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In vitro Targeting and Cytotoxicity of Adriamycin in Liposomes Bearing Monoclonal Antibody against Rat or Human gp125 Cell Proliferation-associated Antigen

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Chemoimmunoliposomes (CIL) were prepared by entrapping adriamycin in monoclonal antibody (mAb)-coated liposomes and examined for their binding capacity and cytotoxicity to relevant target tumor cells. Sonicated unilamellar liposomes were coated with B3 and HBJ127 mouse, mAbs, which recognize a rat and a homologous human cell proliferation-associated surface antigen, gp125, respectively, and then adriamycin was entrapped in the liposomes by means of transmembrane Na^+/K^+ gradients using valinomycin. These CIL selectively bound with relevant target tumor cells bearing the corresponding gp125 antigen, such as BC47 rat bladder cancer, FTL-13 rat thymic lymphoma, T24 human bladder cancer and Molt-4 human leukemia cells, although the binding capacities of the CIL to bladder cancer cells were relatively larger than those to lymphoma cells in both rat and human systems. This difference in the target cell binding was found to be attributable to the amount of gp125 antigen expressed on each target tumor cell, as determined by a Scatchard plot analysis. In accordance with the target cell binding capacities of CIL preparations, the CIL displayed much higher cytotoxic activity to bladder cancers than to lymphomas in both rat and human systems. In conjuction with our previous finding that gp125 antigen is expressed on tumor cells but not on resting normal cells, these findings indicate that CIL composed of anti-gp125 mAb will be useful for tumor therapy and that the antitumor efficacy is dependent upon the extent of the antigen expression on target tumor cells.

Key words: Monoclonal antibody — Chemoimmunoliposomes — Cytotoxicity — Amount of antibody binding sites

The recent development of monoclonal antibody (mAb[2]) against a variety of tumor cells has enabled the selective delivery of certain drugs to tumor cells which express the corresponding antigen on the cell surface. Although many methods for drug targeting have been devised, one approach is to employ antibody-coated lipid vesicles or liposomes as a drug carrier. We and others have previously reported that an antitumor drug introduced into mAb-coated liposomes (chemoimmunoliposome(s), CIL) shows higher and more selective activity than the free form of the drug.[1-4] However, it has not been possible to find a mAb suitable for a CIL preparation which may be applicable to the therapy of human tumors because of limitations in the amount of the mAb-defined antigen and tumor specificity of the mAb.

Murine mAbs, B3 to rat and HBJ127 to human cells, have been prepared in our laboratory and were shown to recognize homologous rat and human antigen systems (gp125), which are expressed in a relatively large amount on all tumor cells tested.[5, 6] In this paper, we show that the CIL prepared with anti-gp125 mAb and adriamycin show immunoselective binding and cytotoxicity against gp125 antigen-bearing tumor cells and that the efficacy is dependent upon the amount of the antigen expressed on the target tumor cells.

MATERIALS AND METHODS

Chemicals Iodogen and *m*-maleimidobenzoyl-N-hydroxysuccinimide ester were obtained from Pierce Chemical Co., Rockford, IL., N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP) from Pharmacia Fine Chemicals, Uppsala, and dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, cholesterol and valinomycin from Sigma Chemical Co., St. Louis, MO. Adriamycin was a kind gift from Kyowa Hakko Kogyo, Tokyo. [^{3}H]Thymidine ([^{3}H]TdR), cholesteryl [1-^{14}C]oleate and Na^{125}I were purchased from Amersham Laboratories, Buckinghamshire, England.

Tumor cell line BC47 ACI/N rat bladder cancer cell line[7] has been maintained in our laboratory by *in vitro* culture. FTL-13 F344 rat thymic lymphoma cell line was donated by Dr. T. Ogiu,[8] T24 human bladder cancer cell line by Dr. T. Suzuki, Niigata University, Niigata, and Molt-4 human T lymphoma cell line by Dr. T. Tachibana,

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[2]Abbreviations used in this paper: mAb(s), monoclonal antibody(ies); gp, glycoprotein; CIL, chemoimmunoliposome(s); IL, immunoliposome(s); SPDP, N-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate; [^{3}H]TdR, [^{3}H]thymidine; FCS, fetal calf serum; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PBS, phosphate-buffered saline, pH 7.4.

Tohoku University, Sendai. The bladder cancer cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium (Nissui Pharmaceutical Co., Tokyo) containing 10% heat-inactivated fetal calf serum (FCS) (M. A. Bioproducts, Walkersville, MD), 2 m*M* L-glutamine, 1 μM sodium pyruvate, 10 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), $5\times10^{-5}M$ 2-mercaptoethanol, kanamycin at 60 μg/ml and penicillin at 100 U/ml, and lymphoma cell lines in RPMI1640 medium (Nissui) fortified with the same constituents as above.

mAbs Anti-gp125 mAbs, B3 (IgG_1) for the rat and HBJ127 (IgG_1) for the human, were established in our laboratory as described previously.[5, 6] The mAbs used in this work were prepared from ascites of mice intraperitoneally transplanted with the hybridoma cells, and the antibodies in the ascites were purified by ammonium sulfate precipitation followed by diethylaminoethyl cellulose column chromatography.

Thiolation of mAbs Thiolation of mAbs was performed by using SPDP as previously described.[9] In brief, 20 μl of 15.6 m*M* SPDP in ethanol was added to 2 ml of mAb solution (5 mg/ml) in 0.1 *M* sodium phosphate buffer, pH 7.5, containing 0.1 *M* NaCl. After incubation for 30 min at 23°C, excess reagent and products of low molecular weight were removed by passing the solution through a Sephadex G25 column equilibrated in 0.1 *M* sodium acetate buffer, pH 4.5, containing 0.1 *M* NaCl. After reduction with dithiothreitol (50 m*M*) for 30 min at 23°C, the thiolated mAb was separated from the reaction mixture on a Sephadex G25 column equilibrated in 169 m*M* glutamic acid solution containing 20 m*M* HEPES, pH 6.8 (K^+ buffer).

Preparation of antibody-coated liposomes Antibody-coated liposomes were prepared according to the method described previously.[1, 10] Briefly, a lipid film was prepared from N-(*m*-maleimidobenzoyl)dipalmitoylphosphatidylethanolamine (2.5 μmol), dipalmitoylphosphatidylcholine (25 μmol) and cholesterol (17.5 μmol) in chloroform, mixed with 5 ml of K^+ buffer, and vortexed. The resultant multilamellar liposomes were sonicated to obtain small unilamellar liposomes. Aggregated and large liposomes were removed by centrifugation at 10,000*g* for 10 min, and the upper 4 ml of the liposome suspension was collected. The liposome suspension was treated with 2 mg of thiolated anti-gp125 mAb in 2 ml of K^+ buffer for 60 min at 37°C and then with cysteine (5 mg) in order to block the excess maleimido groups on the liposome surface. To remove unconjugated antibody from, and to generate transmembrane Na^+/K^+ chemical gradients (K^+ inside) in the antibody-coated liposomes (immunoliposome(s), IL), the liposome suspension was passed through a Sepharose CL-4B column equilibrated in 150 m*M* NaCl containing 20 m*M* HEPES, pH 7.5 (Na^+ buffer).[11, 12] Radio-labeled liposomes were prepared by adding cholesteryl [1-^{14}C]oleate to the lipid mixture. Contents of lipid and antibody in IL were determined as described previously.[10]

Incorporation of adriamycin into IL Adriamycin was introduced into IL vesicles according to the method of Mayer *et al.*[12] Briefly, adriamycin (20 μg/μmol lipid) and valinomycin (0.5 μg/μmol lipid) were added to IL possessing the Na^+/K^+ chemical gradient and after incubation for 4 h at 37°C, adriamycin-containing CIL were separated from free drugs by passing the mixture through a Sepharose CL-4B column equilibrated in the Na^+ buffer. The CIL were sterilized and made uniform in size by passing them through a 0.4 μm polycarbonate filter. The adriamycin concentration in the CIL was quantitated by monitoring the absorbance at 480 nm with a Beckman DU-50 spectrophotometer after disruption with 0.5% Triton X-100[12] and the lipid content was assayed as above.

Assay for the binding between IL or CIL and target tumor cells ^{14}C-Labeled liposomes (IL, CIL or cysteine-coated liposomes) were added to 200 μl of tumor cell suspension containing 5×10^5 cells at a final concentration of 300 μM lipid in the culture medium containing 10% FCS. In the competitive inhibition assay, 500 μg of mAb was added to the reaction mixture. After incubation for 30 min at 37°C, the cells were washed three times with phosphate-buffered saline, pH 7.4 (PBS) to remove unbound liposomes. The cell pellets were digested with Soluene (Packard Instrument Co., Downers Grove, IL), and the radioactivity was measured by a standard scintillation technique. All experiments were performed in triplicate.

Scatchard plot analysis for anti-gp125 mAb-defined antigen present on target tumor cells B3 and HBJ127 mAb were labeled with ^{125}I by an iodogen method.[13] Various amounts of the ^{125}I-labeled mAb (specific activity, 1×10^4 cpm/ng) were added to a target tumor cell suspension containing 3×10^5 cells in PBS containing 0.1% bovine serum albumin (200 μl in total). After incubation for 60 min at 4°C, the cells were washed three times with PBS. The radioactivity bound to the cells was counted by the use of a gamma counter. To adjust the nonspecific binding of labeled mAb, target cells were incubated with labeled mAb in the presence of an excess amount (1000-fold of labeled mAb) of unlabeled mAb and the radioactivity in the cells was subtracted from the value obtained for the cells incubated with labeled mAb alone. All these experiments were performed in duplicate and the average values were scored.

Cytotoxicity assay Cytotoxicity of CIL was assessed by measuring [^{3}H]TdR uptake and by visual cell counting. For [^{3}H]TdR assay, various concentrations of adriamycin in free form or in CIL were added to aliquots

(200 μl) of tumor cell suspension containing 1×10^5 cells in the culture medium containing 10% FCS in wells of a Costar round-bottomed 96-well microtiter plate and incubated for 30 min at 37°C. The cells were washed three times with PBS and then suspended in 200 μl of fresh culture medium containing 10% FCS. Aliquots (20 μl) of the cell suspension were distributed in triplicate into a Costar flat-bottomed 96-well plate and then cultured in 200 μl of fresh culture medium for 20 h at 37°C in a humidified CO_2 incubator. The cells in each well were pulsed with [^{3}H]TdR for an additional 4 h and then harvested. The radioactivity of the cells was measured by standard liquid scintillation counting. For the visual cell counting assay, target tumor cells were treated, in the presence (for a competitive inhibition assay) or absence of 250 μg of mAb (the same sort of mAb used for CIL), with CIL as above, and aliquots (20 μl) of the cell suspension were distributed in a Costar 24-well plate in triplicate and cultured in 1 ml of fresh culture medium for 4 days at 37°C in a CO_2 incubator. The cells were harvested and viable cells were determined by means of a trypan blue dye exclusion test.

RESULTS

Suitable conditions for IL and CIL preparations We first examined suitable conditions for the coupling of SPDP to mAb by using B3 mAb. The number of thiol groups introduced into B3 mAb was proportionally increased by increasing the amount of SPDP. However, use of more than a 20-fold molar excess of SPDP over B3 mAb resulted in a decrease in the antigen-binding ability of the mAb and at 40-fold excess, precipitation of the couples mAb occurred (data not shown). Therefore, we decided to employ the molar ratio of SPDP to mAb of 5, where 2.3 thiol groups on average were introduced into a mAb molecule and the mAb showed efficient coupling to maleimido-modified liposomes. IL prepared under these conditions contained 3 μmol lipid/ml and the ratio of mAb protein to lipid was 60 μg to one μmol. Adriamycin was successfully accumulated into the preformed IL in response to a valinomycin-dependent K^+ diffusion potential and the trapping efficiency appoached about 75% (15 μg adriamycin/μmol lipid).

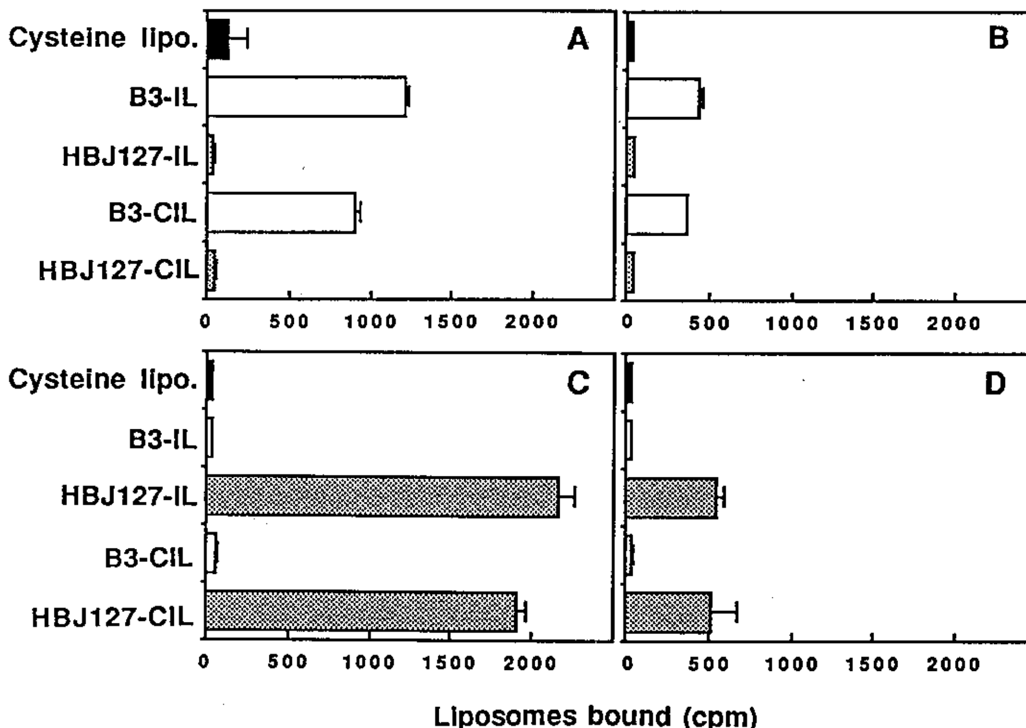

Fig. 1. Binding ability of IL, CIL and cysteine-coated liposomes (cysteine lipo.). ^{14}C-Labeled liposomes (IL or CIL modified with the indicated mAb) were added to 200 μl of target tumor cell suspension containing 5×10^5 cells at a final concentration of 300 μM lipid. After incubation at 37°C for 30 min, the cells were washed and cell-associated radioactivity was determined as described in "Materials and Methods." Target cells: A, BC47; B, FTL-13; C, T24; D, Molt-4.

***In vitro* binding of IL and CIL to tumor cells bearing gp125 antigen** ^{14}C-Labeled IL prepared as above were divided into two portions and used as IL and adriamycin-containing CIL. IL or CIL were incubated with target tumor cells and their binding capacity to tumor cells was determined by measuring the radioactivity bound to the target cells (Fig. 1). IL and CIL preparations modified with the relevant anti-gp125 mAb selectively bound to gp125-bearing bladder cancer and T lymphoma cells in both rat and human systems, although the binding capacities of CIL were relatively small as compared to those of the corresponding IL. Non-specific binding of either IL or CIL was of a similar level to that of cysteine-coated liposomes. As to the binding preference of IL or CIL to bladder cancer and lymphoma cells, the HBJ127-IL and HBJ127-CIL showed larger binding capacity to T24 bladder cancer cells than to Molt-4 T lymphoma cells. Similarly, the B3-IL and B3-CIL bound to BC47 bladder cancer cells in larger amounts than to FTL-13 T lymphoma cells.

The antigen specificity of the binding of CIL to target cells was confirmed by a competitive binding inhibition assay using an excess amount of the corresponding mAb as an inhibitor (Fig. 2). The binding of B3-CIL to BC47 cells was completely blocked by addition of B3 mAb but not by HBJ127 mAb. Similarly, the binding of HBJ127-CIL to T24 cells was selectively inhibited by addition of HBJ127 mAb. These results indicated that the binding of CIL to target tumor cells is principally mediated by a specific antigen-antibody interaction.

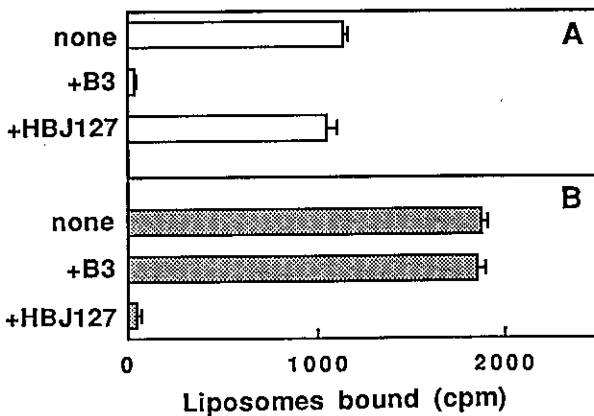

Fig. 2. Competitive inhibition of CIL-target cell binding with mAb. CIL and target tumor cells were incubated for 30 min in the presence or absence of B3 or HBJ127 mAb as described in "Materials and Methods." After washing, the radioactivity bound to the cells was determined. A, B3-CIL and BC47 rat bladder cancer cells; B, HBJ127-CIL and T24 human bladder cancer cells.

Scatchard plot analysis for the binding of mAb to tumor cells As mentioned above, the CIL containing adriamycin showed higher binding capacity to bladder cancer cells than to T lymphoma cells in both human and rat systems. This difference would be due to differences in the amount and/or the antibody affinity of the anti-gp125 mAb-defined antigen present on the target tumor cells. To determine the number and antibody affinity of the gp125 molecules present on the surface of each tumor cell, we performed Scatchard plot analysis using ^{125}I-labeled anti-gp125 mAb (Fig. 3). In the rat system, the number of B3 mAb-binding sites (molecules) on a BC47 bladder cancer cell was 3.4×10^5, which was three times that in the case of an FTL-13 lymphoma cell (1.1×10^5), although the binding affinities of B3 mAb to both tumors were equivalent ($Ka=1.3\times10^9$ M^{-1}). In the human system, the number of the HBJ127 mAb-binding molecules on a T24 bladder cancer cell was 8.4×10^5, about

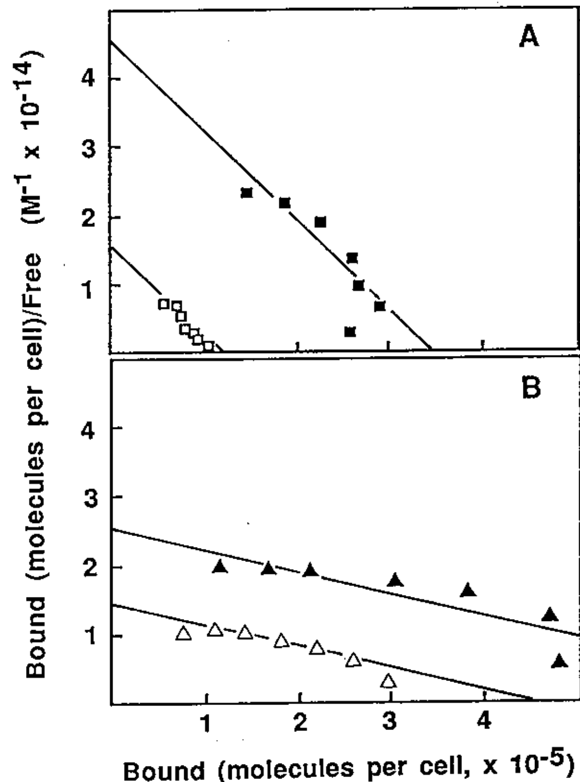

Fig. 3. Scatchard plot analysis of anti-gp125 mAb binding. ^{125}I-Labeled mAb was added to 200 μl of tumor cell suspension containing 3×10^5 cells. After incubation for 60 min at 4°C, the cells were washed and the radioactivity bound to the cells was counted as described in "Materials and Methods." A: ■, B3 mAb bound with BC47 cells; □, B3 mAb bound with FTL-13 cells. B: ▲, HBJ127 mAb bound with T24 cells; △, HBJ127 mAb bound with Molt-4 cells.

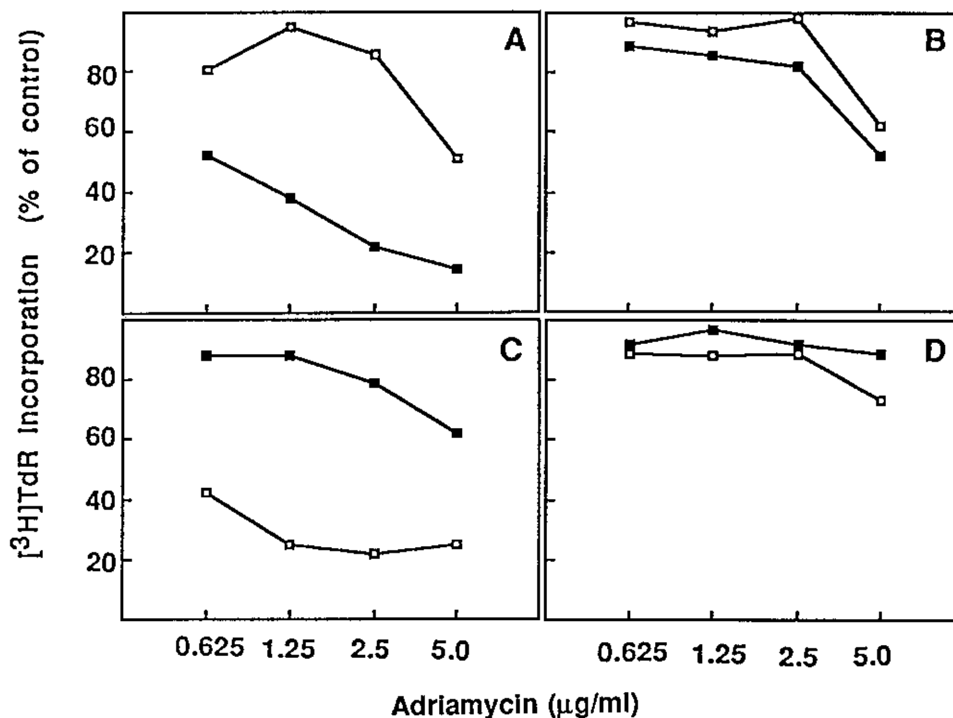

Fig. 4. *In vitro* cytotoxicity of CIL. Target tumor cells were incubated with CIL for 30 min at 37°C. After washing, the cells were further incubated for 24 h and then [^{3}H]TdR incorporation was determined as described in "Materials and Methods." These CIL contained 15 μg of adriamycin per 1 μmol of lipid. ■, B3-CIL; □, HBJ127-CIL. Target cells; A, BC47; B, FTL-13; C, T24; D, Molt-4.

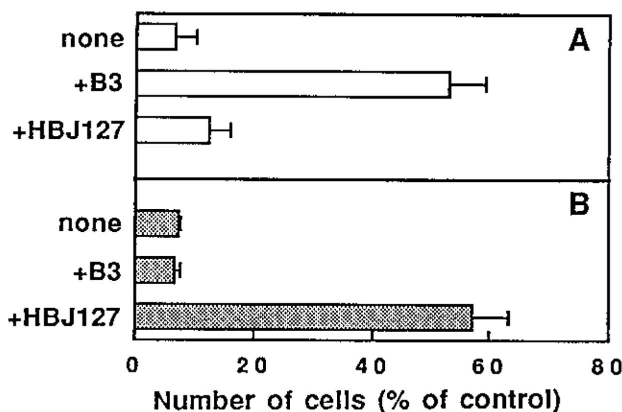

Fig. 5. Competitive inhibition of cytotoxicity of CIL with mAb. CIL and target tumor cells were incubated for 30 min in the presence or absence of B3 or HBJ127 mAb as described in "Materials and Methods." After washing, the tumor cells were further cultured for 4 days, and viable cells were determined by means of a trypan blue dye exclusion test. A, B3-CIL and BC47 rat bladder cancer cells; B, HBJ127-CIL and T24 human bladder cancer cells.

twice as many as that of a Molt-4 lymphoma cell (4.4×10^5). The binding affinities of HBJ127 mAb to these cells were equivalent ($Ka=0.29\times 10^9$ M^{-1}).

***In vitro* cytotoxicity of CIL** Tumor cells were incubated with free adriamycin, B3-CIL or HBJ127-CIL for 30 min. The cells were washed, cultured for 24 h in a fresh medium, and then examined for the activity of cellular DNA synthesis. Under these conditions, the concentrations (μg/ml) of free adriamycin required for 50% inhibition of cellular DNA synthesis in tumor cells were as follows: BC47, 0.27; FTL-13, 0.26; T24, 0.42; Molt-4, 0.54. The mAbs alone were not cytotoxic to all target cells (data not shown). Cytotoxicity of CIL is depicted in Fig. 4. B3-CIL and HBJ127-CIL displayed selective and potent cytotoxicity to the rat and human bladder cancer cells, respectively, while the cytotoxicity of these CIL was very low against T lympoma cells.

The antigen specificities in the cytotoxic reaction with CIL were confirmed by competitive inhibition assay using an excess amount of mAb as an inhibitor (Fig. 5). The CIL were added to antigen-bearing bladder cancer cells and incubated in the presence or absence of mAb

for 30 min. After washing, the cells were cultured for 4 days, and viable cells were determined by means of a trypan blue dye exclusion test. Cytotoxicity of the B3-CIL to BC47 cells was inhibited by addition of B3 mAb but not HBJ127 mAb. Similarly, cytotoxicity of the HBJ127-CIL to T24 cells was inhibited by addition of HBJ127 mAb but not B3 mAb. These results indicate that CIL bind to target tumor cells by virtue of antigen-antibody reaction, leading to killing of the target tumor cells.

DISCUSSION

The purpose of our study was to develop CIL that could effectively inhibit antigenic tumor cell growth. In the present study, we successfully prepared CIL by coupling thiolated anti-gp125 mAbs with maleimido modified small unilamellar liposomes followed by introduction of adriamycin into the liposomes by utilizing the response to valinomycin-dependent K^+ diffusion potential. The CIL bound to gp125 antigen-bearing bladder cancer cells and T lymphoma cells in the respective species, but not to antigen-negative counterparts. The amount of CIL bound to bladder cancer cells was greater than that to T lymphoma cells. This difference is attributable to the amount, but not to the affinity to anti-gp125 antibody, of the gp125 antigen, as judged from a Scatchard plot analysis using radiolabeled mAb. Accordingly, the CIL showed efficient and immunospecific cytotoxicity against bladder cancer cells but very weak activity toward lymphoma cells.

With regard to the mechanism of CIL cytotoxicity, there are several factors that control the efficacy of CIL: 1) stability of liposomes, 2) binding ability to target cells, 3) rapidity of the release of the drug from the cell-bound CIL and 4) extent of the internalization of the cell-bound CIL into the cells. It has been reported that the ligand-mediated internalization of immunotoxins or CIL is requisite for the effective delivery of cytotoxic compounds to target cells.[2, 3, 14, 15] Alternatively, Ho *et al.* reported that IL of a cartain lipid composition rapidly released their contents after binding with the corresponding antigen on target cell.[16] In the present system, the CIL were practically stable and efficiently bound with the target tumor cells, leading to a cytotoxic effect on the tumor cells. However, to explain the marked difference in the cytotoxic activities of the CIL to bladder cancer and lymphoma cells, further studies, e.g. on endocytosis in target tumor cells, will be required.

In this study, we demonstrated that the gp125 antigen systems on bladder cancer cells in human and rat can be specific target antigens for CIL. Although studies on the *in vivo* antitumor activity of the present CIL are still ongoing, the present work suggests that adriamycin-containing liposomes coated with anti-gp125 mAb may be applicable to the therapy of bladder cancer, at least by local application, because gp125 antigen is highly expressed in bladder cancer tissue but not in the normal portion of the bladder.

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