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Depletion of Intracellular NAD$^+$ and ATP Levels during Ricin-Induced Apoptosis through the Specific Ribosomal Inactivation Results in the Cytolysis of U937 Cells

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Our previous studies demonstrated that ricin induces the apoptotic death of U937 cells as evidenced by DNA fragmentation, nuclear morphological changes, and increases in caspase-like activities. In this study, we have found that intracellular NAD$^+$ and ATP levels decrease in ricin-treated U937 cells and that this decrease is followed by the ricin-mediated protein synthesis inhibition. The PARP inhibitor, 3-aminobenzamide (3-ABA), prevents the depletion in NAD$^+$ and ATP levels and concomitantly protects U937 cells from the lysis that follows ricin treatment. Hence, the protective action of 3-ABA is due to the inhibition of PARP and does not result from its other pharmacological side effects. Moreover, the enzymatic activity of PARP gradually increases and reaches a maximum level after ricin exposure for 3 h, whereas no significant change in activity was observed in untreated cells. However, 3-ABA has no effect on ricin-mediated DNA fragmentation. In addition, immunoblot analysis revealed that significant PARP cleavage occurred more than 12 h after ricin addition, while DNA fragmentation reached a maximum level within 6 h of incubation. Thus, in the case of ricin-induced apoptosis, it appears that PARP cleavage is not an early apoptotic event associated with the onset of apoptosis. Our results suggest that multiple apoptotic signaling pathways may be triggered by ricin-treatment. Probably, the pathway leading to cell lysis *via* PARP activation and NAD$^+$ depletion is independent of the pathway leading to DNA fragmentation in which caspases may be profoundly involved. Other protein synthesis inhibitors, including diphtheria toxin and cycloheximide, were less effective in terms of inducing DNA fragmentation and cytolysis, even at concentrations that cause significant inhibition of protein synthesis. Thus, a specific ricin action mechanism through which ribosomes are inactivated may be responsible for the apoptotic events induced by ricin.

Key words: 3-aminobenzamide, apoptosis, cytolysis, poly(ADP-ribose) polymerase, ricin.

Ricin, a toxic lectin (66 kDa) present in castor bean consists of two structurally and functionally different subunits (A and B). These subunits are linked together through a single disulfide bond. The A subunit (32 kDa) inactivates ribosomes by enzymatically removing a specific adenine residue from the 28S RNA of the 60S ribosomal subunit. The B subunit binds to cell surface carbohydrates containing galactose or *N*-acetylgalactosamine residues (*1–3*). Recent studies have demonstrated that ricin induces cell lysis and DNA fragmentation in a process reminiscent of programmed cell death or apoptosis (*4–6*). Although the mechanism of cell lysis or apoptosis by ricin is still unclear, it has been reported that ricin-induced apoptotic changes are prevented by cycloheximide and 3-methyladenine, a specific inhibitor of autophagy (*4*). Thus autophagy may be important in ricin-induced apoptosis.

On the other hand, recent studies have demonstrated that cysteinyl aspartate-specific proteases, which are designated as caspases, play crucial roles in the apoptotic signal transduction pathway (*7, 8*). Fourteen caspases have been reported so far (*9*), and multiple caspases are activated in cells undergoing apoptosis. Especially, caspase-3 and caspase-6 have been shown to be the major active caspases responding to various apoptotic stimuli (*10, 11*). Consistent with these findings, increased caspase-3– and -6–like activities have been found in ricin-treated U937 cells (*12*). Furthermore, we have found that carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane (Z-Asp-CH_2-DCB), a broad inhibitor of caspase family proteases, and dichloroisocoumarin (DCI), a highly specific serine protease inhibitor, completely block ricin-induced apoptosis in human leukemia U937 cells (*12*). These results suggest that caspases and serine proteases are involved in ricin-induced apoptosis.

Caspases activated during apoptosis, especially caspase-3, cleave various critical cellular substrates, such as actin and lamin, resulting in dramatic morphological changes in cells undergoing apoptosis (*13–15*). Several lines of evidence indicate that poly(ADP-ribose) polymerase (PARP), a

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Abbreviations: PARP, poly(ADP-ribose)polymerase; 3-ABA, 3-aminobenzamide; LDH, lactate dehydrogenase; Z-Asp-CH_2-DCB, carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl) oxy]methane; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; PBS, phosphate buffered saline; CHA, cycloheximide.

DNA-binding protein that is activated by DNA strand breaks to participate in DNA repair, is also a substrate for caspase-3 or caspase-7 (*16*). Intranuclear PARP is selectively activated by DNA strand breaks to catalyze the addition of long branched chains of poly(ADP-ribose) from its substrate nicotinamide adenine dinucleotide (NAD^+) to a variety of nuclear proteins, most notably PARP itself (*17*). The depletion of NAD^+ that accompanies PARP activation results in lower intracellular ATP levels, since the cells consume ATP in an effort to resynthesize NAD^+. This energy crisis may culminate in cell death. Recent studies have demonstrated that 3-aminobenzamide (3-ABA), a PARP inhibitor, has an inhibitory effect on apoptosis (*18, 19*), suggesting the involvement of PARP in the progression of apoptosis. In this study, we investigated the role of PARP in ricin-induced apoptosis and eventual cell death. Our results show that the depletion of intracellular NAD^+ and ATP occurs with similar time course as DNA fragmentation in ricin-treated U937 cells, and cytolysis is observed after these events are completed. Since 3-ABA strongly prevents the cytolysis of ricin-treated U937 cells, PARP activation and subsequent NAD^+ depletion may be a direct cause of ricin-induced cytolysis, whereas 3-ABA has no effect on ricin-induced DNA fragmentation. Based on comparative studies with other protein synthesis inhibitors, we suggest that the specific way in which ribosomes are inactivated by ricin is responsible for the potent apoptotic induction and subsequent cell lysis.

MATERIALS AND METHODS

Materials and Cell Culture—Ricin was isolated from small castor beans as described by Mise *et al.* (*20*). The caspase family protease inhibitor, Z-Asp-CH_2-DCB, was obtained from Peptide Institute, Osaka. [^{3}H]Leucine (60 Ci/mmol) was obtained from NEN Research Products (Boston, MA). [^{32}P]NAD^+ (25 nCi/nmol) was obtained from ICN Pharmaceuticals. 3-Aminobenzamide (3-ABA) was obtained from Sigma Chemical (St. Louis, MO). A human myeloid leukemia U937 cell line was obtained from Riken Cell Bank, Tsukuba. Cells were cultured in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air.

Measurement of Protein Synthesis Inhibition—Cells in 48-well plates (5×10^5 cells/well), each well containing 100 μl of RPMI-1640 medium containing 35 μM bovine serum albumin (BSA), were incubated at 37°C with the indicated concentrations of ricin or other protein synthesis inhibitors. After 3 h incubation at 37°C, the medium was replaced with leucine-free medium containing 1 μCi/ml [^{3}H]leucine by centrifugation, and the cells were incubated for 45 min at 37°C. The incorporation of [^{3}H]leucine into perchloric acid/phosphotungstic acid-insoluble materials was determined as described previously (*12*). In the case of cycloheximide (CHA), the rate of protein synthesis was measured in the presence of the indicated concentrations of CHA. The results are expressed as percentages of the incorporation in control cells incubated without toxin but otherwise treated in the same way. For time course analysis, cells were incubated with ricin (10 ng/ml) for the indicated periods of time at 37°C, and then the rate of protein synthesis was measured by incubating the cells with leucine free medium containing 10 μCi/ml [^{3}H]leucine for 10 min at 37°C as described above.

Cytolytic Assay—The cytolytic activity of ricin and other toxins was measured by the LDH release assay (*21*). In brief, cells (2×10^4 cells/well) were incubated in a 96-well plate in RPMI-1640 medium containing 35 μM BSA with or without 20 mM 3-ABA for 1 h at 37°C, followed by the addition of varying concentrations of ricin. After incubation at 37°C, the plate was centrifuged at 1,500 ×*g* at 4°C for 10 min. The supernatant (50 μl) was collected from each well and incubated with an equal volume of 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) reagent at 37°C for 10 min. The reaction was stopped by the addition of 100 μl of 0.1 *N* HCl and then the absorbance was measuned photometrically at 570 nm. The results were expressed as the percentage release of total cellular contents.

Diphenylamine Assay—Cells (5×10^6 cells/dish) in RPMI-1640 medium containing 35 μM BSA were incubated with ricin or other toxins at the concentrations indicated for various periods of time. The cells were then washed once with phosphate buffered saline (PBS) and lysed in 1 ml of ice-cold lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, 20 mM EDTA). The samples were subsequently centrifuged for 30 min at 13,000 ×*g* to separate DNA fragments (supernatant) from intact DNA (pellet). The DNA contents of the supernatant and pellet fractions were determined using diphenylamine reagent.

Detection of DNA Fragmentation—Cells (5×10^6 cells/dish) in RPMI-1640 medium containing 35 μM BSA were preincubated in the presence or absence of 20 mM 3-ABA for 1 h at 37°C. After further incubation with 10 ng/ml of ricin in the presence or absence of 3-ABA for the indicated period of time, the cells were washed once with PBS and lysed in 1 ml of lysis buffer (0.2 M NaCl, 10 mM EDTA, 20 mM Tris, 1% sodium dodecyl sulfate, pH 8.0) as described previously (*22*). The lysate was incubated with 250 μg/ml of proteinase K for 15 h at 37°C. The solution was then extracted with phenol/chloroform, and precipitated with 2.5 volumes of ethanol for 20 min at –83°C, and the precipitate was dried by evaporative centrifugation. The DNA was then dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 100 μg/ml RNase (DNase free) and incubated for 1 h at 37°C. The DNA in each sample was analyzed by electrophoresis in a 2.0% agarose gel, stained with ethidium bromide, and then photographed on a UV illuminator.

Intracellular NAD^+ Level—The NAD^+ levels were measured by an enzymatic cycling assay (*23*). Cells (2×10^6 cells/dish) were treated with or without 10 ng/ml of ricin for various times at 37°C, after which the cells were harvested by centrifugation and extracted with 0.4 M perchloric acid. After neutralization with 2.5 M $KHCO_3$, cell debris was removed by centrifugation. An aliquot of the supernatant (10 μl) was added to 1 ml of reaction mixture (2 mM PES, 0.5 mM MTT, 600 mM ethanol, and 200 μg of alcohol dehydrogenase in 120 mM Na/Bicine buffer, pH 7.8), and incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 ml of 12 mM iodoacetate. The absorbance was measured at 570 nm, and the results are expressed as percentages of control values.

Intracellular ATP Level—The ATP levels were measured by chemiluminescence assay as previously reported (*24*). In brief, cells (2×10^6 cells/dish) treated with or without ricin

were harvested by centrifugation and extracted with 0.4 M perchloric acid. After neutralization and removal of the cell debris as described above, the supernatant was mixed with luciferase-luciferin solution, and its chemiluminescence was measured using the 1254 luminometer (Bio-Orbit), and the results are expressed as percentages of control values.

Measurement of Poly (ADP-Ribose) Polymerase Activity—Poly (ADP-ribose) polymerase activity was measured by the incorporation of [^{32}P]NAD$^+$ into acid-insoluble materials as previously described (*25*). In brief, ricin-treated cells (2×10^5 cells/ml) were washed once with PBS, resuspended in ice-cold permeation buffer (10 mM Tris-HCl buffer, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, and 0.5 mg/ml BSA), and left for 15 min on ice. The cells were then mixed with assay mixture (50 mM Tris-HCl buffer, pH 8.5, 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mg/ml BSA, and 210 μM [^{32}P]NAD$^+$), and incubated 30 min at 25°C. TCA solution (final 10%) was added to the reaction mixture, and the TCA-insoluble materials were collected on a glass filter (Whatman GF/C). The radioactivities of the insoluble materials were measured with a liquid scintillation counter.

Immunofluorescence Microscopy—Poly(ADP-ribose) in ricin-treated U937 cells was detected by an immunofluorescence technique. Cells (4×10^6 cells/dish) in RPMI-1640 medium containing 35 μM BSA were incubated with or without 10 ng/ml of ricin for 4 h at 37°C. The cells were then fixed with 2% formaldehyde in PBS for 10 min at room temperature and washed with PBS containing 10% FBS (10% FBS/PBS). For permeabilization, cells were treated with methanol at 0°C for 1 min, and then washed with 10% FBS/PBS. The cells were incubated with the primary antibody (528815, Calbiochem-Novabiochem) diluted 1:500 with 10% FBS/PBS containing 0.15% saponin for 1 h. After washing with 10% FBS/PBS, the cells were incubated with the FITC-labeled secondary antibody (Wako Chemical) in FBS/PBS/0.15% saponin for 1 h. Thereafter, the cells were washed and viewed using an Olympus BX-60 fluorescence microscope.

Western Blot Analysis of PARP—Ricin-treated cells (2×10^6 cells/dish) were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 7.4, 1% Triton X-100, 0.1% SDS, 0.1 mM *N*-tosyl-L-lysine chloromethyl ketone, 0.2 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 1 μM pepstatin). Twenty micrograms of protein per lane was subjected to SDS-PAGE analysis, transferred to a PVDF membrane, and hybridized with anti-PARP rabbit polyclonal antibody as previously reported (*26*). Protein bands were visualized with biotin-conjugated goat ant–rabbit immunoglobulin G by the chemiluminescence system.

RESULTS

Time-Course Analysis of Ricin-Mediated Protein Synthesis Inhibition and Apoptotic Events in U937 Cells—Ricin inhibits cellular protein synthesis followed by receptor-mediated endocytosis (*27*). Our previous study demonstrated that 10 ng/ml of ricin is sufficient to achieve almost complete inhibition of protein synthesis in U937 cells. Thus, we used this concentration of ricin throughout the experiments. Time-course analysis revealed that ricin require 2 h to inhibit protein synthesis in U937 cells (Fig. 1A). DNA fragmentation, a typical hallmark of apoptosis began immediately after protein synthesis was inhibited (Fig. 1B). Moreover, we have found that the kinetics of the activation of the caspase-3–like activity in ricin-treated U937 cells was similar to that of DNA fragmentation (*12*). Thus, it is likely that ricin-mediated protein synthesis inhibition may be a trigger for apoptotic induction. In contrast to these relatively early events, cytolysis, as measured by LDH release assay, was observed much later after DNA fragmentation had reached the maximum level (Fig. 1C).

Intracellular Levels of NAD$^+$ and ATP in Ricin-Treated U937 Cells—Increasing evidence suggests that the consumption of NAD$^+$ and then ATP during apoptotic progress may lead to cell lysis. To ascertain whether or not this is the case for ricin-induced cytolysis, we measured intracellular NAD$^+$ and ATP levels in ricin-treated U937 cells. As shown in Fig. 2, the decrease in NAD$^+$ and ATP levels began after 3 h incubation with ricin, and 5 h later became undetectable, while cell lysis did not occur up to 9 h incubation as judged by the LDH release assay (Fig. 1C) and the trypan blue exclusion test (data not shown). Thus, the decrease in NAD$^+$ and ATP levels may be due to intracellular consumption rather than leakage from the cells.

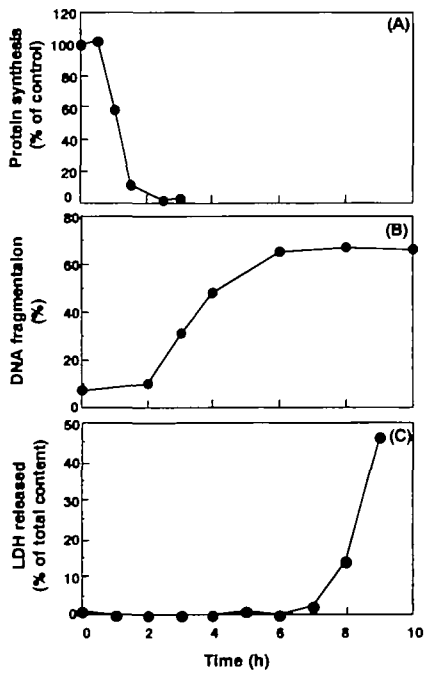

Fig. 1. **Time course analysis of protein synthesis (A), DNA fragmentation (B), and cytolysis (C) in ricin-treated U937 cells.** (A) Cells in 48-well plates (5×10^5 cells/well) were incubated with 10 ng/ml of ricin in RPMI-1640 medium containing 35 μM BSA for the indicated periods of time, and then protein synthesis was measured as described under "MATERIALS AND METHODS." (B) Cells (2×10^6 cells/dish) were incubated with 10 ng/ml ricin in RPMI-1640 medium containing 35 μM BSA for the indicated periods of time at 37°C. DNA fragmentation in the ricin-treated cells was assayed with diphenylamine as described under "MATERIALS AND METHODS." (C) Cells in 96-well (2×10^4 cells/well) plates were incubated with 10 ng/ml of ricin in RPMI-1640 medium containing 35 μM BSA for the indicated periods of time at 37°C. Cytolysis was assayed by LDH release as described under "MATERIALS AND METHODS." Each point represents the average of duplicate measurements.

Effects of 3-ABA on Ricin-Induced Cell Lysis in U937 Cells—To investigate the involvement of PARP in the depletion of NAD^+ and ATP in ricin-treated cells, we examined the effect of 3-ABA, a PARP specific inhibitor. As shown in Table I, 20 mM 3-ABA significantly prevented the decrease in both NAD^+ and ATP. To confirm further the activation of PARP in ricin-treated U937 cells, we measured the enzymatic activity of PARP by the incorporation of [^{32}P]NAD^+ into the acid-insoluble materials. As shown in Fig. 3A, a gradual increase in the activity after a 1 h lag time was observed in ricin-treated cells, while there was no significant change in activity in untreated cells during the period examined. The specific accumulation of poly(ADP-ribose) in ricin-treated U937 cells was also confirmed by immunofluorescence analysis using anti-poly(ADP-ribose) (Fig. 3B). These results indicate that PARP is activated in ricin-treated U937 cells, and this leads to the depletion of NAD^+ and ATP. Consistent with these results, 3-ABA profoundly protected against ricin-induced cell lysis (Fig. 4). Therefore, it appears that the depletion of NAD^+ and ATP levels *via* PARP activation is one of the major factors responsible for ricin-induced cell lysis. On the other hand, 20 mM 3-ABA had no effect on the inhibition of protein synthesis by ricin in U937 cells (Table II).

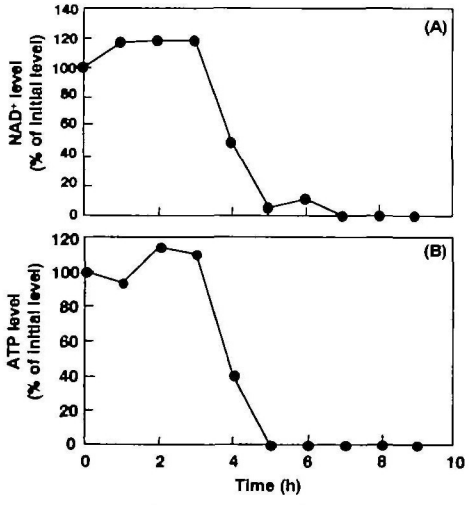

Fig. 2. **Intracellular NAD^+ and ATP levels in ricin-treated U937 cells.** (A) Cells (2×10^6 cells/dish) were treated with 10 ng/ml ricin in RPMI-1640 medium containing 35 μM BSA for the indicated periods of time at 37°C, and analyzed for NAD^+ content as described under "MATERIALS AND METHODS." (B) In parallel, intracellular ATP levels were measured by the luciferase assay. Each point represents the average of duplicate measurements.

TABLE I. **Effects of 3-aminobenzamide (3-ABA) on NAD^+ and ATP levels in U937 cells after 5 h exposure to ricin.**

Experimental conditions	NAD^+ (pmol/10^6 cells)	ATP (nmol/10^6 cells)
None	407.3 ± 11.4	241.7 ± 2.9
+ Ricin	15.8 ± 6.8	0.0 ± 0.8
+ Ricin, + 3-ABA	482.4 ± 0.0	285.8 ± 4.0

Cells were preincubated with or without 20 mM 3-ABA for 1 h in RPMI-1640 medium containing 35 μM BSA at 37°C. After the addition of ricin (10 ng/ml), the cells were incubated for another 5 h at 37°C. Intracellular NAD^+ and ATP were measured as described under "MATERIALS AND METHODS."

Effect of 3-ABA on DNA Fragmentation in Ricin-Treated U937 Cells—DNA fragmentation and chromatin condensation are generally considered to be characteristic apoptotic changes (*28*). In our previous report, agarose gel electrophoretic analysis of DNA extracted from ricin-treated cells showed typical DNA ladder formation (*12*). In this study,

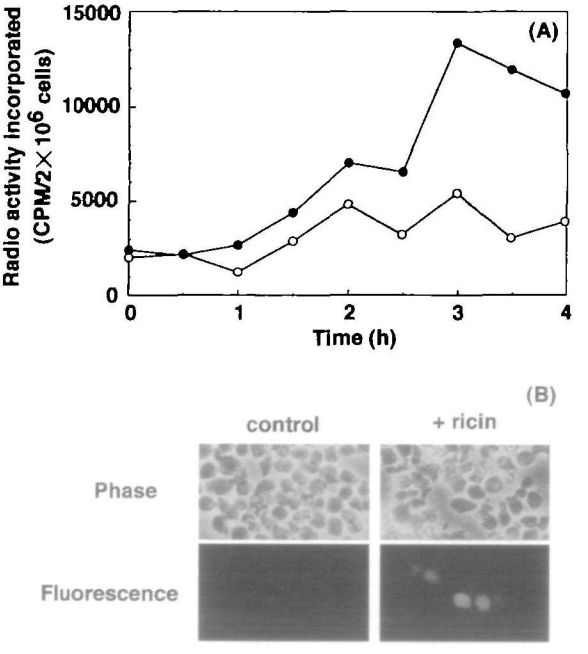

Fig. 3. **Poly(ADP-ribose)polymerase activity in ricin-treated U937 cells.** (A) Cells (2×10^5 cells/ml) were treated with (●) or without (○) ricin (10 ng/ml) in RPMI-1640 medium containing 35 μM BSA for the indicated periods of time at 37°C, and then PARP activity was measured as described under "MATERIALS AND METHODS." Each point represents the average of duplicate measurements. The experiment was repeated several times with basically similar results. (B) Immunofluorescence detection of poly(ADP-ribose) synthesized in ricin-treated U937 cells. Cells (4×10^6 cells) were treated with or without ricin (10 ng/ml) in RPMI-1640 medium containing 35 μM BSA for 4 h at 37°C, and then cells were fixed with 2% formaldehyde in PBS and processed for immunofluorescence as described in "MATERIALS AND METHODS."

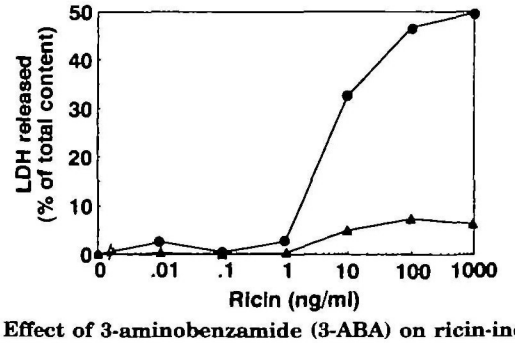

Fig. 4. **Effect of 3-aminobenzamide (3-ABA) on ricin-induced cell lysis in U937 cells.** Cells (2×10^4 cells/well) in 96-well plate in RPMI-1640 medium containing 35 μM BSA were incubated with or without 20 mM 3-ABA for 1 h at 37°C, followed by the addition of varying concentrations of ricin. After 15 h incubation at 37°C, the cells were subjected to LDH release assay as described under "MATERIALS AND METHODS." Control (●); + 3-ABA (▲).

we also found that incubating U937 cells for 9 h with 10 ng/ml of ricin resulted in DNA ladder formation (Fig. 5). The effect of 3-ABA on ricin-induced DNA fragmentation was examined by agarose gel electrophoresis. As shown in Fig. 5, 3-ABA failed to prevent ricin-induced DNA fragmentation, despite the strong protective effect against cell lysis caused by ricin (Fig. 4).

Immunoblotting Analysis of PARP in Ricin-Treated U937 Cells—To examine whether or not PARP cleavage occurs in ricin-treated U937 cells, total cellular proteins were extracted at different time points and subjected to immunoblotting analysis using a anti-PARP antibody that recognizes both the parent PARP (113 kDa) and the 89 kDa cleavage product. As shown in Fig. 6, 12 h after ricin addition, a substantial amount of PARP was cleaved into an 89 kDa fragment, resulting in the almost complete cleavage of the parental 113 kDa PARP to the 89 kDa fragment after 15 h incubation.

Comparison of Apoptosis-Inducing Ability of Ricin and Other Protein Synthesis Inhibitors—To examine whether

TABLE II. **Effect of 3-aminobenzamide (3-ABA) on the protein synthesis inhibitory activity of ricin in U937 cells.**

Experimental conditions	Protein synthesis (% of control)
None	100.0
+ Ricin	7.1 ± 1.8
+ Ricin, + 3-ABA	4.7 ± 8.7

Cells were preincubated with or without 20 mM 3-ABA for 1 h in RPMI-1640 medium containing 35 μM BSA at 37°C. After the addition of ricin (10 ng/ml), the cells were incubated for another 3 h at 37°C. Protein synthesis was measured as described under "MATERIALS AND METHODS."

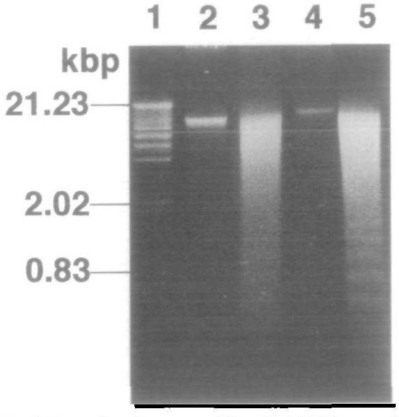

Fig. 5. **Effect of 3-aminobenzamide (3-ABA) on ricin-induced DNA fragmentation in U937 cells.** Cells (2×10^6 cells/ml) were incubated in the presence or absence of 20 mM 3-ABA in RPMI-1640 medium containing 35 μM BSA for 1 h at 37°C, followed by the addition of ricin (10 ng/ml). Cells were incubated for another 9 h at 37°C, and DNA was extracted and analyzed in a 2.0% agarose gel as described under "MATERIALS AND METHODS." Lane 1, molecular size standards (kbp); lane 2, control untreated cells; lane 3, + ricin; lane 4, + 3-ABA; lane 5, + 3-ABA, + ricin.

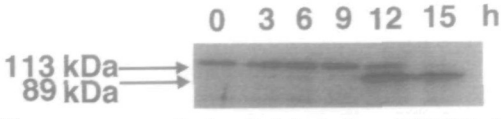

Fig. 6. **Time course analysis of ricin-induced PARP cleavage in U937 cells.** Cells (2×10^6 cells/dish) were treated with 10 ng/ml of ricin in RPMI-1640 medium containing 35 μM BSA at 37°C for the indicated periods of time (0, 3, 6, 9, 12, 15 h), and then cell lysates were prepared for immunoblotting with anti-PARP antibody as described under "MATERIALS AND METHODS."

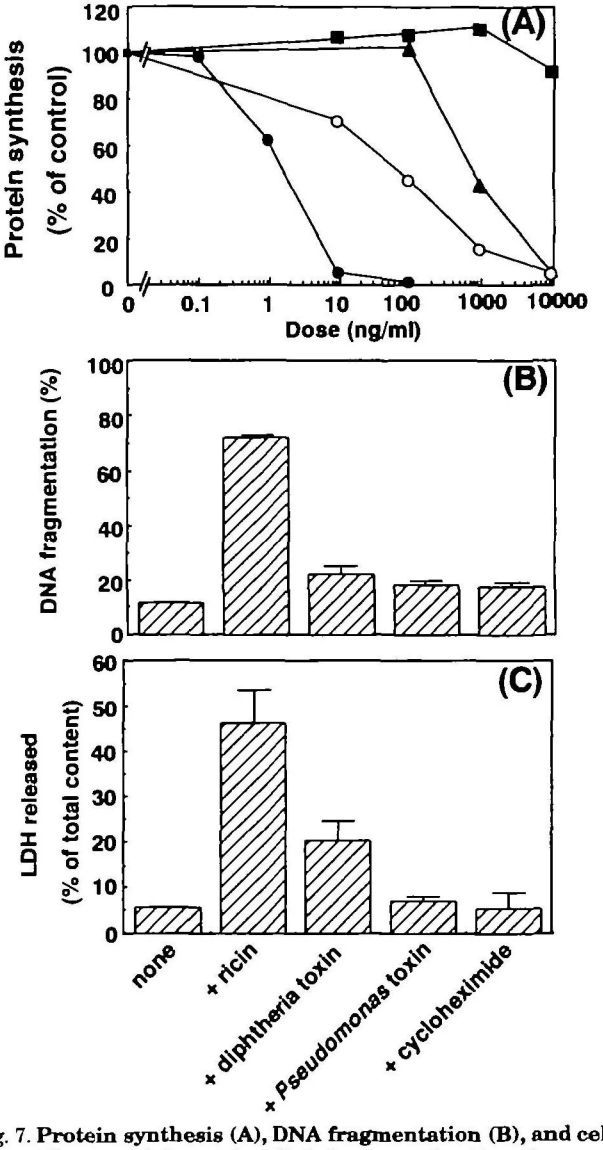

Fig. 7. **Protein synthesis (A), DNA fragmentation (B), and cell lysis (C) caused by ricin, diphtheria toxin, *Pseudomonas* toxin, and CHA in U937 cells.** (A) Cells in 48-well plates (5×10^5 cells/well) were incubated with varying concentrations of ricin (●), CHA (○), *Pseudomonas* toxin (■), or diphtheria toxin (▲) in RPMI-1640 medium containing 35 μM BSA for 3 h and then protein synthesis was measured as described under "MATERIALS AND METHODS." (B) Cells (2×10^6 cells/dish) were treated with 10 ng/ml of ricin, 10 μg/ml of CHA, 10 μg/ml of *Pseudomonas* toxin, or 10 μg/ml of diphtheria toxin in RPMI-1640 medium containing 35 μM BSA for 6 h. DNA fragmentation in cells treated with each toxin was assayed with diphenylamine as described under "MATERIALS AND METHODS." (C) Cells in 96-well plates (2×10^4 cells/well) were treated with 10 ng/ml of ricin, 10 μg/ml of CHA, 10 μg/ml of *Pseudomonas* toxin, or 10 μg/ml of diphtheria toxin in RPMI-1640 medium containing 35 μM BSA for 15 h. Cytolysis was assayed with LDH release as described under "MATERIALS AND METHODS." Each point represents the average of duplicate measurements.

other toxins that inhibit cellular protein synthesis also induce apoptosis in U937 cells to a similar extent as ricin, we tested diphtheria toxin, *Pseudomonas* toxin, and CHA, which act by differ biochemical mechanisms than ricin. The dose-response curves show that ricin has a stronger protein synthesis inhibitory activity than the other toxins tested (Fig. 7A). *Pseudomonas* toxin had no significant inhibitory effect on protein synthesis in U937 cells up to 10,000 ng/ml, suggesting that this cell line is highly resistant to *Pseudomonas* toxin. Consistent with this notion, *Pseudomonas* toxin failed to induce DNA fragmentation and cytolysis. The concentrations of toxins required to reduce protein synthesis to low levels, approximately 10% or less of the control level, were: ricin, 10 ng/ml; diphtheria toxin, 10,000 ng/ml; and CHA, 10 μg/ml. Even at these concentrations, diphtheria toxin and CHA induced clearly less DNA fragmentation and cytolysis than ricin. Especially, CHA produced no significant DNA fragmentation or cytolysis in U937 cells despite its significant inhibition of protein synthesis.

DISCUSSION

It has been shown that ricin, as well as other protein toxins such as diphtheria toxin and *Pseudomonas* toxin, can cause apoptotic cell death as judged by DNA fragmentation and target cell lysis (*12*). Although the mechanism of the ricin-mediated inhibition of protein synthesis is well defined, ricin-mediated apoptosis or cytolysis is largely unknown. In the present study, we investigated the mechanism of cell lysis produced by exposure to ricin and showed that there is an increase in PARP activity and depletion of intracellular NAD^+ and ATP in ricin-treated U937 cells (Figs. 2 and 3). PARP is an abundant nuclear enzyme, which, in response to DNA damage, undergoes automodification by forming poly(ADP-ribose) polymers using NAD^+ (*29*). Although it has been believed that PARP is involved in DNA repair, its exact physiological functions remain unclear. In addition to the beneficial effects of PARP activation, it has been suggested that PARP activation is an important mechanism leading to cell death. Namely, the prolonged activation of this enzyme leads to an excessive consumption of NAD^+ resulting in depletion of the ATP pool (*30*), which has been proposed as a mechanism for DNA damage-induced cell death in many cell types (*31*, *32*). It seems likely that this is also the case for ricin-induced cell lysis, since a PARP inhibitor, 3-ABA, preserved cellular NAD^+ and ATP and prevented ricin-induced cell lysis (Table I and Fig. 4).

It has been shown that various apoptosis-inducing stimuli can trigger both apoptotic and necrotic pathways (*33*). Necrosis and apoptosis are generally recognized as two different mechanisms of cell death, regulated by intracellular ATP levels. The apoptotic pathway requires high levels of ATP, while low ATP levels shift the cell death mode from apoptosis to necrosis (*34*, *35*). Therefore, it is possible that ricin can also induce necrosis as well as apoptosis in the same cells. Since DNA fragmentation in ricin-treated cells was initiated after protein synthesis was reduced to a very low level, the inhibition of protein synthesis may the trigger apoptotic pathway, which in turn activates PARP as a relatively early event. Thereafter, the cells would eventually undergo secondary necrosis. In ricin-treated cells, the release of LDH began 8 h after the addition of ricin, a time at which DNA fragmentation had already reached a maximum level (Fig. 1). The lag time between DNA fragmentation and delayed cell lysis may reflect the shift in the death mechanism from apoptosis to necrosis. Moreover, 3-ABA failed to prevent DNA fragmentation in ricin-treated cells despite its strong ability to inhibit cell lysis. Thus, these results suggest that the apoptotic pathway characterized by DNA fragmentation and the subsequent necrotic pathway leading to cytolysis are not co-linked, but are independent of each other in ricin-mediated cell death. Consistent with our results, it has been reported that PARP inhibition by 3-ABA and nicotinamide prevents H_2O_2-induced necrosis but has no effect on apoptosis (*24*).

Regarding the behavior of PARP in ricin-treated cells, PARP activity was found to reach a maximum level after 3 h of ricin exposure just prior to the initiation of the decrease in the intracellular NAD^+ level. The reason for the existence of this lag time between PARP activation and the actual depletion of NAD^+ is unclear, but might be due to the assay method for PARP activity in which the cells were permealized. Probably, the activity detected by the *in vitro* system does not completely reflect the *in vivo* activity, although there is no doubt about the activation of PARP and subsequent depletion of NAD^+ level during the course of ricin-induced apoptosis. Furthermore, we found that PARP cleavage occurs in U937 cells after 12-15 h of ricin exposure (Fig. 6). The onset of this event was delayed 5–6 h from the completion of ricin-induced DNA fragmentation, even though our previous study demonstrated that caspase-3–like activity reaches its maximum level in U937 cells after 4–6 h of ricin exposure (*12*). Furthermore, a caspase-3–specific tetrapeptide inhibitor, DEVD-CHO, had no significant effect on ricin-mediated DNA fragmentation and cytolysis despite its strong ability to inhibit increased caspase-3 activity (*12*). A membrane-permeable inhibitor, DEVD-fmk, also failed to prevent ricin-induced DNA fragmentation even at a very high concentration (100 μM) (data not shown). Thus, these results suggest that the activation of the caspase-3–like activity may be redundant, and that a caspase-3–independent signaling pathway exists in ricin-induced apoptosis. This is one of the ricin-specific apoptotic features, since caspase-3 is generally known to play an important role in many apoptotic systems. Regarding the time course of PARP cleavage, it has been reported that significant PARP cleavage is induced in U937 cells after 1.5 h of exposure to tumor necrosis factor or UV light (*36*). Therefore, the initiation of PARP cleavage may depend on apoptotic stimuli, and a delay in PARP cleavage is another characteristic feature of ricin-induced apoptosis. Such delayed PARP cleavage may result in the transition from apoptosis to secondary necrosis accompanied by cell lysis. In other words, this result also suggests that the prolonged maintenance of PARP activity is responsible for the induction of secondary necrosis at a late stage of apoptosis. This notion is consistent with the recent finding that fibroblasts expressing caspase-resistant PARP exhibit accelerated cell death due to the induction of necrosis (*37*).

Because the primary activity of ricin in cells is to inhibit protein synthesis by inactivating the ribosomes (*38*), it seems reasonable to postulate that the ricin-mediated protein synthesis inhibition leads to apoptosis concomitant with DNA fragmentation. However, the biological significance of the inhibition of protein synthesis in cells undergoing apoptosis remains controversial. For instance, it has

been reported that a well-known protein synthesis inhibitor, CHA, can induce apoptosis through the inhibition of protein synthesis under certain experimental conditions, and a delay or inhibition of apoptosis has been reported (*39*). In addition, it has been shown that macrophage adherence prevents ricin-induced apoptosis without affecting the inhibition of protein synthesis by ricin (*40*). In this study, we investigated whether other toxins or reagents (CHA, diphtheria toxin and *Pseudomonas* toxin) that inhibit cellular protein synthesis also induce apoptosis in U937 cells. After 3 h treatment with each toxin, protein synthesis was strongly inhibited in a concentration-dependent manner by all except *Pseudomonas* toxin (Fig. 7A). However, significant DNA fragmentation and cell lysis were observed only in ricin-treated cells (Fig. 7, B and C). These results suggest that the inhibition of protein synthesis itself is not important, but the means by which protein synthesis is inhibited is important; *e.g.*, ricin inactivates ribosomes by acting on 28S ribosomal RNA, while diphtheria toxin inactivates elongation factor EF-2, and CHA binds to the 60S ribosomal subunits and blocks the EF-2–dependent translocation step. Regarding the relationship between protein synthesis inhibition and the stress-signaling pathway, Iordanov *et al.* have recently reported a novel stress signaling pathway in mammalian cells (ribotoxic stress response) (*41*) that is initiated by damage to the 28S ribosomal RNA and leads to the activation of stress-activated kinase (SAPK/JNK1). They also proposed that the 28S ribosomal RNA functions as a sensor for stress induced by a subset of agents that inhibit protein synthesis. Furthermore, it has been reported that ricin shows a strong ability to activate SAPK/JNK1, but *Pseudomonas* toxin completely fails to activate the kinase, and diphtheria toxin causes only marginal activation under conditions at which all these toxins strongly inhibit cellular protein synthesis (*41*). Therefore, it can be speculated that the potent ability of ricin to induce apoptosis may be explained in part by its enzymatic action on 28S ribosomal RNA, and, thereby, stress-related signals leading to apoptosis may be triggered. Further studies are required to clarify the involvement of MAP kinase activation in the ricin-mediated induction of apoptosis.

In conclusion, our results suggest that ricin induces two distinct death pathways in U937 cells, one apoptosis, as evidenced by DNA fragmentation, and the other delayed necrosis caused by NAD^+ and ATP depletion through PARP activation. The potent ability of ricin to induce such apoptotic events may be the result of the specific action of its A chain on 28S ribosomal RNA.

REFERENCES

1. Middlebrook, J.L. and Dorland, R.B. (1984) Bacterial toxins: cellular mechanisms of action. *Microbiol. Rev.* **48**, 199–221
2. Goldstein, J.L., Brown, M.S., Anderson, R.G., Russel, D.W., and Schneider, W.J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1**, 1–39
3. Sandvig, K., Olsnes, S., Brown, J.E., Petersen, O.W., and van Deurs, B. (1989) Endocytosis from coated pits of Shiga toxin: A glycolipid-binding protein from *Shigella dysenteriae* 1. *J. Cell Biol.* **108**, 1331–1343
4. Sandvig, K. and van Deurs, B. (1992) Toxin-induced cell lysis: Protection by 3-methyladenine and cycloheximide. *Exp. Cell Res.* **200**, 253–262
5. Oda, T., Komatsu, N., and Muramatsu, T. (1997) Cell lysis induced by ricin D and ricin E in various cell lines. *Biosci. Biotechnol. Biochem.* **61**, 291–297
6. Oda, T., Komatsu, N., and Muramatsu, T. (1998) Diisopropylfluorophosphate (DFP) inhibits ricin-induced apoptosis of MDCK cells. *Biosci. Biotechnol. Biochem.* **62**, 325–333
7. Nicholson, D.W. and Thornberry, N.A. (1997) Caspases: killer proteases. *TIBS Rev.* **22**, 299–306
8. Villa, P., Kaufmann, S.H., and Earnshaw, W.C. (1997) Caspases and caspase inhibitors. *TIBS Rev.* **22**, 388–393
9. Ahmad, M., Srinivasula, S.M., Hegde, R., Mukattash, R., Fernandes-Alnemri, T., and Alnemri, E.S. (1998) Identification and characterization of murine caspase-14, a new member of the caspase family. *Cancer Res.* **58**, 5201–5205
10. Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Callamt, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T.T., Yu, V., and Miller, D.K. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37–43
11. Ghibelli, L., Coppola, S., Rotilio, G., Lafavia, E., Maresca, V., and Ciriolo, M.R. (1995) Non-oxidative loss of glutathione in apoptosis *via* GSH extrusion. *Biochem. Biophys. Res. Commun.* **216**, 313–320
12. Komatsu, N., Oda T., and Muramatsu, T. (1998) Involvement of both caspase-like proteases and serine proteases in apoptotic cell death induced by ricin, modeccin, diphtheria toxin, and *Pseudomonas* toxin. *J. Biochem.* **124**, 1038–1044
13. Mashima, T., Naito, M., Noguchi, K., Miller, D.K., Nicholson, D.W., and Tsuruo, T. (1997) Actin cleavage by CPP-32/apopain during the development of apoptosis. *Oncogene* **14**, 1007–1012
14. Mashima, T., Naito, M., Noguchi, K., Miller, D.K., Nicholson, D.W., and Tsuruo, T. (1998) Caspase-3-induced gelsolin fragmentation contributes to actin cytoskeletal collapse, nucleolysis, and apoptosis of vascular smooth muscle cells exposed to proinflammatory cytokines. *Eur. J. Cell Biol.* **77**, 294–302
15. Geng, Y.J., Azuma, T., Tang, J.X., Hartwig, J.H., Muszynski, M., Wu, Q., Libby, P., and Kwiatkowski, D.J. (1998) Fas-induced DNA fragmentation and proteolysis of nuclear proteins. *Genes Cells* **3**, 297–306
16. He, J., Whitacre, C.M., Xue, L.Y., Berger, N.A., and Oleinick, N,L. (1998) Protease activation and cleavage of poly(ADP-ribose) polymerase: an integral part of apoptosis in response to photodynamic treatment. *Cancer Res.* **58**, 940–946
17. de Murcia, G., Schreiber, V., Molinete, M., Saulier, B., Poch, O., Masson, M., Niedergang, C., Menissier, and de Murcia, J. (1994) Structure and function of poly(ADP-ribose) polymerase. *Mol. Cell Biochem.* **138**, 15–24
18. Walisser, J.A. and Thies, R.L. (1999) Poly(ADP-ribose) polymerase inhibition in oxidant-stressed endothelial cells prevents oncosis and permits caspase activation and apoptosis. *Exp. Cell Res.* **251**, 401–413
19. Tanaka, Y., Yoshihara, K., Tohno, Y., Kojima, K., Kameoka, M., and Kamiya, T. (1995) Inhibition and down-regulation of poly (ADP-ribose) polymerase results in a marked resistance of HL-60 cells to various apoptosis-inducers. *Cell. Mol. Biol.* **41**, 771–781
20. Mise, T., Funatsu, G., Ishiguro, M., and Funatsu, M. (1977) Isolation and characterization of ricin E from castor beans. *Agric. Biol. Chem.* **41**, 2041–2046
21. Thomas, D., Marie-Luise, and Lohmann-Matthes (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immuno. Method* **15**, 61–69
22. Oda, T., Komatsu, N., and Muramatsu, T. (1997) Inhibitory effect of dideoxyforskolin on cell death induced by ricin, modeccin, diphtheria toxin, and *Pseudomonas* toxin in MDCK cells. *Cell Struct. Funct.* **22**, 545–554
23. Carl, B. and Marcella, S. (1973) An improved cycling assay for nicotinamide adenine dinucleotide. *Anal. Biochem.* **53**, 452–458

24 Watson, A.J., Askew, J.N., and Benson, R.S. (1995) Poly(Adenosine diphosphate ribose) polymerase inhibition prevents necrosis induced by H_2O_2 but not apoptosis. *Gastroenterology* **109**, 472–482

25. James, L.S., Georgina, W.S., Donna, M.C., Sosamma, J.B., and Nathan A.B. (1982) Poly(adenosine diphosphoribose) polymerase inhibitors stimulate unscheduled deoxyribonucleic acid synthesis in normal human lymphocytes. *Biochemistry* **21**, 1813–1821
26. Tewari, M., Quan, L.T., O' Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S., and Dixit, V.M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801–809
27. Olsnes, S., Refsnes, K., and Phil, A. (1974) Mechanism of action of the toxic lectins abrin and ricin. *Nature* **249**, 627–631
28. Annette, J.H., Christopher, A.S., Leslie, C.S., Victoria, E.A.S., Vanessa, L.L., Aedin, C.C., and Gwyn, T.W. (1996) Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.* **236**, 1–26
29. Landahl, T., Satoh M.S., Poirier G.G., and Klungland, A. (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *TIBS.* **20**, 405–411
30. Sims, J.L., Berger S.J., and Berger N.A. (1983) Poly (ADP-ribose) polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5'-triphosphate pools in DNA-damaged cells: mechanism of stimulation of unscheduled DNA synthesis. *Biochemistry* **22**, 5188–5194
31. Berger, N.A. (1985) Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat. Res.* **101**, 4–15
32. Cherney, B.W., McBride, O.W., Chen, D., Alkhatib, H., Bhatia, K., Hensley, P., and Smulson, M.E. (1987) cDNA sequence, protein structure, and chromosomal location of the human gene for poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA* **84**, 8370–8374
33. Coppola, S., Nosseri, C., Maresca, V., and Ghibelli, L. (1995) Different basal NAD^+ levels determine opposite effects of poly-(ADP-ribosyl) Polymerase inhibitors on H_2O_2-induced apoptosis. *Exp. Cell Res.* **221**, 462–469
34. Eguchi, Y., Shimizu, S., and Tsujimoto, Y. (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res.* **57**, 1835–1840
35. Leist, M., Single, B., Castoldi, A.F., Kuhnle, S., and Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med.* **185**, 1481–1486
36. Susan, C.W., Ute, S., Hong, W., David, H.K., Jamil, W.T., and James, W.L. (1997) Activation of CPP32-like proteases is not sufficient to trigger apoptosis: Inhibition of apoptosis by agents that suppress activation of AP24, but not CPP32-like activity. *J. Exp. Med.* **186**, 1107–1117
37. Zdenko, H. and Zhao-qi, W. (1999) Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. *Mol. and Cell. Biol.* **19**, 5124–5133
38. Endo, Y. and Tsurugi, K. (1986) Mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. *Nucleic Acids Symp.* Ser. **17**, 187–190
39. Chow, S., Peters, I., and Orrenius, S. (1995) Reevaluation of the role of de novo protein synthesis in rat thymocyte apoptosis. *Exp. Cell Res.* **216**, 149–159
40. Khan, T. and Waring, P. (1993) Macrophage adherence prevents apoptosis induced by ricin. *Eur. J. Cell Biol.* **62**, 406–414
41. Iordanov, M.S., Pribnow, D., Magun, J.L., Dinh, T.-H., Pearson, J.A., Chen, S.L.-Y., and Magun, B.E. (1997) Ribotoxic stress response: Activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the α-sarcin/ricin loop in the 28S rRNA. *Mol. Cell. Biol.* **17**, 3373–3381