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International Journal of Pharmaceutics 237 (2002) 129–137

international journal of pharmaceutics

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Targetability and intracellular delivery of anti-BCG antibody-modified, pH-sensitive fusogenic immunoliposomes to tumor cells

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Received 9 August 2001; received in revised form 18 January 2002; accepted 23 January 2002

Abstract

We prepared tumor-specific immunoliposomes by coupling anti-BCG monoclonal antibodies to pH-sensitive fusogenic liposomes modified with succinylated polyglycidol (sucPG), in order to obtain efficient binding to, and endocytotic internalization into, the tumor cells. Mouse colon carcinoma 26 cells, which are known to share a common antigen with BCG, were used in in vitro experiments. BCG-sucPG immunoliposomes showed fusion ability under acidic conditions. Fluorescence microscopic observation indicated that BCG-sucPG immunoliposomes bound to colon 26 tumor cells and induced receptor-mediated endocytosis at 37 °C. Fusion assay by resonance energy transfer using *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) diacyl phosphatidylethanolamine and *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine suggested that fusion between BCG-sucPG immunoliposomes and endosomal and/or lysozomal membrane did occur. These results imply that the BCG-sucPG immunoliposomes transfer their content into the cytoplasm by fusing with the endosomal and/or lysozomal membrane after recognition of target cells and subsequent internalization into the cells by endocytosis. © 2002 Published by Elsevier Science B.V.

Keywords: Liposome; pH-sensitive liposome; Immunoliposome; Drug delivery system; BCG

1. Introduction

Liposomes have been extensively studied as a carrier for both hydrophobic and hydrophilic

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0378-5173/02/$ - see front matter © 2002 Published by Elsevier Science B.V.

PII: S0378-5173(02)00044-3

drugs. Generally, the capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal tissues (Jain and Gerlowski, 1986). Therefore, circulating small liposomes extravasate from blood vessels and accumulate passively in the tumor tissue. In order to achieve more specific targeting, modification of the liposomes with various target-specific ligands has been tested (Lee et al., 1996; Harrington et al., 2000a,b). We prepared immunoliposomes by conjugation of anti-BCG monoclonal antibodies, in order to obtain specific targetability to the tumors, upon which a common antigen with BCG is expressed. On the other hand, when the liposomes are used as a gene transfer vector, they are required to release their contents into the cytoplasm without degradation by the lysosomal enzymes after having been taken up by the cells through an endocytotic pathway. It is thought that pH-sensitive liposomes, which become fusogenic under acidic conditions, may fuse with the endosomal membrane when they are exposed to the acidic pH (Lee et al., 1996) of the endosome, resulting in release of their contents into the cytoplasm.

Kono et al. have developed a new type of pH-sensitive liposome, egg yolk phosphatidylcholine liposomes bearing succinylated polyglycidol (sucPG), that is a polyethylene glycol derivative having carboxyl groups, and showed that fusion ability of the liposomes increases under weakly acidic and acidic conditions (Kono et al., 1994, 1997). In the present study, we investigated the binding efficiency of anti-BCG antibody-coupled sucPG liposomes (BCG-sucPG liposomes) to mouse colon carcinoma 26 (colon 26) cells, which share a common antigen with BCG (Sasaki et al., 1985). Furthermore, the uptake of the BCG-sucPG liposomes by the cells and the intracellular fusion activity of these liposomes were also estimated.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC) was kindly donated by Nippon Oil and Fats (Tokyo, Japan). sucPG (Fig. 1) was synthesized as described (Kono et al., 1994). The most frequent molecular weight of the polyglycidol obtained was estimated to be 11 500 by HPLC on an Asahipak GS-510 column (Asahi, Japan). 1-Ethyl-3-(dimethyl aminopropyl)carbodiimide (EDC), *N*-hydroxysulfosuccinimide (S-NHS) and IODO-GEN were obtained from Sigma (Rockford, IL, USA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (DiI) was purchased from Lambda (Graz, Austria). *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidyl ethanolamine (Rh-PE) and *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) diacyl phosphatidyl ethanolamine (NBD-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) and ^{125}I were obtained from New England Nuclear Japan (Tokyo, Japan).

2.2. Cell culture

Colon 26 mouse adenocarcinoma cells were grown in RPMI1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 2.5% HEPES buffer and antibiotics (1% PC/SM). They were cultured in a 37 °C cell culture incubator in a humidified atmosphere containing 5% CO_2.

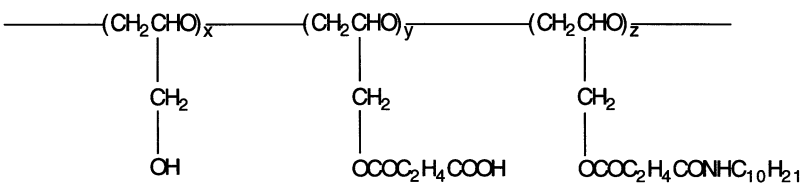

Fig. 1. Chemical structure of pH-sensitive polyglycidol derivative (sucPG), which is sucPG bearing *n*-decylamine, $x:y:z = 4.4:88.8:6.8$.

2.3. Preparation of anti-BCG mouse monoclonal antibodies

XVIII G1, an anti-BCG monoclonal antibody hybridoma cell line, was a gift from Dr Jacqueline De Bruyn (Pasteur Institute of Brussels, Belgium). Cells were grown in E-RDF medium (Kyokuto, Tokyo, Japan) under serum-free conditions. The monoclonal antibody was purified from culture supernatant by protein A-agarose affinity chromatography (ImmunoPure IgG Purification Kit, Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The purity of the antibody was confirmed by electrophoresis (data not shown).

2.4. Preparation of immunoliposomes

SucPG liposomes were prepared from egg PC and sucPG (4:1, w/w) by a hydration method followed by extrusion (Lipex Biomembranes, Canada). SucPG and egg PC were dissolved in methanol in a round-bottomed glass tube and the organic solvent was evaporated. The lipid film was vacuum-desiccated. Phosphate-buffered saline (PBS; pH 7.4) was added and the lipid was allowed to hydrate. If necessary, 100 mM calcein solution or acetate buffer (pH 7.4) was used as the aqueous phase. The lipid suspension was extruded five times through two stacked polycarbonate membrane filters (Nuclepore, CA) with 400 and 200 nm pores, respectively, and ten times through filters with 100 nm pores to form liposomes of 130 nm in mean diameter. A trace amount of ^{3}H-CHE or DiI was added for binding assay, and NBD-PE and Rh-PE were added for fusion assay.

Coupling of antibodies to the plain sucPG liposomes was performed as described (Maruyama et al., 1995). Briefly, to 300 μl of the plain liposomes (7 μmol lipids) in PBS, 120 μl of 0.25 M EDC in water and 120 μl of 0.25 M S-NHS in water were added, and the mixture was stirred for 10 min at room temperature. Anti-BCG monoclonal antibody (100 μg) with a trace amount of ^{125}I-labeled antibody was then added and the whole was incubated for 2 h at room temperature with stirring. The immunoliposomes were separated from the unbound antibody on a Bio-Gel A15M column. The lipid concentration and the coupling efficiency of antibody were estimated by the phosphorus assay and the radioactivity of ^{125}I, respectively. The average number of antibody molecules per liposome was calculated by using the above values and the following assumptions: molecular weight of antibody is 150 000; the average number of phospholipid molecules per a liposome is estimated by the method of Enoch and Strittmatter (1979). The average number of antibody molecules per a liposome was calculated to approximately 20.

2.5. Binding ability of anti-BCG antibodies to colon 26 cells

Specific binding of the purified antibody to colon 26 cells was monitored by immunostaining. Colon 26 cells (2×10^4) were seeded in dishes and incubated. The primary anti-BCG monoclonal antibody was added to the tumor cells, followed by addition of the secondary antibody, biotin-labeled anti-mouse IgG. Colon 26 cells not treated with primary antibody were prepared as negative controls. Peroxidase-labeled streptoavidin was then added and stained cells were investigated microscopically (Histofine SAB-PO kit, Nichirei, Tokyo, Japan).

2.6. pH-sensitivity of sucPG liposomes

Calcein-containing sucPG liposomes were prepared for leakage assay. Calcein-loaded liposomes were dispersed to a lipid concentration of 5.1×10^{-4} M in acetate buffer of preadjusted pH (pH 4.0, 5.0, 6.0 or 7.4) and the fluorescence change was monitored after incubation for 30 min at 37 °C. Excitation and monitoring wavelengths were 495 and 520 nm, respectively. The percent leakage of liposomes was defined as

$$\%\text{leakage} = \left(\frac{F^{t} - F^{0}}{F^{f} - F^{0}}\right)100$$

where F^0 and F^t mean the initial and intermediate fluorescence intensities of liposomes, respectively. F^f is the fluorescence intensity after the addition of Triton X-100 (final concentration 0.15%) in acetate buffer of preadjusted pH (pH 4.0, 5.0, 6.0

or 7.4). Calcein leakage from egg PC liposomes was calculated similarly (Kono et al., 1993).

2.7. Binding efficiency of BCG-sucPG liposomes to colon 26 cells

Colon 26 cells (1×10^4) in RPMI 1640 medium with 10% fetal bovine serum were seeded in dishes and incubated for 24 h (37 °C, 5% CO_2) prior to binding assay. ^{3}H-CHE-labeled BCG-sucPG liposomes were then added at 100 μg as lipid amount and the mixture was incubated at 4 or 37 °C for 90 min. To examine the specificity of the receptor-mediated binding, cells were incubated with excess dose of anti-BCG monoclonal antibody (50 μg) at 4 °C for 30 min, and then binding assays of ^{3}H-CHE-labeled BCG-sucPG liposomes were performed in medium containing excess dose of antibody (50 μg) at 4 °C for 90 min. After washing the cells with PBS (4 °C), the suspension was centrifuged three times ($180 \times g$, 3 min), and the supernatant was removed. After lysis with 200 μl of 0.2 N NaOH, radioactivity was determined in Hionic-Fluor scintillation mixture in an Aloka LSC-3000 counter (Aloka Co., Tokyo, Japan).

On the other hand, BCG-sucPG liposomes containing DiI were incubated with colon 26 cells in PBS for 30 min at 4 or 37 °C. Liposome binding to cells was investigated with a fluorescence microscope.

2.8. Fusion of sucPG liposomes and BCG-sucPG liposomes

Fusion of liposomes was determined by measuring the fluorescence energy transfer between NBD-PE and Rh-PE (Kono et al., 1997). The egg PC liposomes containing 1 mol% of NBD-PE and Rh-PE were mixed in a 1:1 molar ratio with the probe-free sucPG liposomes to give a lipid concentration of 1.8×10^{-4} M. The suspension was excited at 450 nm and fluorescence emission spectra were measured at various time intervals at pH 4.0, 5.0, 6.0 and 7.4. The ratio of fluorescence intensity at 530 nm to that at 590 nm was then calculated. The ratio of fluorescence intensity of the egg PC liposomes containing 1 or 0.5 mol% NBD-PE and Rh-PE was also calculated to establish a standard curve. Apparent concentration of these fluorophores in the membrane was then calculated using this standard curve. The percent fusion was defined as

$$\%\text{fusion} = \left(\frac{C_t - C_0}{C_f - C_0}\right)100$$

where C_0 (1 mol%) and C_t mean the initial and intermediate concentrations of the fluorophores, respectively. C_f (0.5 mol%) represents the concentration at which complete fusion occurs.

Fusion of egg PC or BCG-sucPG liposomes after 30 min incubation was evaluated similarly (Kono et al., 1993).

2.9. Intracellular fusion ability of BCG-sucPG liposomes

Fusion between BCG-sucPG liposomes and cellular membranes was detected by measuring resonance energy transfer between NBD-PE and Rh-PE (Kono et al., 1997). Colon 26 cells were seeded in 35-mm dishes and incubated for 24 h (37 °C, 5% CO_2). After 90 min incubation with BCG-sucPG liposomes containing 5 mol% of NBD-PE and 1 mol% of Rh-PE, the tumor cells were washed with PBS (4 °C) and examined under a fluorescence microscope with 515 nm long-pass filter.

3. Results

3.1. pH-sensitivity of sucPG liposomes

To estimate the pH-sensitivity of sucPG liposomes, calcein leakage from sucPG liposomes and egg PC liposomes was determined. Leakage of calcein from sucPG liposomes was approximately 90% or more at pH 4.0 and 4.5, and was below 30% at above pH 5.0. On the other hand, leakage from the egg PC liposomes was below 20% in the experimental pH region (Fig. 2).

3.2. Binding efficiency of BCG-sucPG liposomes to colon 26 cells

As shown in Fig. 3, anti-BCG antibody-cou-

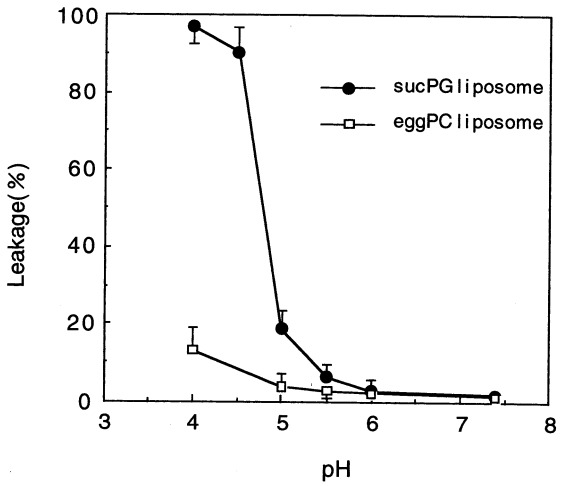

Fig. 2. pH-dependence of calcein-leakage from sucPG liposomes. Calcein-loaded liposomes were dispersed to a lipid concentration of 5.1×10^{-4} M in 10 mM acetate buffer of preadjusted pH and the fluorescence change was monitored after incubation for 30 min at 37 °C. Data are expressed as mean ± S.D. ($n = 3$).

pled sucPG liposomes showed higher targeting ability to colon 26 cells than did nontargeted sucPG liposomes at 4 °C. The presence of 50 μg of free antibody decreased significantly the degree of cell binding of BCG-sucPG liposomes. These results revealed that the binding of BCG-sucPG liposomes to colon 26 cells is indeed receptor specific. Increasing the incubation temperature to 37 °C induced remarkable enhancement of association of BCG-sucPG liposomes with the target cells. This high association at 37 °C was considered to endocytotic uptake of BCG-sucPG liposomes by colon 26 cells after binding. After incubation of DiI-labeled BCG-sucPG liposomes with colon 26 cells, diffuse fluorescence of DiI was observed in the cells at 37 °C, whereas fluorescence was seen only on the cell surface at 4 °C (Fig. 4). These results also indicate the specific binding of the BCG-sucPG liposomes to the cell surface and subsequent internalization into the cells by receptor-mediated endocytosis at 37 °C.

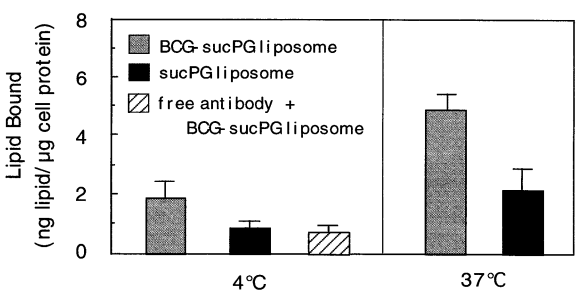

Fig. 3. Association of BCG-sucPG liposomes with colon 26 cells. Colon 26 cells (1×10^4) and ^{3}H-CHE labeled BCG-sucPG liposomes (100 μg as lipid amount) were incubated at 4 or 37 °C for 90 min. Blocking tests of BCG-sucPG liposomes-cell binding were performed with excess dose (50 μg) of free anti-BCG monoclonal antibody. Radioactivity was then determined in Hionic-Fluor scintillation mixture. Data are expressed as mean ± S.D. ($n = 3$–5).

3.3. Fusion of sucPG liposomes and BCG-sucPG liposomes

Interaction of sucPG liposomes or BCG-sucPG liposomes with egg PC liposomes was investigated to see whether anti-BCG antibody bound to the surface of sucPG liposomes reduced the fusion ability of sucPG liposomes. The fusion ability of these liposomes was examined by detecting dilution of the fluorescence probes. Fusion of sucPG liposomes after 30 min incubation was 60% at pH 4.0, but was below 20% at above pH 5.0 (data not shown). Compared with the fusogenic activity of sucPG liposomes, that of BCG-sucPG liposomes was similar at pH 4.0 and higher at pH 5.0. Fusogenic activity of egg PC liposomes was below 30% in the experimental pH region (Fig. 5).

3.4. Intracellular fusion ability of BCG-sucPG liposomes

As the pH in endosomes is reported to be approximately 4.5–5.5, BCG-sucPG liposomes, which show fusion ability under acidic conditions, are expected to fuse with the endosomal and/or lysozomal membrane. In order to confirm the occurrence of fusion between BCG-sucPG liposomes and the endosomal and/or lysozomal membrane after uptake by colon 26 cells, fusion assay by resonance energy transfer using NBD-PE and Rh-PE was performed. As shown in Fig. 6A, orange fluorescence (mixed fluorescence of NBD-PE and Rh-PE) was observed on the cell surface,

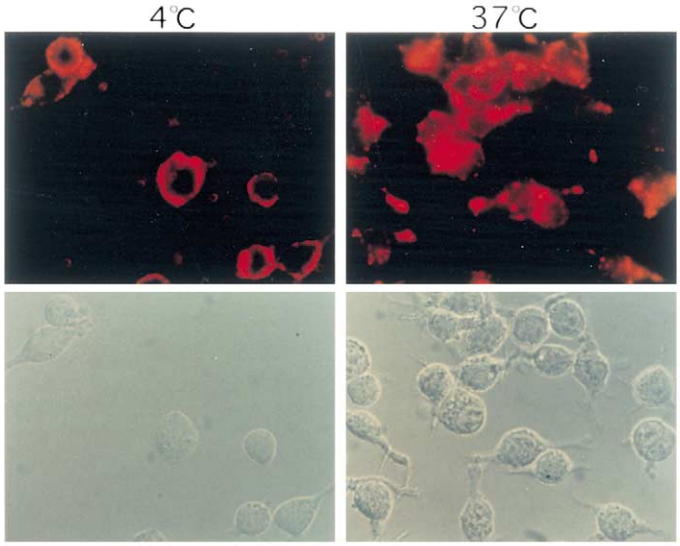

Fig. 4

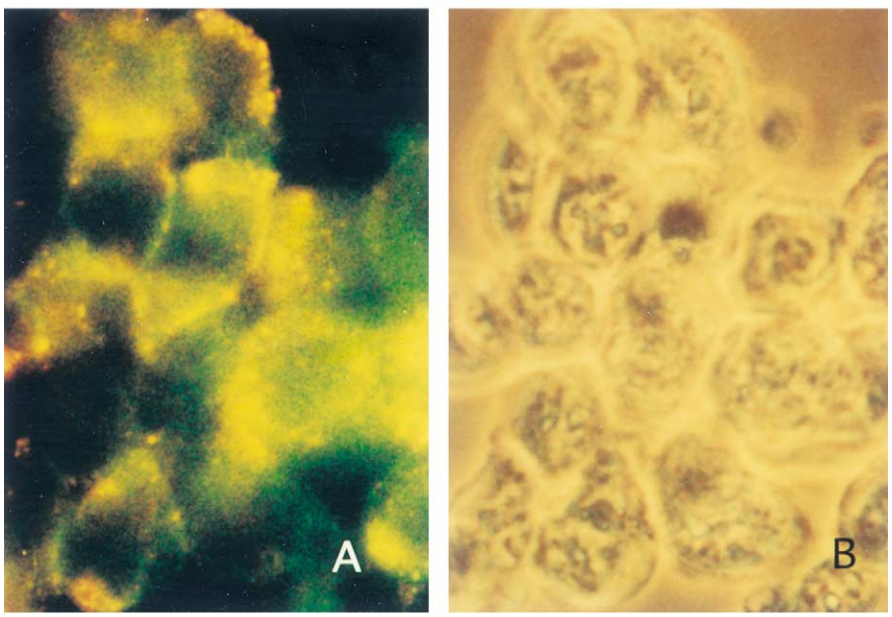

Fig. 6

Fig. 4. Fluorescence and phase-contrast micrographs of colon 26 cells incubated with BCG-sucPG liposomes containing DiI. The incubation was performed in PBS for 30 min at 4 or 37 °C.

Fig. 6. Intracellular fusion assay of BCG-sucPG liposomes determined by fluorescence or phase contrast microscopy. Fluorescence micrographs (A) and phase contrast micrographs (B) of colon 26 cells incubated with BCG-sucPG liposomes containing NBD-PE and Rh-PE. The incubation was performed in PBS for 90 min at 37 °C.

and yellow fluorescence (fluorescence of NBD-PE) was seen in the cells, indicating that the BCG-sucPG liposomes had fused with the endosomal and/or lysozomal membrane after internalization into the tumor cells through an endocytotic pathway.

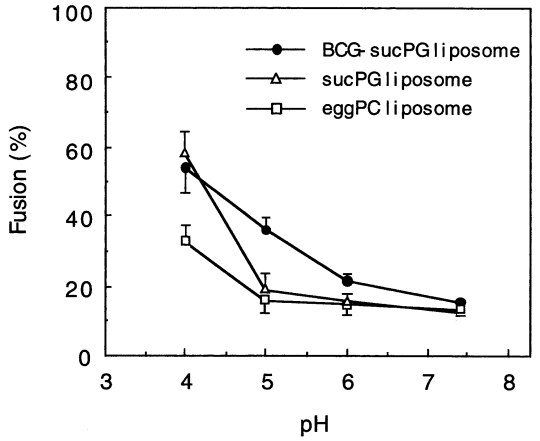

Fig. 5. Fusion of liposomes determined by measuring the fluorescence energy transfer between NBD-PE and Rh-PE. Effect of BCG modification on the fusogenic activity of sucPG liposomes. The incubation was performed in 10 mM acetate buffer for 30 min at 37 °C. Data are expressed as mean ± S.D. ($n = 3$).

4. Discussion

Drug delivery to specific cells by immunoliposomes represents a potentially attractive mode of therapy. In this study, we chose anti-BCG antibody as a targeting ligand and evaluated the in vitro ability of anti-BCG antibody-coupled sucPG immunoliposomes to target colon 26 cells, which are known to share a common antigen with BCG (Sasaki et al., 1985). Another purpose of the study was to test the fusion ability of BCG-sucPG liposomes after uptake by the tumor cells by receptor-mediated endocytosis, which is important if these liposomes are to be used as a gene transfer vector.

Specific binding ability of the purified anti-BCG antibody to colon 26 cells was confirmed by immunostaining. SucPG liposomes, coupled with the anti-BCG antibodies, also showed specific targetability to the tumor cells and were considered to be internalized into the cells by receptor-mediated endocytosis (Figs. 3 and 4). However, difference in cell association between BCG-sucPG liposome and sucPG liposome at 4 °C was not apparent compared with the difference at 37 °C. This is possibly due to another pathway to be taken up into cells, such as direct fusion between the plasma membrane and the liposomes. The fusion ability of sucPG liposomes was not reduced by anti-BCG antibody attached to the surface of the liposomes (Fig. 5). SucPG liposomes show fusogenic activity under acidic conditions, possibly due to an increase in hydrophobic interactions between succinate groups and the lipid membrane and the formation of hydrogen bonding between unionized carboxyl groups of the polymer and the phosphodiester groups of the liposome surface, followed by dehydration involving oxyethylene units of polyglycidol and free water which exists on the liposome surface and contributes to its stability (Kono et al., 1994). The fusion rate of BCG-sucPG liposomes was higher than that of sucPG liposomes at pH 5.0, probably due to reduction of the stability of the liposomal membrane as a result of the coupling of anti-BCG antibody. SucPG liposomes showed fusogenic activity even after having been taken up into the cells (Fig. 6), indicating the possibility of releasing their contents into the cytoplasm without degradation by lysosomal enzymes.

Common antigenic determinants with BCG, which was identified as a 64 kDa stress protein, are known to be widely distributed among various types of tumor cells, such as B16, DHM-28, YAC-1 and Meth-A in the mouse, line10 hepatoma in the guinea pig, human bladder carcinoma HT1197, HT1376 and human malignant lymphoma. However, the cells prepared from the normal liver, kidney, and intestine of animals did not react with the BCG antibody (Sasaki et al., 1985, 1986). In order to provide target specificity to liposomes, conjugation of monoclonal antibodies specific to cancer cells has been tried. However, preparation of monoclonal antibodies specific to an individual cancer cell line is difficult. From this point of view, anti-BCG antibody, which shows cross-reactivity with various types of tumor cells, could be a useful ligand for tumor-specific delivery of liposomal drugs. It is thus of interest to examine the distribution of common antigens with BCG on other kinds of human tumor cell lines.

Maruyama et al. have recently reported that transferrin (TF)-pendant-type PEG-liposomes, in which TF is covalently linked to the distal terminal of PEG chains on the external surface of PEG-liposomes, showed a prolonged residence time in the circulation and low RES uptake in colon 26 tumor-bearing mice, resulting in enhanced extravasation of the liposomes into the solid tumor tissue (Maruyama et al., 2001). However, it has also been shown that conjugation of intact IgG molecules to the distal terminal of PEG chains enhances RES uptake by an Fc receptor-mediated mechanism (Maruyama et al., 1995). This problem can be overcome by conjugation of antibody Fab′ fragments instead of whole IgG to the PEG terminal (Maruyama et al., 1997). Therefore, PEG-liposomes, conjugated at PEG terminals to anti-BCG antibody Fab′ fragments, are expected to have potential as carriers of chemotherapeutic agents in vivo.

Recently, Kono et al. reported that sucPG-complexes, prepared by mixing the lipoplex with sucPG-modified liposomes bearing transferrin as a target specific ligand, exhibited high transfection ability achieved through their binding to the cellular receptors (Kono et al., 2001). These negatively charged complexes minimize nonspecific interaction with cells and their cytotoxicity is less than the plain lipoplex, so that they are expected to be useful as nonviral vectors in vivo. BCG-sucPG liposomes are also considered to be useful in such formulation.

In conclusion, we have designed novel immunoliposomes by conjugation of anti-BCG monoclonal antibody to a new type of pH-sensitive liposomes (sucPG liposomes). The formulation retains specific antigen-binding ability to target cells and the liposomes were considered to undergo receptor-mediated endocytosis. Moreover, these liposomes are indicated to be fused with the endosomal and/or lysozomal membrane after uptake by tumor cells. Such liposomal formulations are expected to be useful for endocytotic internalization of anti-cancer drugs or bioactive materials including plasmid DNA. Feasibility studies of their use in liposome-mediated targeting would be worthwhile.

Acknowledgements

The authors wish to thank Kunitsugu Katayama and Yusuke Sekine (Teikyo University) for technical assistance. The advice of Dr Jin-ichi Sasaki (Department of Bacteriology, Hirosaki University School of Medicine) and Dr Shoji Ogihara (The Fourth Department of Internal Medicine, Tokyo Medical University) are gratefully acknowledged. A part of this work was supported by Grants-in-Aid for Scientific Research (No. 08672568 and 13470190 to Kazuo Maruyama, No. 12780649 to Tomoko Takizawa) from the Ministry of Education, Science and Culture, Japan and a Grant-in-Aid for Cancer Research (No. 12-1 to Kazuo Maruyama) from the Ministry of Health, Labor and Welfare, Japan.

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