Abstract. Background: Adriamycin (ADM) is a potent antitumor drug that induces apoptosis (AP) in tumor cells. AP is modulated by caspases and by mitogen-activated protein kinases (MAPK) as well as by the mitochondrial membrane potential (ΔΨm). We studied the participation of these systems in peritoneal macrophages from ADM-treated mice. Materials and Methods: Balb/c mice were either treated with ADM (5mg/kg, i.p.) or with 0.85% NaCl solution (controls). One hour later, peritoneal cells were harvested and cultured for 28 h. AP was evaluated by ethidium bromide and acridine orange staining; ΔΨm was monitored using a MitoCapture stain Kit; DNA integrity was assessed by electrophoretic analysis. Animals were treated (i.p.) 1 h before ADM administration with Z-LEHD-FMK, Z-DEVD-FMK, or Z-VAD-FMK (caspase-9, caspases-3,7,10 and general caspase inhibitors, respectively) or with PD169316 (a MAPK p38 inhibitor). Results: ADM induced a higher rate of AP and the characteristic electrophoretic DNA ladder pattern. Mice treated with caspases inhibitors plus ADM showed significant reductions in AP and DNA laddering; in contrast, no differences were observed in mice treated with PD169316 plus ADM in comparison with ADM alone. ADM also induced early loss of the ΔΨm. Conclusion: In these experimental conditions, ADM induced AP in a mainly caspase-9-dependent manner and this was related to a reduction in the ΔΨm.

Macrophages play a major role in mammalian homeostasis by a variety of mechanisms such as phagocytosis, releasing cell signalling molecules, presenting cell antigens and destruction of tumour cells (1,2). Under physiological conditions, monocyte precursors from the bone marrow are needed to replace normal tissue macrophages (3).

Adriamycin (doxorubicin: ADM) is a highly potent antineoplastic agent for combating human and murine tumours, but with limited application because of the risk of developing cardio and myelotoxicity through the generation of oxygen-derived free radicals. In murine models, ADM stimulates macrophage activities such as phagocytosis, increased interleukin-1 secretion and antitumour activity (4-8), and induces ultrastructural changes in peritoneal macrophages that are related to free radical production (9).

Despite extensive study, the mechanisms for the antitumour activity of ADM are not understood and are still controversial. The nature of the cytotoxic effects of this drug has been related to DNA binding or intercalation, to alkylation, topoisomerase II inhibition, free radical generation and cell membrane damage (10-13). In addition to these mechanisms, ADM is able to induce apoptosis (AP) (14-18) by poorly understood mechanisms. In vitro study with tumour cell lines indicated that ADM induces AP in a Fas- and Fas ligand (FasL)-independent manner (19-20) in which the disruption of the inner mitochondrial membrane potential (ΔΨm) proceeds the nuclear signs of AP and, in Jurkat cells, the activation of different cysteine- and aspartate-specific proteases called caspases (21).

According to their substrate specificity, sequence homology and biochemical function, caspases may be classified into three major subfamilies: an interleukin-1 beta converting enzyme (ICE)-like subfamily and activators and effectors. The first family is mainly involved in inflammation and the other two are implicated in AP. However, caspases are not the only enzymes related to AP. Other enzymes such as mitogen-activated protein kinase (MAPK) have also been implicated in the induction of AP. Three main families of...
MAPK (ERK, JNK/SAPK and p38) have potent signal transduction systems (22). Among them, the p38 kinase family can regulate the induction of AP, with or without caspase activation (23).

We have established a model for the induction of AP by in vivo ADM administration in therapeutic doses in peritoneal murine macrophages. We have also reported the antiapoptotic effects of some antioxidant agents such as (+)-a-tocopherol, superoxide dismutase and lipopolysaccharide, which suggest the participation of oxygen-derived free radicals in AP (14).

In this study, we investigated the participation of mitochonridia and the involvement of caspase cascades and p38 MAPK in apoptotic cell death induced in murine peritoneal macrophages by in vivo treatment with ADM. Our results showed an early loss of ∆ψm in peritoneal macrophages (Sector Salud # 1921). This medium will be referred as RPMI-S.

To obtain peritoneal macrophages, mice were injected (i.p.) with 1 ml of sterile fluid thioglycollate broth (Merck México # 15867). Four days later, mice were injected with ADM as described above and sacrificed 1 h later. Peritoneal cell exudates were obtained by washing the abdominal cavity with 7 ml of RPMI-S at 4°C (pH 7.2). They were then washed three times in the same solution and re-suspended at a density of 1 x 10⁶ cells/ml in RPMI-S. Peritoneal macrophages were isolated by plastic surface adherence in 24-well plates (Costar Cambridge, MA, USA). One milliliter of peritoneal cell suspension was placed in each well and incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO₂/95% air. At the end of incubation, non-adhering cells were eliminated by aspiration and the wells were washed three times with RPMI-S at 37°C and re-suspended in 1 ml of the same medium. The resulting cultures were 97% pure macrophages as determined by morphology and by staining for non-specific esterase; viability was confirmed by trypan blue (Sigma México # 468) exclusion (>95%). The macrophage monolayers were cultured for 27 h after ADM injection as suggested by a previous pilot study for optimal ex vivo AP development.

Administration of inhibitors. Inhibition of AP was modulated using the following inhibitors: the general caspase inhibitor, benzoyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone (Z-VAD-FMK) (Biorad # 160-3192); and the inhibitor of caspases -3, -7 and -10, benzoyloxycarbonyl-Asp-Glu-Val-Asp-(OMe)-fluoromethyl ketone (Z-DEVD-FMK) (Biorad 170-3180); a preferential caspase-9 inhibitor, benzoyloxycarbonyl-Leu-Glu-(OMe)-His-Asp-fluoromethyl ketone (Z-LEHD-FMK) (Biorad # 161-3192); and the β and δ isoform p38 MAPK inhibitor [4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole] (PD169316) (Calbiochem # 513030 San Diego, CA, USA). The caspases and p38 MAPK inhibitors were first dissolved in 100 μl of dimethyl sulfoxide (DMSO) (Sigma # D8481) and later in 0.85% sterile NaCl solution at final concentrations of 100, 200, 400 and 600 μM. A volume of 0.5 ml of each concentration of inhibitors (50, 100, 200 and 300 nM/mouse, respectively) was injected (i.p.) 30 min before ADM administration. The control animals received the same DMSO/NaCl solution without inhibitors. To confirm the specificity of the inhibitors, in some groups caspase inhibitors were inactivated by three cycles of heat (90°C for 30 min) and freezing.

Quantification of apoptotic and cell viability. Cells were stained with ethidium bromide (Sigma # E-8751) and acridine orange (Sigma # A-2886) (100 μg/ml each in PBS). Acridine orange is used to stain the cells that have undergone AP, whereas ethidium bromide differentiates between viable and non-viable cells. Two hundred cells were counted and the numbers of each of the following four cellular states recorded: live cells with normal nuclei, bright green chromatin, and organized structure (LN); apoptotic cells with highly condensed or fragmented bright green-yellow chromatin (A); dead cells with normal nuclei, bright red chromatin, and organized structure (DN); and dead cells with apoptotic nuclei, and bright orange chromatin that was highly condensed and fragmented (DA). Indices of various cellular states were calculated as follows: Apoptotic index (AI) = (A + DA)/(LN + A + DN + DA) x 100

Live cell index (LCI) = (LN)/(LN + A + DN + DA) x 100

Necrotic cell index (NCI) = (DN)/(LN + A + DN + DA) x 100

Mitochondrial monitoring. Disruption of the mitochondrial ∆ψm is one of the earliest intracellular events that occurs following induction of AP. The MitoCapture Apoptosis Detection Kit (Biovision # K250-100 Mountain View, CA, USA) provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. The method was used according to the manufacturer’s instructions. The kit uses a cationic dye (MitoCapture) that fluoresces differently in healthy versus apoptotic cells. Two hundred cells were counted and the number of each of the following two cellular states was recorded: in live cells (LC), dye accumulates and polymerizes in the mitochondria, giving off a bright red fluorescence; in apoptotic cells (AC), dye cannot aggregate in the mitochondria because of the lost mitochondrial transmembrane potential and thus remains in the cytoplasm in its monomeric form, fluorescing green. The fluorescent signals can be easily detected by fluorescence microscopy using a band-pass filter. Indices of various cellular states based on these measurements were calculated as follows: 2690
Mitochondrial apoptotic index (MAI) = AC/(LC + AC) x 100
Mitochondrial live cell index (MLCI) = LC/(LC + AC) x 100

DNA preparation and analysis. To visualize the DNA ‘ladder pattern’ characteristic of its fragmentation into 180-300 bp sequences following AP, peritoneal macrophage DNA was prepared according to Gustincich (24). Briefly, peritoneal macrophages were lysed, denatured and deproteinized. DNA was precipitated, purified and run in 2% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed with UV transillumination. A molecular weight marker kit of DNA (Gibco# 15628-019 Frederick, MD, USA) with a 100 bp fragmentation pattern was employed as a positive control.

Statistical analysis. Results are presented as means±standard deviations of at least seven independent observations. The non-parametric Mann-Whitney U-test was used to assess statistical significance (p<0.05) between the groups.

Results

The highest apoptotic index (AI) measured by ethidium bromide and acridine orange staining was achieved 27 h after ADM injection (AI 81.33±10.37) and was still high after 28 or 30 h of culture (Figure 1). The peritoneal macrophages from the untreated control group showed an AI of 5.60±3.27 at almost all points tested. Moreover, a typical pattern of apoptotic DNA was observed at 25 h after ADM administration by electrophoretic analysis (Figure 2, lane 3) and was absent in the control group (Figure 2, lane 2). This suggests that ADM induced AP in a time-dependent manner. For all subsequent studies we used peritoneal macrophages 27 h after ADM injection.

AP can be induced in peritoneal macrophages by several pathways. One of these is the activation of caspase cascades. Thus, the next step was the study of a potential role of caspases in the ADM-induced AP. The results are shown in Figures 3A-C. Experimental and control mice groups were pre-treated (i.p.) with different concentrations of caspase inhibitors 30 min before ADM administration. Results are expressed as cell indices (AI, LCI or NCI). In the untreated control group, AI was 5.6±3.2, LCI 79.1±6.0 and NCI 15.3±5.2. For the ADM-treated group AI was 81.3±10.3, LCI 2.9±3.0 and NCI 17.9±13.8, and these were all statistically different when compared to the control group (p<0.001). AP induced by ADM was inhibited in a dose-dependent manner by all three caspase inhibitor compounds tested (Figures 3A-C). Moreover, an increase in live cells analysed by LCI was observed at optimal concentrations of caspase inhibitors (42.00±2.82; 22.00±5.65; 34.66±6.11 for general caspases at 200 nM; caspases-3, -7 and -10 at 300 nM and caspase-9 inhibitors at 100 nM: p<0.001 compared with the ADM group). However, we also observed an increase in the NCI at nearly all concentrations of caspase inhibitors used. These last results could be explained by a toxic effect of caspase inhibitors at high concentrations as described previously (25).
The optimal concentrations of the caspase inhibitors differed, as shown in Figure 4, where results are expressed in percentage inhibition of AP. The highest inhibitions were observed at 100 nM for Z-VAD-FMK and Z-LEHD-FMK, and 200 nM for Z-DEVD-FMK. These probably reflect the different contribution of specific caspases in our model.

To test the specificity of caspase inhibition, we pre-treated animals with denatured enzyme inhibitors before ADM treatment. Non-significant differences were obtained for indices (LCI, NCI or AI) when compared to those from ADM-treated mice. These results confirmed the specific activity of the inhibitors and excluded the possibility of DMSO solvent interference (data not shown).

As inhibition of AP with caspase inhibitors did not reach 100%, we tried to test an independent caspase pathway using the p38 MAPK inhibitor (Figure 3D). For no concentrations of PD169316 tested did we observe any inhibition of AI when compared to the ADM-treated group. Similar results were obtained when we compared the LCI and NCI indices.
Inhibition of AP by caspase inhibitors was also analysed using DNA electrophoresis. Results for optimal concentrations of inhibitors are shown in Figure 5. Typical DNA ladder patterns were observed when animals were treated with ADM alone (lane 3) or when caspase inhibitors were inactivated (lanes 7, 10 and 13 for Z-VAD-FMK, Z-DEVD-FMK and Z-LEHD-FMK, respectively). These typical apoptotic DNA profiles were not detected in animals pre-treated with caspase inhibitors (lanes 6, 9 and 12). No inhibition of AP was observed when peritoneal macrophage DNA from the p38 MAPK inhibitor-treated animals was analysed (data not shown).

The results strongly suggest that the AP induced by ADM depends on caspase activation, and that implication of caspase-9 activation seems to be more important. The p38 MAPK pathway is probably not involved in the AP induced by ADM. As in vitro models have shown that incubation of Jurkat tumour cells with ADM induces disruption of ΔΨₘ before the nuclear signs of AP (21), we also investigated mitochondrial membrane integrity in our experimental conditions. The ΔΨₘ was determined by MitoCapture staining and compared with the AI calculated using the ethidium bromide and acridine orange technique. As shown in Figure 6, disruption of ΔΨₘ was observed after 12 h of culture (MAI 78.00±2.82) and reached maximum values within 24 h of culture (88.00±3.65). By contrast, the AI determined by morphology is about 20 after 12 h of culture, but similar values measured by both methods are observed after 27 h of culture. Thus, the MitoCapture technique allowed an early detection of the disruption of mitochondrial membrane integrity induced by ADM treatment.

Peritoneal macrophages from experimental and control groups were harvested, purified by plastic adherence and cultured in RPMI-S culture medium at 37°C in a humidified atmosphere containing 5% CO₂ / 95% air for 27 h.

Discussion
During the past 20 years, explanations about the mechanism of cytotoxicity induced by ADM have been related to DNA intercalation/binding, inhibition of topoisomerase II, free radical generation and damage to cell membranes (21). However, recently, ADM as well as other antitumour drugs has been reported to induce AP (26, 27). Results presented here showed that ADM treatment induced AP in thioglycollate-treated peritoneal macrophages. The AP induced by ADM is partially dependent on caspase activity, as demonstrated by inhibition of AP after injection of caspase inhibitors.
Immunological effects of ADM have also been reported by our group. ADM induces an increase in phagocytic activity, secretion of oxygen-derived free radicals, in the secretion of IL-1 in normal peritoneal macrophages, as well as a higher destruction of tumour cells in vivo and in vitro (5-7, 9). We have also reported that in vivo ADM treatment induces AP in peritoneal murine macrophages (14). Here we aimed to elucidate the mechanisms by which ADM induced AP in murine peritoneal macrophages.

The ADM concentration plays an important role in the induction of AP. Concentrations of up to 100 μM achieved similar cytotoxic effects in cultures of MOLT-4 ALL cells, but acted via different mechanisms. ADM (1 μM) induced AP, which is dependent on RNA synthesis and involved oxidative stress. However, an ADM concentration greater than 3 μM – a dose pharmaceutically unachievable in vivo – induced free radical generation, lipid peroxidation and cell death without internucleosomal DNA fragmentation and significant inhibition of RNA synthesis (28). DNA strand-breaks in MOLT-4-ALL cells occurred in the presence of 1 to 5 μM ADM to a similar extent, but showed dose-dependence at higher concentrations (28). In this ex vivo study with murine peritoneal macrophages, AP induced by in vivo ADM administration is reached with effective doses of 5 mg/kg. These doses increase the lifespan of tumour-bearing mice (5, 8). This is consistent with a previously established mechanism of ADM action in which oxygen-derived free radicals were related to ADM toxicity and ADM was considered as a stimulus for the activation of AP (14). Higher doses of ADM (10 mg/kg) induced necrosis in MOLT-4 ALL cells (28) as well as in our previous observations.

An early event of many types of AP models is the opening of mitochondrial pores or megachannels, a process known as the mitochondrial permeability transition, which is related to the disruption of the ΔΨ_m (29). AP induced by ADM in Jurkat cells also caused disruption in ΔΨ_m and caspase activation (30-32). Pre-treatment of cells with peptide-based inhibitors has proven useful to evaluate the implication of caspases in AP, although their specificity seems to be less extended than previously recognized (33-36). Our results here showed that, under these experimental conditions, AP is caspase-dependent and that the main caspase involved is probably caspase-9. This is supported by the observation that the general caspase inhibitor (Z-VAD-FMK) showed the same degree of inhibition as the specific caspase-9 inhibitor (Z-LEHD-FMK). This specificity was confirmed by the lack of AP induction using denatured inhibitors. These observations are in agreement with in vitro experiments where caspase-9 plays an important role in ADM-induced AP in Jurkat cells (21).

An interesting point is the observation that ADM induces early loss of the ΔΨ_m. This suggests that mitochondria are important targets for the action of ADM. Moreover, these data support those related to caspase-9, which is activated after mitochondrial cytochrome c release by the loss of ΔΨ_m. It is important to point out that these results did not exclude the implication of other caspase-independent mechanisms (21). This is highlighted by our results showing that total AP inhibition could not be achieved after injection of caspase inhibitors. Inhibition of all caspases downstream of mitochondria may prevent the development of the classical apoptotic features but not cell death, because the activity of apoptotic inductor factors are not blocked by caspase inhibitors. Such factors induce an atypical nuclear pattern of AP through a caspase-independent mechanism (37-41). Another possible explanation was suggested by Gamen et al. (21) who hypothesized that an unknown caspase activity could be induced by ADM incubation in vitro.

In this work, we also investigated the participation of p38 MAPK in AP induced by ADM. This enzyme related to AP is highly expressed in macrophages (22). However, p38 MAPK did not seem to play a role in our experimental conditions, because the p38 MAPK inhibitor did not alter the AI induced by ADM.

In conclusion, our results clearly showed that the in vivo AP induction by ADM is dependent on both caspases and mitochondrial disruption. This evidence, together with previously reported data, may help in the design of chemotherapy regimens with rational molecular bases.

Mice were treated with ADM (5 mg/kg of weight i.p.); 1 h later peritoneal macrophages were harvested and purified by plastic adherence and cultured in RPMI-S culture medium at 37°C in a humidified atmosphere containing 5% CO2 / 95% air for 27 h. The AI was determined by ethidium bromide and acridine orange staining and the ΔΨ_m disruption index (MAI) was determined using MitoCapture staining.

**Figure 6. Adriamycin induces mitochondrial potential disruption and apoptosis in murine peritoneal macrophages.**
Acknowledgements

Many thanks to Ms. Sonia Roman Maldonado for revising the manuscript.

References


Received November 4, 2003
Accepted February 6, 2004