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Antitumor Effect of Diphtheria Toxin A-Chain Gene-containing Cationic Liposomes Conjugated with Monoclonal Antibody Directed to Tumor-associated Antigen of Bovine Leukemia Cells

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Monoclonal antibody c143 against tumor-associated antigen (TAA) expressed on bovine leukemia cells was conjugated to cationic liposomes carrying a plasmid pLTR-DT which contained a gene for diphtheria toxin A-chain (DT-A) under the control of the long terminal repeat (LTR) of bovine leukemia virus (BLV) in the multicloning site of pUC-18. The specificity and antitumor effects of the conjugates were examined *in vitro* and *in vivo* using TAA-positive bovine B-cell lymphoma line as the target tumor. *In vitro* studies with the TAA-positive cell line indicated that luciferase gene-containing cationic liposomes associated with the c143 anti-TAA monoclonal antibody caused about 2-fold increase in luciferase activity compared with cationic liposomes having no antibody, and also that the c143-conjugated cationic liposomes containing pLTR-DT exerted selective growth-inhibitory effects on the TAA-positive B-cell line. Three injections of pLTR-DT-containing cationic liposomes coupled with c143 into tumor-bearing nude mice resulted in significant inhibition of the tumor growth. The antitumor potency of the c143-conjugated cationic liposomes containing pLTR-DT was far greater than that of normal mouse IgG-coupled cationic liposomes containing pLTR-DT as assessed in terms of tumor size. These results suggest that cationic liposomes bearing c143 are an efficient transfection reagent for BLV-infected B-cell lymphoma cells, and that the delivery of the *pLTR-DT* gene into BLV-infected B-cells by the use of such liposomes may become a useful technique for gene therapy of bovine leukosis.

Key words: Gene therapy — Cationic liposomes — Monoclonal antibody — Bovine leukemia virus — Diphtheria toxin

Enzootic bovine leukosis (EBL) is a contagious lymphoproliferative disease whose etiologic agent is a retrovirus, the bovine leukemia virus (BLV).[1)] BLV induces both lymphocytosis, which is characterized by an increased number of B lymphocytes in the peripheral blood, and lymphosarcoma in various lymph nodes in a later stage.[1)] The tumor cells have tumor-associated antigen (TAA) on the cell surface.[2–4)] One of the most characteristic features of BLV is that the viral genome encodes a specific transcriptional activator called p34,[5,6)] as is the case with human T-cell leukemia virus (HTLV) type I and II.[7,8)] The transactivation by p34 is assumed to be mandatory for the promoter activity of the long terminal repeat (LTR) of BLV (BLV-LTR), and hence for the expression of viral genes.

Diphtheria toxin A-chain (DT-A) has two unique characteristics in that it specifically modifies mammalian elongation factor 2, leading to cellular death at extremely low concentrations, and that it can not enter the living cell unless it is associated with the B-chain.[9,10)] DT-A-mediated cell death is cell cycle-independent, making it particularly attractive for application to cytoreductive gene therapy.[11)] Accordingly, the gene coding for DT-A, controlled by cell- or tissue-specific regulatory elements, has been used as a molecular tool for a novel approach to the treatment of cancer[12–17)] and acquired immunodeficiency syndrome (AIDS).[18–20)] We previously demonstrated that the promoter activity of BLV-LTR is highly specific to BLV-infected cells,[14)] and that the growth of BLV-infected

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cells was effectively suppressed by the delivery of the gene for DT-A under the control of BLV-LTR (pLTR-DT) to the cells *in vitro*[14, 15] and *in vivo*.[16]

Liposomes are bilayer vesicles composed of amphiphilic phospholipids. They are non-toxic, biodegradable and poorly immunogenic. Furthermore, liposomes-entrapped materials are protected from enzymatic attack until they reach the target sites. The potential usefulness of liposomes as a carrier for gene transfer has attracted considerable interest during the last few years.[14, 15, 20–27] In *in vitro* and *in vivo* experiments, we demonstrated that the number of viable BLV-infected cells was markedly reduced by treatment with pLTR-DT-containing cationic liposomes composed of *N*-(α-trimethylammonioacetyl)didodecyl-D-glutamate chloride (TMAG), dioleoyl phosphatidylethanolamine (DOPE) and dilauroyl phosphatidylcholine (DLPC) (1:2:2, molar ratio).[15, 16] EBL caused by BLV is a B-cell lymphoma. It is well known that lymphoid cells are resistant to liposome-mediated gene transfer.[28, 29] In order to transfer the gene encapsulated in liposomes to specific cells, appropriate cytophilic ligands such as monoclonal antibody are available.[21] Previously, we produced a monoclonal antibody, c143, which identified a TAA on tumor cells from cattle with EBL.[30] Furthermore, we also reported that c143 monoclonal antibody could be coupled to liposomes without alteration of its specificity toward EBL tumor cells and that c143 monoclonal antibody-bearing liposomes containing an antitumor drug could significantly inhibit EBL tumor growth in nude mice.[31] These observations suggest that c143 monoclonal antibody-conjugated cationic liposomes would be suitable for *DT-A* gene transfer in BLV-infected B-lymphocytes.

In this paper, in order to examine the usefulness of c143 monoclonal antibody-bearing cationic liposomes containing pLTR-DT for the gene therapy of EBL, the antitumor effect of these liposomes was investigated *in vitro* and *in vivo* using a TAA-positive bovine B-cell lymphoma line as the target cells.

MATERIALS AND METHODS

Materials *N*-Hydroxysuccinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from Pharmacia Biotech (Pharmacia Biotech, Uppsala, Sweden). A stock solution (20 m*M*) was made in ethanol and stored at −20°C. TMAG was a gift from Sogo Pharmaceutical Co., Ltd., Tokyo; DOPE was purchased from Avanti Polar Lipids, Birmingham, AL; DLPC and dipalmitoylphosphatidylethanolamine (DPPE) were from Sigma Chemical Co. (Sigma Chemical Co., St. Louis, Mo); 4,6-diamidino-2-phenylindole (DAPI) and dithiothreitol (DTT) were from Nacalai Tesque, Inc., Kyoto. 3-(2-Pyridyldithio)-propionyldipalmitoylphosphatidylethanolamine (DTP-DPPE) was prepared by reacting SPDP with DPPE as described by Barbet *et al*.[32] Plasmids pSRα/L-AΔ5′ (a gift from Dr. Y. Takebe, National Institute of Health, Tokyo), which allows the expression of firefly luciferase driven by the SRα promoter, and pLTR-DT[14] were amplified in *Escherichia coli* JM 109 and purified by the cesium chloride gradient centrifugation method.

Cells The bovine lymphoid cell line BLSC-KU-17 (KU-17), established from a tumor of an animal with EBL, the mouse myeloma cell line P3-X63-Ag8-U1 (P3U1), and a feline cell line transformed with mouse sarcoma virus, C8, were used as targets for transfection. KU-17 cells have B cell surface markers and TAA identified by c143 on the cell surface, and produce BLV.[33] P3U1 and C8 cells do not possess TAA recognized by c143 on the cell surface. KU-17 and P3U1 cells were maintained in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal calf serum and antibiotics. C8 cells were grown in Eagle's minimum essential medium (Nissui) supplemented with 10% fetal calf serum and antibiotics.

Monoclonal antibody Monoclonal antibody c143 had been previously characterized and its specificity for common TAA expressed on all EBL tumor cells has been confirmed.[4, 30] IgG of c143 monoclonal antibody was fractionated from mice ascitic fluid by precipitation with 50% ammonium sulfate, followed by affinity chromatography on Protein-A Sepharose CL-4B (Pharmacia Biotech).

Modification of monoclonal antibody IgG fraction of c143 (2 mg) was dissolved in 1 ml of 0.01 *M* Hepes buffer (pH 7.45) containing 0.145 *M* NaCl, and treated with 0.1 m*M* SPDP under nitrogen gas. The reaction mixture was kept for 30 min at room temperature and the mixture was applied to a Sephadex G-50 (Pharmacia Biotech) column pre-equilibrated with 0.1 *M* acetate buffer (pH 4.5) containing 0.145 *M* NaCl. The protein peak fraction (1 ml) was reduced with DTT (50 m*M*, final concentration) under nitrogen gas. After 30 min incubation at room temperature, the mixture was applied to a Sephadex G-50 column pre-equilibrated with Hepes buffer (0.01 *M*, pH 7.45, containing 0.145 *M* NaCl). The first main peak fraction (1 ml) was stored at 4°C under nitrogen gas until use.

Preparation of liposomes Cationic liposomes entrapping plasmids were prepared as follows: TMAG (0.05 μmol), DLPC (0.1 μmol), and DOPE (0.1 μmol) were each dissolved in chloroform-methanol (2:1, v/v) and the solutions were mixed in a conical flask. The mixture was evaporated by the use of a rotary evaporator and a vacuum pump, then 125 μl of sterilized phosphate-buffered saline (PBS; pH 7.2) containing 10 μg of plasmid was added to the residue. The lipid film was vortexed for a few minutes, and untrapped plasmids were removed by centrifugation at 12,000*g* for 5 min.

The antibody-conjugated cationic liposomes containing pLTR-DT for *in vitro* and *in vivo* experiments were prepared from a lipid film, which consisted of a mixture of TMAG (1 μmol), DLPC (2 μmol), DOPE (2 μmol), and DTP-DPPE (0.1 μmol), and 200 μg of plasmid as described above.

DNA determination The amount of DNA entrapped in liposomes was determined by the method described previously[15, 16]: 200 μl of 1.5 *M* NaCl/30 m*M* ethylenediaminetetraacetic acid (EDTA, pH 8.0) was added to a 20 μl suspension of liposome-entrapped plasmid or standard DNA solution containing 0.5–2 μg of DNA, followed by vortex mixing. The resulting solutions were mixed with 300 μl of a mixture of chloroform and methanol (2:1, v/v). After centrifugation at 12,000*g* for 5 min, the aqueous phase (200 μl) was mixed with 1 ml of 10 m*M* Tris-HCl buffer (pH 7.0) containing 100 ng/ml DAPI, 100 m*M* NaCl and 100 m*M* EDTA, and the fluorescence of DNA-DAPI complexes was subsequently measured at 450 nm with excitation at 360 nm, using a Hitachi F-3010 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo).

Coupling of modified IgG to cationic liposomes The liposome suspension was added to the modified IgG and the mixture was gently shaken overnight at 4°C under nitrogen gas. The protein-bearing cationic liposomes were separated from uncoupled protein by centrifugation at 12,000*g* for 20 min.

Liposome-mediated transfection KU-17 cells were seeded at a density of 1×10^5 cells per well in 24-well tissue culture plates (Corning Inc., Corning, NY) and cultured for 24 h at 37°C. After cultivation, 500 μl of medium was removed, and 500 μl of a fresh culture medium suspension of antibody-conjugated cationic liposomes containing pSRα/L-AΔ5′ was added to each well, then culture was continued. After 48 h cultivation at 37°C, cells were harvested and used for luciferase assay.

Luciferase assay Transfection efficiency was evaluated by means of luciferase assay using pSRα/L-AΔ5′. After 48 h of incubation, the cells were washed 3 times with PBS, covered with 50 μl of cell culture lysis reagent (25 m*M* Tris-phosphate, pH 7.8, containing 2 m*M* DTT, 2 m*M* 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100), and incubated for 15 min at room temperature. The cell suspension was centrifuged briefly in a microcentrifuge (700*g*, 3 min). The supernatant was then subjected to assay for luciferase activity and protein quantitative analysis.

The enzymatic activity of luciferase was measured using a luciferase assay system kit (Promega Co., Madison, WI) as follows: 100 μl of luciferase assay reagent [20 m*M* Tricine, 1.07 m*M* $(MgCO_3)_4Mg(OH)_2\cdot5H_2O$, 2.67 m*M* $MgSO_4$, 0.1 m*M* EDTA, 33.3 m*M* DTT , 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP] was added to 15 μl of the supernatant in a 96-well microplate (MS-8496K, Sumitomo Bakelite, Tokyo). Integrated light output was measured for 1 min with a Micro Lumino Reader (Corona MLR-100, Corona Electric Co., Ltd., Hitachinaka). Luciferase activity was expressed as unit/μg protein/min.

The protein concentration of the cell lysate was quantitated using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) and bovine plasma gamma globulin was used as a standard.

***In vitro* studies with c143-conjugated cationic liposomes containing pLTR-DT** A preliminary study of liposome-mediated transfection was performed to determine the optimal dose of pLTR-DT for the *in vitro* experiments. The growth of KU-17 cells was inhibited to the extents of about 70, 60, and 25% by 1, 0.8, and 0.4 μg of pLTR-DT-containing cationic liposomes, respectively. So, 0.8 μg of pLTR-DT was considered to be the optimal dose for the *in vitro* studies.

KU-17 cells (at a concentration of 1×10^5/ml of culture medium) were distributed in triplicate into each well of 24-well tissue culture plates (Corning) and cultured for 24 h at 37°C. After cultivation, an aliquot of the medium (10 μl) was removed, and 10 μl of unconjugated cationic liposomes containing 0.8 μg of pLTR-DT (DT-A-L), c143-conjugated cationic liposomes containing 0.8 μg of pLTR-DT (DT-A-c143-L), normal mouse IgG-conjugated cationic liposomes containing 0.8 μg of pLTR-DT (DT-A-mIgG-L), or c143-conjugated cationic liposomes containing 0.8 μg of pUC18 plasmid DNA (pUC18-c143-L) was added into each well. After that, the cells were cultured. The same liposome-mediated gene transfection of P3U1 and C8 cells was also carried out using DT-A-L (containing 0.8 μg of pLTR-DT) or DT-A-c143-L (containing 0.8 μg of pLTR-DT).

***In vivo* antitumor effect of c143-conjugated cationic liposomes containing pLTR-DT** Our previous *in vivo* experiments[16] showed that a significant antitumor effect of cationic liposomes containing pLTR-DT was observed against BLV-infected tumor cells when a dose of 20 μg of pLTR-DT-containing cationic liposomes was directly injected into the tumor. In this experiment, therefore, we used cationic liposomes containing 20 μg of pLTR-DT.

BALB/c nude mice were inoculated subcutaneously at the back with a single cell suspension of KU-17 culture cells (2×10^8 cells). When tumor growth was observed (about 2 weeks after injection), mice were divided randomly into 3 groups and treatment with c143-conjugated liposomes containing pLTR-DT was started. The mice were anesthetized with diethyl ether. Each group of animals (5 mice) received 3 injections of DT-A-c143-L (containing 20 μg of pLTR-DT), DT-A-mIgG-L (containing 20 μg of pLTR-DT) or no treatment at 2-day intervals (days 2 and 4 after the first injection), through the heart in a volume of 100 μl per mouse. The mice were killed on

day 6 of DT-A-c143-L treatment and the effect of the treatment was evaluated based on the tumor size. Tumor size was measured with calipers and the volume was calculated from the equation v (mm^3)$=0.4\times a\times b^2$, where a and b are the major and minor diameters, respectively.[31] The significance of differences between the volume of tumors in experimental and control mice was evaluated by means of Student's t test.

RESULTS

Transfection efficiency of c143-conjugated cationic liposomes The ability of cationic liposomes coupled with c143 monoclonal antibody to mediate gene transfer in TAA-positive bovine leukemia cells was assessed by means of the luciferase assay. The luciferase activity measured in KU-17 cells transfected with c143-conjugated and unconjugated cationic liposomes containing pSRα/L-AΔ5′ is shown in Fig. 1.

The cationic liposomes coupled with c143 monoclonal antibody gave highly efficient transfection into KU-17 cells. On the other hand, the transfection activity of the cationic liposomes uncoupled with the monoclonal antibody was significantly lower than that of the coupled cationic liposomes, i.e., about 2-fold less. This result indicates that the coupling of the monoclonal antibody to cationic liposomes elicits an enhancement in gene delivery and expression.

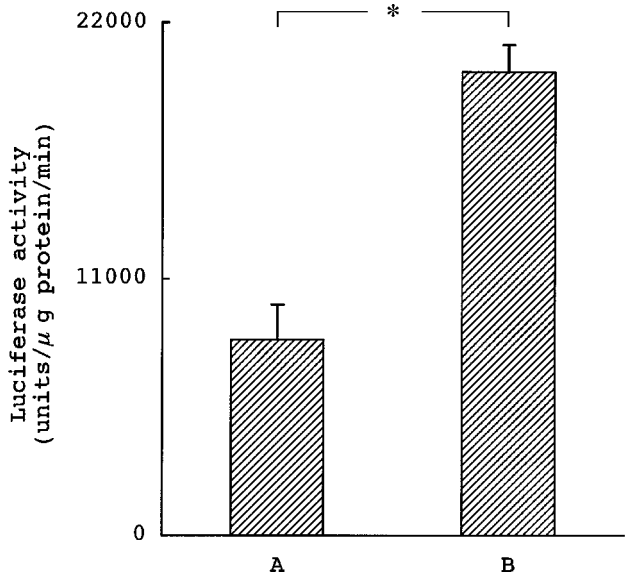

Fig. 1. Transfection activity of c143 monoclonal antibody-conjugated and unconjugated cationic liposomes. KU-17 cells were transfected with 0.8 μg of pSRα/L-AΔ5′ plasmid in monoclonal antibody-unconjugated (A) and conjugated (B) cationic liposomes, then incubated for 48 h and subjected to luciferase assay. Results are the mean±SE of triplicate experiments. ∗ $P<0.005$ as compared with the unconjugated cationic liposomes by Student's t test.

Growth-inhibitory effect toward bovine leukemia cells of c143-conjugated cationic liposomes containing pLTR-DT In order to examine whether c143-conjugated cationic liposomes containing pLTR-DT could effectively inhibit the growth of TAA-positive bovine leukemia cells, KU-17 cells were treated for 24 h with DT-A-L, DT-A-c143-L, DT-A-mIgG-L, or pUC18-c143-L. Control cells received no treatment.

As shown in Table I, in the nontreated control cells and cells treated with c143-conjugated cationic liposomes containing pUC18 plasmid DNA instead of pLTR-DT (pUC18-c143-L), cell growth was rapid, and the numbers

Table I. Suppressive Effect of c143-conjugated Cationic Liposomes Containing pLTR-DT on the Growth of KU-17 Cells

Treatment	Number of cells (×10^5)	
	Day 0	Day 2
Nontreated control	1	2.73±0.15[a)]
DT-A-L	1	1.60±0.09[b)]
DT-A-c143-L	1	1.10±0.06[c, d, e)]
DT-A-mIgG-L	1	1.80±0.15[f)]
pUC18-c143-L	1	2.37±0.23

a) Data are expressed as mean±SE of three experiments in triplicate.
b) $P<0.003$ as compared with nontreated control cells by Student's t test.
c) $P<0.0005$ as compared with nontreated control cells by Student's t test.
d) $P<0.008$ as compared with DT-A-L-treated cells by Student's t test.
e) $P<0.02$ as compared with DT-A-mIgG-L-treated cells by Student's t test.
f) $P<0.02$ as compared with nontreated control cells by Student's t test.

Table II. Effect of c143-conjugated Cationic Liposomes Containing pLTR-DT on the Growth of TAA-negative Cells

Cells	Treatment	Number of cells (×10^5)	
		Day 0	Day 2
P3U1	Nontreated control	1	9.55±0.26[a)]
	DT-A-L	1	9.77±0.26
	DT-A-c143-L	1	9.33±0.13
C8	Nontreated control	1	1.67±0.12
	DT-A-L	1	1.40±0.10
	DT-A-c143-L	1	1.57±0.12

a) Data are expressed as mean±SE of three experiments in triplicate.

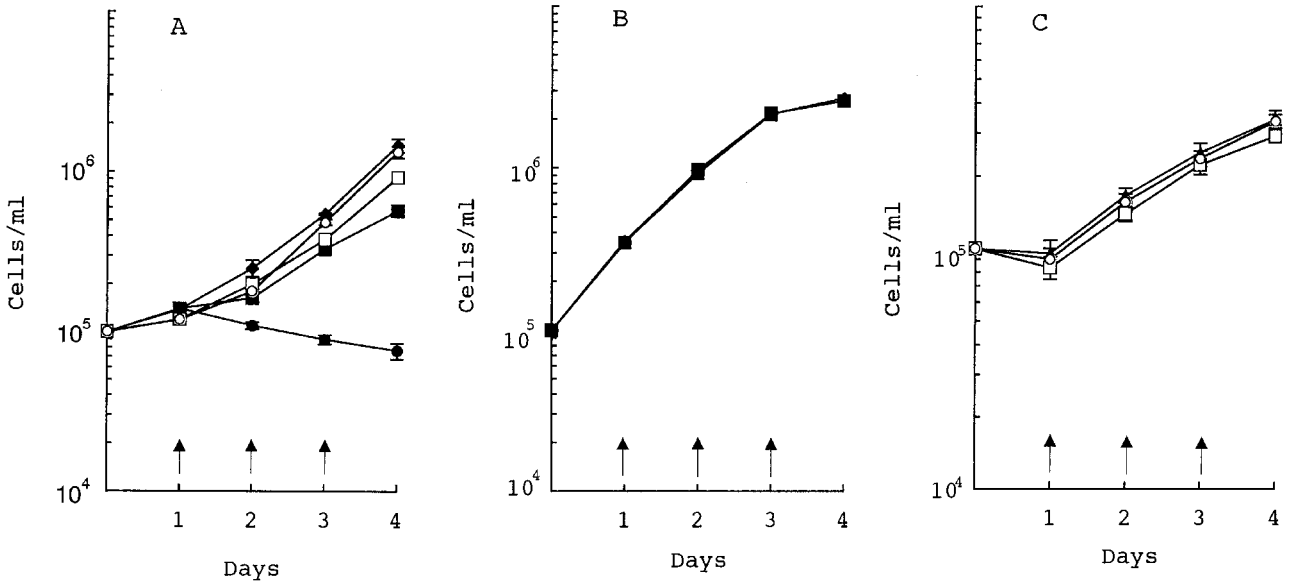

Fig. 2. Suppressive effect of successive transfection with pLTR-DT-entrapped cationic liposomes coupled with c143 monoclonal antibody on the growth of KU-17, P3U1, and C8 cells. (A) KU-17 cells (1×10^5 cells) were transfected with DT-A-L (■), DT-A-c143-L (●), DT-A-mIgG-L (□), or pUC18-c143-L (○) at times 24, 48, and 72 h (▲). (B) P3U1 cells (1×10^5 cells) were transfected with DT-A-L (■) or DT-A-c143-L (●) at 24, 48, and 72 h (▲). (C) C8 cells (1×10^5 cells) were transfected with DT-A-L (□) or DT-A-c143-L (○) at 24, 48, and 72 h (▲). At 24, 48, 72, and 96 h, the number of viable cells was counted. Control KU-17, P3U1, and C8 cells (▲) received no treatment. Data are expressed as mean±SE of three experiments in triplicate.

of cells were 2.73±0.15 and 2.37±0.23×10^5 cells, respectively. There was no significant difference in growth inhibition between pUC18-c143-L and nontreated control cells. On the other hand, the numbers of cells treated with DT-A-L (1.60±0.09×10^5 cells, P<0.003), DT-A-c143-L (1.10±0.06×10^5 cells, P<0.0005) and DT-A-mIgG-L (1.80±0.15×10^5 cells, P<0.02) were significantly decreased as compared with that of the nontreated control cells. Moreover, DT-A-c143-L suppressed the growth of KU-17 cells more strongly than DT-A-L (P<0.008) or DT-A-mIgG-L (P<0.02). There was no significant difference in growth inhibition between the DT-A-L-treated and DT-A-mIgG-L-treated cells. Neither empty cationic liposomes nor such liposomes coupled with c143 monoclonal antibody significantly suppressed the growth of KU-17 cells (data not shown). These results suggest that cationic liposomes coupled with c143 monoclonal antibody were not toxic to the TAA-positive KU-17 cells, and that the growth of KU-17 cells was effectively inhibited when the monoclonal antibody was conjugated to cationic liposomes containing pLTR-DT.

The growth-inhibitory effect of DT-A-c143-L against P3U1 and C8 cells, which do not express TAA reactive with the c143, was also estimated. As shown in Table II, treatment with DT-A-L and DT-A-c143-L did not result in growth inhibition of P3U1 and C8 cells. In these cases, there was no significant difference in cell growth between the DT-A-L-treated, DT-A-c143-L-treated, and nontreated cells.

Furthermore, the effects of successive treatment of KU-17, P3U1, and C8 cells with DT-A-c143-L were studied. In this experiment, TAA-positive KU-17 cells were treated with DT-A-L, DT-A-c143-L, DT-A-mIgG-L, or pUC18-c143-L, while TAA-negative P3U1 and C8 cells were treated with DT-A-L and DT-A-c143-L, at 24, 48, and 72 h. Viable cells were counted at 24, 48, 72, and 96 h.

As shown in Fig. 2A, pUC18-c143-L did not suppress the growth of KU-17 cells. In contrast, DT-A-c143-L strongly suppressed the cell growth. Although DT-A-L and DT-A-mIgG-L showed significant inhibition of the cell growth as compared with the nontreated control, their growth-inhibitory effects on KU-17 cells were significantly inferior to that of DT-A-c143-L. There was no significant difference in growth inhibition between DT-A-L-treated and DT-A-mIgG-L-treated cells, as can be seen in Table I. In TAA-negative cells, the growth of P3U1 and C8 cells was not suppressed by treatment with DT-A-L or DT-A-c143-L (Fig. 2, B and C). No significant differences were observed in cell growth between the DT-A-L-treated, DT-A-c143-L-treated, and nontreated cells. These results indicate that treatment with DT-A-c143-L specifically inhibits the growth of TAA-positive bovine

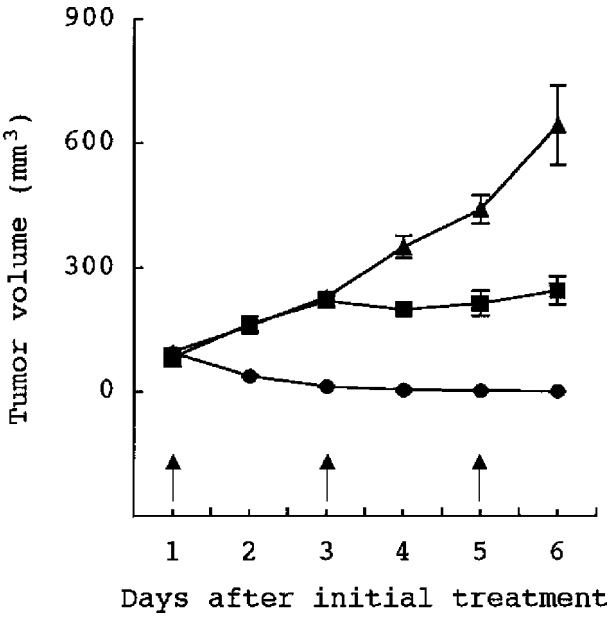

Fig. 3. Antitumor effect of pLTR-DT-containing cationic liposomes coupled with c143 monoclonal antibody. BALB/c nude mice were inoculated with cultured KU-17 cells (2×10^8 cells/mouse). When the tumor volume reached about 90 mm^3, the mice were given three intracardiac injections (▲) of c143 monoclonal antibody-conjugated cationic liposomes containing 20 μg of pLTR-DT (DT-A-c143-L) (●) or normal mouse IgG-conjugated cationic liposomes containing 20 μg of pLTR-DT (DT-A-mIgG-L) (■) in a volume of 100 μl per mouse. Control mice (▲) received no treatment. Tumor size was measured every day. Data represent mean±SE of tumor volume in 5 different mice.

leukemia cells and has no cytotoxic effect on TAA-negative cells.

***In vivo* antitumor effect of c143-conjugated cationic liposomes containing pLTR-DT** The antitumor effect of DT-A-c143-L was studied *in vivo* by examining its suppressive effect on tumor growth in nude mice inoculated with KU-17 cells. The tumors in most of the inoculated nude mice appeared after a similar time interval and were similar in size. When the tumor volume reached about 90 mm^3, the mice were separated into 3 groups and each mouse was given 3 injections of DT-A-c143-L or DT-A-mIgG-L through the heart. Control mice received no injection. After injection, the tumor size was measured every day. The mice were killed on day 6 after the initial treatment and tumor size was compared before treatment and at the end of the treatment (on day 6).

Fig. 3 shows the tumor growth pattern of animals from each group. In the nontreated control mice, tumor growth was rapid. On the other hand, tumor growth in the mice treated with DT-A-c143-L was significantly inhibited as compared with that in the nontreated control group or DT-A-mIgG-L-treated group. The mean tumor volumes in the group treated with DT-A-c143-L, in that treated with DT-A-mIgG-L, and in that nontreated at day 6 were 0.43±0.1, 258.8±38.5, and 777.1±96.9 mm^3, respectively. The values of mean tumor volume in the groups treated with DT-A-c143-L ($P<0.001$) and DT-A-mIgG-L ($P<0.01$) were significantly smaller than that of the nontreated control group. Furthermore, tumor growth in the group treated with DT-A-c143-L was significantly decreased ($P<0.002$) as compared with that of the DT-A-mIgG-L-treated group.

DISCUSSION

Monoclonal antibody c143 used in the present study was shown to be specific to TAA expressed on tumor cells from cattle with EBL.[30, 34] Further study demonstrated, however, that TAA recognized by c143 monoclonal antibody is expressed very weakly in lymphoid cells of BLV-free normal cattle.[4, 35] The bovine lymphoid cell line BLSC-KU-17, derived from an EBL tumor, has TAA on its cell surface and about 90% of the cells react with c143 monoclonal antibody *in vitro*.[33] To confirm the specific targeting of c143 monoclonal antibody to TAA-positive cells *in vivo*, ^{125}I-labeled c143 monoclonal antibody $F(ab')_2$ fragment was injected into athymic nude mice bearing BLV-induced bovine lymphoid cells.[36] The fragments were preferentially localized in tumor tissues, but not in normal tissues, as determined by differential counting of tissue radioactivity and by whole-body scintigraphy.[36] The finding of specific biodistribution of c143 monoclonal antibody in nude mice bearing EBL tumor led to the idea of utilizing it for gene therapy.

Liposomes, artificially generated lipid vesicles that can entrap drugs within their aqueous compartment and/or in their lipid bilayer, have been regarded as a useful drug delivery system[37, 38]; their potential for gene transfection has been reported.[14, 21–26] However, selective and efficient transfection of the gene to target cells is very important for *in vitro* and *in vivo* applications. One approach to increase the selectivity and the efficiency of gene delivery by liposomes is to conjugate appropriate cytophilic ligands such as monoclonal antibodies to the liposomes. In this study, we investigated *in vitro* and *in vivo* growth inhibition of TAA-positive bovine leukemia cells transfected with *DT-A* gene by the use of cationic liposomes coupled with c143 monoclonal antibody.

We found that the activity of luciferase expressed in KU-17 cells was about 2 times higher when the plasmid was encapsulated into cationic liposomes coupled with c143 monoclonal antibody than it was when uncoupled cationic liposomes were used (Fig. 1). This indicates that targeting of cationic liposomes with c143 monoclonal antibody can enhance gene transfer and expression in

bovine lymphoid tumor cells carrying TAA, and also that cationic liposomes coupled with monoclonal antibody can be an efficient tool for gene transfer into cells.

In vitro experiments demonstrated the suppressive effect of c143-conjugated cationic liposomes containing pLTR-DT (DT-A-c143-L) on the growth of TAA-positive KU-17 cells. The growth of KU-17 cells was significantly inhibited by the administration of DT-A-c143-L as compared with that of the DT-A-mIgG-L-treated cells ($P<0.02$) or nontreated control cells ($P<0.0005$) (Table I). In addition, this experiment demonstrated the augmentation of the growth-inhibitory effect of cationic liposomes containing pLTR-DT by coupling the c143 monoclonal antibody to the liposomes. Cell growth was significantly inhibited by DT-A-c143-L as compared to that of cells treated with DT-A-L ($P<0.008$). However, no significant difference in cell growth between the DT-A-L-treated and DT-A-mIgG-L-treated cells was observed. Moreover, neither DT-A-c143-L nor DT-A-L suppressed the growth of the TAA-negative cells tested, P3U1 and C8 cells (Table II and Fig. 2, B and C). These results indicate that targeting of cationic liposomes with c143 monoclonal antibody can enhance *DT-A* gene transfer and expression in KU-17 bovine lymphoid tumor cells carrying TAA, and selectively inhibit the growth of TAA-positive cells. It has been reported that about 90% of KU-17 cells showed a positive reaction with c143 monoclonal antibody.[33] In this study, in addition, we found that cationic liposomes coupled with monoclonal antibody were more effective in gene transfer to lymphoid cells than the uncoupled cationic liposomes (Fig. 1). These findings strongly suggest that the augmented inhibitory effect of DT-A-c143-L on the growth of KU-17 cells was due to the delivery of the *DT-A* gene into the cells as a result of specific interaction between TAA on the KU-17 cells and the monoclonal antibody on the liposomal surface. Furthermore, the successive treatment of KU-17 cells with DT-A-c143-L, DT-A-L, or DT-A-mIgG-L resulted in significant inhibition of cell growth in the DT-A-c143-L-treated cells when compared with that in the DT-A-L-treated or DT-A-mIgG-L-treated cells (Fig. 2A). On the other hand, multiple treatment with DT-A-L or DT-A-c143-L did not cause a significant growth inhibition of P3U1 or C8 cells (Fig. 2, B and C). These results indicate that repeated exposure to DT-A-c143-L has higher potential for the transfer of genes to TAA-positive cells as compared with DT-A-L or DT-A-mIgG-L and does not exert any lethal effect against TAA-negative cells.

In addition to the *in vitro* studies, c143-conjugated cationic liposomes containing pLTR-DT (DT-A-c143-L) were also assessed for their antitumor effect on EBL tumor *in vivo*. *In vivo* studies demonstrated the therapeutic effect of DT-A-c143-L. When tumor volumes were compared at 6 days after the initial treatment, the mean tumor volume of the DT-A-c143-L-treated group ($0.43\pm0.1 mm^3$) was smaller than that of the DT-A-mIgG-L-treated group (258.8 ± 38.5 mm^3) or the nontreated control group (777.1 ± 96.9 mm^3) (Fig. 3), indicating a remarkable therapeutic effect of DT-A-c143-L. We recently demonstrated a similar *in vivo* antitumor effect of pLTR-DT-containing cationic liposomes on BLV-infected tumor cells.[16] When the cationic liposomes containing pLTR-DT were directly injected into tumors transplanted into nude mice, the tumor growth was significantly inhibited. On the other hand, cationic liposomes containing pUC-18 plasmid showed no such effect. These results suggest that cationic liposomes can be utilized for DT-A gene delivery into BLV-infected tumor cells *in vivo*, and that the growth-inhibitory effect of pLTR-DT-containing cationic liposomes on BLV-infected tumor cells can be ascribed to diphtheria toxin produced in the tumor cells transfected with the gene. Accordingly, the specific delivery of the *DT-A* gene into BLV-infected tumor cells by the use of monoclonal antibody-conjugated cationic liposomes should be a useful technique for gene therapy of EBL. Previously, we reported that when c143 monoclonal antibody was injected into athymic nude mice bearing EBL tumor, the antibody was preferentially localized in tumor tissues and not in normal tissues.[36] In the present study, therefore, we consider that c143-conjugated cationic liposomes containing pLTR-DT (DT-A-c143-L) preferentially bound to the TAA-positive KU-17 tumor cells *in vivo*, and inhibited the growth of the tumor cells more efficiently than pLTR-DT-containing cationic liposomes conjugated with normal mouse IgG (DT-A-mIgG-L) (Fig. 3).

For clinical application, the effect of DT-A-c143-L treatment on long-term survival has to be studied. In this experiment, however, we did not examine the long-term survival after the therapy. Nevertheless, injections of DT-A-c143-L into tumor-bearing nude mice significantly inhibited the tumor growth. Approximately 96% reduction of the original tumor volume occurred within 3 days after the primary injection (total 2 injections). At 5 days after the primary injection (total 3 injections), furthermore, about 99.5% tumor reduction was seen. In the group of mice given DT-A-mIgG-L injections, on the other hand, tumor progression was delayed, in comparison with nontreated control mice, especially after the second administration (Fig. 3). Since the results presented here clearly demonstrate the superiority of DT-A-c143-L in terms of tumor reduction, we expect that prolongation of survival would be much greater in mice given DT-A-c143-L injections than in those given DT-A-mIgG-L injections.

The activity of DT-A-c143-L against bovine leukemia cells in nude mice indicates that this therapeutic method (gene therapy) may be applicable to the bovine system. The *DT-A* gene directed by BLV-LTR (pLTR-DT) works

only in BLV-infected cells, but not in BLV-uninfected cells,[14] since the activation of BLV-LTR is highly dependent on the presence of the BLV p34 trans-activator.[5, 6] It has been shown that BLV does not express the gene products at a detectable level in freshly collected lymphocytes or tumor cells from the infected host.[39, 40] This indicates that DT-A-c143-L might not be effective against peripheral blood lymphocytes of healthy BLV-infected cattle and tumor cells of cattle with EBL. However, Kono *et al*. have reported that transcription of *BLV* genes increases in parallel with the development of persistent lymphocytosis (PL).[41] PL is characterized by an increase in the number of peripheral blood lymphocytes, and most cattle with PL have been shown to possess TAA on their lymphocytes,[34] but to have no clinical signs. Thus, PL may be amenable to treatment by the delivery of the *DT-A* gene into BLV-infected cells with cationic liposomes coupled to c143 monoclonal antibody. Since PL is thought to be an early stage of EBL, gene therapy using DT-A-c143-L may be useful to stop the progress of the disease.

Furthermore, the present study on the therapeutic effect of c143 monoclonal antibody-bearing cationic liposomes containing pLTR-DT seems to be an excellent model for application to the human system. Recent advances in molecular virology have established the importance of virally encoded transactivators such as *tax* of HTLV-I,[7, 8] *tat* of human immunodeficiency virus (HIV),[42, 43] pX of hepatitis B virus,[44] and VP16 of herpes simplex virus.[45] In some virus-related cancers, specific viral and/or cellular gene expression is considered to be critical for stable transformation and/or tumorigenicity of the cell. Human papilloma virus (HPV) E6-E7 oncoproteins, for example, are associated with the malignant transformation of infected cells.[46, 47] Therefore, the HPV early promoter region could be taken as a *DT-A* gene fusion. On the other hand, Maxwell *et al*. showed that *DT-A* gene under the control of immunoglobulin enhancer/promoter was a selective toxic agent towards B-lymphocytes, including malignant B-cell lines.[13] More recently, it was suggested that HIV-regulated expression of *DT-A* fragment gene is a potential gene therapy approach to AIDS.[18–20] It is, therefore, possible that the present strategy directed towards BLV-infected B-cell lymphoma cells can be applied to human diseases where expression of a specific gene expression is likely to be therapeutic.

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