case of PEM, the cytotoxicity was higher by i. p. injection (Fig. 3). As to effector lymphocytes, TIL showed a high cytotoxicity in i. v. of TSTA (15 μg of CBE) alone and especially in liposome-borne TSTA injections. On the other hand, CTL obtained from spleen did not necessarily show the high cytotoxicity that TIL showed in the same condition. These results suggested the superiority of TSTA, especially when encapsulated in liposome on antimetastatic tumors, and a distinct influence of administration route on its effectiveness.

DISCUSSION

A large number of preclinical studies have shown that the therapeutic effect of antineoplastic agents can be potentiated by the immune responses of the host against immunogenic tumors (10, 12, 22). It has also been demonstrated that various biological response modifiers (BRM) and TSTA can engender the antitumor immunity of the host (11, 19, 24). To obtain this type of immunotherapeutic

Table 2. *Number of spontaneous lung metastatic colonies with or without immunotherapy.*

		No. of lung metastatic colonies ± SE		
		7w	8w	10w
Tumor resection				
(−)		13±3	52±12	88±26
(+)		28±4	78±28	94±30
Treatment				
CBE[a]	iv		14±5 (72)[c]	20±11 (77)
	ip		24±6 (54)	48±12 (45)
Lipo-CBE[b]	iv		6±3 (88)	8±6 (91)
	ip		26±8 (50)	56±10 (36)

Groups of 6 mice were examined as described previously. The number of spontaneous lung metastatic colonies were counted on weeks 7, 8 and 10 after MCA-F-M4 cells inoculation into the right hind footpad.

[a] Immunized with 15 μg of CBE.

[b] Incorporated 15 μg of CBE protein in negatively charged liposomes.

[c] % tumor growth inhibition against the tumor non-resection group as a control.

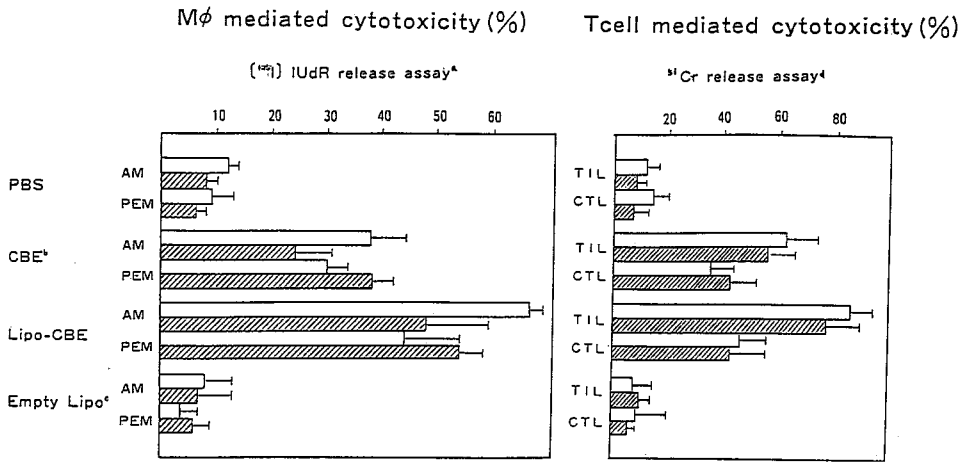

Fig. 3 Each immunogen was injected into 3 mice twice weekly i. v. (□) or i. p. (▨) for 2 weeks. Three days after the last injection, Mφ and spleneic lymphocytes were harvested 24 h later and cultured for the cytotoxicity assay.

[a] [125I] IUdR-labeled MCA-F-M4 target cells (1×10^4) were cultured with AM (alveolar Mφ) or PEM (peritoneal Mφ) for 72 h at 37℃. *Columns*, percentage of cytotoxicity; *bars*, SE.

[b] Concentration of CBE was 15 μg.

[c] Instead of CBE, 15 μg ovalbumin was used.

[d] Cytotoxic activity of CTL and TIL propagated by rIL-2 was assessed in ^{51}Cr release assay for 6 h incubation at an E/T ratio of 100. *Columns*, percentage of cytotoxicity; *bars*, SE.

synergism, however, the activity of the host's effector cells must be directed specifically against tumor cells. Our study was designed to test for differences in antitumor efficacy among administration routes of TSTA and between the effectiveness of TSTA and liposome-borne TSTA as well. As we reported previously (20), TSTA extracted by butanol treatment were effective for immunotherapy in a murine model of local recurrence; also, examinations of the effect of combination chemoimmunotherapy with TSTA and cyclophosphamide showes that such a regimen afforded better protection against postsurgical tumor growth. TSTA incorporated into lipid vesicles induced an effective antitumor immune response in the tumor recurrence system. Liposome-borne TSTA was found to be far more efficient in eliciting the antitumor responce than TSTA alone in the retardation of recurrent tumors and the prolongation of survival time (22). The results of the present study concerning antitumor activity using TSTA and liposome-borne TSTA were consistent with previous data.

Liposomes have been commonly used as a material for coating and microencapsulating various drugs. The drugs thus employed so far have been tumoricidal chemicals and liposome-borne chemicals easily entrapped by Mϕ. One of the advantages of liposome for targeting a drug to Mϕ is that it works as a strong opsonin; thus, liposome has been uned as a safe plasma expander *in vivo*. In the murine experiments, we demonstrated that *in vivo* effector cells such as Mϕ, TIL, and CTL showed favorable antitumor activity against tumor cells after TSTA or liponome-borne TSTA administration. Systemic administratinon of liposome-borne activators succeeded in delivering activators to reticuloendothelial cells *in vivo* (24). Fidler and his collaborators demonstrated the induction of tumoricidal activity in mouse Mϕ by liposome containing lymphokines (2, 15), muramyl dipeptide, and its lipophilic derivative (3, 25). Liposome-borne Mϕ activating factors (MAF) showed stronger cytotoxic activity in humans than free MAF did. As to other effector cells *in vivo*, such as TIL and CTL, a similar result was obtained by our previous report (19).

In the present study, it was shown that the cytotoxicity of TIL depended on the TSTA administration route; i. v. administration was clearly the most favorable method for TIL and i. p., for CTL obtained from spleen . Our previous studies of tumor destruction by sensitized CTL have provided various immunological evidence regarding tumor-host response. Further, it was also suggested that HMC restriction was necessary for CTL to propagete and produce a strong destructive response against tumor cells (17, 21). This supports that T-cell recognition against weak TSTA in tumor-bearing host could preferentialy produce numerous tumor-specific cytotoxic effects by stimulation using TSTA. It is, however, still unclear as to the datails of the complicated immunological

relationships between tumor cells and effector cells. Further immunological, oncological, and genetic studies of tumor and host relationships are needed in the near future.

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