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A newly developed immunoliposome – an egg phosphatidylcholine liposome coated with pullulan bearing both a cholesterol moiety and an IgMs fragment

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An improved methodology for providing a more stable and targetable drug carrier has been developed. This method involves the synthesis of a newly designed immunoliposome by coating the outermost surface of large oligolamellar vesicles of egg phosphatidylcholine with the polysaccharide pullulan, modified to carry both cholesterol, as the hydrophobic anchor, and the monoclonal antibody fragment (anti-sialosyl Lewis^x, IgMs) as the sensory device. Compared with the binding of pullulan-coated liposomes, that of this immunoliposome to specific cells in vitro was significantly increased by factors of 447 to PC-9 and 295 to KATO-III, but only by a factor of 148 to the less specific cell, 3LL. This strong and specific binding of the immunoliposome to the cell surface of PC-9 was also confirmed by a fluorescence-microscopic investigation using the immunoliposome, which bore the hydrophobic fluorescent probe, terbium trisacetylacetonate, in the liposomal membrane.

Introduction

Liposomes have gained acceptance as potential drug carriers since they have many advantages, especially for water-soluble drugs, over other drug delivery systems. Several basic problems, however, must be overcome before the liposomal drug carrier can be employed in clinical practice. First, greater stability must be attained both to achieve longer storage periods and for in vivo administration. Secondly, targetability for specific cells or tissues must be developed.

Many approaches have been tried in order to obtain the targetability of liposomes in drug delivery systems. These approaches include noncovalent association of cell-specific antibodies with liposomes, covalent attachment of a polyclonal or monoclonal antibody to liposomes, and usage of glycoprotein-bearing liposomes, or natural and synthetic glycolipid-containing liposomes [1]. The following factors are required for the preparation

Abbreviations: egg PC, egg phosphatidylcholine; IgM, immunoglobulin M; IgMs, subunit of immunoglobulin M; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; MLV, multilamellar vesicles.

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of an efficient targetable liposome: (1) a sufficient quantity of the sensory device must be bound to the liposomal surface; (2) the binding between liposome and sensory device must be stable, although noncovalent binding is more convenient for several reasons such as biodegradability and/or immunogenicity; (3) the targetable property of the sensory device should remain unchanged even after its conjugation to the liposomes; (4) the integrity of the liposomes should be preserved during the process of conjugation [2–4] and before their arrival at the targeted cell or tissue after in vivo administration; and finally (5), the drug-bearing liposomes are required to be effectively internalized into the targeted cells after binding to them [5–8]. Many approaches have been tried for satisfying all or some of the above mentioned requirements and many basic in vitro experiments have been performed [1–8]. Unfortunately, however, only a few has been carried out in vivo [9,10] and none of those have been successful.

Very recently, we have developed an improved technique for producing liposomes which are mechanically and chemically stable against biochemical and physicochemical stimuli [11,12]. This technique involves coating the outermost surface of egg PC liposomes with naturally occurring polysaccharide derivatives such as mannan or amylopectin [11,12]. These polysaccharide-coated liposomes showed a unique targetability to specific tissues such as lung tissue and to specific cells such as alveolar macrophages [13,14]. Interestingly, when the pullulan derivative was employed to coat liposomes, however, although relatively high coating efficiency and very stable liposomes were obtained, we could not attain any significant targetability [11,12].

On the basis of our successful results in a previous study in which we made liposomes stable, even in vivo, by coating the liposomal surface with a naturally occurring polysaccharide derivative, we have developed a new and improved technique for safely binding the sensory device, a monoclonal antibody fragment, to the liposome. This method involves covalent binding of an IgMs fragment to the pullulan derivative and subsequent coating of the outer surface of large liposomes with the immunopolysaccharide derivative so obtained.

Materials and Methods

Chemicals. Egg phosphatidylcholine was isolated and purified from fresh egg yolk as described in Refs. 15–18. Monochloroacetic acid, tetrasodium ethylenediaminetetraacetate (EDTA), ethylenediamine dihydrochloride, 1-ethyl-3-(3-dimethylamino)propylcarbodiimide hydrochloride, cholesteryl chlorofomate, and γ-maleimidobutyryloxysuccinimide were purchased from Wako Pure Chemical Ind. Ltd. Tokyo, Japan. Other organic and inorganic reagents were commercially available as analytical grade.

Monoclonal antibody fragment, IgMs. Using the method developed by Köhler and Milstein [19], mouse monoclonal antibody IgM (CSLEX1) was obtained by immunization with human stomach cancer tissue (32-op-T-ST) [20]. An ascitic fluid of CSLEX1 antibody, which was kindly supplied by Professor Paul I. Terasaki, UCLA, U.S.A., was precipitated by adding saturated ammonium sulfate and then subjected to extensive dialysis against 0.15 M phosphate-buffered saline (pH 7.2). The resulting crude aqueous IgM solution was applied to a Sephacryl S-300 column (diameter 1.5×43 cm, Pharmacia Fine Chemicals, Japan) equilibrated with 0.15 M phosphate-buffered saline (pH 7.2). The pure IgM antibody obtained was radiochemically labelled with ^{125}I (specific activity $5.8 \cdot 10^8$ cpm $\cdot$ mg^{-1}) using the chloramine T method [21].

The method reported by Miller and Metzger [22] was used to reduce IgMs by cysteine to yield an IgMs fragment bearing the SH group: IgM (0.75 mg) was reduced by 0.1 ml of 0.5 M cysteine in 1.0 ml of 0.1 M phosphate-buffered saline (pH 8.0) at 20.0 °C for 10 min. The resulting reaction mixture was submitted to a Sephadex G-25 column (1.0×10 cm) preequilibrated with 0.1 M phosphate-buffered saline (pH 6.0) containing 5 mM EDTA, and 60% of the protein was recovered. The concentration of IgMs was determined either from the count rate of the ^{125}I-labelled material or from the absorbance at 280 nm ($\varepsilon^{H_2O} = 1.29$ g$^{-1} \cdot$ cm$^{-1} \cdot$ l).

Synthesis of the pullulan derivatives. Cholesterol derivatives of pullulan were synthesized as described elsewhere. First, carboxymethylated pullulan was obtained by reacting pullulan (molecu-

lar weight 50000, 3.0 g) with sodium monochloroacetate (2.9 g) in 50 ml of 1 M NaOH. After adjusting the pH of the aqueous carboxymethylated pullulan solution to 4.7, to the resulting solution was added ethylenediamine (4.0 g) in the presence of 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (0.72 g) as the coupling agent. The degree of substitution of ethylenediamine in aminoethylcarbamylmethyl pullulan was determined by elemental analysis.

Cholesterol moiety was introduced by adding a water-free DMF solution (10 ml) containing cholesteryl chloroformate (0.8 mg) into a water-free dimethylsulfoxide solution (30 ml) containing aminoethylcarbamylmethyl pullulan (1.0 g). The reaction was carried out at 80 °C for 20 h. The degree of substitution of cholesterol moiety was determined by ^{1}H-NMR. The pullulan derivative so obtained was substituted by 3.3 free aminoethylcarbamylethyl residues and 1.5 of cholesterol moieties per hundred glucose units. The above processes are given in Fig. 1.

Conjugation of the IgMs fragment to aminoethylcarbamylethyl pullulan on the liposomal surface. In order to conjugate the antibody fragment bearing the SH group (IgMs) to pullulan, the maleimide group was first introduced to the free aminoethylcarbamylmethyl group in the polysaccharide. To a 30 ml solution of water-free dimethylsulfoxide containing both cholesterol-bearing pullulan (300.0 mg) and γ-maleimidobutyryloxysuccinimide (50.0 mg) were added 5.0 ml of water-free pyridine and the reaction was carried out at room temperature. If insoluble materials appeared, the reaction mixture was warmed gently to below 50.0 °C. The reaction was usually completed after about 48 h, and was confirmed by the fluorescamine test [23]. The reaction mixture was poured into 300 ml of ethanol and kept overnight, and the precipitates afforded were collected by filtration using a Kiriyama funnel. The precipitates were redissolved in water and the water-insoluble materials were filtered off. The filtrate was submitted to lyophilization; yield, 121.0 mg (42%). Coating of

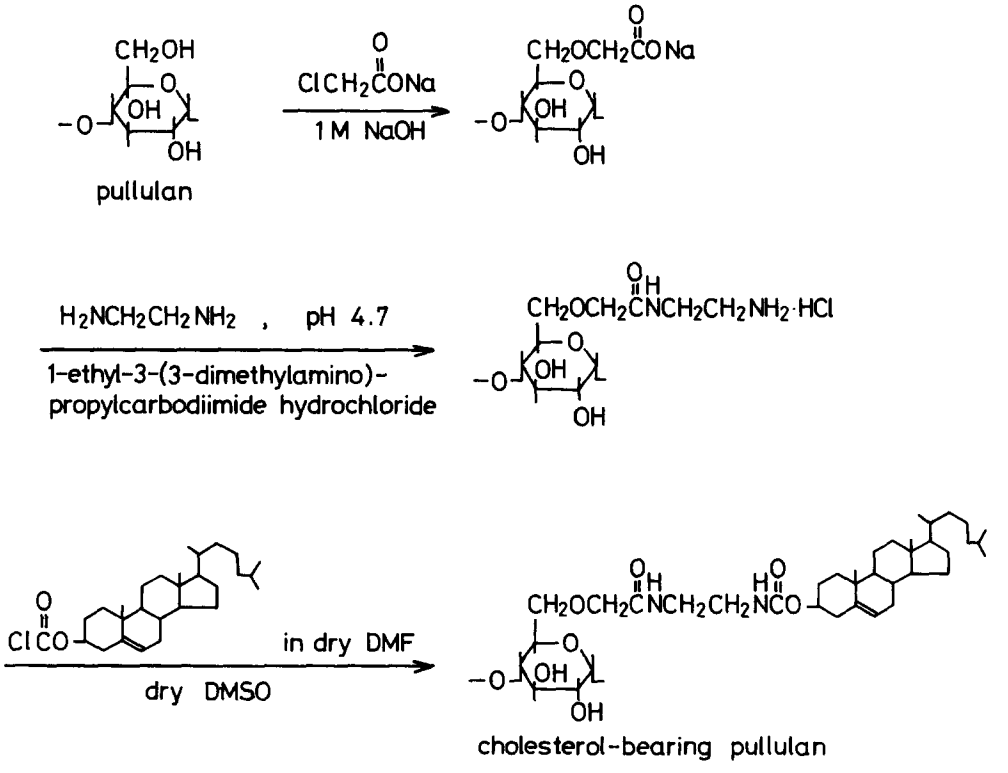

Fig. 1. Chemical processes of replacement of the cholesterol moiety to pullulan.

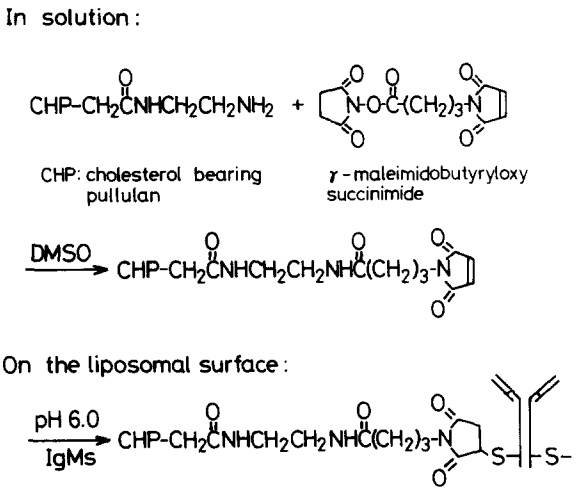

Fig. 2. Chemical processes of conjugation of the IgMs fragment to the cholesterol moiety-bearing pullulan derivative.

liposomes with the pullulan derivative so obtained was made by essentially the same procedure as that adopted for preparation of conventional liposomes [18,24,25]. To prepare a thin film, a chloroform solution of egg PC (30.0 mg) was evaporated under reduced pressure using a rotary vacuum evaporator. Thereafter, the procedure was carried out under a nitrogen atmosphere. The thin film was swollen and dispersed in 4.0 ml of 0.1 M aqueous phosphate-buffered solution (pH 6.0) containing 5.0 mM EDTA. The resulting suspension held in an ice-bath was subjected to ultrasonic irradiation using a Tomy UR-200P probe type sonifier at 25 W for 5 min at 30 s intervals. (If necessary, the liposomes were labelled by adding 10–20 μCi of di[^{14}C]palmitoylphosphatidylcholine (New England Nuclear, Boston, MA) into the presonified liposome suspension and by resonicating.) An aqueous solution of the pullulan derivative (3.0 mg/1.0 ml), which carries both γ-maleimidobutyryloxy and cholesterol moieties, was added into the whole liposomal suspension and the reaction mixtrue was kept for 1 h at 23.0 °C under gentle stirring. That all the pullulan derivative employed were quantitatively bound to the liposomal surface has been ascertained in advance using fluorescent-probe labelled pullulan. Therefore, it was not necessary to isolate the unbound pullulan at this stage. To the whole pullulan-coated liposomal suspension so obtained (approx. 40 μmol as phospholipid) were added 3.5 ml of 0.1 M phosphate buffer containing the antibody fragment (IgMs, 3.0 nmol): the final volume of the resulting solution was 7.5 ml and the pH was approx. 6.0. (If necessary, the antibody fragment labelled by ^{125}I was employed.) The mixture was incubated at 4.0 °C for 20 h. Subsequently, in order to block any unreacted IgMs, 100 μl of 0.1 M *N*-ethylmaleimide were added and the mixture was further incubated at 20.0 °C for 90 min. The whole reaction mixture was then submitted to a Sepharose 4B column (1.8 × 40.0 cm) which was preequilibrated with 0.1 M phosphate buffer (pH 6.0) containing 5.0 mM EDTA, by which both unreacted and blocked IgMs fragments were eliminated. For each fraction eluted out, the protein and lipid contents were determined from the specific activities of ^{125}I and ^{14}C, respectively.

Cell lines. The human stomach cancer cell line, KATO-III, which was established by Sekiguchi [26], was kindly provided by Professor H. Watanabe, the First Department of Pathology, School of Medicine, Niigata University, Japan. The human lung cancer cell line, PC-9, which was established by Hayata, Tokyo Medical College, Japan [27], was a personal gift from Dr. Hayata. The mouse Lewis lung carcinoma line, 3LL, was supplied from H. Ogawa (Chugai, Japan). All cell lines used were maintained in culture in RPMI-1640 supplements with 15% fetal calf serum (FCS), penicillin, and streptomycin.

Assay of binding of the liposomes to the target cells. The binding of the immunoliposome to the various cancer cells was examined by using ^{14}C-labelled immunoliposome. A 50.0 μl suspension of egg PC liposome labelled with di[^{14}C]palmitoylphosphatidylcholine (vide supra) was added at various concentrations (0.47, 2.3, and 11.6 μg of lipid) to $1.5 \cdot 10^5$ cancer cells in 1.0 ml RPMI-1640 containing 10% fetal calf serum. After incubation at 37 °C for 1 h, the cell suspensions were centrifuged at 300 × *g* for 10 min at the same temperature. Then, unbound liposomes in the cell-pellet obtained were washed out thrice with 0.1 M phosphate-buffered saline (pH 7.4). 5 ml of an aqueous counting scintillant cocktail (ACS II, Amersham, Japan) were added and the count rate of ^{14}C in the cell-pellet was measured on a β-ray scintillation counter (Aloka, LSC-903).

Fluorescent microscopic observations. Liposomes, in which the lipophilic fluorescent probe (terbium trisacetylacetonate, a gift from Professor L. Varallino, Virginia Commonwealth University) was embedded, were added to PC-9 in RPMI-1640 with 10% fetal calf serum. After incubation at 37 °C for 30 min, the cells were washed thrice with 0.1 M phosphate-buffered saline (pH 7.4). The cell-pellets were mounted and visualized, on an Olympus BH-2 fluorescence microscope, with ultraviolet excitation.

Results and Discussion

Polysaccharide-coated liposomes as the matrix of the carrier

As previously reported, by coating of the outermost surface of liposomes with a naturally occurring polysaccharide which bears hydrophobic substituents, such as simple palmitoyl and cholesterol moieties, capable anchoring into the lipid bilayer membrane, causes the liposomes to become very tough and stable against external stimuli such as the changes in osmotic pressure, ionic strength, and/or pH and against the attack of serum, plasma, lipases or lipoxygenases in vivo [11–14]. Most fortunately, this technique of polysaccharide-coating is more favorable for the larger sizes of liposomes (large unilamellar vesicles), which therefore more conveniently encapsulate a large amount of water soluble drugs, such as adriamycin, in the interior water pool, than do the smaller liposomes. Therefore, such polysaccharide-coated liposomes were considered to be a very good candidate for use as a targetable cancer therapy.

Until now, in conjugation of monoclonal antibody to liposomes, antibody proteins of high molecular mass (around 50 000–200 000) have been directly bound to relatively small lipid molecules (mol. wt. approx. 500–800) and mixed with naturally occurring lipids to form liposomes [1]. Therefore, even if these were to show relatively high specificity to target cells in vitro, they would be easily destructed by various stimuli in vivo. Previous unsuccessful results have been mostly attributable to the in vivo instability of the prepared immunoliposomes after administration. If the antibody protein were bound to a polysaccharide having a molecular mass comparable with that of the protein, and the liposomes were then coated by this monoclonal antibody conjugated polysaccharide, one should be able to prepare a more stable immunoliposome, even in vivo. For this reason we decided to employ the polysaccharide-coated liposomes as the matrix of the carrier onto which we could attach an excellent sensory device, the monoclonal antibody, the whole assembly being capable of, first, transporting a sufficient quantity of an effective antitumor drug and, secondly, releasing it on the target.

Of the polysaccharide derivatives which we have investigated, pullulan was the best candidate because of its relatively high coating efficiency and diminished targetability [11,12]. The polysaccharide itself should not interfere with the targetability of the attached monoclonal antibody.

The monoclonal antibody fragment as the sensory device of the carrier

The CSLEX1 antibody employed in this work reacts not only with the human stomach cancer tissue used in the immunization but also with the cell lines of leukemia (HL-60 and U937), stomach cancer (KATO-III and MKN-28), colon cancer (C-1, M7609, COLO205, WiDr, and SK-CO-1), lung cancer (PC-1, PC-3, PC-9, and QG-56), esophageal cancer (TE-1), and breast cancer (SK-BR-2III and BT-20). The epitope that the antibody recognizes is sensitive to neuraminidase and sialosyl Lewis^X (NeuAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ (Fuc $\alpha 1 \rightarrow 3$) GlcNAc $\beta 1 \rightarrow 3$ Gal). The antigen of CSLEX1 is distributed in various cancer tissues and cell lines and is found at high frequencies even in the sera of patients with a number of cancers [20,28].

Conjugation of IgMs to the pullulan derivative

Conjugation of IgMs to the pullulan derivative has been carried out using an almost identical procedure to that developed by Hashimoto and his co-workers [29], who introduced first the thiol-reacting maleimide group into a lipid molecule and then coupled it with the SH-bearing fragment of an antibody on the liposomal surface. In this work, we first introduced the thiol-reacting maleimide group into the pullulan bearing aminoethylcarbamylmethyl group, then coated the

outermost surface of egg PC large oligolamellar vesicles with the polysaccharide derivative, and finally coupled the antibody fragment IgMs, now bearing the SH group, onto the surface of the liposome. The chemical procedures of the conjugation processes are illustrated in Fig. 2.

After conjugation, the immunoliposome was isolated by gel-filtration on a Sepharose 4B column, the separation being monitored by radiochemical analyses of both ^{125}I on the antibody fragment and ^{14}C on the lipid. Obvious overlapping of the antibody fragment with the fractions containing the liposome is observed. We observed, of course, the noncovalent binding of IgMs to the liposome when IgMs was incubated with the pullulan-coated liposome without the coupling device for IgMs. However, the extent of noncovalent binding of the protein was negligibly small (less than 2.5%). The efficiency of conjugation determined from both the concentration of recovered protein and from the radioactivity of ^{125}I in the liposome fraction was on average about 50%.

The method which we presently employ in the conjugation of a sensory device to a drug carrier is the so-called 'hinge method' [30]. Compared with the non-hinge method which directly couples an antibody to a membrane protein or lipid using glutaraldehyde or periodate, the hinge method furnishes much better coupling efficiency and specific activity of the attached antibody [30].

The most important finding to be noted in this work is that no significant loss of the drug which has been preencapsulated in the liposome is observed during the conjugation and subsequent gel-filtration procedures. This finding was verified by employing a liposome in which adriamycin was encapsulated. For example, when adriamycin was encapsulated in the pullulan derivative-coated MLV and then IgMs was conjugated to pullulan on the surface of the liposome, 93% of encapsulated adriamycin was retained through the conjugation procedures. On the other hand, when adriamycin was encapsulated in a conventional MLV without any polysaccharide coat and the MLV so obtained was incubated in the absence of IgMs under the same condition as that used for the conjugation of IgMs to pullulan, the loss of adriamycin was approx. 26%. When the conventional MLV, which were loading adriamycin, were coincubated with the protein, the loss of adriamycin increased up to 63%.

In addition, the effect of structural stabilization of liposome by coating with the polysaccharide was examined in the presence of 18% (v/v) human serum employing carboxyfluorescein release technique [11,12] (Fig. 3, right-hand-side). Comparing with the case of spontaneous release of carboxyfluorescein from interior to exterior (Fig. 3, left-hand-side), the addition of serum increased the actual extent of release of encapsulated carboxyfluorescein. However, the relative release of carboxyfluorescein was significantly suppressed by coating the liposome with the polysaccharide even in the presence of serum. The effect of the polysaccharide coat on the liposomal surface during physicochemical and biochemical lyses of liposome has been demonstrated from various aspects [1,11–14].

If one assumes that the diameter of the liposome is about 1090 Å (on the basis of electron

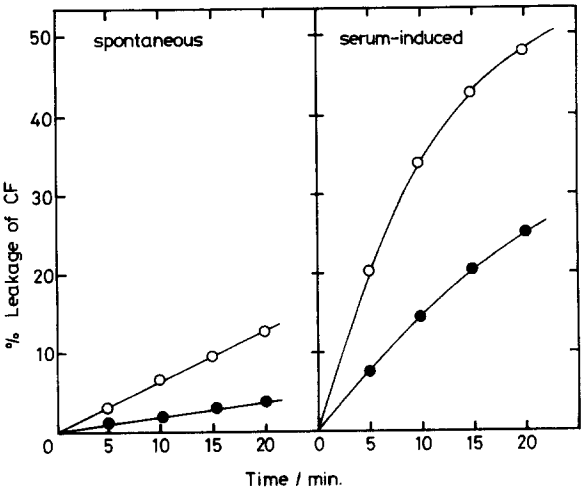

Fig. 3. Spontaneous (left-hand-side) and serum-induced (right-hand-side) release of carboxyfluorescein encapsulated in a conventional (○) and pullulan derivative-coated (●) small unilamellar liposomes in a 20 mM Tris-buffered solution containing 200 mM sodium chloride (pH 7.4) at 37.0°C. Small unilamellar liposomes ([egg PC] = $1.0 \cdot 10^{-4}$ M), which contain 200 mM carboxyfluorescein in their interior aqueous phase, were formed and coated with cholesterol moiety bearing pullulan of one tenth to lipids (by wt.). Carboxyfluorescein release was monitored according to the same technique described previously [1,11,12].

microscopic observations) and that the molecular mass of the IgMs employed is 185000 [22], one can calculate that approx. 35 IgMs molecules are bound to one vesicle, a value which agrees with that estimated from the concentration ratio of protein to lipid in the synthesized immunoliposome, namely 32 ng of protein per μg of lipid. This value further leads to the estimation that one IgMs molecule is sitting on an area of 490 Å^2 at the liposomal surface.

Cell specificity of the newly developed immunoliposome

PC-9, KATO-III, and 3LL were selected as the target tumor cells for investigation of the cell specificity of our immunoliposome. The first two of these cell lines are more specific for CSLEX1, while the third cell line, 3LL, is considered to be less specific [20,28]. Fig. 4 shows relative numbers of the immunoliposome bound to the cells, estimated from the count rates of ^{14}C and ^{125}I incorporated in the cell-pellets after incubation for 60 min at 37.0 °C. The pullulan-coated liposome, without the sensory device, did not show any significant binding to the cell lines. Conversely, the binding of the immunoliposome to specific cells was dramatically increased by factors as high as 447 to PC-9 and 295 to KATO-III compared with the pullulan-coated liposome, but only by a factor of 148 to the less specific cell, 3LL. The result was in good agreement with that for parent IgM and IgMs in the absence of the liposome.

This specific binding of the immunoliposome to the target cell was further confirmed by fluorescence microscopic observation. For this purpose, we selected PC-9 as the target cell and employed the immunoliposome in whose lipid membranes the hydrophobic fluorescent probe, terbium trisacetylacetonate [14], was embedded. The photograph (Fig. 5) indicates that the cell surface of PC-9 is strongly stained by the fluorescent probe.

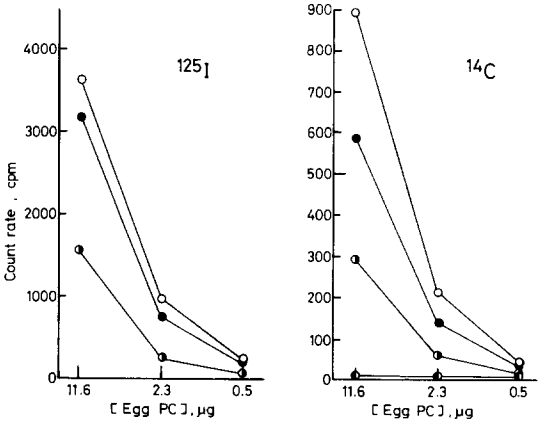

Fig. 4. Specific binding of the immunoliposome and non-specific binding of the pullulan-coated liposome to various cells as a function of lipid concentration. A 50.0 μl suspension of the immunoliposome or the pullulan-coated liposome (◐), which was doubly labelled with [^{14}C]DPPC and/or [^{125}I]IgMs, was added to $1.5 \cdot 10^5$ cancer cells (○, PC-9; ●, KATO-III; and ◑, 3LL) in 1.0 ml of RPMI-1640 containing 10% fetal calf serum. After incubation for 1 h at 37.0 °C, unbound liposomes were washed out thrice with 0.1 M phosphate-buffered saline by centrifugation. The radioactivity remaining in the target cells was counted by both γ-ray (for ^{125}I) (left-hand-side) and β-ray (for ^{14}C) (right-hand-side) spectrometry.

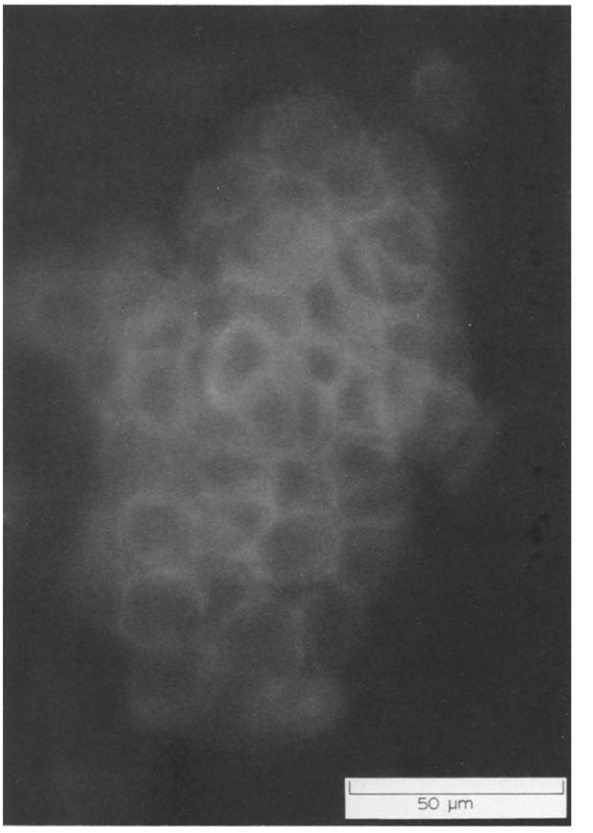

Fig. 5. Fluorescence microscopy photograph (×560) of PC-9 incubated with the immunoliposome labelled by the hydrophobic fluorescent probe (terbium trisacetylacetonate). Only the cell surface is stained.

In conclusion, we have prepared a very stable and targetable drug carrier by coating the outermost surface of large unilamellar liposomes with the pullulan derivative which bears both a cholesterol moiety, as the hydrophobic anchor, and an IgMs fragment, as the sensory device. When we encapsulated adriamycin in our newly developed immunoliposome, we obtained reliable results of its targetability in vivo and of its treatment of human lung cancer disease in experimental mice. These successful results will be reported separately (Hirato, M., et al.).

Acknowledgments

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