Induction of Apoptosis in WEHI 231 Cells by Cationic Liposomes

Yukihiko Aramaki,[1,2] Shuhei Takano,[1] Hidetoshi Arima,[1] and Seishi Tsuchiya[1]

Received October 29, 1999; accepted January 18, 2000

Purpose. Liposomes are of considerable interest as drug carriers and immunoadjuvants. However, few investigators have studied the changes exerted by liposomes in the cells with which they interact. The purpose of this study was to investigate whether liposomes induce apoptosis in B cells.

Methods. The mouse immature B cell line WEHI 231 cells and mouse splenic B cells were treated with liposomes, and the induction of apoptosis was evaluated by monitoring changes in DNA content, DNA fragmentation and chromatin condensation by flow cytometry, agarose gel electrophoresis and by morphological investigation.

Results. Cationic liposomes induced apoptosis in WEHI 231 cells, but neutral and anionic liposomes did not. A contact time of 30 min between WEHI 231 cells and cationic liposomes was sufficient to induce apoptosis, and 80% of the cells showed hypodiploid DNA content. Apoptosis induced by cationic liposomes composed of stearylamine was inhibited by addition of the oxidant scavenger, N-acetyl-cysteine.

Conclusions. Cationic liposomes induced apoptosis in WEHI 231 cells, and the production of reactive oxygen species is important in the regulation of apoptosis induced by cationic liposomes. It is well known that cationic liposomes show cytotoxicity, and apoptosis may be one of the causes of this toxicity.

KEY WORDS: apoptosis; cationic liposome; B cell; WEHI 231; reactive oxygen species.

INTRODUCTION

Liposomes are of considerable interest as drug carriers because many substances can be encapsulated in their aqueous and lipid phases. Intravenously injected liposomes are rapidly taken up by the reticuroendotherial system including the liver and spleen (1). Since this characteristic is advantageous for directing antigens or immunomodulating agents to macrophages, many investigators have used liposomes as immunoadjuvants (2,3). However, few investigators have studied the changes exerted by liposomes in the cells with which they interact.

Recently, we reported that liposomes activate macrophage function, Fcγ-receptor-mediated phagocytosis of IgG-opsonized sheep red blood cells, both *in vitro* and *in vivo* (4,5). Liposomes did not activate macrophages directly, and sugar chain-modified α2-macroglobulin with a mannose residue at the terminal of the sugar chain was essential for the activation. Modified α2-macroglobulin was produced by hydrolysis of the sugar chain of α2-macrogloburin by B cell membrane glycosidases, which were activated by the interaction of liposomes and B cells (6). However, it was still unclear what changes liposomes exert in B cells.

Apoptosis plays a major role in development, homeostasis, and in many disease processes including cancer and acquired immunodeficiency syndrome (7). Unlike necrotic cell death, apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and eventual disintegration into membrane-enclosed apoptotic bodies (8,9). Clonal deletion is an important phenomenon in development of the normal immune system, and B cells that have susceptibility to self-antigen are deleted by apoptosis (10). WEHI 231 is an immature B cell line and serves as a model system for studying the complex interplay of signaling events mediating murine B cell survival, apoptosis and proliferation (10–12).

In this study, we investigated the effects of liposomes on the induction of apoptosis in the immature mouse B cell line WEHI 231 and mouse splenic B cells. Treatment of WEHI 231 cells with cationic liposomes clearly induced apoptosis as indicated by DNA fragmentation and morphological observations. Reactive oxygen species (ROS) were suggested to be involved in apoptosis induced by cationic liposomes.

MATERIALS AND METHODS

Materials

WEHI 231 cells were provided by American Type Culture Collection (Rockville, MD). Balb/c mice were purchased from SLC Co. Ltd. (Shizuoka, Japan). Phosphatidylcholine (PC) was a kind gift from Nippon Oil and Fat Co. Ltd. (Tokyo, Japan). Phosphatidylserine (PS), propidium iodine (PI), and stearylamine (SA) were obtained from Sigma Co. Ltd. (St. Louis, MO). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes (Eugene, OR). All other cationic amphiphiles were obtained from Sigma Co. Ltd.

Preparation of Liposomes

Liposomes were prepared by vortexing and were passed through a membrane filter (0.45 μm; Iwaki Co. Tokyo, Japan) before use. Lipid compositions of liposomes were PC:cholesterol = 1:1 (PC-liposomes, neutral liposomes), PC:PS:cholesterol = 1.5:0.5:2.0 (PS-liposomes, anionic liposomes), and PC:SA:cholesterol = 1.5:0.5:2.0 (SA-liposomes, cationic liposomes).

Cell Culture

WEHI 231 cells were incubated with three kinds of liposomes, PC-, PS- or SA-liposomes, at the indicated concentrations in RPMI 1640 medium containing 10% FCS.

Mouse splenic B cells were prepared as described by Suzuki *et al.* (13). Briefly, the spleens were teased through sterile stainless steel screens (mesh No. 150) into ice-cold RPMI 1640 medium containing 10% FCS. The single-cell suspensions were treated with Tris-(hydroxymethyl) aminomethan-buffered ammonium chloride to lyse contaminating erythrocytes and then washed three times with RPMI 1640 medium containing FCS. The splenic macrophages were removed by adherence to

[1] School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.

[2] To whom correspondence should be addressed. (e-mail: aramaki@ps.toyaku.ac.jp)

0724-8741/00/0500-0515$18.00/0

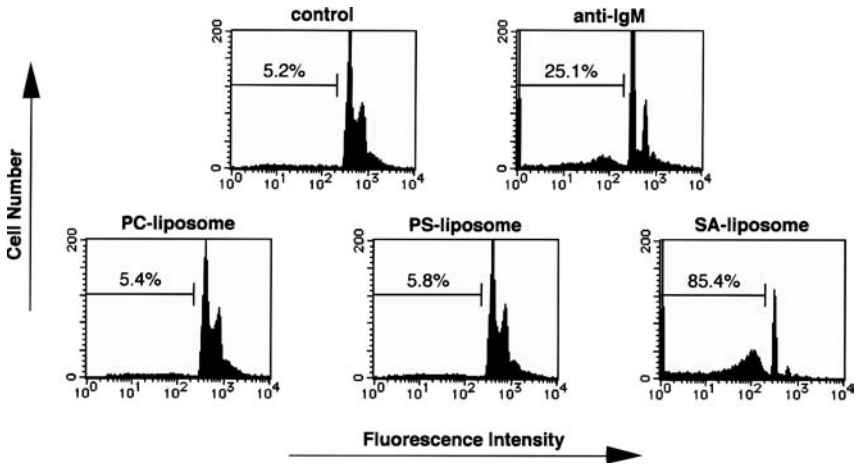

Fig. 1. Flow cytometric analysis of DNA content in WEHI 231 cells treated with liposomes. WEHI 231 cells treated with anti-IgM (10 μg/mL) or three kinds of liposomes (0.5 μmol lipid/mL) for 24 hr were stained with propidium iodide, and subjected to flow cytometry. Percentages in insets indicate cells showing hypodiploid DNA content.

plastic Petri dishes, and non-adherent cells were prepared. The B cell population was obtained by depletion of T cells by treatment with anti-Thy-1.2 serum and rabbit complement (Cederlane Lab. Ltd., Ontario, Canada) according to the method of Illera *et al.* (14). Splenic B cells were treated with liposomes at the indicated concentrations for 24 hr.

DNA Content

Liposomes- or anti-IgM antibody-treated WEHI 231 cells ($1 \times l0^6$ cells) were fixed with 70% ethanol at 4°C over night. The cells were then centrifuged at 500×g for 5 min and ethanol was thoroughly removed. The cell pellets were resuspended in phosphate-citrate buffer and incubated at room temperature for at least 30 min. After centrifugation at 1000×g for 5 min, the cell pellets were suspended in RNase A solution (100 μg/mL, Amresco Inc., Dallas, TX) and incubated at 37°C for 20 min to deplete RNA. RNase A-treated cells were suspended in 1.0 mL of propidium iodide solution (PI, 50 μg/mL) and the cells were subjected to flow cytometry (Becton Dickinson, FACSCalibur). The same procedure was employed for splenic B cells.

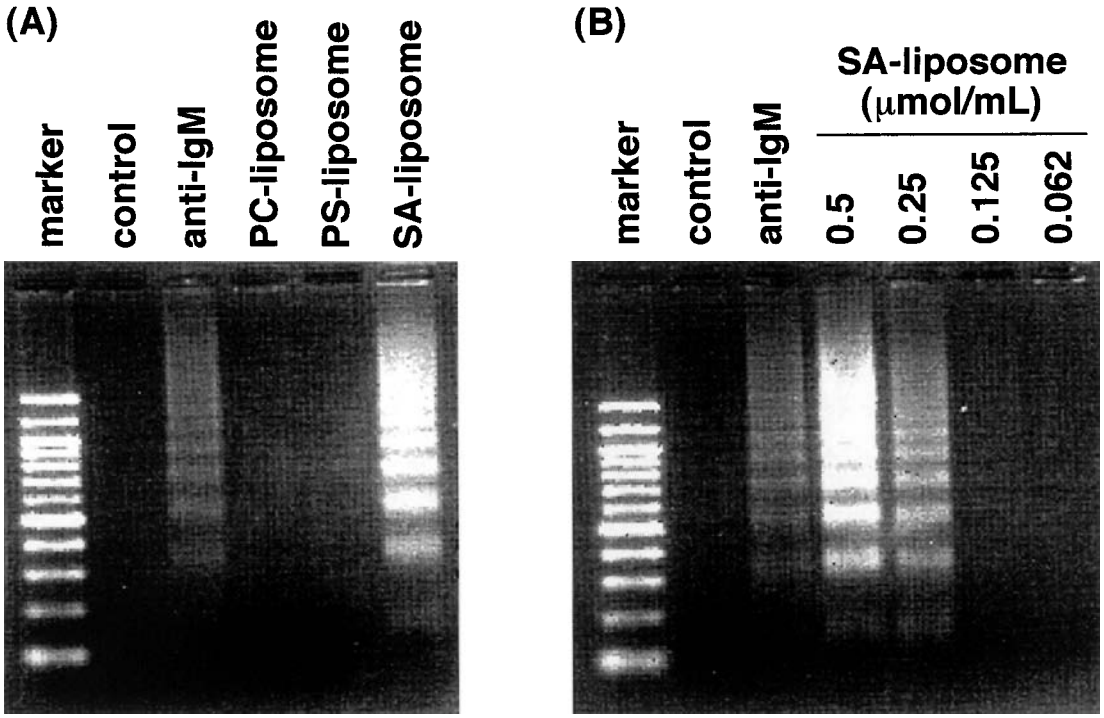

Fig. 2. Agarose gel electrophoresis of DNA extracted from WEHI 231 cells treated with liposomes. (A) WEHI 231 cells were treated with anti-IgM (10 μg/mL) or three kinds of liposomes (0.5 μmol lipid/mL) for 12 hr, and then DNA fragmentation was analyzed by gel electrophoresis. (B) Effects of liposomal dose of SA-liposomes (0.062 ~ 0.5 μmol lipid/mL). control: WEHI 231 cells were treated with buffer solution.

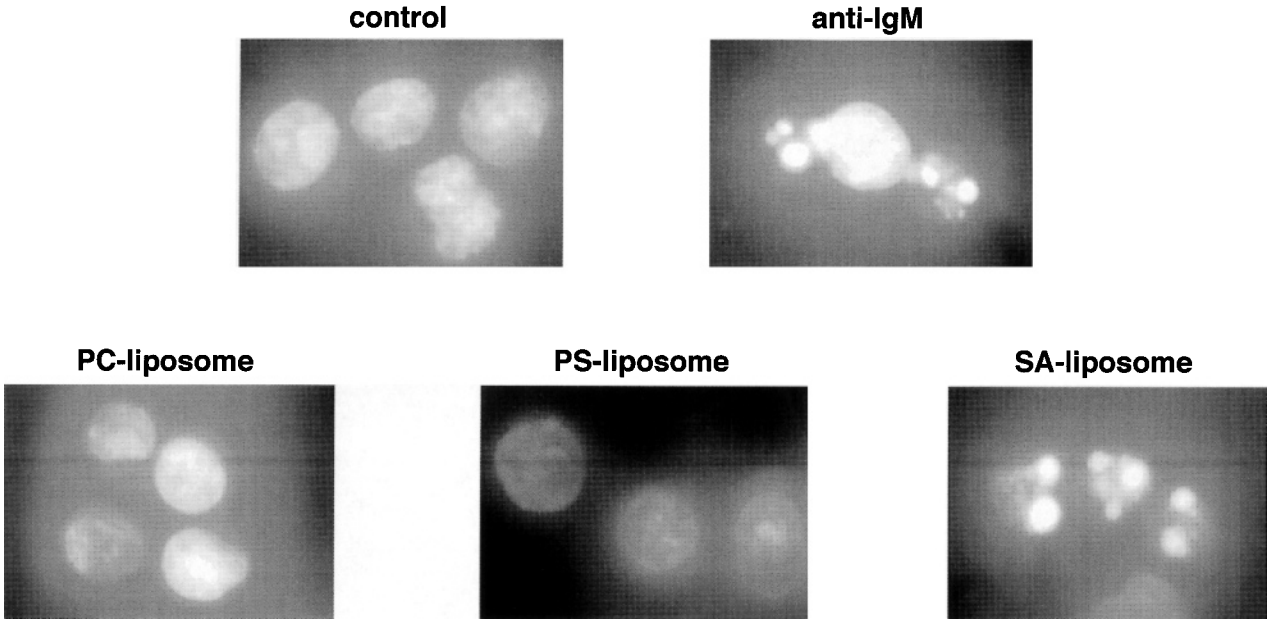

Fig. 3. Fluorescence micrographs of WEHI 231 cells treated with liposomes. WEHI 231 cells treated with anti-IgM (10 μg/mL) or three kinds of liposomes (0.5 μmol lipid/mL) for 48 hr were stained with Hoechst 33342, and chromatin condensation was examined by fluorescence microscopy (×400).

DNA Fragmentation

Liposome- or anti-IgM antibody-treated WEHI 231 cells (2.5×10^6 cells) were lysed by lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100) at 4°C for 45 min. The cell lysate was incubated with RNase A (400 μg/mL) at 37°C for 60 min to digest RNA, and then further incubated with Proteinase K (400 μg/mL, Merck,) for 60 min to digest protein. DNA was extracted with phenol:chloroform:isoamylalchol = 25:24:1 according to the method of Fukuda *et al.* (15). The extracted DNA was dissolved in TE buffer (10 mM Tris buffer, pH 7.4, containing 1 mM EDTA), and subjected to 2% agarose gel electrophoresis as described previously (16).

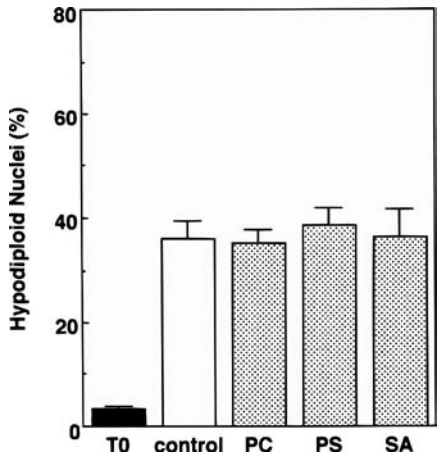

Fig. 4. Apoptosis induced by liposomes in splenic B cells. Freshly prepared splenic B cells (To) or those cultured with three kinds of liposomes (0.5 μmol lipid/mL) for 24 hr were stained with propidium iodide for flow cytometry. NS: splenic B cells were treated with buffer solution for 24 hr. Results represent the means ± SD of three experiments.

Morphological Assessment

Liposome- or anti-IgM antibody-treated WEHI 231 cells ($2.5 \times l0^6$ cells) were fixed with 2% glutaraldehyde solution at room temperature for 2 hr. After washing twice with PBS, the cells were stained with the DNA dye Hoechst 33342 (160 μM), and morphological changes and chromatin condensation were examined by fluorescence microscopy (Olympus VFS-R, Tokyo Japan).

Liposome Association and ROS Generation

WEHI 231 cells (5×10^5 cells/mL) were incubated with DiI-labeled liposomes (0.5 μmol/mL) at 37°C for 1 hr. The cells were washed with PBS and then the association of liposomes with the cells was measured by flow cytometry.

To investigate ROS generation, WEHI 231 cells were preloaded with 5 μM of 2′,5′-dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak) for 15 min. The cells were incubated with liposomes (0.5 μmol/mL) at 37°C for 30 min, and then the changes in fluorescence intensity were investigated by flow cytometry.

RESULTS AND DISCUSSION

Cationic Liposomes Induce Apoptosis in WEHI 231 Cells

Apoptosis is characterized by chromatin condensation, DNA fragmentation, and eventual disintegration into membrane-enclosed apoptotic bodies (2–3). The induction of apoptosis in B cells by liposomes was evaluated by monitoring DNA content, DNA fragmentation, and chromatin condensation. The changes in DNA

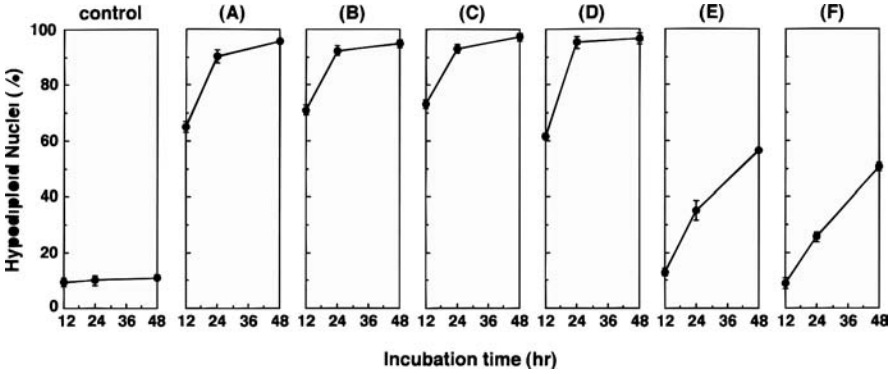

Fig. 5. Effects of cationic liposomes composed of various cationic lipids on apoptosis of WEHI 231 cells. WEHI 231 cells were treated with cationic liposomes (0.5 μmol lipid/mL) for 12, 24, and 48 hr, and the percentage of cells showing DNA degradation was examined by flow cytometry. Results represent the means ± SD of three experiments. (A), SA-liposomes (PC:SA:Chol = 1.5:0.5:2 (mol)); (B), DTAB-liposomes (PC:DTAB:Chol = 1.5:0.5:2); (C), TTAB-liposomes (PC:TTAB:Chol = 1.5:0.5:2); (D), CTAB-liposomes (PC:CTAB:Chol = 1.5:0.5:2); (E), TMAG-liposomes (PC:TMAG:Chol = 1.5:0.5:2); (F), DDAB-liposomes (PC:DDAB:Chol = 1.5:0.5:2).

content of WEHI 231 cells following treatment with liposomes (0.5 μmol lipid/mL) were examined by flow cytometry. As shown in Fig. 1, 85% of WEHI 231 cells treated with cationic liposomes (SA-liposomes) for 24 hr had hypodiploid DNA content. In contrast, less than 6% of the cells had hypodiploid DNA content after treatment with neutral (PC-) or anionic liposomes (PS-liposomes), and should the same value as controls. Since cross-linking of the sIgM on WEHI 231 cells by anti-IgM induces apoptosis (5), the effects of anti-IgM on DNA content were examined as a positive control; 25% of cells showed hypodiploid DNA content. To clarify the length of contact time between WEHI 231 cells and SA-liposomes required to induce apoptosis, the cells were treated with SA-lioposomes for 5 min ∼ 12 hr, then liposomes were removed by washing, and the cells were incubated for up to 12 hr. The contact time between WEHI 231 cells and SA-liposomes sufficient to induce apoptosis was shown to be 30 min by flow cytometry, and 80% of these cells had hypodiploid DNA (data not shown).

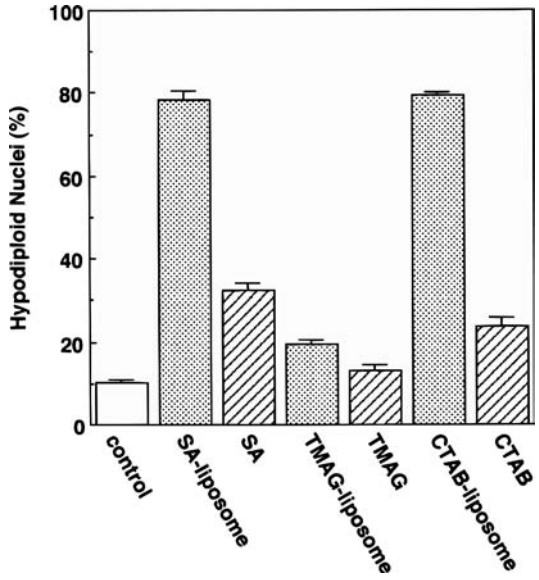

Fig. 6. Effects of cationic lipids on apoptosis of WEHI 231 cells. WEHI 231 cells were treated with cationic liposomes (0.5 μmol lipid/mL) or cationic lipids composed of liposomes (the same amount as cationic lipids composing liposomes) for 12 hr, and the percentage of cells showing DNA degradation was examined by flow cytometry. Results represent the means ± SD of three experiments.

In cells undergoing apoptosis, nuclear DNA is fragmented into the size equivalent of DNA into mono- or oligonucleosomes (17). DNA was extracted from WEHI 231 cells treated with liposomes, and DNA fragmentation was examined by 2% agarose gel electrophoresis. As shown in Fig. 2 (A), DNA ladder formation was observed in DNA extracted from SA-liposomes-treated and anti-IgM-treated cells, but no DNA fragmentation was observed in that from PC- or PS-liposomes-treated cells. DNA ladder formation was clearly observed when the cells were treated with SA-liposomes at concentrations of more than 0.25 μmol lipid/mL (Fig. 2(B)).

Chromatin condensation was observed in WEHI 231 cells treated with SA-liposomes, but not in those treated with PC- or PS-liposomes (Fig. 3). From these findings (Figs. 1–3), we concluded that SA-liposomes induce apoptosis in the immature B cell line, WEHI 231.

Hypercross-linking of sIgM by anti-IgM antibody induced apoptosis in mature as well as immature B cells (18). Previously, we reported that liposomes interact with splenic B cells through sIgM (6). Therefore, the effects of liposomes on the induction of apoptosis in splenic B cells were examined. As shown in Fig. 4, hypodiploid DNA content of freshly prepared B cells was 3%, and this increased to 37% after incubation for 24 hr. Hypodiploid DNA contents of B cells treated with liposomes were the same to those of controls, suggesting that no apoptosis was induced by treatment with liposomes irrespective of liposomal charge (Fig. 4). These findings indicated that sIgM does not contribute to SA-liposome-induced apoptosis in WEHI 231 cells. To clarify whether the difference in the apoptotic effect of SA-liposomes on both types of cells may be due to liposomal uptake, the association of SA-liposomes to WEHI 231 cells and splenic B cells was investigated by flow cytometry using DiI-labeled liposomes. The association of SA-liposomes to

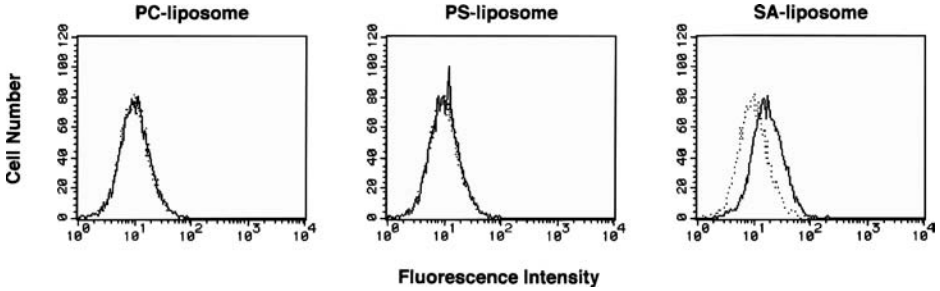

Fig. 7. Flow cytometric analysis of intracellular peroxide levels in WEHI 231 cells treated with liposomes. WEHI 231 cells pre-loaded with 5 μM DCFH/DA for 15 min were treated with three kinds of liposomes (0.5 μmol lipid/mL) for 20 min, and subjected to flow cytometry. Dotted line; untreated control, solid line; liposome-treated.

WEHI 231 cells was higher than that of splenic B cells, suggesting that apoptosis induced by SA-liposomes is closely related to the uptake (data not shown).

Lipid Composition of Liposomes

The effects of cationic liposomes composed of various cationic amphiphiles on the apoptosis of WEHI 231 cells were investigated. As shown in Fig. 5, all of the cationic liposomes induced apoptosis in the cells, suggesting that cationic charge is important for the induction of apoptosis of WEHI 231 cells. Furthermore, when WEHI 231 cells were treated with cationic lipids, SA, CTAB, or TMAG, the extents of apoptosis were lower than those observed with liposomes composed of each cationic lipid (Fig. 6), indicating that cationic lipids act as apoptosis inducers when incorporated into the liposomal membrane. However, the potency to induce apoptosis differd depending on the lipid composition; cationic liposomes composed of SA, DTAB, TTAB or CTAB strongly induced apoptosis, while those composed of TMAG or DDAB did so only weakly. The differences between cationic amphiphiles with varying potency to induce apoptosis may have been due to the number of acyl chains; cationic amphiphiles strongly inducing apoptosis had one acyl chain, while those that weakly induced apoptosis had two.

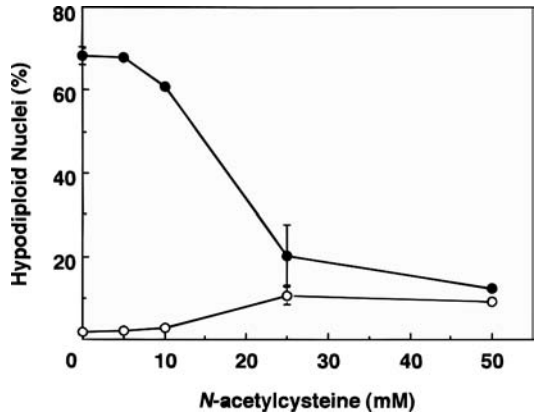

Fig. 8. Effects of N-acetyl-cysteine on apoptosis induced by liposomes. WEHI 231 cells (5×10^5/mL) were treated with SA-liposomes (0.5 μmol lipid/mL) in the presence of N-acetyl-cysteine at indicated concentrations. After 12-hr incubation, the percentage of cells showing DNA degradation was examined by flow cytometry. Results represent the means ± SD of three experiments. open circles: control, closed circles: SA-liposome-treated.

Contribution of Reactive Oxygen Species

Oxidative stress is one of the causes of apoptosis (8,19,20). The production of ROS after treatment of WEHI 231 cells with liposomes was estimated fluorometrically using DCFH-DA as a substrate according to the method of Kohno *et al.* (21). As shown in Fig. 7, the fluorescence intensity of cells was increased by treatment with SA-, but not PC- or PS-liposomes, indicating the generation of ROS following SA-liposome treatment. N-Acetyl-cysteine (NAC) has been shown to be an oxidant scavenger and to increase intracellular GSH levels (22). Thus, the effects of NAC on the induction of apoptosis by cationic liposomes were investigated. As shown in Fig. 8, apoptosis of WEHI 231 cells by cationic liposomes was reduced by NAC in a dose-dependent manner, and complete inhibition was observed at 25 mM NAC. This concentration of NAC did not interfere the binding of cationic liposomes to WEHI 231 cells. These findings suggested that ROS production and intracellular thiol levels are important in the regulation of apoptosis induced by cationic liposomes.

Since DNA is readily forms a complex with cationic liposomes via electrostatic interactions, cationic liposomes are candidates as non-viral vectors (23,24). Intravenously injected liposomes are rapidly taken up by macrophages in the liver and spleen (7), and this characteristic is advantageous for directing antigens to macrophages. It has been reported that cationic liposomes function as adjuvants enhancing humoral and cellular immunity (25). Recently, we found that cationic liposomes induce apoptosis in mouse splenic macrophages and mouse macrophage cell line RAW264.7 cells (26). Cationic liposomes were shown to induce apoptosis not only immature B cells but also macrophages, and thus care must be taken when cationic liposomes are used as non-viral vectors or immune adjuvants. Cationic liposomes show cytotoxic effects (27), and apoptosis may be one of the causes of this cytotoxicity.

ACKNOWLEDGMENTS

We are especially grateful to Dr. T. Hirano (Tokyo University of Pharmacy and Life Science) for helpful discussions and technical advice, and to Misses E. Yamazaki and Y. Hoshino for excellent technical assistance.

REFERENCES

1. M. J. Ostro. *Liposomes:* from Biophysics to Therapeutics. Marcel Dekker, New York, 1987.
2. G, Gregoriadis. Immunological adjuvant: a role for liposomes. *Immunol. Today* **11**:89–97 (1990).
3. C. R. Alving. Liposomes as carriers of antigens and adjuvants. *J. Immunol. Methods* **140**:1–13 (1991).
4. Y. Aramaki, M. Murai, and S. Tsuchiya. Activation of Fc receptor-mediated phagocytosis following the intraperitoneal administration of liposomes. *Pharm. Res.* **11**:518–521 (1994).
5. M. Murai, Y. Aramaki, and S. Tsuchiya. Identification of the serum factor required for liposome-primed activation of mouse peritoneal macrophages. *Immunology* **86**:64–70 (1995).
6. M. Murai, Y. Aramaki, and S. Tsuchiya. Modification of α2-macroglobulin into a macrophage-activating factor through the action of liposome-stimulated B-cell membranous glycosidases. *Immunology* **86**:58–63 (1995).
7. H. Steller. Mechanisms and genes of cellular suicide. *Science* **267**:1445–1448 (1995).
8. A. Gorman, A. McGowan, and T. G. Cotter. Role of peroxide and superoxide anion during tumour cell apoptosis. *FEBS Lett.* **404**:27–33 (1997).
9. M. Ruff. Cell suicide for beginners. *Nature* **396**:119–122 (1998).
10. A. Norvell, L. Mandik, and I. J. Monroe. Engagement of antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J. Immunol.* **154**:4404–4413 (1995).
11. T. L. Rothstein. Signals and susceptibility to programmed death in B cells. *Current Opt. Immunol.* **8**:362–371 (1996).
12. T. Tsubota, J. Wu, and T. Honjo. B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signaling through CD40. *Nature* **364**:645–648 (1993).
13. I. Suzuki, K. Kitamura, H. Kiyono, T. Kurita, R. R. Green, and J. R. McGhee. Isotype specific immunoregulation: evidence for a distinct subset of T contrasuppressor cells for IgA responses in murine Peyer's patches. *J. Exp. Med.* **164**:501–516 (1986).
14. V. A. Illera, C. E. Perandones, L. L. Stunz, D. R. Mower, and R. F. Ashman. Apoptosis in splenic lynmphocytes: regulation by protein kinase C and IL-4. *J. Immunol.* **151**:2965–2973 (1993).
15. T. Fukuda, D. Kitamura, I. Taniuchi, Y. Maekawa, L. Benhamou, P. Sarthou, and T. Watanabe. Registration of surface IgM-mediated apoptosis in an anti-IgM-resistant variant of WEHI 231 lymphoma cells by HS1, a protein-tyrosine kinase substrate. *Proc. Natl. Acad. Sci. USA* **92**:7302–7306 (1995).
16. J. Gong, F. Traganos, and Z. Darzynkiewicz. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* **218**:314–319 (1994).
17. M. J. Arends, R. G. Morris, and A. H. Wyllie. Apoptosis: the role of the endonuclease. *Am. J. Pathol.* **136**:593–608 (1990).
18. S. L. Parry, J. Hasbold, M. Holman, and G. B. Klaus. Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL-4 and anti-CD40. *J. Immunol.* **152**:2821–2829 (1994).
19. H. D. Um, J. M. Orenstein, and S. M. Wahl. Fas mediated apoptosis in human manocytes by a reactive oxygen intermediate dependent pathway. *J. Immunol* **156**:3469–3477 (1996).
20. M. S. Williams and P. A. Henkart. Role of reactive oxygen intermediates in TCR-induced death of T cell blasts and hybridomas. *J. Immunol.* **157**:2395–2402 (1996).
21. T. Kohno, Y. Yamada, T. Hara, H. Mori, M. Yamamura, M. Tomonaga, Y. Urata, S. Goto, and T. Kondo. Relation of Oxidative stress and glutathione synthesis to CD95 (Fas/APO-1)-mediated apoptosis of adult T cell leukemia cells. *J. Immunol.* **156**:4722–4728 (1996).
22. M. Proederer, F. Staal, P. A. Raju, S. W. Ela, L. A. Herzenberg, and L. A. Herzenberg. Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cystein. *Proc. Natl. Acad. Sci. USA* **87**:4884–4888 (1990).
23. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz. J. P. Northrop, G. M. Ringold, and M. Danielsen. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:8850–8854 (1987).
24. G. Zhang, V. Gurtu, T. H. Smith, P. Nelson, and S. R. Kain. Cationic lipid for rapid and efficient delivery of plasmid DNA into mammalian cells. *Biochem. Biophys. Res. Commun.* **236**:126–129 (1997).
25. T. Nakanishi, J. Kunisawa, A, Hayashi, Y. Tsutsumi, K. Kubo, S. Nakagawa, H. Fujiwara, O. Hamaoka, and T. Mayumi. Positively charged liposome functions as an efficient immuno-adjuvant in inducing immune response to soluble antigens. *Biochem. Biophys. Res. Commun.* **240**:793–797 (1997).
26. Y. Aramaki, S. Takano, and S. Tsuchiya. Induction of apoptosis in macrophages by cationic liposomes. *FEBS-Lett.* **460**:472–476 (1999).
27. X. Gao, D. Jaffurs, P. Robbins, and L. Huang. A sustained, cytoplasmic transgene expression system delivery by cationic liposomes. *Biochem. Biophys. Res. Commun.* **200**:1201–1206 (1994).