# Induction of Cell Death by Saponin and Antigen Delivery

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***Purpose.*** Saponin is the major component in the formation of immune stimulating complex (ISCOM), a potent adjuvant able to induce both humoral and cellular immune reactions. The immunogenicity induced by saponin, however, has been unclear. The objective of this study was to investigate the apoptotic and necrotic effects induced by saponin in EL4 mouse lymphoma cells, expected to be a possible mechanism of the cytotoxic T-lymphocyte (CTL) effect elicited by the ISCOM.

***Methods.*** EL4 cells were treated with saponin, and viability of the cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase release assays. Fluorescence microscopy was used to detect the morphological changes by staining saponin-treated cells with Hoechst 33342. Extent of apoptosis and necrosis was determined by Annexin V-FITC/propidium iodide staining, followed by flow cytometric analysis. Dendritic cells were cultured with either saponin–protein complexes or saponin-treated cells and analyzed by flow cytometry.

***Results.*** Treatment of EL4 cells with saponin resulted in concentration-dependent cytotoxicity and the appearance of the hypodiploid DNA peak. Cells treated with saponin showed highly condensed chromatin when stained with fluorescent DNA-binding dye Hoechst 33342. Analysis of EL4 cells by flow cytometry after Annexin V/propidium iodide staining demonstrated that saponin induced both apoptosis and necrosis. Pretreatment of EL4 cells with zVAD-fmk, a broad-range caspase inhibitor, did not prevent cell death induced by saponin, indicating the non-caspase-dependent cell death. Dendritic cells were shown to phagocytose both the antigen-saponin complexes and the saponin-induced dead cells.

***Conclusions.*** Results obtained in this study demonstrated that saponin induced both apoptosis and necrosis in EL4 cells. These events are critical for antigen processing and presentation.

**KEY WORDS:** antigen delivery; apoptosis; ISCOM; necrosis, saponin.

## INTRODUCTION

The vaccine adjuvants were designed for efficient delivery of antigens and to potentiate the immune response. The classical model of antigen processing and presentation states the segregated pathways for presentation of exogenous and endogenous antigens: Exogenous antigens are presented to induce antibodies and $CD4^+$ major histocompatibility complex (MHC) class II–restricted helper T cells; endogenous antigen is processed in association with $CD8^+$ MHC class I molecules for generation of cytotoxic T-lymphocyte (CTL) effect (1–3). This model was challenged by several experimental evidences illustrating the induction of MHC class I–restricted CTLs by soluble antigens, with concurrent MHC class II antigen presentation, in the presence of vaccine adjuvants (4–6). It was recognized that antigen presentation in association with MHC class I molecules may be facilitated by incorporation of exogenous antigens into the immunostimulating complexes (ISCOM) (6), followed by accessing to the endogenous pathway by presenting antigens to antigen-presenting cells (APCs). This speculation, however, has not been justified, and the mechanism of immunogenity exerted by the vaccine adjuvants, though partially elucidated, still remains unclear.

Quillaja saponin is a mixture of complex triterpenoids extracted from Quillaja bark *Saponaria milina* and is used in veterinary vaccines for formulating the immunostimulating complex (ISCOM) (7–12), which consists of saponin, phospholipids, surfactants, cholesterol, and the antigen. The vaccine adjuvants containing saponin were shown to stimulate pro-inflammatory cytokines and to elicit a potent acquired cell-mediated immunity (6,13,14). It was suggested that the efficient internalization of ISCOM by the APCs is an important factor for enhancing the immunogenicity of ISCOM-containing antigens (6). This speculation, however, was found to account only partly for the immunostimulating effects elicited by the vaccine adjuvants containing surface-active agents.

Apoptosis and necrosis are two modes of cell death culminating with cessation of biological activity. Apoptosis is a process of programmed cell death, often characterized by condensation of the nuclear chromatin, DNA fragmentation, cytoplasmic blebbing, cell shrinkage, and exposure of phosphatidylserine (PS) residues on the outside of the plasma membrane (15,16). Necrosis, on the other hand, is associated with inflammation and is a degenerative process, resulting in mitochondrial swelling, rupture of the plasma membrane, and release of cytoplasmic constituents, including proteolytic enzymes (17). Apoptotic cells usually can be recognized via membrane receptors and then engulfed by scavenger phagocytes. In normal cells, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located on the inner leaflet. Entry of apoptosis was shown to lead to a loss of phospholipid asymmetry, with exposure of PS on the outer leaflet (18), which can subsequently be recognized by APCs, including macrophages, and trigger specific immune response (18,19). These cell death events are later recognized to be involved in antigen acquisition processes and to provide signals for the activation of dendritic cells (DCs), the professional APCs critical for triggering primary T-cell response and subsequent stimulation of the CTL effect. About almost three decades ago, experimental evidence demonstrated the cross-priming phenomenon in which bone marrow–derived APCs were shown to acquire exogenous antigens and present them in the context of MHC class I molecules (20). Until recently, this phenomenon was partially elucidated by studies employing DCs, demonstrating that antigens were acquired by these potent APCs from apoptotic cells (21). Cross-presentation of

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**ABBREVIATIONS:** Ag, antigen; APCs, antigen-presenting cells; CFSE, 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T-lymphocyte; DCs, dendritic cells; FITC, fluorescein isothiocyanate; ISCOM, immune stimulating complex; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OVA, ovalbumin; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.



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antigens to $CD8^+$ T cells was found to take place after phagocytosis of apoptotic cells by immature DCs, which provide antigenic signals for MHC class I presentation. Optimal cross-presentation of an antigen, however, required an additional step of DC maturation induced by necrosis (22).

Here in this study, we present the experimental evidence showing that saponin, a commonly used surface-active agent used for formulating vaccine adjuvants, induced both apoptosis and necrosis in EL4 thymoma cells. These experimental findings suggest novel mechanisms for the design of vaccine adjuvants for induction of CTLs in the context of MHC class I pathway of antigen delivery.

## MATERIALS AND METHODS

### Materials

Saponin extracted from Quillaja bark was purchased from the Sigma Chemical Co. (St. Louis, MO, USA) without any further purification. Murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) and murine interleukin-4 (mIL-4) were purchased from PeproTech EC Ltd. (London, UK). The general caspase inhibitor zVAD-fmk was obtained from R&D Systems (Minneapolis, MN, USA). Annexin V-FITC kit was purchased from Bender MedSystems (Vienna, Austria). 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probe (Eugene, OR, USA). PKH26 Red Fluorescent Cell Linker Kit was obtained from Sigma. PE-conjugated anti-mouse CD11c antibody was obtained from BD Biosciences (Palo Alto, CA, USA).

### Cells

EL4 mouse thymoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% horse serum.

### MTT Assays

The colorimetric assay based on the formation of formazan from the tetrazolium salt, requiring mitochondria enzymes from the living cells, was used for the viability of the cells (23). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma. EL4 cells at $1 \times 10^5$/100 μl/well in the 96-well plates were treated with the test agents for 24 h. Control cells were treated with the same volume of deionized water (DI) as the stock of the test agent for examination of the solvent effect. The experiments were carried out in triplicate. Ten microliters of 5 mg/ml MTT was added to the cells, followed by incubation for 1 h. Cells were treated with 10% Triton X-100 for 10 min, and the plates were then read at 550 nm on a SPECTRmax PLUS microplate reader (Molecular Devices, Sunnyvale, CA, USA). The survival rate of cells was analyzed from a calibration curve established from known numbers of viable cells.

### Lactate Dehydrogenase Release Assays

The release of lactate dehydrogenase (LDH), using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega Corp., Madison, WI, USA), was used to determine the cytotoxicity of saponin (24). In brief, EL4 cells at $5 \times 10^3$/100 μl per well in triplicate wells were treated with various concentrations of saponin, incubated at 37°C for 24 h. The cells were then centrifuged at room temperature for 5 min. Fifty microliters of the supernatant were added to 50 μl of substrate mix provided by the manufacturer and incubated in the dark for 30 min, followed by addition of 50 μl stop solution. The absorbance at 490 nm was monitored by using the SPECTRmax PLUS microplate reader (Molecular Devices). LDH release (percentage of total lysate) from saponin-treated cells was calculated by comparison of the absorbance with that of the control cells treated with 0.8% (v/v) Triton X-100, following the instruction of the manufacturer.

### Analysis of Cell Cycle by Propidium Iodide Staining

To examine the DNA content after treatment with saponin, the apoptotic nuclei in the cells were stained with propidium iodide (PI) using the modified Nicoletti method (25). Briefly, EL4 cells were treated with various concentrations of saponin for 24 h, followed by fixation with 70% ethanol at –20°C for more than 30 min. The cells were then centrifuged, and the pellets were resuspended at room temperature with 200 μl of phosphate buffer containing 0.2 M $Na_2HPO_4$ and 0.1 M citric acid (pH 7.8) for 30 min. The cells were then centrifuged and stained with 1 ml PI staining solution (80 μg/ml PI, 1% Triton X-100, 100 μg/ml RNAse A) and analyzed by flow cytometry using a FACSCalibur (Becton-Dickson, Immunocytometry System, San Jose, CA, USA). The data were analyzed using the CellQuest software.

### Examination of Cell Morphology by Confocal Spectral Microscopy

The bisbenzimidazole dye Hoechst 33342 is known to bind specifically to DNA, and its fluorescence is greatly enhanced upon binding (26). To examine the apoptotic cell morphology, EL4 thymoma cells were treated with 300 μg/ml saponin for 24 h and stained with Hoechst 33342 (0.1 μg/ml), followed by incubation at 37°C for 15 min. Cells were then centrifuged, washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min, and examined under a Leica TCS SP2 confocal spectral microscope, (Heidelberg, Germany) equipped with a UV laser 351 nm/364 nm.

### Annexin V and Propidium Iodide Staining

Exposure of phosphatidylserine on the outer leaflet of the plasma membrane is a surface change common to many apoptotic cells. Annexin V (Ann V) preferentially binds to negatively charged phopholipids such as phosphatidylserine (PS), thus apoptotic and necrotic cells can be distinguished by flow cytometry using fluorescein-conjugated Annexin V in combination with PI (27). Briefly, $1 \times 10^5$ EL4 cells were treated with various concentrations of saponin for various time periods and centrifuged. Cells were then washed with cold PBS and stained at room temperature with Annexin V-FITC and PI for 10 min using the Annexin V-FITC kit (Bender MedSystems, Vienna, Austria), followed by flow cytometric analysis. The extent of apoptosis and necrosis was determined as the percentage of Ann $V^+/PI^-$ and Ann $V^+/PI^+$ cells, respectively.

### Effect of zVAD-fmk

To study the effect of zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone)—a general caspase inhibi-

tor—on saponin-induced apoptosis, EL4 cells at $1 \times 10^6$/ml were either untreated or pretreated with 50 μM zVAD-fmk (R&D Systems Inc., Minneapolis, MN, USA) at 37°C for 4 h, followed by treatment with 200 μg/ml saponin at 37°C for 24 h. Cells were then stained with Annexin V and PI as described above and analyzed by flow cytometry.

### Dendritic Cells Isolation

Male C57BL/6J mice 6–8 weeks old were obtained from the Animal Breeding Center at the Medical College of National Taiwan University. Bone marrow DCs were generated from femurs and tibias of the mice according to established procedures (28). DCs were incubated in 24-well plates for 6 days by incubation in RPMI-1640 medium containing 50 μM β-mercaptomethanol, 20 μM HEPES, 10% fetal bovine serum, and antibiotics, supplemented with mGM-CSF and mIL-4. After every 2 days of incubation, medium plus nonadherent cells were removed, and fresh medium containing mGM-CSF and mIL-4 was added. On day 6, nonadherent cells were considered to be immature bone marrow–derived DCs. DC phenotype was confirmed by staining with antibodies and subsequent analysis by flow cytometry.

### Phagocytosis of Antigen–Saponin Complexes or Saponin-Induced Apoptotic Cells by the Dendritic Cells

To prepare fluorescently labeled antigen (Ag), ovalbumin (OVA) was dissolved in 0.1 M sodium carbonate (pH 9.0) and labeled with fluorescein isothiocyanate (FITC) at 4°C for 8 h. The reaction was stopped by the addition of 50 mM $NH_4Cl$ and then incubated at 4°C for 2 h, followed by gel filtration through a Sephadex G-50 column and elution with PBS. Protein fractions with absorbance ratios at 495nm/280 nm between 0.3 and 1.0 were collected, followed by protein assays using BioRad protein assay kit (Hercules, CA, USA). To form the saponin–protein complexes, 10 μl saponin (30 μg/ml) was mixed with OVA-FITC containing 1 μg ovalbumin.

For induction of apoptosis, EL4 cells at $1 \times 10^6$/ml were washed with PBS and labeled with 1 μM CFSE, an intracellular fluorescent dye, at 37°C for 10 min, followed by three washes with DMEM. These cells were treated with 300 μg/ml saponin for 24 h for induction of apoptotic cell death.

DCs at day 6 were centrifuged and dyed red at room temperature with PKH26-GL for 5 min, followed by washes three times with the culture medium. DCs at $5 \times 10^5$/ml/well were then treated with either a mixture containing 10 μl saponin (30 μg/ml) and 1 μg FITC-labeled OVA for 2 h or cocultured with $1 \times 10^6$ CFSE-labeled EL4 cells, pretreated with 300 μg/ml saponin for 24 h, followed by flow cytometric analysis.

## RESULTS

Quillaja saponin is a major component in the formation of ISCOM, a potent adjuvant in inducing both humoral and cellular immune responses when physicochemically complexed with the antigens. Here in this study, we attempted to examine cell death induced by saponin, as related to the antigen delivery in the DCs, the most potent APCs. The cytotoxic effect of saponin was determined by using the MTT and LDH release assays, while apoptosis and necrosis were evaluated by Annexin V/PI staining and further confirmed by confocal microscopy.

### Cell Viability Assays

To investigate the cytotoxic effect of saponin, a commonly used component in vaccine adjuvants, EL4 cells were incubated with increasing concentrations of saponin for 24 h, followed by MTT and LDH release assays. Figure 1 shows that cell viability was dramatically reduced with increasing concentrations of saponin. Results obtained from the MTT assays, based on cleavage of tetrazolium ring in active mitochondria by various dehydrogenase enzymes, indicated that treatment of EL4 cells with 200 μg/ml saponin resulted in approximately 40% cell survival (Fig. 1A). Similar results were obtained in saponin-treated EL4 cells by the LDH assay (Fig. 1B), showing concentration-dependent decrease of cell viability after treatment with saponin.

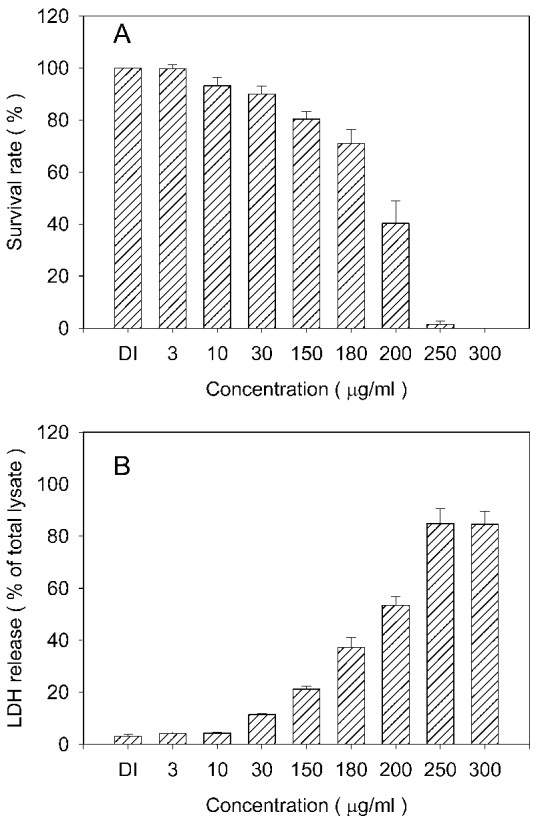
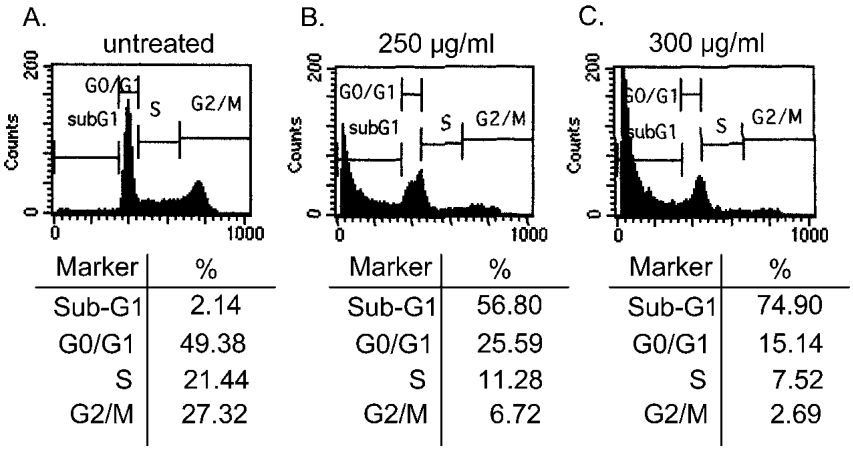



**Fig. 1.** Cell viability assays by (A) MTT method. EL4 cells at $1 \times 10^5$/well in 96-well plates were treated with varying concentrations of saponin for 24 h. MTT reagent was added for 1 h, and cells were lysed with 1% Triton X-100, followed by measurement of 550 nm absorbance. The survival rate was calculated based on the calibration curves, established from known numbers of cells. (B) LDH release assay. EL4 cells at $5 \times 10^3$/well in 96-well plates were treated with varying concentrations of saponin for 24 h. Cells were then centrifuged. Fifty microliters of the supernatant was mixed with 50 μl substrate mix and incubated for 30 min, followed by the addition of 50 μl stop solution, and the absorbance was read at 490 nm. Percentage of total lysate was calculated by comparison with the control cells treated with 0.8% Triton-X-100, as described in "Materials and Methods." Control cells were treated with the same volume of deionized water (DI) as the stock of the test agent. Data are presented as mean ± SE for three separate experiments in triplicate.




| Marker | % |
|---|---|
| Sub-G1 | 2.14 |
| G0/G1 | 49.38 |
| S | 21.44 |
| G2/M | 27.32 |

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| Sub-G1 | 56.80 |
| G0/G1 | 25.59 |
| S | 11.28 |
| G2/M | 6.72 |

| Marker | % |
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| Sub-G1 | 74.90 |
| G0/G1 | 15.14 |
| S | 7.52 |
| G2/M | 2.69 |

**Fig. 2.** Analysis of DNA content of EL4 cells by PI staining after treatment with saponin. EL4 cells at $1 \times 10^6$/well were either (A) untreated, or treated with (B) 250 μg/ml, (C) 300 μg/ml saponin for 24 hours, fixed with 70% ethanol, treated with 0.2 ml DNA phosphate buffer, and stained with 80 μg/ml PI for 30 min at room temperature, followed by flow cytometric analysis. The experiment was repeated three times with similar results.

### Determination of Cellular DNA Content in the Saponin-Treated Cells

DNA cleavage is a characteristic feature of apoptosis and provides a basis for identification of apoptotic cells by using flow cytometry. In the current study, fluorochrome staining of DNA strand breaks by PI was used to measure the DNA content in the saponin-treated cells. Flow cytometric analysis of ethanol fixed, PI-stained EL4 cells after saponin treatment showed a concentration-dependent increase of the hypodiploid DNA peak and a concomitant decrease of diploid DNA content (Fig. 2), demonstrating clearly that DNA degradation occurred after treatment of the cells with saponin.

### Morphological Examination by Fluorescence Microscope

Morphological features of apoptotic cell death, characterized by chromatin condensation and DNA fragmentation, can be detected using fluorescence microscopy by staining the cells with DNA-binding dye Hoechst 33342. Figure 3 shows the appearance of EL4 cells, either untreated or treated with 300 μg/ml saponin for 24 h, by confocal fluorescence microscopy. In contrast to the untreated control cells, saponin-treated EL4 cells showed an intense staining of hypercondensed chromatin, a characteristic feature of apoptotic nuclei. Some cells exhibited fragmented DNA (as indicated by the arrowhead). These results confirmed the apoptotic effect induced by the treatment of saponin.

### Analysis of Apoptosis and Necrosis by Annexin V and Propidium Iodide Staining

To examine the apoptotic and necrotic effects, cells were treated with saponin, stained with Annexin V and PI, followed by flow cytometric analysis. Figure 4 demonstrates that treatment of EL4 cells with saponin, up to 300 μg/ml, resulted in a concentration-dependent apoptosis and necrosis, with the maximum cell death occurring at 250 μg/ml saponin.

### Effect of zVAD-fmk

To examine the effect of zVAD-fmk (a general caspase inhibitor) on saponin-induced apoptosis, EL4 cells were ei-

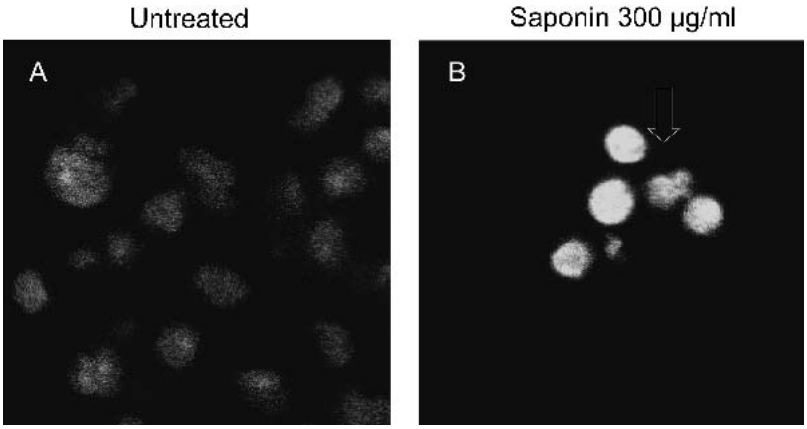


**Fig. 3.** Photomicrographs of EL4 cells after saponin treatment. EL4 cells were either (A) untreated or (B) treated with 300 μg/ml saponin for 24 h, then stained with Hoechst 33342 (0.1 μg/ml) and incubated at 37°C for 15 min. Cells were then washed with PBS, fixed with 4% paraformaldehyde, and examined by a Leica confocal microscope.

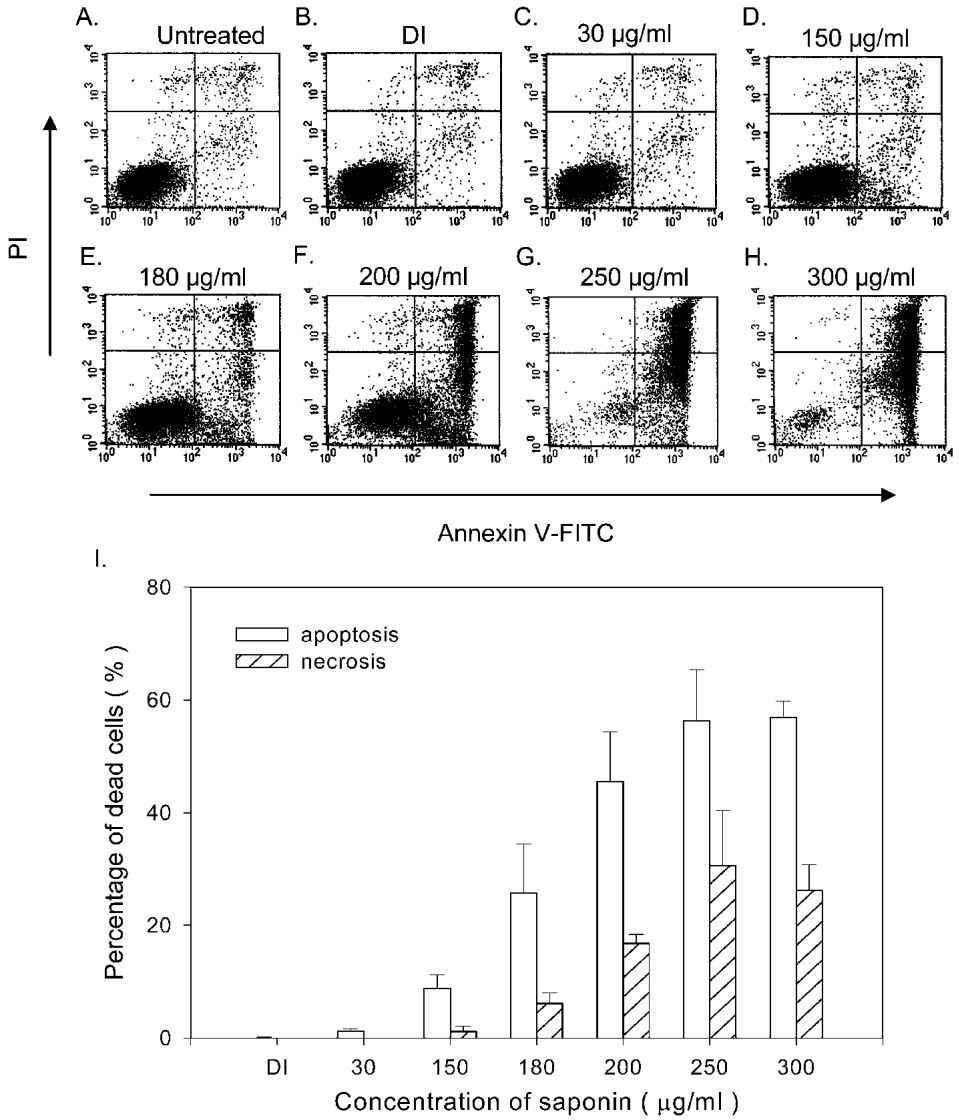


**Fig. 4.** Analysis of apoptosis and necrosis in saponin-treated EL4 cells by flow cytometry after Annexin V and PI staining. EL4 cells were cultured in a culture medium containing various concentrations of saponin for 24 h. Cells were washed and stained with Annexin V and PI, followed by flow cytometric analysis. Results presented in (A)–(H) are representative of three separate experiments. Deionized water (DI) was used instead of the stock solution of test agent. Data shown in (I) are the mean ± SE for three independent experiments.

ther untreated or pretreated with 50 μM zVAD-fmk for 4 h prior to treatment with saponin. Analysis by flow cytometry after Annexin V and PI staining demonstrated that saponin-induced apoptosis cannot be blocked by the pretreatment of this inhibitor (Fig. 5), suggesting a caspase-independent pathway of apoptosis induced by saponin.

### Phagocytosis of Antigen–Saponin Complexes or Apoptotic Cells by Dendritic Cells

To examine whether DCs phagocytose Ag–saponin complexes or saponin-induced dead cells, OVA was labeled with FITC and formed complexes with saponin, while EL4 cells were induced to undergo cell death by treatment of saponin. Dendritic cells were cocultured with either OVA–FITC/saponin complexes or fluorescently labeled EL4 cells, followed by flow cytometric analysis. Figure 6 shows that the

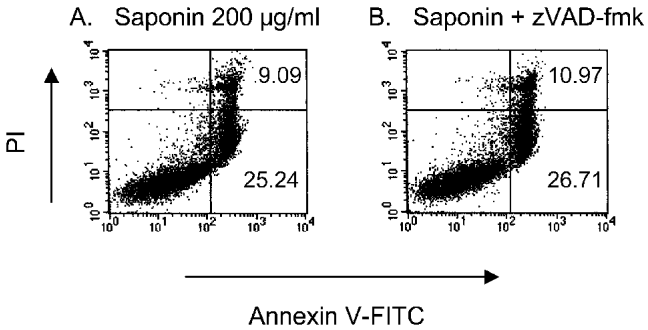


**Fig. 5.** Effect of zVAD-fmk on saponin-induced apoptosis in EL4 cells. EL4 cells at $1 \times 10^6$/well were preincubated with 50 μM zVAD-fmk prior to treatment with 200 μg/ml saponin for 24 h. Cells were then washed and stained with Annexin V and PI, followed by flow cytometric analysis.

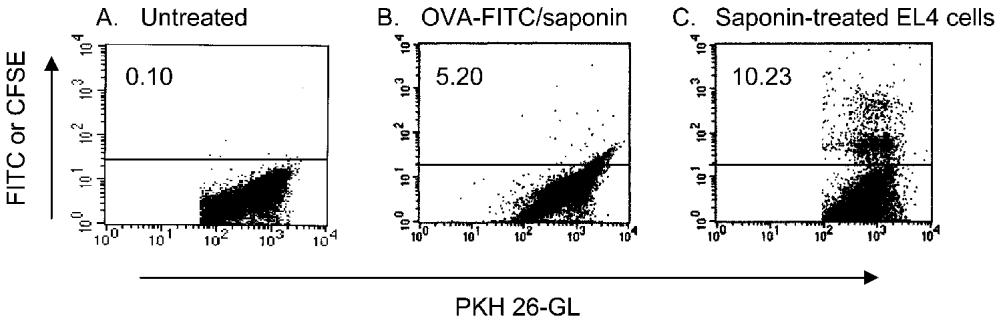


**Fig. 6.** Internalization of Ag–saponin complexes or saponin-induced apoptotic EL4 cells by DCs. DCs cultured at day 6 were stained with PKH26-GL dye, plated in a 24-well plate, and either (A) untreated or (B) treated with a mixture containing 10 μl saponin (30 μg/ml) and 1 μg FITC-labeled OVA for 2 h; or (C) co-cultured for 4 h with CFSE-stained EL4 cells, pretreated for 24 h with 300 μg/ml saponin, followed by flow cytometric analysis.

extent of Ag–saponin complexes, containing 30 μg/ml saponin and 1 μg/ml ovalbumin, phagocytosed by the DCs was approximately 5.2%. On the other hand, approximately 10.2% dead cells, induced by treatment with 300 μg/ml saponin, were phagocytosed by the DCs within 4 h. These results indicated that DCs phagocytose both Ag–saponin complexes and dead cells, suggesting the effects of saponin on antigen delivery.

## DISCUSSION

The development of vaccines requires not only the presence of appropriate antigens, but also effective delivery systems to optimize both the humoral and cell-mediated immune responses. Although vaccine adjuvants have been used for over 70 years, many adjuvants produce undesirable side effects that have precluded them from clinical use in humans. In addition, most vaccine adjuvants were developed empirically. Poor understanding of the mechanisms of adjuvant actions presents obstacles to further development of appropriate vaccine delivery systems.

The pathways through which the antigens are processed determine the immune response against cytosolic antigens. Conventionally, exogenous antigens are presented at the cell surface in association with class II molecules whereas endogenous antigens enter a different pathway in the context of MHC class I molecules for eliciting CTLs. Although saponin-based ISCOMs have been used for several decades, and the triterpenoid compounds present in the ISCOMs were shown to be potent inducers of cytokine production including IL-1, IL-6, and IL-12 (13,29,30), the adjuvant action as related to the class I–restricted antigen presentation has never been clear. It was suggested that APCs uptake the antigen-containing ISCOMs and the peptides derived from the ingested antigens may gain access to the cytosol, thereby being processed and loading onto both MHC class I and class II molecules (6). Similar findings have also been reported, showing class I–restricted CTL induced by other vaccine adjuvants containing soluble antigens (4,5). This evidence suggested the possibility of processing exogenous soluble antigens in a cytosolic class I pathway by the use of vaccine adjuvants. Cross-presentation is a mechanism for effectively priming class I–restricted response to peripheral antigens, whereby APCs take up the antigens and present the resulting fragments in association with MHC class I molecules (31). DCs, the most potent APCs, have been shown to phagocytose apoptotic cells and present the acquired Ag to the CTLs (21). Unfortunately, this pathway of antigen delivery has been ignored by most studies on vaccine adjuvants.

In the current study, we presented experimental evidence showing that saponin induced both apoptosis and necrosis in EL4 thymoma cells. Apoptotic cell death is usually preceded by DNA fragmentation, membrane blebbing, and condensation of chromatin. The appearance of sub-diploid DNA peak in the DNA histogram indicated the presence of the DNA fragments. Results shown in Fig. 2 on PI staining demonstrated the presence of reduced DNA content of the apoptotic nuclei in those cells treated with saponin, showing unequivocally the presence of apoptosis. Cells undergoing apoptosis also activate a cascade of molecular events, leading to condensation of the cytoplasm and change of cell morphology. DNA in the condensed chromatin may show hyperchromasia and stain strongly with fluorescence dyes (32). In contrast to the untreated controls, cells treated with saponin exhibited strongly stained nuclei after Hoechst 33342 staining, confirming the apoptotic effect of saponin.

Phosphatidylserine (PS) exposure has been implicated as a mediator of macrophage recognition of apoptotic cells. Annexin V identifies apoptotic cells via binding to PS residues translocated to the cell surfaces during the early apoptosis process (27). Results shown in Fig. 4 demonstrate that saponin induced both early apoptosis and necrosis in EL4 cells in a concentration-dependent manner. Cell death induced by saponin, however, cannot be inhibited by the general caspase inhibitor zVAD-fmk (Fig. 5), indicating a caspase-independent pathway induced by saponin. DCs were shown to phagocytose both the Ag–saponin complexes and the saponin-induced dead cells (Fig. 6).

In summary, we have demonstrated in the current study that saponin induced both apoptosis and necrosis in EL4 thymoma cells. Processing of engulfed apoptotic bodies was shown to yield T-cell epitopes (33), and DCs are known to acquire antigens from apoptotic cells for presentation on MHC class I molecules, leading to CTL (21). Bystander apoptosis and necrosis, on the other hand, triggered DC maturation, resulting in cross-presentation of antigens from apoptotic cells (22,34). Induction of apoptosis and necrosis therefore are crucial in cross-presentation of antigens to MHC class I–restricted CTLs. Based on these rationales and the

results obtained in this study, we propose that cell death, both apoptosis and necrosis, induced by saponin provides additional mechanisms that allow access of the antigen associated with ISCOMs to the cytosol of APCs, particularly to the DCs. It is thus suggested that the mode of adjuvant effect of the ISCOMs is not only contributed by the enhancement of antigen uptake by the APCs due to the incorporation of particulate or soluble protein antigens into the ISCOMs, as suggested by Villacres et al. (6), but also by cell death induced by saponin, which may play some critical roles in the stimulation of DC function and cross-presentation of antigens to the $CD8^+$ T cells, leading to induction of class I–restricted CTLs.

## ACKNOWLEDGMENTS

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