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

Shinya SUZUKI, Takashi MASUKO, Kazuya TAKANASHI, Kazutoshi TAKASHIO and Yoshiyuki HASHIMOTO*
Department of Hygienic Chemistry, Pharmaceutical Institute, Tohoku University, Aoba-yama, Sendai 980, Japan. Received November 27, 1991

A monoclonal antibody (mAb) reactive with a crosslinking reagent, N-(m-maleimidobenzoyl)diethylphosphatidylethanolamine (mMBP), 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 mMBPE. Using this mAb (termed AL-6), the quantity of immunoliposomes bound on target tumor cells was assessed by flow cytfluorometry. The results obtained using fluorescent isothiocyanate (FITC)-conjugated 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 cytfluorometry

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 N2-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), dipalmitoylphosphatic acid (DPPA), and L-cysteine were obtained from Sigma Chemical Co., St. Louis, Mo., m-maleimidobenzoyl-N-hydroxysuccinimido ester (mMBE) from Pierce Chemical Co., St. Louis Mo., and Sepharose CL 6B, diethylaminoethyl (DEAE)-Sephalose and N-hydroxy succinimidy-3-(2-pyridyl)diethylopropionate (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 (mMB-BSA or pMB-BSA) was prepared from m- or p-maleimidobenzoyl-N-hydroxysuccinimido ester and BSA.

Preparation of Modification of Liposomes Murine mAbs, HB127 (immunoglobulin Gl(IgGl)) and HBE10 (immunoglobulin M (IgM)) were prepared from hybridomas established in our laboratory.4,5) HB127 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 (NH4)2SO4 followed by gel filtration on Sepharose CL 6B for IgM or by ion exchange chromatography on DEAE-Sephalose 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.6,7) HB127 IgG was thiocynated by SPDP substitution at a mole ratio of 1:5 according to the method of Carlson 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.7) In brief, multimammall liposomes (MML) prepared from DPPC (25 μmol), cholesterol (17.5 μmol), N-(m-maleimidobenzoyl)di- palmitoylphosphatidylethanolamine (mMBPE) (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 N2 atmosphere for 30 min at below 50°C and the resultant small unilamellar liposomes (SUL) were treated with thiocyan IgGl or IgMs for 1h 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 IgMs, IL prepared by replacing mMBPE with DPPE, pMBPE 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.8) 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.9) 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 (data not shown).

Preparation of Anti-mMBPE mAb

The MLL modified with HBE10 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 P3X63Ag8.653 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

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colony secreting mAb (called AL-6) reactive with mMBPE-containing liposomes was cloned by limiting dilution. The clone was expanded by culture or transplantation into BALB/c mice and the supernatants or ascites 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 a 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 μl), 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 described above. A calibration curve for the quantitative assay was constructed using known amounts of liposomes containing CF.

Flow Cytomfluorometric 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% ethylendiaminetetraacetic 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-Rsmlg, DAKO, Copenhagen, Denmark). After 2-washings with ice-cold PBS, cells were immediately analyzed for their fluorescence intensity using a flow cytometer, FACScan (Becton Dickinson, Mountain View, CA), with excitation at 488 nm and emission at 515–545 nm and the relative fluorescence intensity of 10,000 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 mMBPE, 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 mMBPE (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 mMBPE-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 mMBPE but not with mMBPE-free liposomes (Fig. 1a). As to the lytic sensitivity of mMBPE-containing liposome preparations, mAb- or cysteine-modified liposomes were 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 mMBPE, but not mMBPE-free liposomes, bound on the AL-6-coated plates. However, mMBPE-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 mMBPE 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 mMBPE as well as AL-6 mAb inhibited the binding. mMB-BSA also showed the inhibitory effect but pMB-BSA did not. l-Cysteine, 2-ME and DTT, which were used for modification of liposome preparations containing mMBPE, and unrelated antibody, HBE10, did not inhibit the binding. These findings indicated that AL-6 recognizes m-maleimidobenzoyl (mMB) group as the antigenic determinant. The reaction appeared to be quite specific to mMB, because the materials bearing its para isomer, pMB, did not react with AL-6.

As we found that AL-6 recognized mMB 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-RzmIg (indirect staining) and analyzed for the fluorescence intensity by flow cytometry (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-RzmIg (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.

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 cytometry 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 formazin treatment (a method for cell or tissue fixation) of cells bearing IL on the reactivity of AL-6 to the IL, because the treatment was used to denature AL-6 liposomes leading to the release of a large amount of entraped CF (data not shown). Ku-1 cells bearing IL completely lost the reactivity to FITC-AL-6 by treatment with 5% formazin for 10 min at 25°C, while the reactivity to Rzmlg was not affected by this treatment (data not shown). This finding suggested that the antigenic determinant to AL-6 (mMB 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 entraped CF as the marker showed the total amount of IL on cells, whereas the flow cytometric 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
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 $4.0 \times 10^3$ as calculated by an equation proposed by Enoch and Strittmatter, 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 $2.2 \times 10^8$ M$^{-1}$, which was almost the same value as that of the binding of HBJ127-IgG to KU-1 ($3 \times 10^8$ 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 mMB groups.

References and Notes