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Improvement of Therapeutic Effect by Using Fab′ Fragment in the Treatment of Carcinoembryonic Antigen-positive Human Solid Tumors with Adriamycin-entrapped Immunoliposomes

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To improve the therapeutic efficiency adriamycin entrapped in antibody-conjugated liposomes, Fab′ fragment was used instead of the whole antibody molecule. The murine monoclonal antibody, 21B2, against human carcinoembryonic antigen (CEA) was digested with pepsin, and the thiol residue of intra-heavy chain produced by reduction of $F(ab')_2$ with dithiothreitol was conjugated to liposomes containing adriamycin. The tissue distribution of adriamycin delivered with these liposomes was studied in BALB/c *nu/nu* female mice bearing CEA-positive human gastric cancer strain MKN-45. An increase in delivery of adriamycin to the tumor was observed in the mice given liposomes with Fab′ fragment as compared to those given liposomes with whole antibody. However, the preferential distribution of adriamycin in liposomes to the reticuloendothelial cells remained the same regardless of the use of Fab′ fragment. For investigation of *in vivo* therapeutic effect, three i.v. injections of free adriamycin or adriamycin in liposomes equivalent to 5 mg/kg were given, and adriamycin in Fab′ fragment-conjugated liposomes was found most effective in the inhibition of tumor growth. This was confirmed in terms of actual tumor weights excised and CEA concentration in the blood, as well as by pathological observations. The advantages of using Fab′ fragment instead of whole antibody are discussed.

Key words: Anti-human CEA monoclonal antibody — Immunoliposome — Fab′ fragment — Adriamycin-entrapped liposome — Targeting chemotherapy

Various attempts have been made to deliver anti-tumor agents selectively to tumor cells. Monoclonal antibodies against tumor-associated antigens have been conjugated to drugs directly or to vehicles containing drugs.[1–3] We reported that adriamycin entrapped in liposomes conjugated with monoclonal antibody against human α-fetoprotein showed better therapeutic effects on human hepatoma strain maintained in BALB/c *nu/nu* mice than free adriamycin.[4] However, some disadvantages of immunoliposomes have been pointed out.[4,5] In our previous experiment, the antibodies were conjugated to liposomes with the coupling agent SPDP,[6] and random coupling with SPDP might reduce the binding activity of monoclonal antibodies. Furthermore, accumulation of drugs at high concentration in the RES is generally observed when drugs are administered as immunoliposomes.[5]

In this study, we introduced Fab′ fragment instead of whole antibody for the following reasons. (a) The usage of thiol residue of Fab′ fragment for conjugation with liposomes should leave the antigen-binding activity of the antibody intact, and increase the delivery of drugs to tumor cells. (b) Removal of the Fc portion of antibody should reduce the uptake of immunoliposomes into the RES. (c) Administration of murine antibodies can lead to generation of human anti-mouse antibody, which eventually blocks the targeting function. Antigenicity should be much reduced when the Fc portion of the antibody is removed. We prepared monoclonal antibodies against human CEA, and examined whether the use of Fab′ fragment to prepare adriamycin-entrapped immunoliposomes could increase the therapeutic effects *in vivo* by using CEA-positive human gastric cancer strain MKN-45 inoculated in BALB/c *nu/nu* mice.

MATERIALS AND METHODS

Chemicals Egg PC was obtained from Nippon Fine Chemical Co. (Osaka). Cholesterol, DPPE and DPPA were obtained from Sigma Chemical Co. (St. Louis, MO). DTP-DPPE was prepared by reacting SPDP with

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[6] The abbreviations used are: SPDP, N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate; CEA, carcinoembryonic antigen; RES, reticuloendothelial system; egg PC, egg yolk phosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; DPPE, dipalmitoylphosphatidylethanolamine; DTP-DPPE, 3-(2-pyridyldithio)propionyl-dipalmitoylphosphatidylethanolamine; DTT, dithiothreitol; HRP, horseradish peroxidase.

DPPE as described by Barbet *et al.*[6] SPDP was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). A stock solution (20 m*M*) was made in ethanol and stored at −20°C. DTT was obtained from Sigma Chemical Co. and dissolved at 500 m*M*. HRP was also obtained from Sigma. Adriamycin was used as an antitumor agent and was obtained from Kyowa Hakko Kogyo (Tokyo).

Preparation of anti-human CEA monoclonal antibodies Human CEA antigens were purified from a CEA-producing human gastric cancer strain MKN-45, and BALB/c mice were immunized. Three days after the last injection, spleen cell suspensions were prepared and fused with P3-U1 myeloma cells. Hybridoma cells were selected on the basis of preferential binding to CEA-positive cell line MKN-45, and hybridoma clone 21B2 (IgG_1) was established. The antibodies were obtained as the ascites form, and were purified by passage through a protein A-Sepharose (Pharmacia) column. To obtain Fab′ fragment, the purified antibodies were digested overnight with pepsin (Sigma) at 37°C in a 0.1 *M* acetate buffer solution (pH 3.5) at a molar ratio of 1:40 for enzyme to substrate. $F(ab')_2$ fragment was obtained by elution with a linear gradient from 0 to 0.5 *M* sodium chloride in 0.1 *M* acetate buffer using a positively charged ion exchange resin, Mono S. The molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The antigen-binding activity of Fab′ fragment was confirmed by using Fab′-HRP prepared by the hinge method.[7] MKN-45 cells were fixed with paraformaldehyde, and allowed to react with Fab′-HRP. The color was developed with diaminobenzidine for visualization.

Preparation of liposomes containing adriamycin Egg PC, cholesterol, DPPA and DTP-DPPE, each dissolved in an organic solvent (chloroform:methanol 2:1), were mixed at a molar ratio of 10:5:1:0.16, and the organic solvent was removed by evaporation. One ml of adriamycin (20 mg/ml in water) was added to the dried lipid film, and multilamellar vesicles were prepared by vortex dispersion. The liposomes were sonicated into small unilamellar vesicles with a sonicator (type UCD-110, Japan Biotech, Tokyo), and unencapsulated adriamycin was removed by gel filtration on a Sephadex G-50 column.

Conjugation of the monoclonal antibody to the liposomes surface The whole antibody was conjugated to liposomes as described previously[4] with a slight modification originally described by Barbet *et al.*[6] The antibody (1–2 mg/ml) was treated with SPDP at the final concentration of 0.1 m*M* for 30 min at room temperature and then transferred to acetate buffer (0.1 *M* pH 4.5, 0.145 *M* NaCl) by gel filtration through a Sephadex G-50 column. Protein-bound dithiopyridine was treated with 50 m*M* DTT for 40 min at room temperature, and again eluted through a Sephadex G-50 column with an acetate buffer solution. The free thiol-bearing protein thus activated was immediately mixed with the liposomes suspension, and after adjustment of the pH to 8.0 with 1 *M* sodium borate, the mixtures were allowed to react at room temperature for 24 h. For *in vivo* experiments, the protein-bearing, adriamycin-containing liposomes were administered without further separation from uncoupled protein. For the conjugation of Fab′ fragment to Liposomes, $F(ab')_2$ fragment (1 mg/ml) was reduced with DTT to Fab′ fragment, which was coupled to a adriamycin-containing liposomes as described above. The amount of adriamycin entrapped in liposomes was determined from the fluorescence intensity (excitation at 490 nm and emission at 590 nm) after lysis of the liposomes with 0.3 *N* HCl-50% ethanol.[8, 9]

Tumor cells The human CEA-positive gastric cancer cell line, MKN-45, was maintained in culture flasks (Falcon 3024, Becton Dickinson, Oxnard, CA) in complete RPMI 1640 medium supplemented with 5% fetal calf serum (Lot No. 40701, Commonwealth Serum Labo., Victoria, Australia).

Tissue distribution studies on adriamycin MKN-45 cells maintained *in vitro* were harvested, washed extensively with Hanks' solution and adjusted to 2×10^7 cells/ml. Two million cells in 100 μl were inoculated into the back of female BALB/c *nu/nu* mice (Nihon Clea Co., Tokyo). When the estimated tumor weight (calculated as $1/2\times\text{length}\times\text{width}^2$)[10] reached about 300 mg, the mice were randomly divided into the following groups of 5 animals each, (a) free adriamycin, (b) adriamycin in liposomes with whole antibody, (c) adriamycin in liposomes with Fab′ fragment and (d) adriamycin in liposomes conjugated with normal mouse IgG. Mice received various forms of adriamycin equivalent to 7.5 mg/kg. At 1, 4, 8 h after the injection, the mice were anesthetized by ether inhalation, and blood was collected from the retro-orbital venous plexus. Various organs including liver, spleen, kidney, lung, heart and tumor were excised immediately, rinsed in saline and weighed. The organs were homogenized in 0.3 *N* HCl-50% ethanol with a high-speed mixer (Ultradisperser LK21, Yamato Scientific Co., Tokyo), and then the homogenates were centrifuged at 20,000*g* for 20 min at 4°C. The adriamycin concentration in the supernatant was determined fluorometrically.[8, 9] Tissue homogenates from nontreated mice were prepared by the same procedure, and the values thus obtained were subtracted from those of the experimental group.

Therapeutic effect of adriamycin in liposomes *in vivo* Female BALB/c *nu/nu* mice were inoculated s.c. with 2×10^6 cells in 100 μl on both sides of the back. When the estimated tumor weight reached about 300 mg, the mice were randomly divided into five group (a) saline,

(b) free adriamycin, (c) adriamycin in liposomes without antibody conjugation, (d) adriamycin in liposomes with whole antibody, and (e) adriamycin in liposomes conjugatd with Fab′ fragment. Each group of animals received various forms of adriamycin equivalent to 5 mg/kg through the tail vein at 4 day intervals. The mice were killed on day 9 after the adriamycin treatment, and the therapeutic effect was evaluated, based on the tumor weight, CEA concentration (determined by enzyme-linked immunosorbent assay) in the blood and histological observations. The significance of the difference among experimental groups was examined by means of Student's t test, and a P value of less than 5% was regarded as significant.

RESULTS

Retention of antigen-binding activity in Fab′ fragment The molecular weight of anti-CEA antibody 21B2 after pepsin digestion was about 100 kd as determined by SDS-PAGE, and it shifted to 30 kd under reducing conditions (data not shown). These values corresponded well to those of $F(ab')_2$ and Fab′ fragment of anti-CEA antibody. It was confirmed that Fab′ fragment conjugated to HRP could bind to CEA-positive MKN-45 cells *in vitro*, whereas Fab′ fragment prepared from normal mouse IgG fraction could not (unpublished observation).

Tissue distribution studies on adriamycin It was investigated whether the use of Fab′ fragment instead of whole antibody would modify the pattern of tissue distribution of adriamycin in liposomes as compared to our previous work.[4] Adriamycin in various forms, equivalent to 7.5 mg/kg, was injected i.v. into nude mice which had been inoculated with MKN-45 cells. At 1, 4, and 8 h after injection, various organs were removed by dissection and the concentrations of adriamycin were determined fluorometrically. Free adriamycin was rapidly eliminated from the circulating blood and was undetectable at 1 h after administration. In contrast, when adriamycin was administered as the liposomes-entrapped form, it was cleared slowly. However, no marked differences were observed among three types of liposomes (Fig. 1). The adriamycin levels in the tumor MKN-45 were very low and the differences were hard to evaluate. However, in the mice given free adriamycin and adriamycin in the liposomes conjugated with normal mouse IgG, the adriamycin levels were rather high at 1 h, and then gradually decreased. In contrast, adriamycin levels increased slightly at 4 h in the mice given liposomes with specific antibodies. In the mice given Fab′ fragment-conjugated liposomes, the adriamycin concentration was 2.65 ± 1.45 μg/g at 4 h as compared to 1.17 ± 0.80 μg/g with the

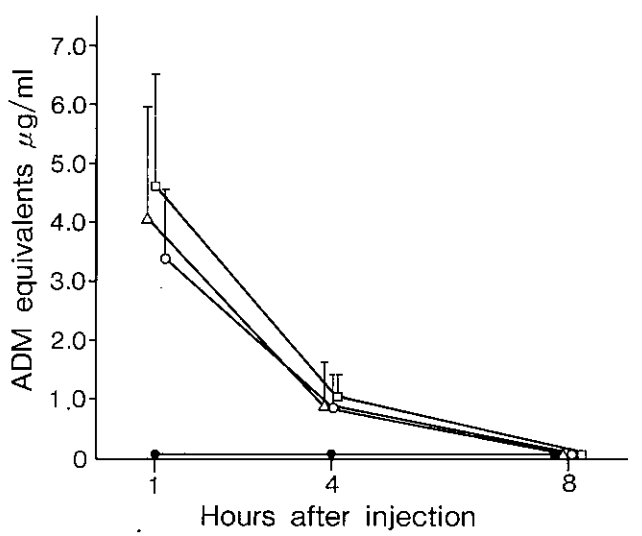

Fig. 1. Adriamycin levels in the serum. Adriamycin (7.5 mg/kg) in various forms [free adriamycin (●), liposomes conjugated with Fab′ fragment (□), liposomes conjugated with whole antibody (△), liposomes conjugated with normal mouse IgG (○)] was injected i.v. into BALB/c *nu/nu* female mice which had been inoculated with MKN 45. At indicated times, blood was collected and the concentration of adriamycin was determined fluorometrically. (N=5)

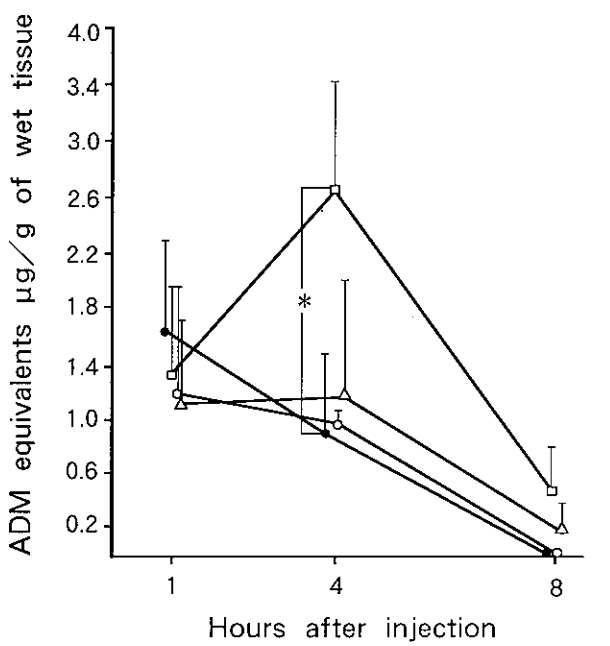

Fig. 2. Adriamycin levels in the tumor MKN 45. The adriamycin levels were determined at indicated times using the mice described in the legend to Fig. 1. (N=5) Free adriamycin (●), liposomes conjugated with Fab′ fragment (□), liposomes conjugated with whole antibody (△), and liposomes conjugated with normal mouse IgG (○).

liposomes bearing whole antibody or 0.88 ± 0.60 μg/g with free adriamycin administration ($P < 0.1$) (Fig. 2). In the liver and spleen of mice injected with liposome-entrapped forms the concentration of adriamycin was markedly higher than that after free adriamycin administration. However, the adriamycin levels were not significantly different among the three types of antibody-conjugated liposomes (Fig. 3). Furthermore, in the heart and lung, the adriamycin levels were reduced by liposome delivery as compared to the free form (data not shown) as reported previously,[4, 8, 11, 12] but no difference was observed between liposomes with Fab′ fragment and those with whole antibody.

Therapeutic effect of adriamycin entrapped in liposomes with Fab′ fragment of anti-CEA antibody When the growth of MKN-45 attained about 300 mg (estimated tumor weight), the mice received three i.v. injections of adriamycin in various forms equivalent to 5 mg/kg. The calculation of relative mean tumor weight revealed strong inhibition of tumor growth when adriamycin was administered in antibody-conjugated liposomes (Fig. 4), and according to the National Cancer Institute assessment criteria ($T_{RW}/C_{RW} < 42\%$),[10] a significant effect was observed only with adriamycin in the liposomes with Fab′ fragment. The therapeutic effect was much more evident when the actual tumor weights were measured after excision (Table I). The effect of adriamycin in Fab′

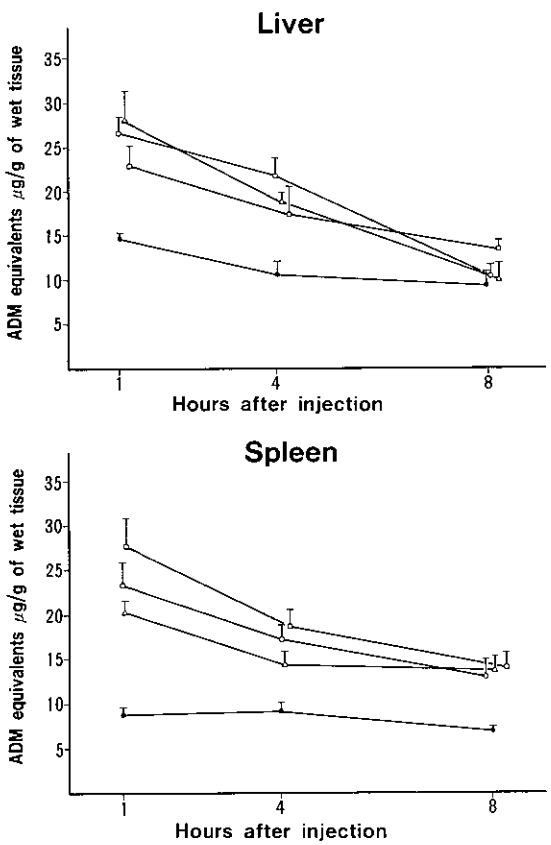

Fig. 3. Adriamycin levels in liver and spleen. The donor mice were the same as in Fig. 1. Free adriamycin (●), liposomes conjugated with Fab′ fragment (□), liposomes conjugated with whole antibody (△), and liposomes conjugated with normal mouse IgG (○).

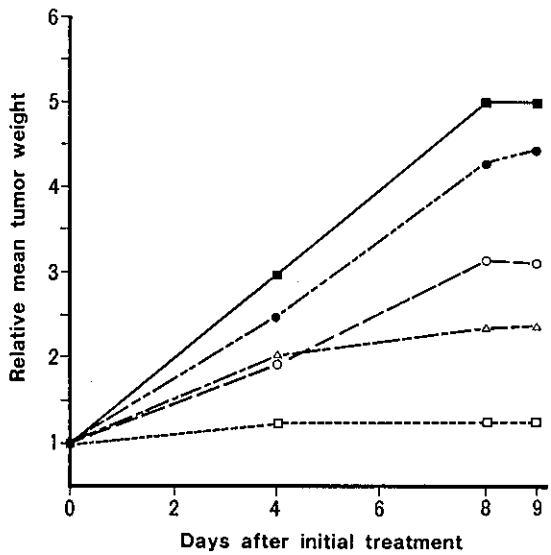

Fig. 4. Effect of adriamycin administered in various forms [saline alone (■), free adriamycin (●), adriamycin in Fab′ fragment-liposomes (□), adriamycin in whole antibody-liposomes (△), adriamycin in liposomes without antibody conjugation (○)] on the growth of MKN 45 inoculated on the backs of BALB/c *nu/nu* mice. (N=6)

Table I. Tumor Weights of Xenotransplanted Tumors from Killed Mice

Group	n	Tumor weight (mg±SD)	T/C (%)
Control	6	1455±133	—
Free ADM	6	1191±410	81.9
Lip-ADM	5	823±475	56.5
Lip-ADM=Ab	6	627±270[a]	43.1
Lip-ADM=Fab′	6	254±75[b]	17.5

Xenografts and treatments were as described in the legend to Fig. 4. On day 9 of the treatment, the mice were killed and the excised tumors were weighed. The results are expressed as tumor weights and the ratios of tumor weights (T/C), where T is tumor weight of the treatment group and C that of the control group. ADM: adriamycin.

a) Not significant compared to Lip-ADM.

b) $P < 0.01$ compared to Lip-ADM and $P < 0.05$ compared to Lip-ADM=Ab.

Table II. CEA Concentration in Serum and Adriamycin Levels in Excised Tumors

Group	n	CEA (ng/ml) (mean±SD)	ADM (μg/g of tissue) (mean±SD)
Control	6	315.5±159.3	—
Free ADM	6	306.5±202.6	0.77±0.24
Lip-ADM	5	188.8±120.6	1.32±0.83
Lip-ADM=Ab	6	56.0±37.0[a]	1.59±0.61[c]
Lip-ADM=Fab′	6	51.8±11.5[b]	2.64±1.92[d]

Xenografts and treatments were as described in the legend to Fig. 4. On day 9 of the treatment, the mice were killed, blood was collected, and the tumors were excised. CEA concentration in the serum and the adriamycin (ADM) levels in the tumors were determined.
a) $P<0.05$ compared to free ADM but not significant to Lip-ADM.
b) $P<0.05$ compared to free ADM but not significant to Lip-ADM.
c) $P<0.05$ compared to free ADM.
d) $P<0.05$ compared to free ADM but not significant to Lip-ADM=Ab.

fragment liposomes was significantly higher than those of free adriamycin ($P<0.01$), of adriamycin in liposomes ($P<0.01$) or even of adriamycin in liposomes conjugated with whole antibody ($P<0.05$). At the termination of the experiment, we also examined the CEA concentration in the serum and the adriamycin levels in the excised tumors (Table II). The serum CEA concentration was significantly lower in the mice given liposomes with whole antibody and Fab′ fragment ($P<0.05$) than in the untreated control mice or mice given free adriamycin, but not significantly lower than that in the mice given adriamycin in liposomes without antibody conjugation. These results are parallel to those on the inhibition of tumor growth. Furthermore, the concentration of adriamycin from excised tumors was significantly higher ($P<0.05$) both in the animals given liposomes with whole or fragmented antibodies as compared to that in mice given free adriamycin. Finally, the therapeutic effect of adriamycin was confirmed by histological examinations. In the case of free adriamycin, degeneration or necrosis of tumor cells was hardly observed, and in the mice given liposomes with whole antibody, destruction of cancer cells and nests was evident but viable cells still remained. In contrast, the mice given liposomes with Fab′ fragment showed a wide area of destruction and necrosis of cancer cells and nests, and hardly any viable cells were observed (data not shown).

DISCUSSION

We have demonstrated here that Fab′ fragment-conjugated liposomes could deliver adriamycin more efficiently than whole antibody-conjugated liposomes, and also that they have a superior therapeutic effect on CEA-positive MKN-45 tumors inoculated into BALB/c *nu/nu* mice. Previously we reported that adriamycin-entrapping liposomes carrying monoclonal antibodies against α-fetoprotein could block tumor growth of α-fetoprotein-positive human tumor cells,[4] and we pointed out that Fab′ fragment might be better for conjugation. In the case of whole antibody, we need coupling agents such as SPDP. However, in this case the coupling between the antibody and the surface of liposomes is random, and some antibodies can not bind to the antigen. Furthermore, the concentration of SPDP used for pretreatment of antibodies is critical, and at high concentration, aggregation of antibodies can occur. In our preliminary experiments, when anti-CEA antibody 21B2 was pretreated with 0.05 m*M* or 0.2 m*M* SPDP, the binding of antibody to CEA-coated plates was reduced to 77% or 28%, respectively, as compared to the antibody without SPDP treatment. In contrast, Fab′ fragments do not need to be treated with SPDP. They are conjugated to the liposomes via the thiol residue of the intra-heavy chain of antibody, and therefore all of the Fab′ fragments can participate in binding to the target cells. These features improve the efficiency of targeting. Another disadvantage of SPDP has also been reported, i.e., SPDP-treated phosphatidylethanolamine is susceptible to cleavage in the serum. It might be better to replace SPDP with N-succinimidyl-(4-*p*-maleimidophenyl)butyrate.[13]

Introduction of Fab′ fragments onto liposomes has been described by several investigators, and its use *in vivo* has mostly focused on targeting to red blood cells. Agrawal *et al.*[14] and Peeters *et al.*[15] reported that chloroquine in liposomes bearing Fab′ or $(Fab')_2$ fragment could efficiently deliver drugs to red blood cells infected with malaria parasites. Recently, Nassander *et al.*[16] demonstrated that immunoliposomes conjugated with Fab′ fragment to ovarian carcinoma cell selectively bind to target cells as compared to those bearing irrelevant fragments and found that the density of Fab′ antibody on the surface of liposomes in important for binding to the tumor cells. However, the therapeutic effects of such liposomes have not been reported, and to our knowledge the present paper is the first to describe the therapeutic effects on solid human tumors.

Liposomes are widely applied as vehicles for targeting therapy,[17-19] and the advantages of using liposomes have been well documented, including (a) liposomes are non-toxic and biodegradable, (b) drugs can be entrapped without modification, (c) liposomes can carry a large dose of drugs as compared to the direct conjugation of antibodies and drugs, (d) drugs are protected from enzymatic degradation or immunological attack in the blood stream, and so on. Furthermore, in the case of

adriamycin, the level of adriamycin in the heart was reduced by liposomal delivery, as reported here. This is particularly beneficial, since the cardiotoxicity of adriamycin is the major factor limiting its clinical use.[20] However, some disadvantages of liposomes have also been pointed out. One of these is, as shown in this experiment, the preferential distribution of drugs to reticuloendothelial cells,[21,22] when they are injected into blood vessels. Since Fab′ fragment lacks the Fc portion, it should not activate the Fc receptor of phagocytes and thus the elimination of immunoliposomes by the RES should be reduced.[23,24] Singhal *et al.*[25,26] reported that covalent attachment of $F(ab')_2$ fragment of anti-rat erythrocyte to liposomes reduced the uptake by liver. However, in our case, uptake by the RES was not much different whether Fab′ fragment or whole antibody was used. Furthermore, the clearance of adriamycin from the circulation was also not much changed among the three types of liposomes. In the case of Singhal *et al.*, competition between binding to the surface of red blood cells and uptake by phagocytes occurs in the same compartment, and increase of the binding to red cells would reduce the uptake by the RES. Preferential delivery to the RES *per se* seems to be an intrinsic characteristic of liposomes themselves, and we are now trying a targeting therapy with immunoliposomes containing ganglioside GM_1, which was reported to reduce uptake by the RES.[27]

Finally, another advantage of using Fab′ fragment is reducing the immunogenic activity of murine monoclonal antibodies. Most of the monoclonal antibodies so far used have been derived from mice, and therefore, repeated administration in clinical cases can lead to the generation of human anti-mouse antibody, which eventually decreases the anti-tumor effects and causes a severe adverse reaction.[28] Removal of the Fc portion should reduce the immunogenicity and thus increase the usefulness of immunoliposomes.

In conclusion, we have shown that adriamycin entrapped in liposomes bearing Fab′ fragment can exert a potent therapeutic effect on CEA-positive human solid tumors. Although there still remain many points to be examined in targeting therapy with immunoliposomes, our results should represent a further atep towards clinical trials.

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