

INFLUENCE OF A CONTINUOUS VERY LOW DOSE OF GAMMA-RAYS ON CELL PROLIFERATION, APOPTOSIS AND OXIDATIVE STRESS

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□ We have previously shown a delay of death by lymphoma in SJL/J mice irradiated with continuous very low doses of ionizing radiation. In order to understand the mechanisms involved in this phenomenon, we have irradiated *in vitro* the Raw264.7 monocytic and the YAC-1 lymphoma cell lines at very low-dose rate of 4cGy.month⁻¹. We have observed a transient increase in production of both free radicals and nitric oxide with a transient adaptive response during at least two weeks after the beginning of the irradiation. The slight decrease of Ki67 proliferation index observed during the second and third weeks of YAC-1 cells culture under irradiation was not significant but consistent with the shift of the proliferation assay curves of YAC-1 cells at these same durations of culture. These *in vitro* results were in good agreement with the slightly decrease under irradiation of Ki67 proliferative index evaluated on lymphomatous lymph nodes of SJL/J mice. A significant decrease of YAC-1 cells apoptotic rate under radiation appeared after 4 weeks of culture.

Therefore very small doses of gamma-irradiation are able to modify the cellular response. The main observations did not last with increasing time under irradiation, suggesting a transient adaptation of cells or organisms to this level of irradiation.

Key words: very low dose; gamma rays; cell proliferation; apoptosis; oxidative stress

INTRODUCTION

Many biological effects of low-dose radiation have been tested *in vitro*. Low-doses of radiation have been shown to affect normal cellular response, especially proliferation (Kim *et al.* 2007a, b). In recent years, growing data in the literature suggest that cell response to radiation depend on both dose and dose rate (Tubiana 2005; Ulsh 2012). The low-doses of irradiation inducible adaptive response is partly due to reactive oxygen species which induce an increased anti-oxidant production and/or enhancement of DNA repair (Matsumoto *et al.* 2004). However, adaptive response has been described with priming doses within the range of 1 cGy followed by a high dose in the range of 1 Gy. Thus, it remains

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unclear whether chronic exposure to a very low dose is able to induce an adaptive response (Tapio and Jacob 2007).

For several years, we have perfected a unique model of chronic irradiation to γ -rays able to deliver a continuous low-dose rate irradiation of 10cGy/year. Life-long irradiation of C57Bl/6J female mice was responsible for a delay of death by lymphoma (Courtade *et al.* 2002) although it had no effect on life span. A slight but not significant life lengthening was also observed using the lymphoma-prone mice SJL/J strain (Lacoste-Collin *et al.* 2007). However, at the end of both studies, the total number of lymphomas was identical to control, suggesting a transient “beneficial” effect of low-dose radiation. Different mechanisms may explain these results. Some parameters of cellular and humoral immunity showed variation upon irradiation in these mice (Courtade *et al.* 2001; Lacoste-Collin *et al.* 2007) though they were insufficient alone to explain these observations. The delay of death by lymphoma could be explained by a lower cell proliferation or by an adaptive response to low-dose radiation. Indeed, we observed a slight but not significant decrease in lymphoma cell proliferation in lymph nodes of irradiated SJL/J mice (Lacoste-Collin *et al.* 2007), though we did not explore the adaptive response *in vivo*. To date, it remains uncertain whether chronic very low-doses induce adaptation, and if so, how long it lasts. The phenomenon of low dose hypersensitivity followed by an increased radioresistance has been well described in many cell lines for doses below 1 Gy (Short *et al.* 2001; Chandna *et al.* 2002). *In vivo*, Brooks *et al.*, described in Chinese hamsters an adaptive response to ^{60}Co -induced chromatid exchange with protracted low-dose-rate exposure low-LET ^{144}Ce (Brooks *et al.* 1992). However, no data are available about the cellular response to a continuous very low dose of gamma-irradiation of about 1cGy.month⁻¹. This is mainly due to the difficulty to deliver a very low dose rate. Moreover, very weak effects are observed. Recent data have shown on human lymphoblastoid cells that gene transcription was modulated at doses as low as 1.0 cGy acute (Wyrobek *et al.* 2011).

Based on SJL/J mice *in vivo* proliferation and apoptosis studies, we bring up additional data on the cellular response to a continuous very low dose of γ -irradiation. Using murine cell lines, we showed that such a very low gamma irradiation is able to induce oxidative stress, adaptive response and modify GSH content. Then we explored the cellular response to radiation including proliferation and apoptosis.

MATERIALS AND METHODS

Cell lines

Raw 264.7 and YAC-1 cells were purchased from American Type Culture Collection (Rockville, MD, USA). The YAC-1 is a murine virus-induced lymphoma cell line. As our mice pathological models were lympho-

mas, it was important to select such a cell line. YAC-1 cell line was maintained in culture in RPMI-1640 (Sigma-Aldrich, Saint-Quentin Fallavier, France) with 5% FBS at 37°C in a humidified 5% CO₂ incubator.

In order to test whether this level of radiation was responsible for a modification of the oxidative stress level, we selected the Raw264.7 murine monocyte cell line, able to produce high amounts of free radicals after stimulation. This cell line is usually used for oxidative stress studies especially conducted under irradiation either at low or high doses. Raw264.7 were maintained in DMEM medium buffered with 20mM Hepes, 2 mM glutamine, 5% SVF (Sigma-Aldrich) and incubated at 37°C.

Paraffin-embedded samples

Forty paraffin-embedded lymph nodes were purchased from pathogen-free SJL/J female mice used in our previous study (Lacoste-Collin *et al.* 2007). These samples were collected from SJL/J female mice killed at an early stage and a late stage of lymphoma progression (respectively 32 and 42 weeks of life) for experimental purposes. These experimental mice were purchased from Janvier breeding (Le Genest Saint Isle, France) at 3-4 weeks old. Animals were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM). They were separated into two groups, controls and irradiated, and bred as previously described (Lacoste-Collin *et al.* 2007). An animal care committee approved the experiment.

The heaviest animals, reflecting lymphoma development, were chosen for each sacrifice. One half of the heaviest lymph node was kept for lymphocyte fluorescence-activated cell sorter (FACS) analysis (Lacoste-Collin *et al.* 2007) and the other half was fixed in 10% formalin in order to preserve antigenic conformation. After paraffin-embedding, 5 μ m thick sections were prepared and the slides were stained. Forty lymph nodes involved by follicular B-cell lymphoma were selected and classified according to the Bethesda classification (Morse *et al.* 2002). These samples were collected from 20 controls mice and 20 irradiated mice sacrificed at 32 and 42 weeks of life (Lacoste-Collin *et al.* 2007).

Irradiation and dosimetry

As in our previous studies, irradiation was provided by Thorium nitrate (Courtade *et al.* 2002; Lacoste-Collin *et al.* 2007). The natural isotope ²³²Th emits γ -rays of 60 KeV energy and α -rays of 4 MeV energy. Its half-life of 1.4x10¹⁰ years ensured a constant rate of irradiation throughout the experiment. The Thorium nitrate powder was contained in airtight plastic bag. The same device was used for *in vivo* and *in vitro* studies.

For *in vivo* irradiation, plastic bags containing Thorium nitrate were protected by 25-mm thick chipboard. It also attenuated the level of

irradiation to keep it to the required dose of 10 cGy.year⁻¹ γ -rays while stopping α -rays. The cages were placed on the chipboard. Control mice were housed in the same room, 3 m away from the irradiated mice, and isolated by a wooden screen covered by a 1.5-mm-thick sheet of lead. The energy spectrum of the radioactive source and dosimetry performed with thermoluminescent detectors have been already described elsewhere (Courtade *et al.* 2002).

In vitro irradiation was performed with Thorium nitrate as previously described (Lacoste-Collin *et al.* 2011). Thorium nitrate was placed in a sealed plastic bag covered with a piece of cardboard. Culture dishes were placed on the cardboard. Cells were cultured into two different 37°C incubators placed in two different rooms : one for controls and one for irradiated cells. As cells were cultured for limited periods of time compared to the duration of mice irradiation, we choose to increase the radiation level for cell cultures. The dosimetry was performed with radiophotoluminescent dosimeters purchased from the “Radioprotection and Nuclear Safety Institute” (Fontenay aux Roses, France) displaying excellent sensitivity for photons and beta particles. They were placed in petri dishes in the same location of cell culture devices. Mean irradiation was evaluated to 4cGy.month⁻¹ at the level of the cell culture. The dose rate was 0.13cGy.day⁻¹. The total cumulative doses at the time of sampling for each endpoint were 0.9 cGy for 1 week, 1.8 cGy for 2 weeks, 2.7 cGy for 3 weeks, 3.6 cGy for 4 weeks, 4.5 cGy for 5 weeks and 5.4 cGy for 6 weeks of culture. As the doubling time of YAC-1 and RAW 264.7 were respectively 12 and 18 hours, dose per cell cycle were respectively estimated at 0.06 cGy and 0.09 cGy. A daily rotation of culture dishes was performed both for controls and irradiated cells in order to normalize the dose between different dishes. The radiation level of the controls was identical to the background irradiation. A continuous registration of the temperature was performed in order to assess that control and irradiated cells were cultured at the same temperature in the two different incubators.

Free radical production

Control and irradiated Raw 264.7 cells were cultured in the same conditions in the control incubator and under irradiation (incubator with thorium nitrate) for different durations. Exponentially growing Raw 264.7 cells were distributed in 96-well plates at a concentration of 10⁵/well and allowed to attach for two hours. The medium was then removed and washed twice with HBSS medium (without phenol red). Following 30 min incubation, the medium was removed and 150 μ l of HBSS, 100 μ l of 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol, Sigma-Aldrich, 1mM) and a phorbol ester, 12- O-tetradecanoyl-phorbol-13-acetate (TPA) 1mM (Sigma-Aldrich) was then added for 30 minutes. The chemoluminescence response was recorded continuously for 20 min using a thermo-

statically (37°C) controlled luminometer (Wallac 1420 Victor², Finland). The peak values of chemoluminescence were used for calculations. The experiments were conducted at least three times.

Nitric oxide production

Control and irradiated Raw 264.7 cells were cultured for different durations as indicate above. For the experiment, cells were plated in 96-well plates for 24 hours, then LPS (0.3 μ g/ml) and γ -interferon (IFN- γ (Sigma-Aldrich) (5 UI/ml) added to the wells. Control wells contained medium alone. Twenty-four hours later, nitric oxide (NO) production was indirectly evaluated by assaying nitrite (NO₂⁻) (a stable product of NO) concentration in the culture supernatants using the colorimetric Griess reagent (sulfanilamide 1% and 0.1% naphthyl ethylenediamine-dihydrochloride in 2.5% phosphoric acid (Sigma-Aldrich). Using 96-well plates, 100 μ l of cell supernatant was added to 100 μ l of Griess reagent. After 10 min incubation at room temperature the absorbance level was measured at 550 nm with a microplate reader (660 nm as a reference filter, Bioadvance, Emmerainville, France). Measurements were expressed as nM of NO₂⁻ per well using a standard curve of NaNO₂. Each point was performed in triplicate. Protein content was measured in each well after removing the medium and carefully washing the adherent cells twice with PBS. The protein level was evaluated using the Bio-Rad kit (Sigma-Aldrich) and the absorbance read with a microplate reader (Bioadvance) at 550 nm (660 nm as a reference filter).

NO production was expressed as nM of NO₂⁻ / μ g of protein.

Adaptive response to H₂O₂

As adaptive response is inducible and transient, Raw 264.7 cell line to hydrogen peroxide (H₂O₂), cells were cultured for different periods of time (1 to 6 weeks) as indicated above. Twenty-four hours before the experiment, the cells were seeded in 96-well plates at a concentration of 50 000 cells/well. Different concentrations of H₂O₂ ranging from 0.18 to 3mM were added for 4 hours at 37°C. A cell viability assay was then conducted using tetrazolium blue (MTT) (Sigma-Aldrich). The optical density was evaluated using a microplate reader (Bioadvance) at 550nm, using 660nm as a reference filter. The results were expressed as an absorbance ratio corresponding to the absorbance of the cells treated with H₂O₂/untreated cells. The absorbance of cells without H₂O₂ (either control or irradiated cells) was defined as 100%. The absorbance ratios ranged from 100% to 40% with increasing concentrations of H₂O₂. The highest concentration of H₂O₂ (3mM) induced the death of more than 95% of the cells (microscopic examination of the plates). We indicate the results for cells treated with a concentration of 0.75mM H₂O₂. This concentration

corresponds to an absorbance ratio for non-irradiated control cells ranging from 0.55 to 0.7. Each point represents the mean of 9 different wells. The experiments were conducted three times.

Glutathione content

Control and irradiated Raw 264.7 cells were cultured during differents as indicated above. Cell pellets (2.10^6 cells) were homogenized in 300 μ l of distilled water, centrifuged at 3000g, at 4°C for 10 minutes and the supernatant collected. Ten μ l were kept to further assess the protein content (Bio-Rad kit) while an equal volume of 10% metaphosphoric acid (Sigma-Aldrich) was added to the remaining supernatant. The glutathione content was then measured using the GSH-400 spectrophotometric kit (Bioxytec Oxis, Foster City, CA, USA) following the instructions given by the manufacturer. A standard curve was established with various concentrations of a reduced glutathione solution. The results are expressed as μ M of GSH per μ g of protein.

Cell proliferation

Before the start of each experiment, the YAC-1 cells were maintained in logarithmic growth in T25 culture flasks. They were cultured in the same conditions in the control incubator or under irradiation (incubator with Thorium nitrate). At different weeks following the beginning of irradiation, T25 culture flasks were cultured for varying durations. The cells in each flask were then counted with a Coulter counter (Beckmann coulter, Villepinte, France). Each point was obtained in triplicate. The experiments were repeated three times.

Proliferation index labelling

Proliferation index labelling was evaluated by immunohistochemistry using an antibody directed against the cell cycle proliferation-associated antigen Ki-67. The assay was performed on 40 samples of paraffin-embedded B-cell lymphomas (controls and irradiated groups) and on YAC-1 cells fixed in 50% ethanol after 1, 2, 3 and 4 weeks of irradiation. The rat anti-mouse Ki67 (clone TEC-3), Dako, Glostrup, Denmark) combined with biotinylated rabbit anti-rat Ig (Dako, Glostrup, Denmark) was applied on deparaffined sections or YAC-1 cytopins silanized slides. An avidin-biotin peroxidase complex was then used followed by visualization with 3,3'-diaminobenzidine (Dako, Glostrup, Denmark). A counterstain was performed with haematoxylin. Reactive lymph nodes were chosen for positive control and germinal center lymphocytes displayed a nuclear staining. Cell counts were performed at 400X magnification on at least 200 cells by three pathologists.

Apoptosis assay

YAC-1 cells were maintained in culture during different times as described above. For the experiment, they were fixed in 1% paraformaldehyde after 1, 2, 3 and 4 weeks of irradiation and processed as cytospin on silanized slides. Paraffin was removed from 5 μ m sections of formalin-fixed lymph nodes. Sections were rehydrated. Apoptosis assay was processed using the ApopTag® Peroxidase *in situ* apoptosis detection kit according to the manufacturer's instructions (Merck Millipore, Billerica, United States of America). Positive control sections from an hyperplastic lymphadenopathy were run with each experiment. Cell counts were performed at high magnification (X1000) on at least 500 cells by three pathologists.

Statistical analysis

Student's *t* test was applied to compare results between control and irradiated cells.

RESULTS

Oxidative stress and adaptive response to H₂O₂

Free radicals

Raw 264.7 cells were cultured under irradiation and the level of production of free radicals evaluated at different times after the beginning of the irradiation using a chemoluminescent method. In order to normalize the data between experiments, the results were expressed as an irradiated/control ratio of free radical levels. The dots in Figure 1A indicate this ratio as a function of time. We observed that the Raw264.7 cells cultivated under irradiation had a statistically higher capacity of free radicals production when compared to controls. This capacity was maximal after 3 and 4 weeks of irradiation ($p < 0.01$ and $p < 0.05$ respectively) corresponding to total cumulative doses respectively estimated at 2.7 cGy and 3.6 cGy) and returned to control level after 6 weeks (total cumulative dose respectively estimated at 5.4 cGy).

This result indicates that even at low doses, external γ -irradiation transiently increases the cellular ability to produce free radicals.

NO production

Another indicator of oxidative stress is the ability of cells from the macrophage system to produce NO. We tested this production in the Raw264.7 cells in parallel with oxidative data.

As indicated in Figure 1B, there was a 2 fold significant increase ($p < 0.01$) in NO production by cells after 1 and 2 weeks of irradiation (total cumulative doses respectively estimated at 0.9 cGy and 1.8 cGy),

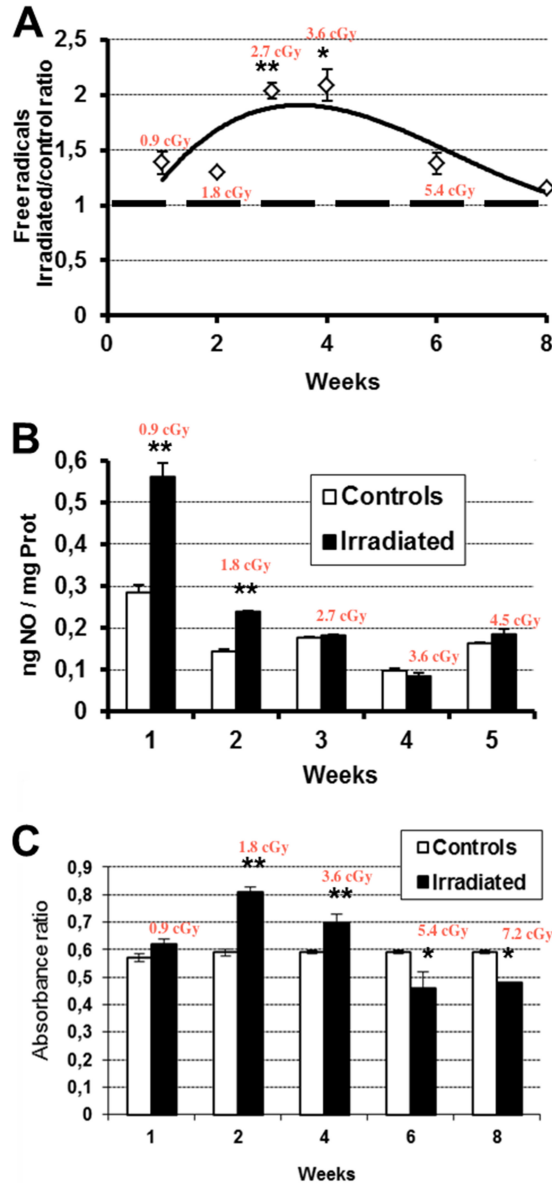


FIGURE 1. Oxidative status and adaptive response to H_2O_2 in Raw 264.7 cells. A: free radical production. Reactive oxygen species production was measured by chemoluminescence in the presence of luminol in a thermostatically controlled luminometer. The dots represent the average irradiated/control ratio of the peak of chemoluminescence emission for 30 min +/- S.E., measured in three experiments. A polynomial tendency curve was drawn in order to follow the results with time. B: NO production. The production of nitrites was measured 24h of LPS later. Values are means \pm S.E. of three separate experiments. C: Adaptive response to H_2O_2 . Cell survival was evaluated using the colorimetric MTT test. The results are expressed as an absorbance ratio of the cells treated with H_2O_2 / cells without H_2O_2 . The absorbance of cells without H_2O_2 was defined as 100%. Each point represents the mean +/- S.E. (n=9) from three different experiments. *p < 0.05, **p < 0.01 indicate a significant difference when compared with the control cells. Total cumulative doses are specified in red for each duration of cell culture under irradiation.

the level returning to control levels from 3 weeks of irradiation (total cumulative dose respectively estimated at 2.7 cGy). This result shows the same tendency already observed for free radical production. However, the increase of NO production under irradiation is earlier and shorter than the production of free radicals by the same cells.

Adaptive response to H₂O₂

The results obtained concerning the production of free radicals may suggest an adaptation of the cells to irradiation. The adaptive response has previously been shown to cross react with different cell mutagens (Dominguez *et al.* 1993). In order to test this hypothesis, we cultured the Raw264.7 cells in the presence of growing concentrations of H₂O₂. Cell toxicity was evaluated by a MTT test. A significant increase ($p < 0.01$) in survival after 2 and 4 weeks of irradiation was observed (total cumulative doses respectively estimated at 1.8 cGy and 3.6 cGy), while a significant decrease ($p < 0.05$) was found after 6 and 8 weeks (total cumulative doses respectively estimated at 5.4 cGy and 7.2 cGy) (Figure 1C).

GSH content

The GSH content displayed no significant change during 6 weeks of culture (data not shown).

In vivo lymphoma cell proliferation and apoptosis

Ki67 index labeling

Ki67 nuclear labeling of lymphomatous cells is illustrated on Figure 2A. The mean values of percentages of positive nuclei are presented in the Figure 2 B. The mean values of percentages of positive Ki67 nuclei were not changed under irradiation at 32 weeks (57.8 ± 5.7 % versus 51.18 ± 6.3 %, *NS*) but significantly lower at 42 weeks (68.8 ± 13.3 % versus 53.57 ± 5.3 %, $P < 0.05$).

Apoptosis assay

The characteristic apoptotic bodies were observed both in extracellular space and in the macrophage cytoplasm. Early-stage apoptosis was also detected as intact cell with nuclear condensation. The mean values of percentages of apoptotic bodies were similar in the two groups at 32 weeks (3 ± 0.8 % in controls and 3.16 ± 0.3 % in irradiated) and at 42 weeks (3.78 ± 0.6 % versus 3.16 ± 0.4 % in controls), without significant changes (data not shown).

In vitro YAC-1 cell proliferation and apoptosis

Cell proliferation

Proliferation curves drawn by cell counting are presented in Figure 3. After one week of culture, there was no modification of the curves profiles

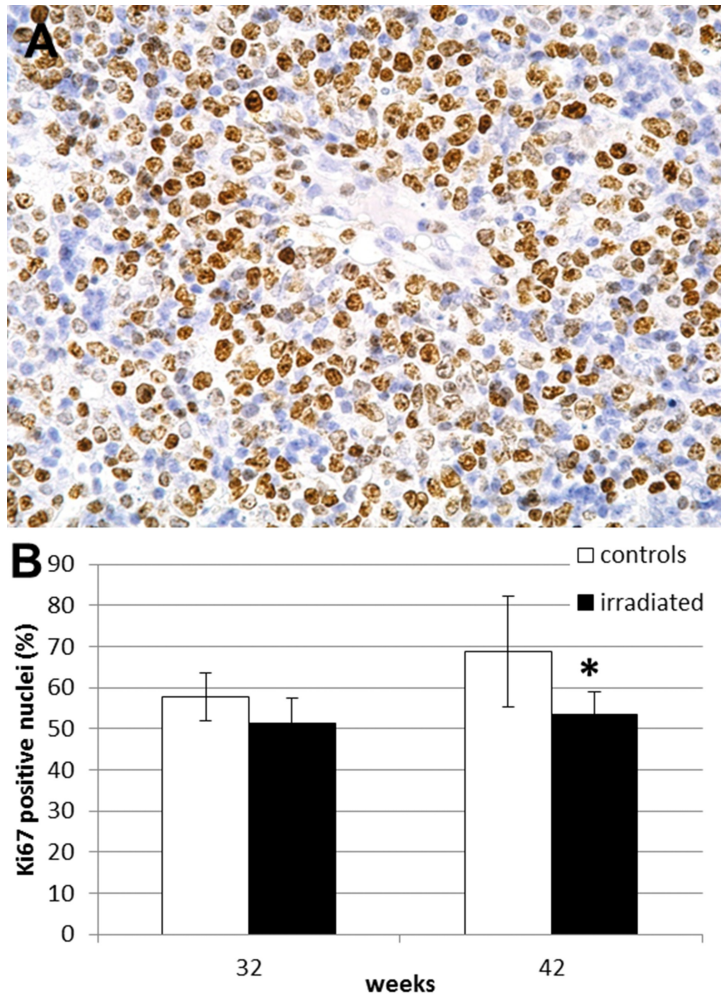


FIGURE 2. Proliferative index and apoptotic rate in lymphomatous lymph nodes. A: Ki67 immunohistochemical nuclear labeling on paraffin-embedded lymphomatous lymph nodes. Percentages of positive dark brown nuclei were evaluated by cell count at high magnification ($\times 400$) on at least 200 cells by three pathologists. B: Mean values of percentages of positive Ki67 nuclei. Values are means \pm S.E. of ten separate specimens. * $p < 0.05$, indicates a significant difference when compared with the control cells.

(data not shown). But after 2 and 3 weeks of culture (total cumulative doses respectively estimated at 1.8 cGy and 2.7 cGy), cell counts were significantly lower under irradiation especially when they were evaluated after 3 days of culture (Figure 3A and 3B). After 4 weeks (total cumulative dose estimated at 3.6 cGy), there was not significant change of cell counts under irradiation (data not shown).

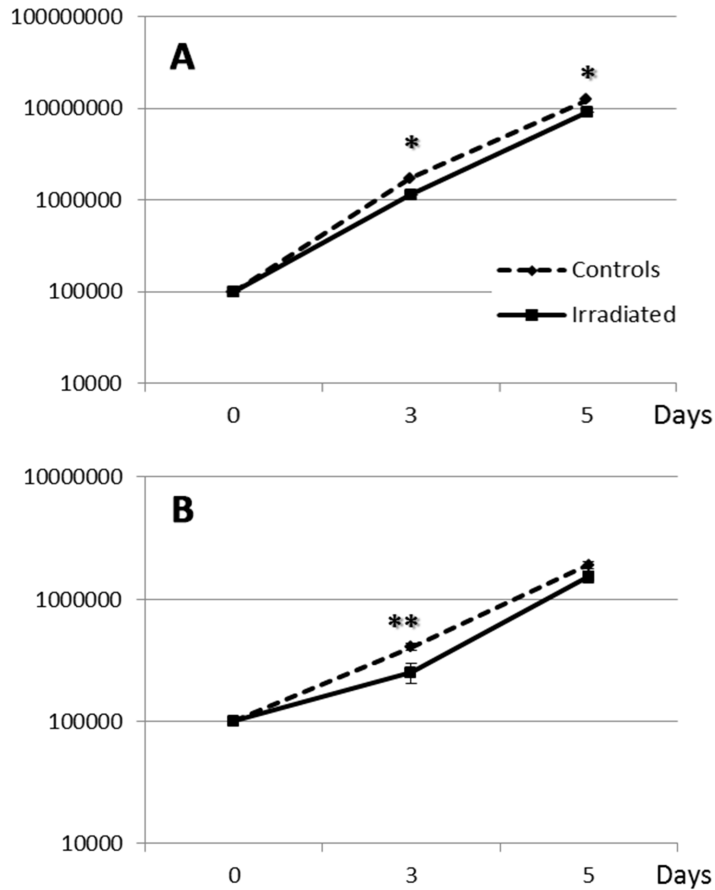


FIGURE 3. Cell proliferation assay with YAC-1 cell line. Cells were seeded at a cell density of 100,000 / 25cm² flask and cultured for different lengths of time before the experiment. Cell counts (logarithmic y-axis) were performed on different days of culture (x axis). Each point represents the mean cell count +/- standard error (S.E.) of three flasks. The cell counts were repeated for 4 successive weeks. Second and third weeks are respectively presented in A and B. The same experiment was conducted three times. *p < 0.05, **p < 0.01 indicate a significant difference when compared with the control cells.

Ki67 index labeling

Ki67 proliferation index was evaluated during the first, second, third and fourth weeks of culture. There was no significant difference between the two groups.

Apoptosis assay

A significant decrease of apoptotic rate under radiation was noticed after 4 weeks of culture at a total cumulative dose estimated at 3.6 cGy (Figure 4).

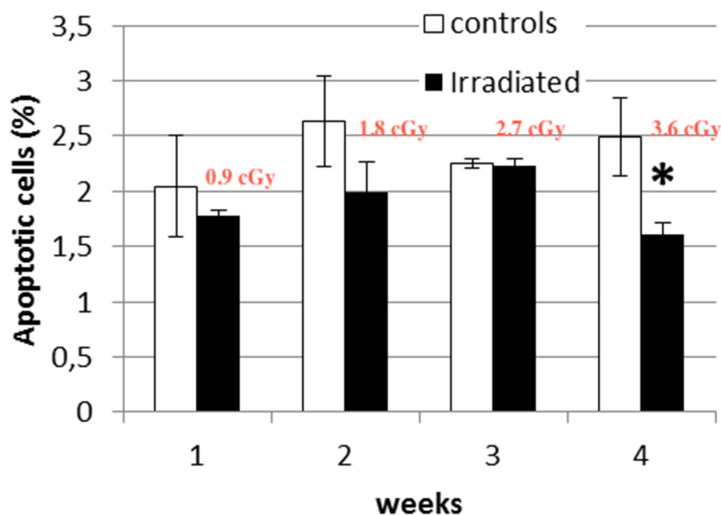


FIGURE 4. Apoptotic rate on YAC-1 cell line. Mean values of percentages of apoptotic bodies. Values are means \pm S.E. of three separate experiments. * $p < 0.05$ indicates a significant difference when compared with the control cells. Total cumulative doses are specified in red for each duration of cell culture under irradiation.

DISCUSSION

The dose of radiation that we used for *in vitro* experiments is very low and very difficult to deliver. It was a cumulative dose of $4\text{cGy}\cdot\text{month}^{-1}$, equating to $0.13\text{cGy}\cdot\text{day}^{-1}$. No data are available in the literature with such a very low dose and dose-rate.

As radiation partly acts by increasing free radical production above the daily level, the first part of this study aimed at determining whether such a low dose of irradiation was able to modify the oxidative status of cells. The Raw264.7 cell line was chosen as it is a monocytic cell line largely used in radiation experiments known to produce high amounts of either free radicals or NO. In monocytic-macrophage cells, exposure to high doses (3 to 6 Gy) (Ibuki and Goto 2003; Ibuki and Goto 2004) stimulates NO production secondary to DNA damage, while exposure to lower doses either *in vitro* (50 cGy, (McKinney *et al.* 1998) or *in vivo* (10 or 20 cGy on mice macrophages), (Nowosielska *et al.* 2006) also enhances cytotoxic activity via increasing NO production. Under continuous irradiation, we observed a transient two-fold increase in the cellular capacity for free radical production, returning to normal levels after 6 weeks. Comparable data were observed as regards to NO production, but the increase of NO was earlier and shorter when compared to free radicals. These results show that the response to continuous irradiation varies with exposure time. It suggests that if a primary transient activation of macrophages is observed, this effect will not last. The maintaining of GSH levels under irradiation without decrease may reasonably be assumed to counterbalance the increase

in the capacity of NO and free radical production. Indeed, GSH plays direct or indirect roles in many biological processes, including the protection of cells from damage caused by free radicals (Meister and Anderson 1983). Antioxidant enzymes have been shown to suppress NO production in macrophages through the inhibition of NF- κ B activation (Han *et al.* 2001). Likewise, antioxidant enzymes contribute to the adaptive response to ionizing radiation (Bravard *et al.* 1999) particularly in the Raw264.7 cell line (Kawarita *et al.* 2003). Our results, however, show no significant change of GSH levels under irradiation and taken alone, cannot explain the decrease in the production of NO or free radicals observed after 3 weeks of exposure. Other mechanisms are most probably involved in this effect. The results of the experiments aimed at demonstrating an adaptive response which was observed after 2 weeks of irradiation and seemed to vanish thereafter. An adaptive response has been demonstrated after a primer dose in the range of 0.1 to 50 cGy followed 24 to 48 hours later by a challenging dose in the range of 1 Gy (Stecca and Gerber 1998; Broome *et al.* 2002). Our “primer” dose is quite lower, evaluated at 0.13cGy.day⁻¹. In theory, this dose corresponds to the lowest level reported to induce an adaptive response. In normal human fibroblasts, Broome *et al.* have demonstrated that priming doses of about 0.01 cGy were ineffective to induce an adaptative response when compared to 0.1 cGy (Broome *et al.* 2002). The cumulative dose received by our cells after 2 weeks was around 2 cGy (estimated at 1.8 cGy). However, as the dose cumulates with increasing time, the adaptive response is no longer observed and disappears after 6 weeks of culture at a total cumulative dose estimated at 5.4 cGy. Interestingly, constantly increased levels of reactive oxygen species or NO have been observed in adapted cells, both factors playing a role in the maintenance process (Tapio and Jacob 2007). The transient increase of production of NO and free radicals by the Raw264.7 cell line may be correlated to the disappearance of the adaptive response with increasing time. Literature data have shown an adaptive response to very low dose irradiation in humans and mice. Occupational exposure was shown to induce an adaptive response in human lymphocytes (Barquinero *et al.* 1995) as well as in patients treated with ¹³¹I (Monsieurs *et al.* 2000). In C57Bl/6 mice, an adaptive response was observed with a priming dose of 5cGy given between 1 and 7 days before the challenging dose, who disappeared when the delay between the priming and the challenging dose increased above one week, in favor of a transient feature (Ito *et al.* 2007). Macrophages play a dual role in carcinogenesis. Radiation-enhanced cytotoxic activity is able to suppress lung metastasis in mice (Nowosielska *et al.* 2006) while mutagenesis is associated with NO production in macrophages (Zhuang *et al.* 1998). It is thus difficult to assess whether an increased capacity of production of NO or free radicals by macrophages is beneficial.

In view of our *in vivo* studies in mice (Courtade *et al.* 2002; Lacoste-Collin *et al.* 2007), rather than being deleterious, it seems that this transient increase in stimulation of macrophages at this dose of radiation may protect the organism, except towards the end of life. The absence of increased life span and the same total rate of lymphomas at the end of life in our animal studies may be witness to the transient stimulating effect of this continuous irradiation on living organisms. The slight lifespan lengthening of SJL/J mice under chronic irradiation that we observed in our last study could be explained by an adaptive response to radiation able to induce extended tumor latency as it has been described by Mitchel *et al.* (Mitchel *et al.* 1999; Mitchel *et al.* 2003; Mitchel *et al.* 2008). Firstly, Mitchel *et al.* showed in the CBA/H mouse strain that a pre-irradiation of 10 cGy of γ -rays applied 24 hours before a high dose of 1 Gy inducing acute leukemia was able to delay the leukemia appearance without changes of the total rate of leukemia or survival (Mitchel *et al.* 1999). Then, in the cancer-prone C57Bl/6 radiation sensitive *Trp53*^{+/-} heterozygous mice developing lymphomas and osteosarcomas, Mitchel *et al.* demonstrated that the level of dose required to induce an adaptive response depends on the tumor type (Mitchel *et al.* 2003). A threshold dose at which an adaptive response was not induced was demonstrated for osteosarcomas. At last, using different schedules of chronic pre-irradiation Mitchel *et al.* suggested that there are minimal and maximal threshold dose for inducing an adaptive response that is tissue-dependent (Mitchel *et al.* 2008).

Indeed, chronic irradiation did not modify the lymphoma incidence and the age of lymphoma appearance (Lacoste-Collin *et al.* 2007). These results are in accordance with an absence of radiation effect on the initiation step of follicular B-cell lymphomas that are MMTV-induced in the SJL/J mice. Then, a longer tolerance of lymphoma masses under radiation related to a slowing of lymphoma growth could rather explain the tendency to mean lifespan lengthening. The progressive decrease of the proliferation rate under radiation between 32 and 42 weeks of life evaluated by Ki-67 immunohistochemical expression on lymphoma masses (respectively 57.8 ± 5.7 % versus 51.18 ± 6.3 %, *NS* and 68.8 ± 13.3 % versus 53.57 ± 5.3 %, $P < 0.05$) without significant change of apoptotic rate (3.78 ± 0.6 % versus 3.16 ± 0.4 % in controls, *NS*) were in good accordance with a slowing of lymphoma growth. The significant decrease of YAC-1 cells counts under irradiation after 2 and 3 weeks of culture at total cumulative dose respectively estimated at 1.8 and 2.7 cGy was an additional tool in favor of the influence of a chronic irradiation on lymphoma growth. However, Ki-67 proliferation index was not changed. Radiation-induced apoptosis in tumor cells by low levels of doses has been rarely studied. In the Kunming mouse strain, 7.5 cGy delivered 7 days after the abdominal implantation of S180 sarcomatous cells lead to a significant increase of tumor cell apoptosis with a tumor size reduction 48 hours after irradi-

ation (Yu *et al.* 2005). It has been demonstrated that such low levels of dose rate are able to modulate the transcription of genes involved in various mechanisms in mammalian cell lines especially in cell cycle and apoptosis. The dose per cell cycle for YAC-1 cells was very low estimated at 0.06 cGy. Amundson *et al.* (Amundson *et al.* 1999; Amundson *et al.* 2003) have demonstrated that doses between 2 and 50 cGy delivered with a low-dose-rate are able to induce a dose-dependent transcription of *CDKN1A* (Cyclin-Dependent Kinase Inhibitor 1 coding for the p21waf1 protein who drives to G1 cell cycle blockage) and *GADD45* (Cyclin-Dependent Kinase Inhibitor 2 coding for a protein involved in G2/M blockage) leading to an alteration of cell cycle progression in a human myeloid leukemia cell line (ML-1). Moreover the apoptosis enhancement observed from 2 cGy was only significant at 25 cGy. Fachin *et al.* (Fachin *et al.* 2007) also demonstrated in normal human lymphocytes that low doses (10, 25 and 50 cGy) modulate oxidative metabolism, cell cycle, DNA repair, cell differentiation and regulation of transcription. *MYC*, *FOS* and *TP53* are the major genes modulated at doses below 1 cGy in human lymphoblastoid cells (Wyrobek *et al.* 2011). The transient effect on proliferation curves of YAC-1 cells and Ki-67 proliferation indexes disappearing at the fourth week of culture at a total cumulative dose estimated at 3.6 cGy also suggests an adaptive response. The significant decrease of the apoptotic rate observed at the fourth week of culture could highlight the induction of anti-apoptotic mechanisms. In a murine lymphoma cell line (EL4), a low dose pre-irradiation at 0.01 Gy 4 hours before a high dose leads to a significant decrease of apoptotic rate and this pre-treatment was compared to the induction of an adaptive response (Kim *et al.* 1997).

In conclusion, a protracted γ -irradiation at a dose rate as low as $0.13\text{cGy}\cdot\text{day}^{-1}$ is able to induce cell responses. However, these responses are mainly transient and may help to understand the *in vivo* observations in animal or human studies.

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REFERENCES

- Amundson SA, Do KT, and Fornace AJ Jr. 1999. Induction of stress genes by low doses of gamma rays. *Radiation Research* 152: 225-31.

- Amundson SA, Lee RA, Koch-Paiz CA, Bittner ML, Meltzer P, Trent JM, and Fornace AJ Jr. 2003. Differential responses of stress genes to low dose-rate γ irradiation. *Molecular Cancer Research* 1: 445-52.
- Barquinero JF, Barrios L, Caballin MR, Miro R, Ribas M, and Subias A. 1995. Occupational exposure to radiation induces an adaptive response in human lymphocytes. *International Journal of Radiation Biology* 67: 187-91.
- Bravard A, Luccioni C, Moustacchi E, and Rigaud O. 1999. Contribution of antioxidant enzymes to the adaptive response to ionizing radiation of human lymphoblasts. *International Journal of Radiation Biology* 75: 639-45.
- Brooks AL, McDonald KE, Kimsey BB, and Kitchin RM. 1992. Radioadaptive response in vivo in Chinese hamsters injected with alpha-(239Pu) or beta-gamma-(144Ce) emitting radionuclides. In: Sugarara T, Sagan LA and T. Aoyama (eds), *Low dose irradiation and biological defense mechanisms*, pp 319-322. Elsevier Science Publishers, Amsterdam
- Broome EJ, Brown DL, and Mitchel REJ. 2002. Dose responses for adaptation to low doses of ^{60}Co γ rays and ^3H β particles in normal human fibroblasts. *Radiation Research* 158: 181-86.
- Chandna S, Dwarakanath BS, Khaitan D, Mathew TL, and Jain V. 2002. Low-dose radiation hypersensitivity in human tumor cell lines: effects of cell-cell contact and nutritional deprivation. *Radiation Research* 157: 516-25.
- Courtade M, Caratero A, Jozan S, Pipy B, and Caratero C. 2001. Influence of continuous, very low-dose gamma-irradiation on the mouse immune system. *International Journal of Radiation Biology* 77: 587-92.
- Courtade M, Billotet C, Gasset G, Caratero A, Charlet JP, Pipy B, and Caratero C. 2002. Life span, cancer and non-cancer diseases in mouse exposed to a continuous very low dose of gamma-irradiation. *International Journal of Radiation Biology* 78: 845-55.
- Dominguez I, Panneerselvam N, Escalza P, Natarajan AT, and Cortes F. 1993. Adaptive response to radiation damage in human lymphocytes conditioned with hydrogen peroxide as measured by the cytokinesis-block micronucleus technique. *Mutation Research* 301: 135-41.
- Fachin AL, Mello SS, Sandrin-Garcia P, Junta CM, Donadi EA, Passos GA, and Sakamoto Hojo ET. 2007. Gene expression profiles in human lymphocytes irradiated in vitro with low doses of gamma rays. *Radiation Research* 168: 650-65.
- Han YJ, Kwon YG, Chung HT, Lee SK, Simmons RL, and Billiar TR. 2001. Antioxidant enzymes suppress nitric oxide production through the inhibition of NF- κ B activation: role of H_2O_2 and nitric oxide in inducible nitric oxide synthase expression in macrophages. *Nitric Oxide* 5: 504-13.
- Ibuki Y, Mizuno S, and Goto R. 2003. Gamma-Irradiation-induced DNA damage enhances NO production via NF- κ B activation in RAW264.7 cells. *Biochimica Biophysica Acta* 17: 159-67.
- Ibuki Y and Goto R. 2004. Ionizing radiation-induced macrophage activation: augmentation of nitric oxide production and its significance. *Cellular and Molecular Biology (Noisy-le-grand)* 50: 617-26.
- Ito M, Shibamoto Y, Ayakawa S, Tomita N, Sugie C, and Ogino H. 2007. Low-dose whole body irradiation induced radioadaptive response in C57BL/6 mice. *Journal of Radiation Research (Tokyo)* 48: 455-60.
- Kawarita Y, Ikekita R, Kurosumi R, and Kojima S. 2003. Increase of intracellular glutathione by low-dose γ -ray irradiation is mediated by transcription factor AP-1 in Raw 264.7 cells. *Biological Pharmaceutical Bulletin* 26: 19-23.
- Kim CS, Kim JM, Nam SY, Yang KH, Jeong M, Kim HS, Lim YK, Kim CS, Jin YW, and Kim J. 2007a. Low-dose of Ionizing Radiation Enhances Cell Proliferation Via Transient ERK1/2 and p38 Activation in Normal Human Lung Fibroblasts. *Journal of Radiation Research (Tokyo)* 48: 407-15.
- Kim CS, Kim JK, Nam SY, Yang KH, Jeong M, Kim HS, Kim CS, Jin YW, and Kim J. 2007b. Low-dose radiation stimulates the proliferation of normal human lung fibroblasts via transient activation of Raf and Akt. *Molecules and Cells* 24: 424-30.
- Kim JH, Hyun SJ, Yoon MY, Ji YH, Cho CK, and Yoo SY. 1997. Pretreatment of low dose radiation reduces radiation-induced apoptosis in mouse lymphoma (EL4) cells. *Archives Pharmacol Research* 20: 212-217.
- Lacoste-Collin L, Jozan S, Cances-Lauwers V, Pipy B, Gasset G, Caratero C, and Courtade-Saïdi M. 2007. Effect of a very low dose of gamma-rays on life span and the immune system in SJL mice prone to B-cell lymphoma. *Radiation Research* 168: 725-32.

- Lacoste-Collin L, Jozan S, Pereda V, and Courtade-Saidi M. 2011. A chronic very low dose of gamma-rays alters cell adhesion. *International Journal of Low Radiation* 8(5/6) : 412-28.
- McKinney LC, Aquilla EM, Coffin D, Wink DA, and Vodovotz Y. 1998. Ionizing radiation potentiates the induction of nitric oxide synthase by IFN- γ and/or LPS in murine macrophage cell lines: roles of TNF- α . *Journal of Leukocyte Biology* 64: 459-66.
- Matsumoto H, Takahashi A, and Ohnishi T. 2004. Radiation-induced adaptive responses and bystander effects. *Biological Sciences in Space* 18: 247-54.
- Meister A and Anderson MN. 1983. Glutathione. *Annual Review of Biochemistry* 52: 611-60.
- Mitchel REJ, Jackson JS, McCann RA, and Boreham DR. 1999. The adaptive response modifies latency for radiation-induced myeloid leukemia in CBA/H mice. *Radiation Research* 152: 273-9.
- Mitchel REJ, Jackson JS, Morrison DP, and Carlisle SM. 2003. Low doses of radiation increase the latency of spontaneous lymphomas and spinal osteosarcomas in cancer-prone, radiation sensitive Trp53 heterozygous mice. *Radiation Research* 159: 320-27.
- Mitchel REJ, Burchart P, and Wyatt H. 2008. A lower dose threshold for the in vivo protective adaptive response to radiation. Tumorigenesis in chronically exposed normal and Trp53 heterozygous C57BL/6 mice. *Radiation Research* 170: 765-75.
- Monsieurs MA, Thierens HM, Vral AM, Van de Wiele C, de Ridder LI, and Dierckx RA. 2000. Adaptive response in patients treated with ¹³¹I. *Journal of Nuclear Medicine* 41: 17-22.
- Morse HC, Anver MR, Fredrickson TN, Haines DC, Harris AW, Harris NL, Jaffe ES, Kogan SC, MacLennan IC, Pattengate PK, and Ward JM. 2002. Bethesda proposals for classification of lymphoid neoplasms in mice. *Blood* 100: 246-58.
- Nowosielska EM, Wrembel-Wargocka J, Cheda A, Lisiak E, and Janiak MK. 2006. Enhanced cytotoxic activity of macrophages and suppressed tumor metastases in mice irradiated with low doses of X-rays. *Journal of Radiation Research (Tokyo)* 47: 229-36.
- Short SC, Kelly J, Mayes CR, Woodcock M, and Joiner MC. 2001. Low-dose hypersensitivity after fractionated low-dose irradiation in vitro. *International Journal of Radiation Biology* 77: 655-64.
- Stecca C and Gerber GB. 1998. Adaptive response to DNA-damaging agents. *Biochemical Pharmacology* 55: 941-51.
- Tapio S and Jacob V. 2007. Radioadaptive response revisited. *Radiation and Environmental Biophysics* 46: 1-12.
- Tubiana M. 2005. Dose-effect relationship and estimation of the carcinogenic effects of low doses of ionizing radiation: the joint report of the Academie des Sciences (Paris) and of the Academie Nationale de Medecine. *International Journal of Radiation Oncology Biology Physics* 63: 317-19.
- Ullsh BA. 2012. The new radiobiology: returning to our roots. *Dose Response* 10: 593-609.
- Wyrobek AJ, Manohar CF, Krishnan VV, Nelson DO, Furtado MR, Bhattacharya MS, Marchetti F, and Coleman MA. 2011. Low dose radiation exposure curves, networks and pathways in human lymphoblastoid cells exposed from 1 to 10 cGy of acute gamma radiation. *Mutation Research* 722: 119-30.
- Yu H, Song A, Fei C, Wang Z, and Qiu W. 2005. Effects of low dose radiation on tumor apoptosis, cell cycle and apoptosis-related protein Bcl-2 in tumor-bearing mice. *Chin-Germ. Journal of Clinical Oncology* 4: 89-92.
- Zhuang JC, Lin C, Lin D, and Wogan GN. 1998. Mutagenesis associated with nitric oxide production in macrophages. *Proceedings of the National Academy of Sciences USA* 95: 8286-91.