Assay of Cell Surface-Bound Immunoliposomes Using Monoclonal Antibody Reactive with a Cross-linking Reagent

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A monoclonal antibody (mAb) reactive with a crosslinking reagent, *N*-(*m*-maleimidobenzoyl)dipalmitoylphosphatidylethanolamine (*m*MBPE), in liposomes was produced from a hybridoma clone established by a fusion between P3X63Ag8.653 mouse myeloma cells and spleen cells from a BALB/c mouse hyperimmunized with the antibody-coated liposomes containing *m*MBPE. Using this mAb (termed AL-6), the quantity of immunoliposomes bound on target tumor cells was assessed by flow cytofluorometry. The results obtained using fluorescein isothiocyanate-coupled AL-6 allowed the enumeration not only of the immunoliposomes bound on all tumor cells but also those on individual target tumor cells. The relevance of this assay method was confirmed by a comparison with another assay method of cell-bound liposomes using immunoliposomes containing carboxyfluorescein in the vesicles.

Keywords crosslinking reagent; monoclonal antibody; immunoliposomes; flow cytofluorometry

Introduction

Antibody-coated liposomes (immunoliposomes, IL) containing a chemotherapeutic agent (chemoimmunoliposome) displayed selective binding and cytotoxic activities to target tumor cells.[1,2] The quantity of liposomes bound to target cells could be assessed, in theory, by a radioisotope (RI) tracer method. RI analysis shows the average amount of cell-bound liposomes but not the amount of those bound to individual target tumor cells. Moreover, there are limitations for the use of RI. Because the expression of tumor-associated antigens is generally heterogeneous among individual tumor cells in tumor tissue, especially in primary tumors, it is desirable to develop a non-RI method capable of measuring the quantity of liposomes bound to individual tumor cells.

IL are usually prepared by coupling antibody with liposomes using a cross linking reagent. Therefore, the production of antibodies that react with the reagent may allow the quantitative immunoassay of cell-bound liposomes leading to the enumeration of those bound on individual cells. Accordingly, we attempted to prepare a monoclonal antibody (mAb) which could bind specifically to a cross linking reagent present in liposomes. In this communication, we report the preparation of such mAb and its application to the quantitative analysis of liposomes bound on target tumor cells.

Materials and Methods

Animals and Tumor Cells Male BALB/c mice were obtained from Funabashi Farm, Chiba, Japan, and used at 8 weeks of age. KU-1 human bladder cancer cells were maintained in Dulbecco's modified Eagle's minimal essential medium (Nissui Pharmaceutical Co., Tokyo), pH 7.4, containing 10% heat-inactivated fetal calf serum (FCS) (M. A. Bioproducts, Walkersville, MD), 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (HEPES), and kanamycin at 60 μg/ml.

Chemicals Dipalmitoylphosphatidylcholine (DPPC) was donated by Nichiyu Liposome Co., Tokyo. Cholesterol, dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidic acid (DPPA), and L-cysteine were obtained from Sigma Chemical Co., St. Louis, Mo., *m*-maleimidobenzoyl-*N*-hydroxysuccinimido ester (*m*MBS) from Pierce Chemical Co., St. Louis Mo., and Sepharose CL 6B, diethylaminoethyl (DEAE)-Sephacel and *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate (SPDP) from Pharmacia Fine Chemicals, Upsala, Sweden and fluorescein isothiocyanate (FITC) from Dojin Chemical Co., Tokyo. 5(6)-Carboxyfluorescein (CF) was obtained from Kodak, Rochester, NY and used after purification by the method of Ralston *et al.*[3] *m*- or *p*-maleimidobenzoyl-bovine serum albumin (*m*MB-BSA or *p*MB-BSA) was prepared from *m*- or *p*-maleimidobenzoyl-*N*-hydroxysuccinimido ester and BSA.

mAbs for Modification of Liposomes Murine mAbs, HBJ127 (immunoglobulin G1(IgG1)) and HBE10 (immunoglobulin M (IgM)) were prepared from hybridomas established in our laboratory.[4,5] HBJ127 recognizes a peptide epitope of the human gp125 antigen which is expressed on human tumor cells but not on normal cells in the resting stage. HBE10 recognizes a sugar epitope of a neutral glycolipid antigen and reacts with several human epithelial carcinomas but not with normal cells.

Hybridoma cells were injected intraperitoneally into BALB/c mice and the ascitic fluid was later collected. mAbs were purified from the ascitic fluid by precipitation at 50% saturation with $(NH_4)_2SO_4$ followed by gel filtration on Sepharose CL 6B for IgM or by ion exchange chromatography on DEAE-Sephacel for IgG. Subunits of HBE10 IgM bearing free SH group (HBE10 IgM-s) were prepared by mild reduction of HBE10 IgM according to the method described earlier.[6] HBJ127 IgG was thiolated by SPDP substitution at a mole ratio of 1 : 5 according to the method of Carlsson *et al.*[7] The average substitution ratio of SPDP was 3.2.

Preparation of IL All IL preparations were done as described by Tanaka *et al.*[2] In brief, multilamellar liposomes (MLL) prepared from DPPC (25 μmol), cholesterol (17.5 μmol), *N*-(*m*-maleimidobenzoyl)dipalmitoylphosphatidylethanolamine (*m*MBPE) (2.5 μmol) and HEPES buffered saline (HBS; 20 mM HEPES and 150 mM NaCl, pH 6.8) or CF solution (100 mM CF, 20 mM HEPES and 80 mM NaCl, pH 6.8) were sonicated under N_2 atmosphere for 30 min at below 50 °C and the resultant small unilamellar liposomes (SUL) were treated with thiolated IgG or IgM-s for 1 h at 37 °C. After blocking the excess maleimide groups on liposomes by treating with L-cysteine, the SUL modified with immunoglobulin were purified by Sepharose CL-6B column chromatography and a void volume fraction containing SUL was used for experiments as IL. In addition to these IL, the MLL modified with IgM-s, IL prepared by replacing *m*MBPE with DPPE, *p*MBPE or DPPA, and SUL treated with L-cysteine, 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) were prepared and used as control liposome preparations.

Contents of lipid and antibody in liposomes were determined as described previously.[6] The diameter of liposomes was assessed by a peak elution volume in Sephacryl S-1000 (Pharmacia) chromatography using polystyrene beads of known diameters as standards.[8] The liposome size was almost the same in the various preparations (70—80 nm in diameter). In all liposome preparations, free maleimide residues were not detected on the liposome surface, as determined with Ellman's reagent using back titration in the presence of a known amount of L-cysteine[9] (data not shown).

Preparation of Anti-*m*MBPE mAb The MLL modified with HBE-10 IgM-s (HBE10-MLL) containing 2.3 μmol of total lipid and 100 μg of HBE10-IgM-s in 150 μl of HBS were emulsified with 150 μl of complete Freund's adjuvant and injected subcutaneously into a mouse once a week for three weeks. Three days after a booster intravenous injection of HBE10-MLL (without adjuvant), spleen cells were taken from the mouse and fused with P3X63Ag 8.653 mouse myeloma cells in 50% polyethylene glycol solution, and the cells were distributed into 96-well tissue culture plates. Hybridoma cells were selected by HAT medium.[4] A hybridoma

colony secreting mAb (called AL-6) reactive with *m*MBPE-containing liposomes was cloned by limiting dilution. The clone was expanded by culture or transplantation into BALB/c mice and the supernatants or ascitic fluid containing AL-6 were used for experiments after purification.

Analysis for Complement Dependent Liposome Lysis An aliquot (50 μl) of liposomes containing CF (0.1 μmol total lipid/ml) in phosphate buffered saline, pH 7.4 (PBS) containing 0.1% BSA (PBS–BSA) was mixed with 50 μl of diluted mAb solution and 50 μl of 1 : 4 diluted guinea pig serum (PBS was used as diluent) and then incubated for 30 min at 37 °C. After the addition of PBS (1.5 ml), the fluorescence intensity of the reaction mixture was measured on a spectrofluorometer (Hitachi Type F1200, Hitachi, Ltd., Tokyo, Japan). The total content of CF in a liposome sample was measured after treating the sample in 0.5% Triton X-100 solution and background (BKG) value was obtained from the samples by treating them with complement alone. CF release (%) was calculated by the following formula: CF release (%) = 100 × (RFI of a sample − BKG)/(RFI of a Triton-treated sample − BKG). RFI, relative fluorescence intensity.

Analysis for Binding of Liposomes to Antibody-Coated Plate Aliquots (20 μl) of AL-6 or HBE10 (30 μg/ml in PBS) were added to wells of a flat-bottomed 96 well tissue culture plate and incubated for 12 h at 4 °C. After discarding the supernatants, wells were treated with PBS–BSA for 1 h at 37 °C and rinsed with PBS. Aliquots (100 μl) of liposomes containing CF (0.5 μmol total lipid/ml) in PBS–BSA were added to the wells and incubated for 2 h at 4 °C. The wells were washed three times with PBS–BSA. Liposomes adsorbed on wells were solubilized with 0.2 ml of 0.5% Triton X-100 in PBS, then incubated for 20 min at 37 °C. The solution was transferred to new test tubes, mixed with 1.5 ml of PBS, and the fluorescence intensities were determined on a spectrofluorometer as desscribed above. A calibration curve for the quantitative assay was constructed using known amounts of liposomes containing CF.

Flow Cytofluorometric Analysis of Liposome Binding FITC-conjugated AL-6 (FITC-AL-6) was prepared by coupling FITC with AL-6. The molar ratio of FITC to AL-6 in the product was about 12 : 1.

Adherent cells were detached with PBS solution containing 0.004% actinase and 0.1% ethylenediaminetetraacetic acid (EDTA) by incubation for 5 min at 37 °C. After washing, cells were treated with various concentrations of IL for 45 min or 4 h at 4 °C under vibration at 15 min intervals. After 3-washings with ice-cold PBS, cells were stained with FITC-AL-6 at a final concentration of 20 μg/ml or 1 : 200 diluted FITC-coupled rabbit anti-mouse immunoglobulins (FITC-RαmIg, DAKO, Copenhagen, Denmark). After 2-washings with ice-cold PBS, cells were immediately analyzed for their fluorescein intensity using a flow cytofluorometer, FACScan (Becton Dickinson, Mountain View, CA), with excitation at 488 nm and emission at 515—545 nm and the relative fluorescence intensity of 10000 viable cells was recorded.

Results and Discussion

Ten days after culture of the cells obtained by the fusion between myeloma cells and spleen cells from a BALB/c mouse hyperimmunized with the antibody-coated liposomes containing *m*MBPE, hybridoma cells grew in 94 of 192 wells used for cell distribution. Twelve of these cultures were found to produce mouse immunoglobulin reactive with HBE10-IgM-s-conjugated liposomes containing *m*MBPE (HBE10-IL) as determined by the complement-dependent liposome lysis assay (data not shown). By limiting dilution, a hybridoma clone which secreted mAb reactive with HBE10-IL but not with *m*MBPE-free liposomes was obtained. The mAb was of IgM class and was termed AL-6.

Antigen specificity of AL-6 was assessed by the following assays: 1) the complement-dependent liposome lysis assay, 2) binding specificity to various liposome preparations, and 3) inhibitory effect of various materials on the binding. The results of complement-dependent lysis of liposomes with AL-6 are shown in Fig. 1. Significant release of CF (liposome lysis) was observed with liposome preparations containing *m*MBPE but not with *m*MBPE-free liposomes (Fig. 1a). As to the lytic sensitivity of *m*MBPE-containing liposome preparations, mAb- or cysteine-modified liposomes were

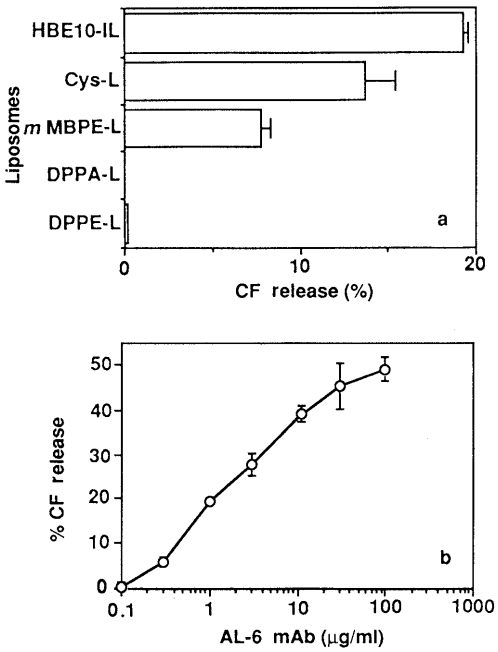

Fig. 1. Complement Dependent Liposome Lysis with AL-6

Various liposome preparations (a) or HBE10-IL (b) were titrated in triplicate with AL-6 (a, 1 μg/ml; b, 0.1—100 μg/ml) in the presence of guinea pig serum as described in materials and methods.

Both columns (a) and symbols (b) represent mean percent of CF release, and bars represent SE of the mean. *m*MBPE-L, liposomes prepared from DPPC/cholesterol/*m*MBPE; Cys-L, *m*MBPE-L conjugated with L-cysteine; DPPA-L, liposomes from DPPC/cholesterol/DPPA; DPPE-L, liposomes from DPPC/cholesterol/DPPE.

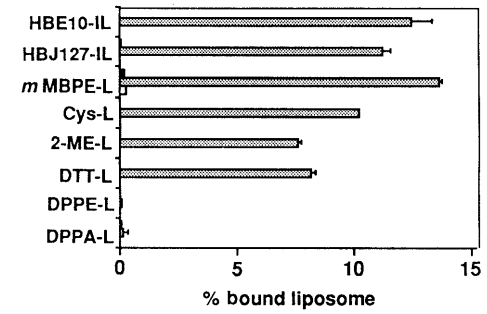

Fig. 2. Binding of Liposomes to an Antibody-Coated Plates

Wells of a tissue culture plate were coated with HBE10, AL-6 or BSA (none), and binding of CF-containing liposomes to the plate was determined in triplicate as described.

Columns and bars represent mean of bound CF (% of added amount) and SE of the mean, respectively. 2-ME-L, *m*MBPE-L conjugated with 2-ME; DTT-L, *m*MBPE-L conjugated with DTT; and others are shown in the legend of Fig. 1. ■, HBE10; ▨, AL-6; □, none.

more sensitive than unmodified liposomes. The reason for this is unclear, but the modification may affect stability of liposome membrane to the lysis. The reaction was dependent on the dose of AL-6 added, as observed with HBE10-IL (Fig. 1b).

The ability of AL-6 to bind to liposome preparations was assessed by measuring the amount of liposomes bound with AL-6 pre-fixed to wells of plastic plates (Fig. 2). All liposome preparations containing *m*MBPE, but not *m*MBPE-free liposomes, bound on the AL-6-coated plates. However, *m*MBPE-containing liposomes did not bind on the plates pre-coated with unrelated mAb. These findings indicated that the binding of AL-6 to liposomes is selective to *m*MBPE in liposomes.

To assess an antigenic determinant recognized by AL-6,

various materials were tested for their inhibitory effect on the binding of HBE10-IL to AL-6-coated wells (Fig. 3). All liposome preparations containing *m*MBPE as well as AL-6 mAb inhibited the binding. *m*MB-BSA also showed the inhibitory effect but *p*MB-BSA did not. L-Cysteine, 2-ME and DTT, which were used for modification of liposome preparations containing *m*MBPE, and unrelated antibody, HBE10, did not inhibit the binding. These findings indicated that AL-6 recognizes *m*-maleimidobenzoyl (*m*MB) group as the antigenic determinant. The reaction appeared to be quite specific to *m*MB, because the materials bearing its *para* isomer, *p*MB, did not react with AL-6.

As we found that AL-6 recognized *m*MB group in liposomes, we applied the mAb for analysis of IL or IL bound with target tumor cells. We selected HBJ127- or HBE10-modified liposomes (HBJ127-IL, HBE10-IL) and KU-1 cells for the experiments, because these liposomes could efficiently bind to KU-1 cells. IL were added to KU-1 cells and incubated for 45 min at 4 °C. After washing, IL bound on KU-1 cells were labeled with FITC-AL-6 (direct staining) or FITC-Rαmlg (indirect staining) and analyzed for the fluorescence intensity by flow cytofluorometry (Fig. 4). KU-1 cells treated with HBJ127-IL or HBE10-IL showed high intensity of fluorescence by staining with FITC-AL-6. Similar results were obtained by staining the cells with FITC-Rαmlg (data not shown). These results indicated that IL bound on target cells could be analyzed by the use of AL-6. As seen in Fig. 4, the addition of relevant antibody to the reaction mixture resulted in decrease of the fluorescence intensity on KU-1 cells, indicating that binding of the IL is antigen specific.

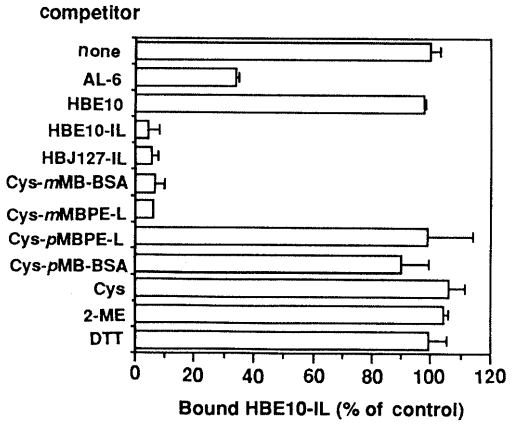

Fig. 3. Inhibition for Binding of HBE10-IL to an AL-6-Coated Plate by mAb, mAb-Modified Liposomes and Other Materials

mAb (10 μM), IL or BSA modified with maleimido derivative (100 μM maleimido equivalent) or a chemical (1 mM) was added with HBE10-IL to an AL-6-coated plate and analyzed in triplicate for the inhibitory effect on CF-entrapped HBE10-IL binding as described.

Columns and bars represent mean of bound CF (% of control) and SE of the mean. Cys-*m*MB-BSA, *m*MB-BSA conjugated with L-cysteine; Cys-*m*MBPE-L, *m*MBPE-L conjugated with L-cysteine (Cys-L); Cys-*p*MBPE-L, liposomes from DPPC/cholesterol/*p*MBPE conjugated with L-cysteine; Cys-*p*MB-BSA, *p*MB-BSA conjugated with L-cysteine; other abbreviations are shown in the legends of Figs. 1 and 2.

Although IL bound on target cells could be analyzed by immunostaining with AL-6, it was not obvious whether or not AL-6 detected those IL that retained an intact liposome form. Since, under the present experimental conditions (incubation at 4 °C), CF leaking from HBJ127-IL was negligible as determined by CF dequenching technique,[10] liposomes bound on target cells appeared to retain the intact liposome form. Therefore, the assay by immunostaining with AL-6 could also reveal the amount of intact liposomes. This was further substantiated by the following experiment: KU-1 cells were incubated with increasing amounts of HBJ127-IL containing CF or CF-free HBJ127-IL for 4 h at 4 °C, and the amounts of liposomes bound on KU-1 cells were assessed by fluorometric assay of CF in the liposomes or by flow cytofluorometry after staining with FITC-AL-6. As shown in Fig. 5, the relative amounts of cell-bound IL assessed by these two methods were similar.

As an additional experiment, we examined the effect of formalin treatment (a method for cell or tissue fixation) of cells bearing IL on the reactivity of AL-6 to the IL, because the treatment resulted in denaturation of liposomes leading to the release of a large amount of entrapped CF (data not shown). KU-1 cells bearing IL completely lost the reactivity to FITC-AL-6 by treatment with 5% formalin for 10 min at 25 °C, while the reactivity to Rαmlg was not affected by this treatment (data not shown). This finding suggested that the antigenic determinant to AL-6 (*m*MB group) in IL would be lost from the denatured liposome surface, although immunoglobulin determinant remained on the liposome surface.

The assay of cell-bound IL using entrapped CF as the marker showed the total amount of IL on cells, whereas the flow cytofluorometric assay using FITC-AL-6 represents the distribution pattern of IL in individual target cells, as indicated by FACS profiles. For example, the fluorescence pattern in Fig. 4 indicates that individual tumor cells were labelled equally with fluorescent liposomes or

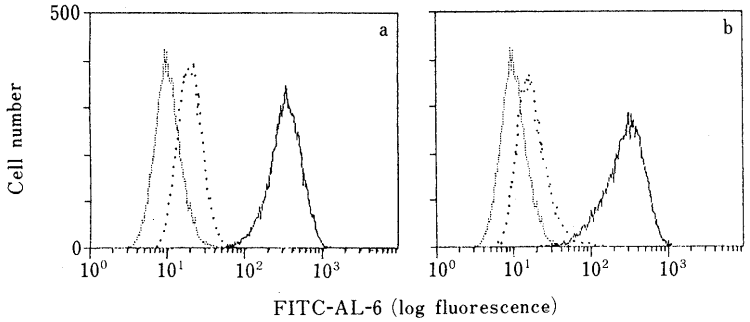

Fig. 4. Fluorescence Histograms of KU-1 Cells Which Had Bound FITC-AL-6

KU-1 cells were treated with HBJ127-IL or HBE10-IL (20 μg liposomal mAb/ml) in the presence or absence of excess amount (1 mg/ml) of relevant mAb, stained with FITC-AL-6, and subjected to flow cytofluorometry as described.

Background fluorescence was obtained from KU-1 cells untreated with IL. a: ------, control; ···, HBJ127-IL + excess HBJ127-IgG; ——, HBJ127-IL. b: ------, control; HBE10-IL + excess HBE10-IgG; ——, HBE10-IL.

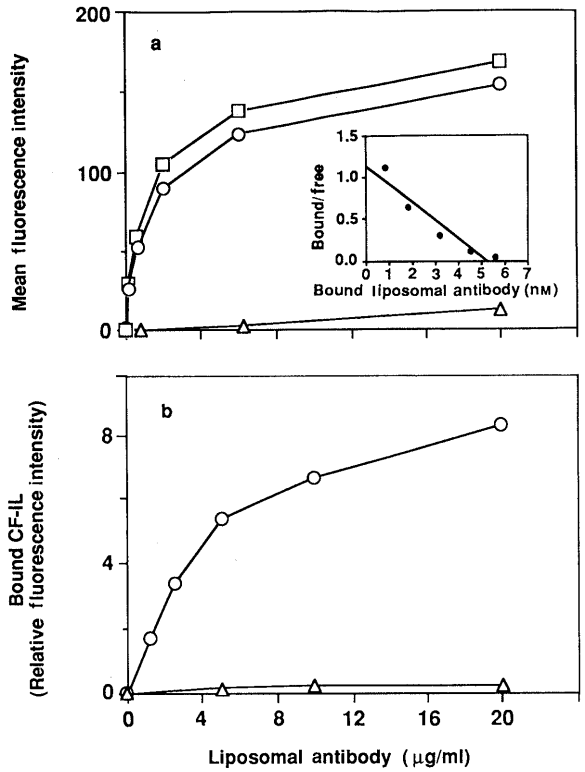

Fig. 5. Binding of HBJ127-IL to KU-1 Cells

a. flow cytofluorometric analysis. KU-1 cells (3×10^5) were incubated for 4 h at 4 °C with increasing concentration of HBJ127-IL in the presence or absence of excess amount of HBJ127 (1 mg/ml). After washing, the cells were stained with FITC-AL-6 or FITC-RαmIg and measured for the fluorescence intensity as described. ○, FITC-AL-6; △, excess HBJ127 plus FITC-AL-6; □, FITC-RαmIg. The inset represents a Scatchard plot of the data shown by ○ by comparing the mean fluorescence intensity at the maximal dose in a and the related value of bound IL in b.

b. fluorometric analysis. KU-1 cells (3×10^5) were treated with HBJ127-IL containing CF and solubilized with 2 ml of PBS containing 0.5% Triton X-100 for 30 min at 37 °C. After centrifugation, the amount of bound IL was assessed by measuring fluorescence intensity of the supernatant. ○, HBJ127-IL; △, HBJ127-IL plus excess HBJ127 mAb. All data were corrected by subtracting background values in untreated cells. Symbols indicate the mean value of duplicate samples.

that individual tumor cells expressed the same density of target antigen, since this pattern displayed nearly normal distribution. If we refer to Fig. 5b (in the maximum dose), number of bound IL per target cell is estimated to be 4.0×10^5 as calculated by an equation proposed by Enoch and Strittmatter,[11] and adaptation of this data to Fig. 5a as a Scatchard plot (inset in Fig. 5a) reveals that the apparent affinity constant of HBJ127-IL to KU-1 cells is $2.2 \times 10^8\ \text{M}^{-1}$, which was almost the same value as that of the binding of HBJ127-IgG to KU-1 ($3 \times 10^8\ \text{M}^{-1}$).

In the present work, we applied AL-6 only for analysis of cell-bound IL, but this mAb will be useful for analysis of any form of liposomes containing *m*MB groups.

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