Immunotherapeutic Effects of Autologous Liposome-borne Tumor-specific Transplantation Antigens in Combination with Cyclophosphamide on Postsurgical Tumor Recurrence in Mice

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SUMMARY

Autologous crude butanol extracts (CBE) from the 3-methylcholanthrene-induced murine fibrosarcoma, MCA-F, incorporated into lipid vesicles (liposome) induced an effective antitumor immune response in an autologous tumor recurrence system. The tumor-specific transplantation antigens (TSTA) expressed on the tumor cell surface were extracted using single-phase aqueous solutions of 2.5%(V/V) 1-butanol, and were used to prepare a liposome-borne activator of antitumor *in vivo* immunity. Liposome-borne TSTA was found to be far more efficient in eliciting an antitumor response than TSTA alone, resulting in a significant retardation of challenge tumor growth on day 27 post challenge ($P<0.01$; 10 μg of CBE, $P<0.005$; 30 μg of CBE), and prolonged the survival rates of challenged mice significantly ($P<0.05$). Furthermore, in the same experimental system the therapeutic effect of multiple immunizations with liposome-borne TSTA suggested that 10 day intervals using 10 μg TSTA yielded optimal survival rates. As a model of local cancer recurrence in man, an autologous tumor recurrence system in mice was devised and the immunother-

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The abbreviations used are:
TSTA, tumor-specific transplantation antigens; CBE, crude butanol extracts;
PC, phosphatidylcholine; PG, phosphatidylglycerol; Chol, cholesterol;
PBS, Dulbecco's phosphate-buffered saline; HBSS, Hank's balanced salt solution;
RES, reticuloendothelial system.

apeutic activity of liposome-borne autologous TSTA with or without a combination chemoimmunotherapy using cyclophosphamide (CY) was examined. The administration of 20 mg/kg CY i. p. and liposome-borne autologous TSTA (15 μg) s. c. on the same day clearly demonstrated that the autologous TSTA encapsulated in liposomes produced a more effective antitumor response against secondary autologous tumor cell challenge following resection of the primary tumor. Other therapy protocols, including a combination therapy of CY and liposome-borne syngeneic TSTA were less effective. Thus, this study suggested that liposome-borne TSTA was effective in the induction of an antitumor immune response and, most of all, that combination chemoimmunotherapy using liposome-borne autologous TSTA and low dose of CY was best for the prevention of secondary tumor growth.

Key words : Autologous tumor-specific transplantation antigens, Crude butanol extracts, Liposome-borne antigens, Cyclophosphamide, Chemoimmunotherapy

INTRODUCTION

Murine tumor cells frequently express a unique cell surface antigens against which an antitumor immune response is directed. Many chemically induced tumors express individually specific TSTA[3] that can lead to tumor rejection by immunized animals(27). In the field of tumor-host immune response, particularly regarding tumor immunotherapy, there has been a need to analyze TSTA biochemically, immunologically and genetically. In parallel with the advance of tumor surface antigens extraction methods using various chemical reagents such as Nonidet-P40, Triton X-100(8) and 3M KCl(12, 28, 29), the role of the TSTA has been clarified gradually. Moreover, in recent years, further understandings of precise TSTA has been attributed to the extraction technique using butanol which showed biochemical pre-eminence in TSTA extraction(18, 37). Especially, by using a single-phase solution(2.5% V/V) of 1-butanol, many interesting biochemical and immunological features of TSTA have been revealed in detail (13-18, 37, 38, 40, 41).

Many experimental trials have been successfully undertaken in mice for the application of active specific immunotherapy using extracted TSTA(9, 10, 28, 44). Furthermore, in order to obtain more significant efficacy of TSTA in antitumor response. Steele *et al.*(36) recently reported that liposome-borne antigens were effective for both immunoprotection and immunotherapeutic protocols, suggesting the possibility of treatment for human cancer using liposome-borne antigens extracted by butanol. The application of liposomes as carrier and adjuvant prop-

erties to deliver activators to reticuloendothelial cells *in vivo* was a great methodical step ahead, especially in the context of the therapy for tumor recurrence. Fidler and his collaborators have contributed much to the induction of tumoricidal activity in mouse macrophages by liposome containing lymphokines (5, 30) or muramyldipeptide (MDP), and its lipophilic derivative(6, 35). These systemic administration of the liposome-borne activators lead to the activation of the tumoricidal properties of macrophages *in vivo* and to the eradication of established spontaneous pulmonary metastases significantly(30). In humans, it was recognized that monocytes were stimulated to the cytotoxic activity against allogeneic tumor cells *in vitro* by 800-1000 times less liposome-borne macrophage activating factor (MAF) than free MAF(11), and that alveolar macrophages also stimulated the tumoricidal activity by liposome-borne MDP efficaciously(34). Therefore, it was suggested that the utility of liposome-containing TSTA might also serve various means of immunotherapy in human cancer.

As previous studies demonstrated, the combination therapy of extracted TSTA and cyclophosphamide (CY) revealed excellent antitumor effects(26). In most of the antitumor chemical agents, CY has been known to be one of the most available drug producing a tumoricidal effect directly and indirectly(49). In particular, low doses of CY have been reported to potentiate the host immune response by selective elimination of suppressor T-cells or their precursors(4, 25). Therefore, suitable low dose of CY provided higher secondary antitumor immune response using the combination therapy with TSTA(26). Recently, we also reinforced the theory with the assay of the combination chemoimmunotherapy of butanol extracted autologous TSTA and low dose of CY in autologous tumor recurrence system(42).

Under the complicated genetic control, the individual TSTA of chemically induced tumors in mice as well as of spontaneously produced tumor in human seemed to be responsible for the antitumor immune response in host(27, 31, 48). As human specific cytotoxic T-cells were restricted to HMC-1 in recognition of autologous tumor cells(22, 23), for the induction of specific antitumor immune response, the genetic accordance of adopted TSTA and T cell lineage which recognized that it might be necessary for more desirable immunoprophylaxic cancer treatment in human.

The purpose of the present investigation was to prove the utility of TSTA encapsulated with liposome in producing an antitumor immune response and to explore the therapeutic efficacy of liposome-borne autologous TSTA combined with CY in autologous tumor recurrence system.

MATERIALS AND METHODS

1. *Animals and tumors*

The 3-methylcholanthrene-induced fibrosarcoma, MCA-F, induced in female C3H/HeJ mice (The Jackson Laboratories, Bar Harbor, ME) was maintained by serial s. c. passage in 4-6 week old specific-pathogen-free female C3H/HeN (MTV^-) mice (Charles River, Kingston, NY) as described previously (13, 29).

2. *Experimental System*

The ability of liposome-borne TSTA to mediate a protective antitumor effect was assessed using the immunoprotection assay(18, 29). In brief, hosts received a single s. c. immunization with liposome-borne antigen 10 days before tumor cell challenge, and the antitumor immune response was determined by measuring tumor growth. In some experiments, mice received two immunizations with liposome-borne antigens at 10 day intervals prior to challenge.

An autologus tumor recurrence system which was the same reported previously(41) was used to examine the efficacy of combination chemoimmunotherapy with liposome-borne TSTA and CY against tumor recurrence. In this assay, 1×10^6 MCA-F viable cells were injected s. c. into the right flank on day -14, and the growing tumors were completely resected when the tumor reached 8 to 10 mm diameter without necrosis or bleeding (day 0). Each of the harvested tumors was dissociated and divided into 2 culture groups to provide materials for autologous CBE and autologous tumor challenge. To obtain CBE, four-fifths of the cells were propagated in culture flasks with Eagle's minimal essential medium supplemented of 10% fetal bovine serum, vitamins, nonessential amino acids, sodium pyruvate, and L-glutamine. CBE was prepared from each individual tumor cell culture after 4 days. CBE was incorporated into liposomes, protein concentrations were determined, and titered recombinant liposome-borne TSTA was injected s. c. into the right flank of autologous mice on day $+7$. Some mice also received 20 mg/kg of CY (cytoxan, Sigma Chemical Co., St. Louis, MO) dissolved in distilled water at a concentration of 20 mg/m*l*, and administered i. p. on day $+7$. The remaining tumor cell cultures were maintained until used for challenge of 5×10^4 viable cells injected s. c. into the left flank on day $+14$. By using both autologous CBE and challenge cells in the autologous host, we considered that this is a model of human tumor recurrence. For syngeneic CBE, we used extracts from MCA-F tumors grown in other mice. Control groups received either PBS or liposomes containing ovalbumin instead of CBE.

Tumor growth was measured on day 15, 21, and 27 post challenge as the average of two perpendicular measurements of tumor diameter, and the mean

tumor diameter of each group was calculated(13, 29). Significant differences in mean tumor diameter of control and treatment groups were determined using the Student-Newman-Keuls multiple comparison test. The tumor growth rate (mm/day) was obtained during days 15 to 27 after challenge, and the tumor growth ratio (TGR) 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 daily observations of the number of mice surviving in each group. The percentage survival was converted to probits and plotted against the $\log_{10}$ of the days fater tumor challenge. Intersection of probit value 5.0 with the regressed survival line yielded the ST_{50} as described previously(29). The ST_{50} values of treated and control group were compared by Chi-square test.

3. *Butanol extraction and preparation of liposomes*

Tumor cell extracts were prepared as described(13-18). Antigen dosage was based on the protein concentration in the CBE estimated by the Pierce protein assay (Pierce Chemical, Rockford, IL), with ovalbumin as a standard. The yield from butanol extraction of 1 to 5×10^7 cultured cells yielded 200 to 400 μg of protein. For combination chemoimmunotherapy assays, butanol dialysis vesicles were prepared from 2.1 mg CBE and 60 μmol lipid as reported previously(16). The lipid composition for negatively charged liposomes was a 7:2:1 molar ratio of PC: PG: Chol. The lipids were combined into 9:1 (V/V) chloroform: methanol, rotoevaporated to dryness, and resuspended in 0.42 ml butanol. The butanol-lipid fraction was admixed with 6 ml CBE (2.1 mg), vortexed vigorously to obtain an opalescent suspension, and dialyzed overnight at 4℃ against 2,000 ml of PBS. The resulting liposomes were washed twice in PBS by centrifugation at 24,900 × g for 10 min at 4℃. The first supernatant was saved for protein determination to estimate the protein incorporated into liposomes. For empty liposomes, 2.1 mg ovalbumin in 6 ml PBS was used instead of CBE. CBE protein incorporation into liposomes was about 35%.

RESULTS

In the first set of experiments we examined the immunoprotective effect of liposome-borne tumor antigens against subsequent s. c. challenge with the parental MCA-F tumor (Table 1). Immunization of mice 10 μg or 30 μg of soluble CBE protein resulted in a significant reduction in tumor diameter ($P<0.005$). Inclusion of CBE protein into negatively charged liposomes enhanced the protective immune response, relative to soluble antigen, at both the 10 μg ($P<0.001$) and 30 μg ($P<0.001$) doses. Immunization with 30 μg of liposome-borne CBE also significantly increased the ST_{50} (51.7, $P<0.05$) when compared to mice

Table 1 *Immunoprotective activity of liposome-borne CBE.*

Immunogen	Immunizing dose (μg protein)	Mean tumor diameter (mm±SE)	P[c]	ST[d]$_{50}$	P[e]
PBS	0	21.5±0.9	—	33.4	—
CBE[a]	10	17.1±0.7	<0.005	43.2	NS
	30	16.1±0.9	<0.001	46.5	NS
Liposome-borne CBE	10	13.0±0.9	<0.001	49.1	NS
	30	11.4±0.9	<0.001	51.7	<0.05
Control liposome[b]	30	22.4±1.2	NS	32.7	NS

Groups of 10 syngeneic C3H/HeJ mice were immunized s. c. with PBS, CBE, liposome-borne CBE or control liposome. Hosts were challenged 10 days later with 1×10^5 MCA-F cells s. c. and tumor outgrowth determined 27 days postchallenge. Liposome preparations contained 60 μmol PC: PG: Chol (7:2:1).

[a] Crude butanol extracts from the MCA-F tumors.

[b] Instead of CBE, ovalbumin was used.

[c] The level of significance was assessed by the Stundent-Newman-Keuls test, as compared with PBS controls. NS is not significant.

[d] ST_{50} was calculated as 50% survival time of inoculated host, in days.

[e] The level of significance was assessed by Chi-square test, as compared with PBS controls.

Table 2 *Immunoprotective activity of liposome-borne CBE as a booster.*

Immunogen	Immunizing dose (μg protein)	Mean tumor diameter (mm±SE)	P[c]		ST[d]$_{50}$	P[e]
PBS	0	22.2±1.7	—	<0.001	30.6	—
CBE[a]	10	17.9±0.7	<0.01	<0.001	41.3	NS
Liposome-borne CBE	10	15.9±0.8	<0.001	<0.025	46.4	NS
	10×2	12.4±1.0	<0.001	—	54.1	<0.01
Control liposome[b]	10	23.0±1.1	NS	<0.001	31.4	NS

Ten syngeneic C3H/HeJ mice were injected s. c. with PBS, CBE, liposome-borne CBE or control liposome on days 0. The group treated twice with liposomal CBE were first immunized on day −10. Hosts were challenged s. c. on day +10 with 1×10^5 MCA-F cells. Tumor growth measurements are for 27 days postchallenge. Liposome preparations were the same as in Table 1.

[a] Crude butanol extracts from the MCA-F tumors.

[b] Instead of CBE, ovalbumin was used.

[c] The level of significance was assessed by the Stundent-Newman-Keuls test, compared with either PBS controls in the left column or the boosted group in the right column. NS is not significant.

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

[e] significance was assessed by Chi-square test, compared to PBS controls.

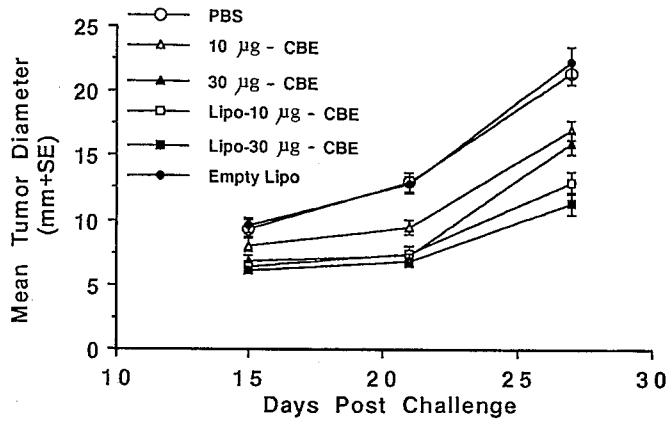

Fig. 1 Growth curve of challenged MCA-F tumor cells: Groups of 10 mice were immunized with PBS (○), control liposome (●), 10 μg (△) 30 μg (▲) of CBE and liposome-borne 10 μg (□) or 30 μg (■) of CBE. Ten days later, hosts were challenged s. c. with 1×10^5 MCA-F tumor cells and growing tumor diameters were serially measured, and mean tumor diameters were calculated (mm±SE).

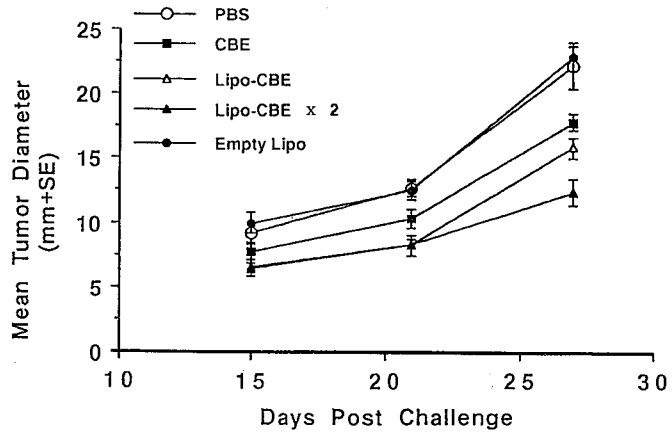

Fig. 2 Growth curve of challenged MCA-F tumor cells in booster effect assay: Five groups of 10 mice were immunized with PBS (○), control liposome (●), 10 μg CBE (■), liposome-borne 10 μg CBE (△) and its boosted group (▲), respectively. The details of the experimental protocol were described in Table 2.

receiving PBS alone. Immunization with control liposomes containing 30 μg of ovalbumin had no effect on tumor growth. In contrast, the liposomes containing 30 μg of CBE showed the best antitumor growth effect; TGR=0.44 (Fig. 1).

Table 3 *Immunoprotective activity of combination therapy with liposome-borne autologous TSTA and CY.*

Therapy group	Mean tumor diameter (mm±SE)	P[f]		ST^{g}_{50}	P[h]
PBS	20.8±0.8	—	<0.001	34.4	—
Autologous CBE[a]	14.6±1.2	<0.001	<0.001	47.7	NS
Liposome-borne autologous CBE[b]	13.1±0.9	<0.001	<0.005	54.0	<0.05
Liposome-borne autologous CBE with CY[c]	8.8±0.7	<0.001	—	63.8	<0.005
Liposome-borne syngeneic CBE[d] with CY	12.3±0.9	<0.001	<0.01	54.3	<0.05
CY	16.1±0.8	<0.001	<0.001	41.8	<0.05
Control liposome[e]	21.7±0.7	NS	<0.001	37.3	NS

Groups of 6 mice were inoculated in the right flank with 1×10^6 MCA-F tumor cells on day −14. Then the growing tumors were resected on day 0, subsequently CBE were extracted and incorporated into liposome. On day +7, various these modified TSTA were injected s.c. with or without CY i.p. While harvested tumor cells were cultured as autologous challenge tumor cells and injected s.c. with 5×10^4 cells were measured and mean tumor diameters were calculatd.

[a] Immunized with 15 μg autologous CBE.

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

[c] 20 mg/kg of CY was administered i.p. on the day of immunization.

[d] Syngeneic CBE was prepared from the parental MCA-F cell line.

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

[f] Significant differences between treated and control group were determined in the left column as well as between liposome-borne autologous CBE with CY and other groups determined in the right column.

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

[h] The level of significance was assessed by Chi-square test, as compared with PBS controls.

We next determined whether the second treatment of liposome-borne tumor antigens would enhance the antitumor immunity (Table 2). Mice were immunized with 10 μg of soluble CBE or liposome-borne CBE on day 0, or on day −10 and 0. In this experiment, incorporation of 10 μg tumor antigen into liposome (TGR=0.72) did not significantly enhance the ability of mice to resist subsequent challenge, when compared to mice receiving 10 μg of soluble CBE (TGR=0.78). However, the boosted treatments of mice with 10 μg liposomal CBE (TGR=0.46) did significantly reduce tumor growth compared to PBS controls ($P<0.001$), mice treated with soluble CBE ($P<0.001$), or mice receiving only a single dose of liposomal CBE ($P<0.025$). As expected, control liposomes were

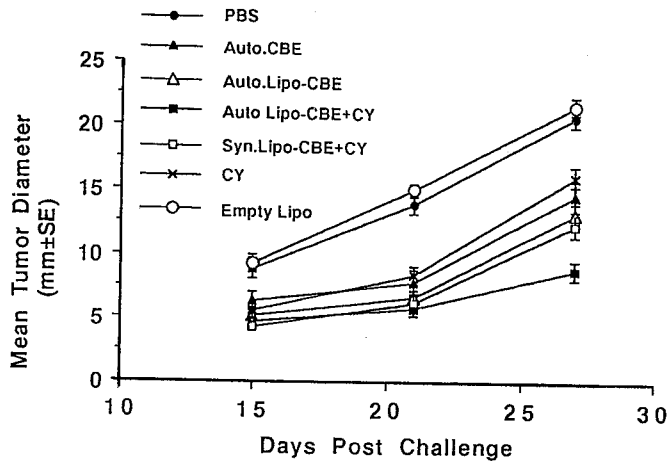

Fig. 3 Growth curve of challenged MCA-F tumor cells in combination therapy: Groups of 6 mice were treated with PBS (●), control liposome (○), autologous CBE (▲), liposome-borne autologous CBE (△), liposome-borne autologous CBE with CY (■), liposome-borne syngenic CBE with CY (□) and CY (×) alone, respctively, as described in Table 3. The details were in Materials and Methods.

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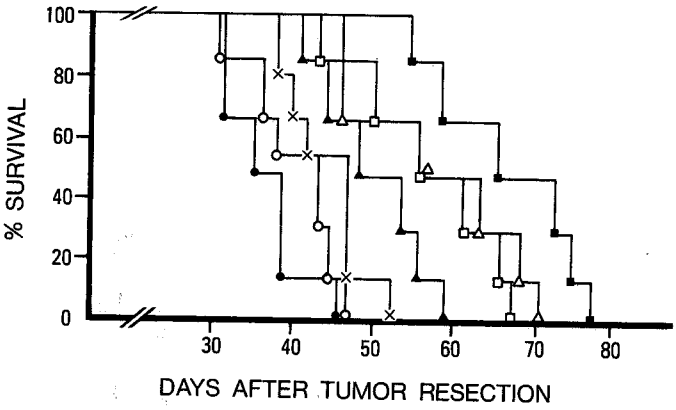

Fig. 4 Percent survivals of treated mice: Groups of 6 mice were treated with PBS (●), control liposome (○), autologus CBE (▲), liposome-borne autologous CBE (△), liposome-borne autologous CBE with CY (■), liposome-borne syngeneic CBE with CY (□) and CY (×) alone as the same protocols as described in Table 3 and observed till their termination.

not effective. A booster immunization with liposome-borne CBE also significantly increased the ST_{50}, compared to mice treated with either PBS or

control liposomes ($P<0.01$). Thus, incorporation of tumor antigen into negatively charged butanol-dialysis liposomes enhances the immunoprotective response relative to soluble CBE in a dose-dependent manner (Fig. 2).

We next asked if immunization with autologous tumor extract incorporated into liposomes would enhance the ability of mice surgically resected of that tumor to resist rechallenge with the same tumor. Immunization with 15 μg of autologous CBE afforded significant protection against autologous tumor challenge (Table 3). Incorporation of autologous CBE into liposomes in combination with low dose CY significantly reduced tumor growth (TGR=0.35) compared to PBS controls ($P<0.001$), soluble autologous CBE ($P<0.001$) or liposome-borne syngeneic CBE plus CY ($P<0.01$). Treatment of curatively resected mice with control liposomes had no effect, while 20 mg/kg CY showed a modest reduction of tumor growth. Analysis of survival times revealed a significant increase in the ST_{50} for both liposome chemoimmunotherapy groups. Mice receiving autologous CBE liposomes plus CY showed nearly double in the ST_{50} (63.8, $P<0.005$) when compared to mice treated with PBS (Table 3). These results demonstrate the superior immunotherapeutic efficacy of liposome-borne autologous CBE in combination with low dose CY for the treatment of a local tumor recurrence model (Fig. 3). Thus, these results clearly suggested that liposome-borne TSTA was more effective to engender antitumor immune response *in vivo*, especially, in an autologous tumor recurrence model, and the combination chemoimmunotherapy of liposome-borne autologous TSTA and saturated dose of CY was sufficiently potent to inhibit second tumor growth, resulting in prolonged survival days (Fig. 4).

DISCUSSION

There are good reasons to expect that immunotherapy will offer considerable therapeutic advantages in the future. Actually, TSTA have been demonstrated on human tumors, as well as animal tumors and have been found to be immunogenic in some cases(27). Many investigators have reported that TSTA induced antitumor response such as inhibiting tumor growth in the host, however, it is still difficult in application of TSTA alone for human cancer immunotherapy because of its difficulties in purification, stabilization, and preservation. At present, the concept of Biological Response Modifiers (BRM) reflects much more expectation that these BMR, which contain a number of agents and approaches, can be exploited therapeutically(20, 21). Also, the concept that the combination of immunotherapy including the use of manifold immunomodulators and chemotherapy might engender a great benefit for cancer treatment which have been elicited by the advantages for the following reasons; the expected advantages of

immunotherapy reside in the potential specificity with its antitumor response that appears to be rather specific in most cases but weak. In contrast, chemotherapy with cytotoxic agents possess strong tumoricidal potency, though it is essentially limited in its antitumor selectively and cause severe damages to normal cells as well. However, these limitation may be rapidly overcome through the utilization of devised combined therapy of immunotherapy and chemotherapy. Consequently, synergistic effects of these combination therapies seem to have produced many favorable therapeutic effects(19).

On the other hand, numerous constant efforts are being made toward improving cancer therapy through the development of new antitumor drugs and immunological agents including various BRM and its new combination treatment modalities. In particular, in order to potentiate the more desirable antitumor immune response using TSTA, considerable attention has been directed toward the possible use of liposomes to bring about an effect on not only specific delivery but also on the functional adjuvants for the induction of cell-mediated immunity *in vivo*(16, 33, 45). For the liposome-mediated adjuvant activity, a depot effect for the enhanced immunogenicity of liposome-borne immunogens has been stressed(43). Subsequently, most of these liposome-borne immunogens were endocytosed by phagocytic cells of the RES including peripheral blood monocytes and local lymphonodes(32). In the experiments, we made use of a liposome-borne TSTA using a 7:2:1 molar ratio of PC: PG: Chol. As previously reported(16), in MCA-induced mouse tumor system, this negatively charged but not uncharged liposomes were quite effective in protecting hosts against supralethal tumor challenge and displayed a relatively high specific activity compared with TSTA alone, suggesting that this type of liposomes were efficient carriers and adjuvants for the induction of antitumor immunity. Besides, owing to its lipophilic characteristics of CBE, the incorporation rate of CBE into liposomes was excellent and by butanol dialysis it became more higher.

In the present immunoprotective assay by using liposome containing CBE, we demonstrated that liposome-borne TSTA was effective in the retardation of challenged tumor cell growth and it was also shown its efficiency in the booster effect significantly ($P<0.001$; v. s. non-boosted group) but not clearly in the dose dependency ($P=NS$; 10 μg CBE v. s. 30 μg CBE). Moreover, administration of liposome-borne TSTA produced marked long survival rates in the boosted treatment ($ST_{50}=54.1$, $P<0.01$; v. s. control) as well as in the 30 μg dose of CBE group ($ST_{50}=51.7$; $P<0.05$; v. s. control), respectively. This might be of interesting to cancer therapy.

In addition, the immunological approach to human cancer therapy must take into account the fact that cancer patients may have diminished antitumor

immune competence either as a consequence of their disease of from previous treatment(3, 47). This reduced immune competence may be partly due to the increased activity of suppressor T-cells; many investigators have proved the existence of suppressor T-cells induced by tumors(1). There is also evidence that tumor cells can themselves produce suppressive factors which protect them against attack by cytotoxic effectors(46). From these points of view, as Greenberg *et al.*(7) mentioned, the choice of CY as a drug for combination chemoimmunotherapy seems to be quite reasonable because of its competent advantage of antitumor effects. One reason is that a relative low dose of CY mainly depresses suppressor T-cells or their precursors selectively and enhance the antitumor effect indirectly(25, 49), and another is that CY has a direct cytotoxic effect against tumor cells(4).

According to our recent data concerning the combination chemoimmunotherapy of CBE and CY in an autologous mouse tumor recurrence system, the combined therapy of autologous extracted TSTA and 20 mg/kg of low dose CY was proved to have the most potent antitumor effect of any treatment cases(42). We examined, as an extent experiment, the efficacy of liposome-borne autologous TSTA combined with CY against autologous recurrent tumor. The result was that the combination immunotherapy of liposome-borne autologous TSTA and low dose of CY produced pre-eminant antitumor immune response against autologous recurrent tumor ($P<0.001$; v. s. control, $P<0.01$; v. s. combined group using liposome-borne syngeneic TSTA) and resulted in the best prolonged survival days ($ST_{50}=63.8$, $P<0.005$; v. s. control).

The autologous tumor recurrence system employed in this assay means the human case of tumor recurrence after operation. Thus, our these data supported strongly that the possibility of utilization of concomitant immunity which induced the inhibitive effect against tumor metastasis under the existence of primary tumor and/or sinecomitant immunity which maintained temporary antitumor immunity after tumor resection. Recently, the studies of tumor destruction by sensitized cytotoxic T-cells have contributed to exhibit various immunological evidence regarding tumor-host response. Interestingly, it was suggested that HMC restriction was necessary for cytotoxic T-cells to propagate and produce a strong destructive response against tumor cells(22, 23). The fact might support that in tumor-bearing host T-cell recognition against weak autologus TSTA could preferentialy produce numerous tumor-specific cytotoxic effectors by means of repeated stimulation by autologous TSTA. It is still unclear as to the details of the immunological complicated relationship between lymphocytes and tumors. However, the current discovery of interleukin 2 enabled various cytotoxic T-cells to propagate *in vitro* for a long time(24, 39). Therefore, it was emphasized that

not only adoptive immunotherapy but combination chemoimmunotherapy using cultured T-cells or modified antigenic immunogens with various cytotoxic drugs has become available in cancer therapy(2). There are several reasons to believe that active specific immunotherapy will offer clinical benefits in the future. Actually, tumor associated antigens have been identified in human tumors, which have been shown in some cases to be immunogenic(27). However, difficulties in isolation, storage, and administration of antigens still pose considerable barriers to wide-spread application. At present the combination of specific immunotherapy with chemotherapy, perhaps in conjunction with immunomodulators, offers the greatest promise because of the intrinsic specificity and selectivity of the antigen -specific approach. In the same line, new light has been shed on the properties of liposome as useful carriers and adjuvants of TSTA, and have examined its antitumor efficacy in using chemoimmunotherapy.

In the study, we have investigated the utility of liposomes as antigen carriers in an animal model of chemoimmunotherapy. Incorporation of autologous tumor antigen extract into negatively charged vesicles, in combination with low dose of CY, offered superior therapeutic benefits in our local recurrence model. The enhanced protective effect observed with autologous extract suggests that the antitumor immune response of the host is affected by the heterogeneous growing tumor, and as well as the clonality of the tumor being influenced by the response of the host.

Liposomes have been used as carriers for the delivery of antigens and adjuvants in the elicitation of cell mediated immunity(16, 33, 45). It is thought that monocytes and macrophages are the primary targets for these liposomes owing to their phagocytic capacity. This propensity to be taken up by macrophages has been exploited to target activators to these cells(16).

The immunological approach to human cancer therapy must take into account the diminished immunocompetence of individuals resulting from their disease or from previous treatment(3, 47). This reduced competence may be due, in part, to the existence of suppressor T-cells and tumor-derived suppressive substances(1, 46). One approach to dealing with the suppressor T-cell problem is the use of low dose CY to decrease their activity(7, 25, 49). Our results clearly indicate a role for suppressor T-cells in the progression of this tumor, and the efficacy of combination chemoimmunotherapy in its treatment.

In conclusion, we have found that butanol-extracted TSTA encapsulated by liposome specifically protected against tumor outgrowth compared with liposome free TSTA, resulting in prolonging host survival days significantly. In addition, in the autologous tumor recurrence system, the therapeutic usefulness of combination chemoimmunotherapy of liposome-borne autologous TSTA and CY was

clearly proved. Such a immunoprotective study of liposomeborne autologous TSTA was carried out in a perfect autologous system. In such a system, genetic difference between tumor and host might be reduced to a minimum or might be eliminated. This system also seems to provide the most convincing evidence for the presence and functioning of TSTA and will contribute to the study of prophylaxis in human cancer recurrence.

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