ANTICANCER RESEARCH *25*: 4091-4100 (2005)

γ-Irradiation Induced Apoptosis in Peritoneal Macrophages by Oxidative Stress. Implications of Antioxidants in Caspase Mitochondrial Pathway

GEORGINA HERNANDEZ-FLORES[1], PIEDAD C. GOMEZ-CONTRERAS[1], JORGE R. DOMINGUEZ- RODRIGUEZ[1], JOSÉ MANUEL LERMA-DIAZ[1], PABLO CÉSAR ORTIZ-LAZARENO[1], RAMON CERVANTES-MUNGUIA[1,2], JOSÉ ERNESTO SAHAGÚN-FLORES[3], SIMONE ORBACH-ARBOUYS[4], DANIEL SCOTT-ALGARA[5] and ALEJANDRO BRAVO-CUELLAR[1,2]

[1]*Centro de Investigacion Biomédica de Occidente, IMSS, Guadalajara;*
[2]*CUALTOS, Universidad de Guadalajara, Tepatitlan;*
[3]*Hospital V. Gomez Farias, ISSSTE, Guadalajara, Jal. México;*
[4]*Service de Pharmacie Hôpital Paul Brousse, Villejuif;*
[5]*Biologie des Rétrovirus, Institut Pasteur, France*

Abstract. *The in vivo and in vitro development of apoptosis induced by γ-irradiation was studied in mouse peritoneal macrophages. The apoptosis index was measured by fluorescence microscopy and DNA electrophoresis. In vivo apoptosis was greatest eight days after 8 Gy total body γ-irradiation. A DNA ladder electrophoretic pattern was only observed in the γ-irradiated group. The participation of reactive oxygen species in apoptosis induction was investigated by pretreating mice with the antioxidants superoxide dismutase, catalase, vitamin E or lipopolysaccharide before γ-irradiation. Measurement of serum lipoperoxides showed oxidative stress in the γ-irradiated mice and the protection given by the antioxidants. These results were confirmed using in vitro cultures of peritoneal macrophages: γ-irradiated groups and antioxidant-pretreated γ-irradiation groups showed results similar to those observed with in vivo irradiation. A loss of mitochondrial membrane potential ($\Delta\psi_m$) was also observed by microscopy in the γ-irradiated cell cultures. Experiments with caspase inhibitors confirmed the participation of caspase 3 and caspase 9.*

Correspondence to: Alejandro Bravo-Cuellar, Ph.D., Centro de Investigacion Biomédica de Occidente, IMSS, Sierra Mojada 800 Col. Independencia, Guadalajara, Jalisco, México, C.P. 44340. Tel: (523) 36189410, e-mail: abravoc@prodigy.net.mx

Key Words: Apoptosis, γ-irradiation, macrophages, reactive oxygen species, antioxidants, caspase inhibitors.

Ionizing irradiation acts mainly through radiolysis of water molecules and induces the production of reactive oxygen species (ROS) such as $O^{\bullet -}{}_2$, H_2O_2, OΔ, OH• and peroxide radicals. Modification of the radiation response through chemical protection of normal tissue has been practiced over the past 50 years. A number of natural and synthetic compounds of diverse structure and presumed mechanism of action, including antioxidant enzymes, interleukin 1 and some vitamins (1-4), have displayed significant protection against ROS generation.

These ROS can perturb the cellular antioxidant mechanism, damaging the major classes of biological macromolecules, including carbohydrates, lipids, proteins and nucleic acids. Cell death results, occuring through the two distinct pathways: necrosis and apoptosis (1-3).

Apoptosis, or programmed cell death, occurs during normal or physiopathological conditions and constitutes a common pathway for cell replacement, tissue remodeling and removal of damaged cells (5, 6). It is a complex process characterized by nuclear chromatin condensation, DNA fragmentation, cell shrinkage and formation of apoptotic bodies. At present, and in addition to the morphological features, apoptosis is defined by complex biochemical processes involving mitochondria and the activation of a family of cysteine aspartic acid-specific proteases (caspases) (7, 8). Apoptosis can be triggered by different stimuli such as glucocorticoids, chemotherapeutic agents, biological stimuli and ionizing radiation (9).

Caspases play an essential role in apoptosis. The caspase family is divided into three subfamilies. The first subfamily of interleukin-1β converting enzyme (ICE, ICE-like proteases) includes caspase 1, caspase 4 (ICE-related-II), caspase 5 (ICE-

related III), and caspase 11, caspase 12 and caspase 13. The second subfamily (Ced-3) includes caspase 3 (CPP32), caspase 6 (Mch2), caspase 7 (Mch3), caspase 8 (Mch5, FLICE), caspase 9 (Mch6, ICE-LAP6) and caspase 10 (Mch4), while the third subfamily includes only caspase 2 (Nedd2) (10, 11).

Two major pathways leading to the activation of the caspase cascade have been identified. The first one is initiated by engagement of death receptors such as Fas or tumor necrosis factor receptors, activating caspase 8. Caspase 8 in turn activates downstream caspases, such as caspase 3, and has been shown to be required for DNA fragmentation and morphological changes in apoptosis. The second pathway involves disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$) with liberation of cytochrome c into the cytosol. Together with Apaf-1 and procaspase 9, this leads to activation of caspase 9, which in turn activates downstream caspases. These enzymes act either directly or indirectly on a wide number of substrates such as structural proteins, proteins related to the cell cycle, DNA cleavage and DNA repair, as well as transcription and translation factors (7-10).

The caspases can be blocked by natural inhibitors such as cytokine response modifier enzyme A, p35 from Baculovirus and several inhibitors of apoptosis, or by synthetic inhibitors, which are variably specific for the different caspases: Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone) is considered a general caspase inhibitor, Z-DEVD-FMK (benzyloxycarbonyl-Asp-Glu-Val-Asp-(OMe)-fluoromethyl ketone), a selective inhibitor of caspases 3, 7 and 10, and Z-LEHD-FMK (benzyloxycarbonyl-Leu-Glu-(OMe)-His-Asp-fluoromethyl ketone), a preferential inhibitor of caspase 9 (11).

γ-Irradiation induces apoptosis in thymocytes by activation of caspase 3 and caspase 6, and mitochondrial damage. Similar findings have been reported in other cells (12), but there is little information about apoptosis induction by γ-irradiation of monocyte-macrophages, which are highly radioresistant cells and play an important role in the innate and specific immune responses, as well as in inflammatory and homeostatic functions (13, 14).

We recently reported that adriamycin, an antitumoral drug, induces apoptosis in peritoneal macrophages and that apoptosis can be inhibited by antioxidant pretreatment, suggesting participation of ROS in this system (15).

In this study, the effects of γ-irradiation on murine peritoneal macrophages were examined both *in vivo* and *in vitro*.

Materials and Methods

Animals. Balb/c (H-2d) 6-week-old male mice were obtained from our breeding facilities at the Centro de Investigacion Biomédica de Occidente, IMSS (Guadalajara, Jalisco, México).

Irradiation. A ^{60}Co-bomb (Departamento de Oncologıa, Centro Médico Nacional de Occidente, IMSS), which delivers a dose of 352 centigrays per minute at a distance of 80 cm, was used.

For *in vivo* studies, mice received different doses of total body γ-irradiation (1, 2, 4, 6, or 8 Gy). Peritoneal macrophages (PM) were harvested from the mice at different times to determine the optimal conditions for apoptosis induction. For *in vitro* studies, cultures containing 1x10^6 PM/mL received a single dose (8 Gy) of γ-irradiation and were put back in the incubator. The cells were harvested every day for 5 days.

Culture medium. RPMI-1640 (R-6504, Sigma, St. Louis, MO, USA) tissue culture medium was supplemented with 10% fetal calf serum (S-2442, Sigma), 10% glutamine (G-6392, Sigma) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, Sector Salud, México 1921). This medium is referred to as RPMI-S.

Peritoneal macrophage cell suspensions after in vivo irradiation. Hank's solution without phenol red, pH 7.4 at 4˚C, was injected into the peritoneal cavity of mice, which was slightly massaged. The peritoneal cell suspension was then aspirated, centrifuged, washed and centrifuged on Ficoll-Hypaque density gradients to obtain mononuclear cells. Contaminating lymphocytes were eliminated by treatment with anti-T and anti-B antibodies plus complement (15). The final cell suspension contained more than 93% of PM as identified by non-specific esterase coloration and phagocytosis. Viability, confirmed by the Trypan blue (Sigma, México 468) exclusion test, was more than 95%.

Peritoneal macrophage cell suspensions for in vitro irradiation. To obtain a large number of PM, mice were *i.p.* injected with 1 mL of sterile fluid thioglycollate broth (Merck, México 15867). Four days later, peritoneal cell exudates were obtained by washing the abdominal cavity as described earlier, the PM were washed 3 times in the same solution and resuspended at a density of 1x10^6 live cells/mL in RPMI-S culture tissue medium. One mL of PM suspension was put in culture using a 8-well plate (Costar, Mexico) in which a sterile coverslip was placed to allow mononuclear phagocytes to adhere and incubated at 37˚C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, non-adherent cells were eliminated by aspiration and the wells were washed 3 times with RPMI-1640 tissue culture medium at 37˚C and resuspended in 1 mL of RPMI-S. Those cultures contained 98% purified macrophages as determined by phagocytosis and non-specific esterase staining. Viability was confirmed by Trypan blue exclusion and was >96%. The cells were then irradiated. After irradiation, the medium was aspirated and the cells resuspended in 1 mL of fresh RPMI-S medium at 37˚C, the PM were cultured in similar conditions and, at the indicated times, the coverslips were recovered for the apoptosis determinations.

Apoptotic, live and necrotic index assessment. The cells were stained with ethidium bromide (E-8751, Sigma) and acridine orange (A-2886, Sigma) (100 μg/mL each in phosphate-buffered saline). Acridine orange visualizes cells which have undergone apoptosis, while ethidium bromide differentiates between viable and non-viable cells. Two hundred cells were counted and the number of each of the following 4 cellular states recorded: i) live cells with normal nuclei (LN), bright green chromatin and organized

structure; ii) apoptotic cells (A) with highly condensed or fragmented bright green-yellow chromatin; iii) dead cells with normal nuclei (DN), bright red chromatin and organized structure and iv) dead cells with apoptotic nuclei (DA) and bright orange chromatin, which was highly condensed and fragmented. The indices of various cellular states were calculated as follows (15):
Apoptotic index (AI): A + DA / LN + A + DN + DA x 100
Live cells index (LCI): LN / LN + A + DN + DA x 100
Necrotic cells index (NCI): DN / LN + A + DN + DA x 100

Mitochondrial membrane potential assessment ($\Delta\psi_m$). Disruption of $\Delta\psi_m$ is one of the earliest intracellular events that occur following the induction of apoptosis. The MitoCapture™ Apoptosis Detection Kit (K250-100, Biovision, USA) provides a simple, fluorescent-based method to distinguish healthy and apoptotic cells by detecting changes in the $\Delta\psi_m$. The manufacturer's instructions were followed. The kit uses a cationic dye that fluoresces differentially in healthy and in apoptotic cells. Two hundred cells were counted and the number of each of the following 2 cellular states recorded: i) in live cells (LC), dye accumulates and polymerizes in the mitochondria, giving a bright red fluorescence; ii) in apoptotic cells (AC), dye cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus remains in the cytoplasm in its monomeric form, giving a green fluorescence (16). Fluorescent signals can easily be detected with fluorescence microscopy using a band-pass filter. The indices of various cellular states were calculated as follows:
Apoptotic index (AI): AC / LC + AC x 100
Live cells index (LCI): LC / LC + AC x 100

DNA isolation and analysis. To visualize the characteristic DNA "ladder pattern" of apoptosis (180-300 bp), PM DNA was prepared according to the Gustincich method (17). Briefly, the PM were lysed, denatured and deproteinized. DNA was precipitated and run in 2% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed with UV transillumination. A molecular DNA weight marker kit (15628-019, Gibco, USA) with 100 bp fragmentation was employed as a positive control.

Lipoperoxide determination. To confirm the existence of oxidative stress in our experimental conditions, lipoperoxides were measured in the serum of control normal and of 8-Gy γ-irradiated mice, by a colorimetric assay (675 nm) with a commercial kit (K-ASSAY LPO-CC, Kamiya Biomedical Company CC-004, Seattle, WA, USA), following the manufacturer's instructions (18). The data are expressed in nM/mL of total lipoperoxides in serum.

Antioxidant protocols for studies after in vivo irradiation. (+)-α-tocopherol acid succinate (Vit. E, T-3126, Sigma) was dissolved in olive oil and given *per os* at a dose of 10 and 30 international units (IU)/Kg of body weight during 3 consecutive days before irradiation. Lipopolysaccharide (LPS) from *E. coli* (O55:B5, L-4005, Sigma) was given as a single dose of 0.15 μg/mouse, 24 hours before irradiation. Superoxide dismutase (SOD, S-2515, Sigma) was administered once at a dose of 3000 or 5000 IU/mouse, one hour before irradiation. Catalase (CAT, C-9322, Sigma) from bovine origin was injected at doses of 1, 2 or 3 mg/Kg one hour before irradiation. LPS, SOD and CAT were dissolved in Hank's solution without phenol red, pH 7.4, immediately before administration and injected *i.p.* in a volume of 0.2 mL.

Antioxidant protocols for studies after in vitro irradiation. Trolox (Vit. E analog soluble, 648471, Calbiochem, USA) was dissolved in 1M $NaHCO_3$ at a concentration of 300 mM and the pH was adjusted to 7.0, aliquoted and stored at –20˚C for less than 8 days. At the time of the test, Trolox, LPS, SOD and CAT were dissolved in Hank's solution without phenol red, pH 7.4, and added to the cultures 1 hour before γ-irradiation, in a standard volume of 0.1 mL to reach a final concentration of 10 mM, 0.15 μg/mL, 250 IU/mL and 1 mg/mL, respectively. To confirm the *in vivo* and *in vitro* specificity of the antioxidants, LPS, SOD and CAT were inactivated by 3 cycles of heat (120˚C for 30 minutes) and frozen; (+)-α-tocopherol acid succinate and Trolox were inactivated by heat at 250˚C for 30 minutes.

Caspase inhibitors administration. Inhibition of apoptosis induction was tested by the addition to the cell cultures of several caspase inhibitors: Z-VAD-FMK, (benzyloxycarbonyl-Val-Ala-Asp-(Ome)-fluoromethyl ketone), a non-specific caspase inhibitor, (170-3196, Biorad, USA); Z-DEVD-FMK (benzyloxycarbonyl-Asp-Glu-Val-Asp-(Ome)-fluoromethyl ketone), a selective inhibitor of caspases 3, 7 and 10, (170-3180, Biorad); and Z-LEHD-FMK (benziloxycarbonyl-Leu-Glu-(Ome)-His-Asp-fluromethyl ketone), a preferential caspase 9 inhibitor (170-3192, Biorad). The caspase inhibitors were first dissolved in 100 μl of dimethyl sulfoxide (DMSO) (D-8418, Sigma) and stored at –80˚C for less than 8 days. They were then diluted in RPMI-S tissue culture medium and added 1 hour before irradiation to the PM cultures in a standard volume of 5 μL to reach a final concentration of 1, 10 and 25 μM/mL. All the wells with cell suspension which were not treated with caspase inhibitors were incubated in the presence of the same concentration of DMSO solution.

Statistical analysis. The results were expressed as the mean±standard deviation (SD). For the *in vivo* experiments, each group was composed of at least 7 independent observations. The *in vitro* experiments were carried out in duplicate and repeated at least 3 times. To determine any statistical significance between the groups, the Student's *t*-test was used. For the dose-response, the linear regression was also calculated.

Results

In vivo experiments

In vivo kinetic and dose-response curves: Mice were irradiated to determine how apoptosis appears in PM. The dose-response and its kinetics after a dose of 8 Gy were studied. The PM were harvested every 24 hours during the 8 days after γ-irradiation.

One and 2 days after irradiation, no morphological or molecular characteristics of apoptosis were seen. Three and 5 days after irradiation, cells with morphological characteristics of apoptosis were present. Eight days after irradiation, the apoptosis index (AI) was 59.6±10.0 and the observed DNA electrophoretic pattern was characteristic of apoptosis (Table IA). Therefore, all subsequent experiments were performed at 8 days post-γ-irradiation.

The radiation doses were varied from 1 to 12 Gy and the AI, live cell index (LCI) and necrotic cell index (NCI)

Table IA. *In vivo apoptosis induction by irradiation: kinetics of its development in peritoneal macrophages after 8-Gy irradiation.*

Days after γ-irradiation	γ-irradiation	Apoptotic index (mean±SD)	DNA pattern
0	–	4.7±1.0	G
1	+	1.5±0.5	G
2	+	8.0±0.5	G
3	+	25.1±12.5◆	G
4	+	30.0±5.3◆	G
5	+	31.4±10.6◆	G
8	+	59.6±10.0◆	L

Table IB. *In vivo apoptosis induction by irradiation: dose-response curve of its development in peritoneal macrophages at 8 days.*

Irradiation dose	Apoptotic index	Live cell index	Necrotic cell index	DNA pattern
0	4.7±1.0	94.0±7.4	3.2±5.9	G
1 Gy	3.0±2.4	94.2±6.0	5.7±4.3	G
2 Gy	13.8±5.5	80.0±5.0	6.1±3.0	G
4 Gy	14.2±9.0	59.0±34.6	13.5±16.5	G
6 Gy	35.4±4.9	36.7±9.5	26.3±8.5	G
8 Gy	59.6±10.0◆	19.4±5.7◆	20.5±6.4◆	L
12 Gy	50.0±10.2◆	20.6±6.8◆	30.2±6.7◆	L

Mice were irradiated at different doses and the PM harvested on the indicated days. Apoptosis was measured morphologically by ethidium bromide and acridine orange staining and by DNA electrophoretic analysis. G: genomic, L: ladder pattern. The results are expressed as the mean±standard deviation (SD) of 7 independent observations. Student's *t*-test: ◆$p<0.001$ *vs.*, respectively, non-irradiated control group.

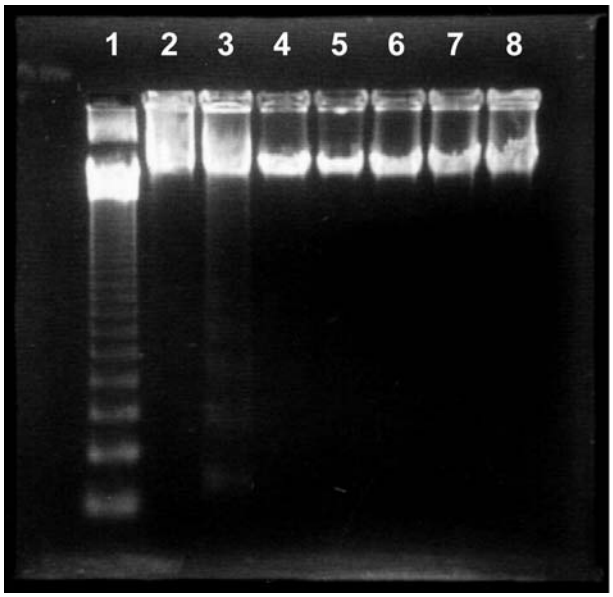

Figure 1. *Lane 1: commercial kit 100 p.b, Lane 2: DNA from non-irradiated group, Lane 3: 8 days after irradiation, Lane 4: 1 day, Lane 5: 2 days, Lane 6: 3 days, Lane 7: 4 days; Lane 8: 5 days. Kinetics of the in vivo apoptosis induction in PM by irradiation: DNA electrophoretic analysis.*

determined (Table IB). The dose of 1 Gy irradiation did not induce appreciable apoptosis. When the irradiation dose was 2, 4 or 6 Gy, the AI increased significantly, but the DNA stayed genomic. In mice irradiated with 8 and 12 Gy, the AI was 59.6±10.0 and 50.0±10.2, respectively ($p<0.01$ *vs.*, non-irradiated control). In those two groups, the electrophoretic DNA ladder pattern was characteristic of apoptosis (Figure 1).

Table IB also shows that, after the 8- and 12-Gy doses, the LCI decreased from 94.0±7.4 (non-irradiated control) to 19.4±5.7 and 20.6±6.8, respectively. In contrast, the NCI increased from 3.2±5.9 (non-irradiated control) to 20.5±6.4 and 30.2±6.7, respectively. Linear regression showed a positive correlation between the radiation dose and the AI ($r=0.92$, $p<0.001$) and between the radiation dose and the NCI ($r=0.88$, $p<0.01$), and a negative correlation between the delivered radiation dose and the LCI ($r=-0.95$, $p<0.001$). In conclusion, significant apoptosis development was observed in PM, 8 days after 8 Gy delivery. These experimental conditions were used for the subsequent studies.

In vivo inhibition of apoptosis by antioxidants: Because ionizing radiation produces ROS, which (among other effects) induce DNA injury and provoke apoptosis *in vitro*, we tested whether, in our experimental conditions, antioxidants given *in vivo* before irradiation could protect against apoptosis. In the first series of antioxidant experiments, either 3000 or 5000 IU/mouse SOD was injected *i.p.* 1 hour before whole body irradiation of the mice. PM were harvested from irradiated and non-irradiated mice, and from SOD-treated irradiated and non-irradiated mice. The AI of PM for irradiated and 3000 and 5000 IU SOD-treated mice were 5 and 25 times lower than the AI for irradiation-only mice (AI=59.6±4.4, $p<0.001$) and was not statistically different from the AI for non-irradiated mice treated with 3000 and 5000 IU SOD or untreated mice (AI=5.0±1.1) (Table II). In addition, in both groups treated with SOD and 8 Gy of ionizing radiation, not only was an inhibition of the AI in the PM from irradiated mice observed, but also an increase of the LCI compared with that of the irradiated groups without SOD pretreatment (LCI=19.4±2.4, $p<0.001$). The integrity of the DNA confirmed the protection given by SOD; with both pretreatments (3000 or 5000 IU SOD), no ladder pattern was observed in the irradiated group.

Mice were also pretreated with CAT (1 mg/kg, 2 mg/kg or 3 mg/kg) given 1 hour before γ-irradiation. The AIs of PM from CAT-pretreated irradiated mice were 6.9, 3.6 and

Table II. *In vivo apoptosis induction by irradiation (8 Gy): modifications after in vivo antioxidants administration.*

Treatment	Irradiation	Apoptotic index	Live cell index	Necrotic cell index	DNA pattern
-	–	5.0±1.1	90.7±4.9	4.2±1.4	G
-	+	59.6±4.4	19.4±2.4	20.4±6.4	L
SOD 3000 IU	+	8.5±4.2◆	51.3±9.0◆	35.1±2.0●	G
5000 IU	+	2.4±1.8◆	65.7±3.9◆	20.0±4.0	G
3000 IU	–	9.5±3.7	77.3±14.4	8.5±4.3	G
5000 IU	–	2.8±1.2	68.5±12.2	21.5±14.3	G
5000 IU*	+	57.8±3.2	23.2±4.1	18.8±3.5	L
CAT 1 mg/Kg	+	8.5±3.8◆	88.3±5.1◆	3.1±2.2◆	G
2 mg/Kg	+	16.4±3.6◆	75.2±3.8◆	8.4±2.7◆	G
3 mg/Kg	+	29.7±3.1◆	61.3±3.3◆	9.0±1.6◆	G
1 mg/Kg	–	2.7±1.8	92.3±5.2	5.5±2.1	G
2 mg/Kg	–	20.3±4.4	65.8±5.0	4.5±8.0	G
3 mg/Kg	–	36.8±3.3	42.2±6.1	21.0±3.4	G
1 mg/Kg*	+	59.3±2.3	20.0±5.5	22.0±3.5	L
VIT.E 10 IU/Kg	+	35.2±4.4◆	53.4±10.3◆	7.1±2.3●	G
30 IU/Kg	+	12.0±1.9◆	68.0±4.4◆	20.0±3.7	G
10 IU/Kg	–	6.1±2.2	85.7±6.8	8.7±5.4	G
30 IU/Kg	–	2.5±1.5	90.5±5.2	5.0±2.7	G
30 IU/Kg*	+	58.0±4.0	15.0±1.1	15.0±2.4	L
LPS 0.15 μg/mouse	+	25.4±4.9◆	62.0±4.4◆	7.4±16.3◆	G
0.15 μg/mouse	–	14.4±9.0	76.7±3.3	8.9±2.5	G
0.15 μg/mouse*	+	49.3±1.2	24.1±3.0	26.5±2.8	L

Mice were pretreated with antioxidant compounds before irradiation and PM harvested 8 days later. G: genomic, L: ladder pattern. Superoxide dismutase (SOD) and catalase (CAT) were given *i.p.* 1 hour before irradiation, lipopolysaccharide (LPS, 0.15mg/mouse) 24 hours before irradiation and (+)-α-tocopherol acid succinate (Vit E) was given for 3 days *per os* before irradiation. *: Heat-inactivated antioxidant. The results represent mean±standard deviation (SD) of at least 7 independent observations. Student's *t*-test ◆$p<0.001$, ●$p<0.05$, respectively, *vs.* irradiated group.

2.0 times lower than the AI of PM from irradiation-only mice (AI=59.6±4.4, $p<0.05$) and was not statistically different from the AI of PM from CAT-injected non-irradiated mice (Table II). However, the AIs were higher than the AI of PM from normal untreated mice (AI=5.0±1.1). The AIs of PM from non-irradiated, 2 mg/kg or 3 mg/kg CAT-injected mice were greater than that of normal PM ($p<0.001$), even though CAT induced protection in irradiated mice. Thus, the best results were observed in the group pretreated with 1 mg/kg of CAT. Interestingly, the higher doses of CAT decreased the LCI in non-irradiated mice, but increased it in γ-irradiated mice ($p<0.05$). A normal DNA electrophoretic pattern was observed in all the groups pretreated with antioxidants.

We then tested whether non-specific scavengers, such as vitamin E or LPS, could also inhibit apoptosis in PM from irradiated mice.

(+)-α-Tocopherol acid succinate was given to mice *per os* over 3 consecutive days before γ-irradiation. The AIs from mice treated with 10 or 30 IU/mouse vitamin E before irradiation were 1.7 and 5.0 times, respectively, lower than the AI of PM from the irradiation-only group of mice (AI=59.6±4.4, $p<0.05$) (Table II). (+)-α-Tocopherol acid succinate given alone to normal non-irradiated mice did not modify the AI of their peritoneal macrophages. The LCI of the (+)-α-tocopherol acid succinate irradiated group was greater than that of the untreated irradiated group. In all the groups treated with (+)-α-tocopherol acid succinate, the DNA pattern remained genomic, in contrast to the irradiated group (positive control), in which a DNA ladder pattern was present.

LPS was given *i.p.* at a dose of 0.15 μg/mouse 24 hours before irradiation. The AI of PM from LPS-injected irradiated mice was 3 times lower than that of the AI from irradiation-only mice (AI=59.6±4.4, $p<0.05$) and was not statistically different from the AI of PM from LPS-injected non-irradiated mice. The DNA pattern of the LPS-pretreated irradiated mice did not show a ladder pattern. The LCI was significantly higher in the LPS-irradiated group (LCI=62.0±4.4) than in the irradiation-only group (LCI=19.4±2.4, $p<0.001$).

To test the specificity of antioxidant inhibition, mice were given heat-inactivated antioxidants before irradiation. No significant differences were observed for AI, LCI and NCI compared with those of the irradiated mice, and a ladder pattern was observed on DNA electrophoretic analysis in each case. These results suggest the participation of ROS in the development of apoptosis in PM after irradiation of mice.

Serum lipoperoxide levels in in vivo-irradiated mice. It is well known that irradiation induces oxidative stress, which can be detected by measuring lipoperoxides (LPO). To confirm the presence of oxidative stress in our experimental conditions, LPO were determined in the sera of irradiated and non-irradiated mice. Eight days after 8-Gy irradiation, the serum LPO concentrations reached a level of 63.3± 2.2 nM/mL, 12 times higher than that of the non-irradiated mice (5.2±0.6, $p<0.001$) (Table III). Because the AI was lower in the groups of antioxidant pre-treated animals, the serum LPO concentration in animals pretreated with the different assayed antioxidants was measured. In mice pretreated with SOD (5000 IU/mouse *i.p.*) 1 hour before irradiation, the serum LPO concentration was 7 times lower ($p<0.001$) than that in control irradiated mice. In mice pretreated with CAT (1 mg/kg *i.p.*) 1 hour before irradiation, the LPO concentration was 14 times lower than that of irradiated mice ($p<0.001$). In mice pretreated with (+)-α-tocopherol acid succinate over 3 days (30 IU/mouse *per os*) before irradiation, the serum LPO concentration was 19 times lower ($p<0.001$) than that of irradiated mice. In

Table III. *Serum lipoperoxide levels from in vivo-irradiated mice pretreated with antioxidants.*

Pretreatment	Irradiation 8 Gy	Lipoperoxides nM/mL (mean±SD)
-	–	5.2±0.6
-	+	63.3±2.2
SOD 5000 IU/ Kg	+	8.8±1.3◆
CAT 1 mg/Kg	+	4.3±1.9◆
VIT E 30 IU/Kg	+	3.3±0.8◆
LPS 0.15 μg/Kg	+	36.7±0.3◆

Mice were pretreated and irradiated as described in Table II. Serum lipoperoxides were measured by a colorimetric assay (675nm) 24 hours after irradiation and expressed in nM/mL. The results represent mean±standard deviation (SD) of at least 7 independent observations. Student's *t*-test, ◆$p<0.001$ for each group *vs.* only irradiated group.

Table IV. *Modifications of irradiation in vitro apoptosis induced by addition of antioxidant compounds.*

Treatment	Irradiation 8 Gy	Apoptotic index	Live cell index	Necrotic cell index	DNA pattern
-	–	15.2±1.7	74.9±4.1	9.8±2.4	G
-	+	56.0±5.3	35.3±11.0	16.7±2.1	L
SOD 250 IU/mL	+	13.2±2.3◆	78.4±1.7◆	8.4±2.0	G
CAT 1 mg/mL	+	17.2±3.0◆	67.4±5.0◆	15.4±2.1	G
TROLOX 10mM	+	27.2±2.3◆	72.8±4.4◆	10.2±1.9	G
LPS 015 μg/mL	+	18.0±3.0◆	66.0±3.0◆	16.0±2.0	G
SOD 250 IU/mL*	+	52.2±2.0	27.6±5.3	18.8±2.3	L
CAT 1 mg/mL*	+	64.0±4.0	26.0±5.6	30.0±5.0	L
TROLOX 10 mM*	+	65.6±5.3	22.2±2.7	12.2±4.8	L
LPS 0.15 μ/mL*	+	57.8±2.4	25.0±3.4	17.2±2.9	L

Antioxidants were added to PM cultures 1 hour before irradiation. The cells were harvested 96 hours later. The results are expressed as the mean±standard deviation (SD) from 3 independent observations done in duplicate. *: heat-inactivated antioxidant. Student's *t*-test: ◆$p<0.001$ *vs.* irradiated group.

mice pretreated with LPS (0.15 μg/mouse *i.p.*) 24 hours before irradiation, the serum LPO was only 1.7 times lower than that of irradiated mice ($p<0.001$).

In vitro experiments

We attempted to confirm our results *in vitro* and to evaluate the role of caspases and of the disruption of $\Delta\psi_m$ in our observations.

Apoptosis in peritoneal macrophage cultures induced by irradiation: PM cultures received 8 Gy irradiation, the cells were harvested at different time-points (2, 24, 48, 96 and 120 hours) and the AI was evaluated morphologically by ethidium bromide and acridine orange staining, and by electrophoretic DNA analysis. The best experimental conditions were 96 hours after irradiation (AI=56.0±5.3, as indicated by the DNA ladder pattern).

To investigate whether antioxidants protect *in vitro* from the induction of apoptosis by irradiation, the following antioxidants were added to the peritoneal macrophages cultures 1 hour before irradiation: SOD (250 IU/mL) or CAT (1 mg/mL), or Trolox, vitamin E soluble analog (10 mM final concentration) or LPS (0.15 μg/mL). The AI, LCI and NCI were evaluated. The AI was 15.2±1.7 in non-irradiated cells and 56.0±5.3 in 8-Gy-irradiated cells ($p<0.001$) (Table IV). In the presence of antioxidants, the AI was similar to the AI of the non-irradiated group, except in the presence of Trolox, which did not completely control apoptosis development: the AI was only half (27.2±2.3) that observed in irradiated cultures, but was higher than in the non-irradiated control group. The LCI and NCI in antioxidant-treated irradiated cells were also similar to those of non-irradiated cells. The DNA ladder pattern was only seen in irradiated cells. The specificity of antioxidants was tested in PM cultures incubated with inactivated antioxidant compounds: values were similar to those observed in irradiated PM cultures, and a DNA ladder pattern was observed, confirming the loss of antioxidant protection.

In vitro apoptosis inhibition by caspases: To study caspase participation, PM cultures were irradiated with 8 Gy and incubated 1 hour later with 3 different inhibitors (general caspase inhibitor, caspases 3, 7 and 10-specific inhibitor, or caspase 9-specific inhibitor) at 3 concentrations (1, 10, or 25 μM). The best results were obtained at the 10 μM caspase inhibitor concentration (only shown).

On the fourth day after irradiation, the 3 assayed caspase inhibitors induced an important diminution of the AI (Table V). The AI of the irradiated PM culture was 56.0±5.3, the AI of cells cultured in the presence of caspase inhibitors was 3 to 3.5 times lower than the AI of the irradiated group. The LCI of the irradiated group (LCI=35.3±11.0) was not modified by the addition of the general caspase inhibitor. In cultures pretreated with the caspases 3, 7 and 10 group inhibitor or the caspase 9 inhibitor, the LCI was 1.9 times higher than that of the non-treated irradiated cells ($p<0.05$). The addition of the general inhibitor increased the NCI, which was 2.5 times higher than for the irradiated group (NCI=16.7±2.1, $p<0.001$). With the other two caspase inhibitors, the NCI was similar to that of the irradiated group. The electrophoretic DNA analysis confirmed the inhibition by caspase inhibitors of irradiation-induced

Table V. *Modifications of irradiation – in vitro – induced apoptosis by caspase inhibitors.*

Treatment	Irradiation 8 Gy	Apoptotic index	Live cell index	Necrotic cell index	DNA pattern
-	–	8.7±2.7◆	72.6±3.0◆	18.7±4.2	G
-	+	56.0±5.3	35.3±11.0	16.7±2.1	L
General	+	18.0±4.2◆	38.5±5.4	41.5±12.3◆	G
3, 7, 10	+	18.0±3.4◆	68.0±3.5◆	14.0±6.0	G
9	+	16.0±4.0◆	68.0±5.0◆	16.0±4.0	G

One hour before irradiation, anticaspase inhibitors diluted in DMSO were added to PM cultures, at a final concentration of 10 μM. The control group was incubated under the same conditions with DMSO. The results are expressed as the mean±standard deviation (SD) of 3 independent observations done in duplicate. Student's *t*-test: ◆$p<0.001$ *vs.* irradiated group.

Table VI. *Mitochondrial membrane potential ($\Delta\psi_m$) modification by caspase inhibitors from in vitro-irradiated peritoneal macrophages.*

Caspase inhibitor	Irradiation 8 Gy	Time after irradiation Apoptotic index mean±standard deviation				DNA pattern
		2 hours	1 day	2 days	4 days	
-	–	0	10.5±3.3	17.5±5.4	25.2±4.6	G
-	+	40.5±4.9	54.6±4.8	61.5±7.4	75.0±2.7	L
General	+	1.7±0.9◆	19.0±2.4◆	24.8±4.2◆	38.5±2.6◆	G
3, 7, 10	+	1.0±0.8◆	20.2±2.1◆	23.5±4.4◆	34.0±1.9◆	G
9	+	2.5±1.0◆	17.7±2.6◆	46.5±5.1	50.2±5.6	G

Caspase inhibitors were added to peritoneal cell cultures, 1 hour before irradiation at a 10 μM concentration in DMSO. The control group was incubated under the same conditions with DMSO. The results are expressed as the mean±standard deviation from 3 independent experiments done in duplicate. Student's *t*-test:◆$p<0.05$ *vs.* irradiated group.

apoptosis: a DNA ladder pattern was observed only in the irradiated control group, whereas in the non-irradiated, or in irradiated and caspase inhibitor-treated groups, the DNA pattern was genomic.

Oxidative stress induced apoptosis through disruption of mitochondrial transmembrane potential ($\Delta\psi_m$): In thymocytes, *in vitro* ionizing radiation treatment induces disruption of $\Delta\psi_m$ before nuclear signs of apoptosis appear (19). Therefore, the mitochondrial membrane integrity was studied under our experimental conditions. PM cultures were irradiated as previously described. $\Delta\psi_m$ was determined by MitoCapture™ staining and related to apoptosis measured by the ethidium bromide and acridine orange technique.

The AI of non-irradiated cells was null 2 hours after the beginning of the culture (Table VI). Four days later, the AI had increased to 25.2±4.6. The AI of irradiated cells was 40.5±4.9 2 hours after irradiation, and on days 1, 2 and 4, the AI had increased to 54.6±4.8, 61.5±7.4 and 75.0±2.7, respectively. When caspase inhibitors were added 1 hour before irradiation, a significant inhibition of AI was observed, being the highest 2 hours after irradiation. In the presence of the general inhibitor, the caspases 3, 7 and 10 group inhibitor and caspase 9 inhibitor, the AIs were 23.8, 40.0 and 16.2 times, respectively, lower than the AI from only irradiated cells (95%, 97% and 93% of the value of irradiated cells). On day 4 after irradiation, the AIs were 1.9, 2.2, and 1.5 times, respectively, lower than the AI from only irradiated cells ($p<0.05$).

The two methods used for apoptosis evaluation [ethidium bromide and MitoCapture™ ($\Delta\psi_m$)] were compared (Table VII). Although the results were similar, $\Delta\psi_m$ was more sensitive. In both groups, the highest values were observed 4 days after irradiation.

Table VII. *Apoptosis comparison by ethidium bromide+acridine orange and MitoCapture dye.*

Technique	Apoptotic index Mean±standard deviation				
	10 min	30 min	1 day	2 days	5 days
Ethidium bromide + acridine orange	4.5±2.5	9.5±2.5	10.3±2.3	31.6±1.5	56.0±5.2
MitoCapture	19.0.±2.3	25.0±2.7	54.6±4.8	61.5±7.4	75.0±2.7

PM cultures were exposed to irradiation (8 Gy) and the cells were harvested at different times. Apoptosis was determined either by ethidium bromide and acridine orange or by MitoCapture staining.

Discussion

Apoptosis induction by irradiation in normal and tumor cells has been previously reported. Most studies have been done *in vitro* with thymocytes and lymphocytes (20, 21), but not with macrophages. These cells play an important role in the immune response and in host homeostasis, inflammation and elimination of tumor cells (14). PM are among the most radioresistant cells: after 100 Gy irradiation, chemotactism and phagocytosis are not modified and their capacity to metabolize, degrade and present antigens is maintained (22).

Resident PM are an easy cell population to harvest: they are quiescent cells and present basal characteristics, different from those of inflammatory or activated macrophages, and are easy to identify (14). For *in vitro* studies, inflammatory macrophages were used mainly to obtain a higher number of cells. A similar sensitivity to irradiation was noted for resident and inflammatory macrophages.

Two different methods were used to evaluate apoptosis: one based on ethidium bromide and acridine orange staining which detects delayed apoptosis (chromatin alterations) and distinguishes between live and necrotic cells; the other based on MitoCapture™ dye fluorescent microscopy to assess the disruption of the $\Delta\psi_m$, indicating the release of cytochrome c by mitochondria, one of the early steps in apoptosis (15, 16).

Apoptosis was confirmed by the presence of a DNA ladder pattern by electrophoresis when the AI was higher than 50. If the AI of the irradiation-treated groups was close to that of the non-irradiated control group and the electrophoretic DNA pattern was genomic, then the cells were considered to be protected from apoptosis by the antioxidant or as having apoptosis inhibited by a caspase inhibitor. This was the case with all the antioxidants and caspase inhibitors we studied.

Three indices were calculated: apoptotic (AI), live cell (LCI) and necrotic cell (NCI) indices (Table IB).

They were affected by antioxidant treatment, suggesting that ROS play a role in apoptosis. Apoptosis may be considered as an adaptive response mechanism to irradiation that reduces the risk of proliferation of genetically-damaged cells (23).

Part of the cell injury after irradiation is explained by the generation of ROS, which alter cellular membranes, inducing lipoperoxidation, damage to mitochondria, nucleic acid mutations and fragmentation, and activation of oncogenes (1-3, 9, 24). Thus, antioxidants may protect at different levels (25).

In vivo, antioxidants were given before animal exposure to irradiation to increase the cellular antioxidant defenses before aggression. The antioxidant doses and protocols have been shown to be effective in other models (26). The 4 antioxidants tested inhibited apoptosis and increased LCI in a dose-dependent manner, showing a protective effect.

Because the mechanisms of action of the 4 antioxidants are different, we suggest that several ROS could be responsible for the induction of apoptosis in our model.

In many biological systems, superoxide anion $O^{\bullet -}_2$ is the first generated radical. SOD transforms it into H_2O_2 and molecular oxygen (27). An important reduction in the AI of peritoneal macrophages from irradiated mice ($p<0.001$) was observed after pretreatment with SOD, indicating participation of $O^{\bullet -}_2$ in the induction of apoptosis. Troy and Shelanski have also reported that a decrease in SOD in PC12 cells to less than 40% of constitutive levels results in rapid death by apoptosis (28). The protective role of SOD against the induction of apoptosis has been reported in mice given anthracyclins (aclacinomycin and adriamycin) (29, 30). Cardiotoxicity is induced by these anthracyclins, due to the generation of ROS. The morphological degenerative changes seen in PM can be inhibited when SOD is given 1 hour before the anthracyclin injection. Autoradiography analysis showed that SOD is captured by PM, suggesting that its protection takes place at the intracellular level (29, 30).

H_2O_2 formed by $O^{\bullet -}_2$ dismutation can also induce apoptosis (31). The toxicity of H_2O_2 was controlled by pretreating the animals with catalase, which transforms H_2O_2 into water and molecular oxygen (32). In this case, a lower AI and higher LCI were observed. We cannot exclude that the better result obtained with the smaller dose of catalase (1 mg/kg) is due to some toxicity of the enzyme, either to its bovine origin or the dose injected. The LCI for PM from non-irradiated mice injected with the highest dose of catalase was lower than the LCI from untreated mice. The AI for PM from catalase-injected (2 or 3 mg/kg) normal mice was higher than for non-treated mice. The apoptosis-inducing activity of catalase may be related to its inhibitory activity on heat shock proteins and transcription factors (33, 34).

(+)-α-Tocopherol acid succinate was the best of our tested antioxidants. A dose-dependent protection was observed, probably due to the ROS inhibition of lipoperoxidation, conferring protection to DNA by hindering the propagation of $OH^{\bullet -}$ radicals (35, 36). It has recently been reported that lipid peroxidation may be an important signaling event that triggers apoptosis in irradiated cells (37).

Finally, LPS, a potent macrophage stimulator (29, 30), is known to have radioprotective capacity (38). The LPS protocol we used stimulates the immune response and protects the host against apoptosis induced by adriamycin in several tissues (15, 29-31). Mice pretreated with LPS 24 hours before adriamycin injection were not only protected against lethal doses of adriamycin, but also showed enhanced immune reactivity. LPS radioprotection is due to interleukin 1 (IL-1) formation induced by LPS stimulation (39, 40).

Because ROS generate LPO when they interact with lipids in cellular membranes (3), we measured them in mice serum and observed that radiation-induced oxidative stress was strongly reduced by the antioxidant pretreatment. The best protection was given by (+)-α-tocopherol acid succinate and catalase (30 UI/mouse per os over 3 consecutive days, and 1 mg/kg 1 hour before irradiation, respectively). These results confirm the oxidative stress present from ROS in our experimental conditions and the protective role of antioxidant compounds. All the results were confirmed *in vitro*.

Caspase inhibitors suppressed radiation-induced apoptosis in PM cultures, allowing us to confirm that the caspase cascade is activated by radiation. The main caspase that was activated was caspase 9. In the caspase activation pathway, caspase 9 is responsible for the activation of caspase 3, caspase 7 and caspase 10 (7, 8).

$\Delta\psi_m$ was disrupted as early as 2 hours after irradiation, and was maximal after 4 days of culture. The caspase inhibitor effects were more potent early and decreased progressively. The enzyme inhibitors may be inactivated by time. Caspase 3 inhibitors showed the same inhibition degree as in the previous experiment, also suggesting that the first target of irradiation is the mitochondria, disrupting $\Delta\psi_m$, cytochrome c release and caspase 9 activation, producing the irreversible events of the apoptotic process (caspase-dependent pathways).

Our results show that γ-irradiation-induced apoptosis in PM is associated with the generation of reactive oxygen species both *in vivo* and *in vitro*, with loss of $\Delta\psi_m$ and with the participation of caspase 3 and caspase 9. These observations may have further clinical application.

References

1 Mishra KP: Cell membrane oxidative damage induced by gamma-radiation and apoptotic sensitivity. J Environ Pathol Toxicol Oncol *23*: 61-66, 2004.

2 Riley PA: Free radicals: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol *65*: 27-33, 1994.

3 Selcuk P, Abacioglu U, Sun I, Konya D, Yüksel M and Pamir NM: Prophylactic effects of magnesium and vitamin E in rat spinal cord radiation damage: evaluation bases on lipid peroxidation levels. Life Sci *75*: 523-530, 2004.

4 Prithivirajsingh S, Story MD, Bergh S A, Geara FB, Ang KK, Ismail SM, Stevens CW, Buchholz TA and Brock WA: Accumulation of the common mitochondrial DNA deletion induced by ionizing radiation. FEBS Lett *571*: 227-232, 2004.

5 Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol *68*: 251-306, 1980.

6 Opferman JT and Korsmeyer SJ: Apoptosis in the development and maintenance of the immune system. Nat Immun *4*: 410-415, 2003.

7 Hengartner MO: The biochemistry of apoptosis. Nature *407*: 770-776, 2000.

8 Cohen GM: Capases: the executioners of apoptosis. Biochem J *326*: 1-16, 1999.

9 Cohen GM, Xiao MS, Roger TS, Michael GO and David D: Identification of a transitional preapoptotic population of thymocytes. J Immunol *151*: 566-574, 1993.

10 Shi Y: Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell *9*: 459-470, 2002.

11 Abraham MC and Shai S: Death without caspase, caspases without death. Trends Cell Biol *14*: 1984-1993, 2004.

12 Ferrer I: Role of caspase in ionizing radiation-induced apoptosis in the developing cerebellum. J Neurobiol *41*: 549-558, 1999.

13 Ma J, Chen T, Mandelin J, Ceponis A, Miller NE, Hukkanen M, Ma GF and Konttinen YT: Regulation of macrophages activation. Cell Mol Life Sci *60*: 2334-2346, 2003.

14 Patrick GJ and Evans Stephen RT: Intraperitoneal macrophages and tumor immunity: a review. J Surg Oncol *75*: 146-154, 2000.

15 Dominguez-Rodriguez JR, Gomez-Contreras P, Hernandez-Flores G, Lerma-Diaz JM, Carranco A, Cervantes-Munguia R, Orbach-Arbouys S and Bravo-Cuellar A: *In vivo* inhibition by antioxidants of adriamicyn-induced apoptosis in murine peritoneal macrophages. Anticancer Res *21*: 1869-1872, 2001.

16 Ogawa Y, Akihito N, Toshihiro KY, Shinji K, Shinji H, Toshiji S, Koichi N, Harumichi S and Shoji Y: Mitochondrial cytochrome c release in radiation-induced apoptosis of human periferial T cell. Int J Mol Med *10*: 263-268, 2002.

17 Gustincich S, Manfiolelli G, Del Sal G, Scheider C and Carminci P: A fast method for high-quality genomic DNA extraction from whole human blood. Biotechniques *11*: 298-300, 1991.

18 Cervantes-Munguia R, Valle-Razo F, Gomez-Contreras, Hernandez-Flores G, Orbach-Arbouys S and Bravo-Cuellar A: Lipoperoxydes sériques chez la femme enceinte et le nouveau-né à terme et prématuré. Presse Med *29*: 788, 2000.

19 Chen Y, Stanford A, Simmons RL, Ford H and Hoffman RA: Nitric oxide protects thymocytes from γ-irradiation-induced apoptosis in correlation with inhibition of p53 upregulation and mitochondrial damage. Cell Immunol *214*: 72-80, 2001.

20 Schmitz A, Bayer J, Dechamps N and Thomas G: Intrinsic susceptibility to radiation-induced apoptosis of human lymphocyte subpopulations. Int J Radiat Oncol Biol Phys *57*: 769-778, 2003.

21 Sellins KS and Cohen JJ: Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J Immunol *39*: 3199-3204, 1987.

22 Neta R: Radiation effects on immune system. *In:* Immunological Encyclopedia. Roitt IM and Delves PJ (eds.). Academic Press, London, pp. 123-1225, 1992.

23 David H: Regulation of T-cell apoptosis by reactive oxygen species. Free Rad Biol Med *36*: 1496-1504, 2004.

24 Jacobson MD: Reactive oxygen species and programmed cell death. TIBS *21*: 83-86, 1996.

25 Joya C, Afshin S and Sten O: Trigggering and modulation of apoptosis by oxidative stress. Free Rad Biol Med *29*: 323-333, 2000.

26 Weiss JF and Landauer MR: Protection against ionizing radiation by antioxidant nutrients and phytochemicals. Toxicology *189*: 1-20, 2003.

27 Hanaoka K, Sun D, Lawrence R, Kamitani Y and Fernandes G: The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis. Biophys Chem *107*: 71-82, 2004.

28 Troy CM and Shelanski ML: Down-regulation of copper/zinc superoxide dismutases cause apoptotic death in neuronal cells. Proc Natl Acad Sci USA *1*: 6384-6387, 1994.

29 Bravo-Cuellar A, Scott-Algara D, Metzger G and Orbach-Arbouys S: Enhanced activity of mouse peritoneal cells after aclacinomycin administration. Cancer Res *47*: 3477-3484, 1987.

30 Bravo-Cuellar A, Homo-Delarche F, Cabbanes J and Orbach-Arbouys S: Enhanced activity of murine peritoneal cells after aclacinomycin injection: characteristics of oxidative burst. Cancer Res *48*: 3440-3444, 1988.

31 Allen RG and Tresini M: Oxidative stress and gene regulation. Free Rad Biol Med *28*: 463-499, 2000.

32 Jonas N and Arnér Elias SJ: Reactive oxygen species antioxidants, and the mammalian thioredoxin system. Free Rad Biol Med *31*: 1287-1312, 2001.

33 Yanardag R, Bolkent S and Kizir A: Protective effects of DL-alpha-tocopherol acetate and sodium selenate on the liver of rats exponed to gamma radiation. Biolog Trace Elem Res *83*: 263-273, 2001.

34 Winterbourn CC, Vissers MC and Kettle AJ: Myeloperoxidase. Curr Opin Hematol *7*: 53-58, 2000.

35 Weiss JF and Landauer MR: Protection against ionizing radiation by antioxidant nutrients and phytochemicals. Toxicology *189*: 1-2, 2003.

36 Peker S, Abaciolgu U, Sun I, Kenya D, Yüksel M and Pamir NM: Prophylactic effects of magnesium and vitamin E in rat spinal cord radiation damage: evaluation based on lipid peroxidation levels. Life Sci *75*: 523-1530, 2004.

37 Manabu U, M Hiroki, Norimasa Y, Noriko F, Jun O, Naohisa M, Tomohisa T, Yuji N and Toshikazu Y: α-Tocopherol prevents apoptosis of vascular endothelial cells *via* a mechanism exceeding that of mere antioxidation. Eur J Pharmacol *456*: 29-37, 2002.

38 Neta R, Douches S and Oppenheim JJ: Interleukin 1 is a radioprotector. J Immunol *137*: 2486-2491, 1986.

39 Kaya M, Palanduz A, Kalayci R, Kemikler G, Simsek G, Bilgic B, Ahishali B, Arican N, Kocyldiz ZC, Elmas I, Kucuk M and Karadeniz A: Effect of lipopolysccharide on the radiation-induced changes in the blood-brain barrier and the astrocytes. Brain Res *1019*: 105-112, 2004.

40 Bravo-Cuellar A, Martın-Ruız JL, Lui XH and Orbach-Arbouys S: Enhanced production of interleukin-1 by mouse peritoneal macrophages after aclacinomycin administration. Immunol Lett *23*: 165-172, 1990.

Received January 13, 2005
Accepted May 30, 2005