THE RBE OF TRITIUM-BETA EXPOSURE FOR THE INDUCTION OF THE ADAPTIVE RESPONSE AND APOPTOSIS; CELLULAR DEFENSE MECHANISMS AGAINST THE BIOLOGICAL EFFECTS OF IONIZING RADIATION.

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ABSTRACT

Adaption to radiation is one of a few biological responses that has been demonstrated to occur in mammalian cells exposed to doses of ionizing radiation in the occupational exposure range. The adaptive response has been well characterized in the yeast *Saccharomyces cerevisiae*, although the doses required to induced the response are higher than in mammalian cells. When yeast cells are primed with sublethal doses of gamma-radiation, they subsequently undergo an adaptive response and develop resistance to radiation, heat and chemical mutagens in a time and dose dependent manner. We have used this model system to assess the relative ability of tritium-beta radiation to induce the adaptive response and examined tritium-induced radiation resistance, thermal tolerance and suppression of mutation. The results show that sublethal priming doses of tritium caused yeast cells to develop resistance to radiation, heat, and a chemical mutagen MNNG. The magnitude and kinetics of the response, per unit dose, were the same for tritium and gamma-radiation. Therefore, the relative biological effectiveness (RBE) of tritium for induction of the adaptive response was about 1.0.

Apoptosis is a genetically programmed cell death or cell suicide. Cells damaged by radiation can be selectively removed from the population by apoptosis and therefore eliminated as a potential cancer risk to the organism. Since we have previously shown that apoptosis is a sensitive indicator of radiation damage in human lymphocytes exposed to low doses, we have used this endpoint to investigate the potency of tritium-beta radiation. Initially, tritium was compared to X-rays for relative effectiveness at inducing apoptosis. The results showed that lymphocytes irradiated *in vitro* with X-rays or tritium had similar levels of apoptosis per unit dose. Therefore the relative biological effectiveness of tritium for induction of apoptosis in human lymphocytes was also about 1.

In the work presented here, we have demonstrated that tritium does not quantititatively differ from X-rays or gamma-rays in inducing the adaptive response in yeast or apoptosis in human lymphocytes.

INTRODUCTION

Accurately determining the relative biological effectiveness (RBE) of tritium has important economic implications for the CANDU nuclear power program. In this paper we examine the RBE of tritium using two sensitive biological endpoints known to respond to sublethal doses of radiation. Ionizing radiation causes harm to living organisms because it can alter the genetic program contained within the DNA of a cell. If the genetic information is damaged or altered, it must be repaired correctly or eliminated. Otherwise the cell may undergo a transformation that could ultimately cause it to become a cancer. Fortunately, cells have evolved mechanisms that protect and repair normal DNA or eliminate misrepaired DNA. Our work focuses on two of these mechanisms: the adaptive response and apoptosis. The adaptive response protects cells from ionizing radiation by enhancing the DNA repair capacity of the cell and consequently reducing the probability of genetic damage. Alternatively, once genetic damage has occurred, the cell can be destroyed by apoptosis, a genetically programmed form of cell death or "suicide". Both mechanisms ultimately can modify the cancer risks associated with exposure to ionizing radiation. In this report we specifically address the relative effects of tritium-beta radiation on these two biological mechanisms.

The adaptive response to radiation is detected as a process induced by radiation whereby the consequences of a subsequent radiation exposure are modified (1). Such an effect has been observed when cells are exposed to either a small or chronic sublethal priming dose of radiation and sometime later are exposed to a second, larger and more harmful, radiation dose. The cellular consequences of the second exposure may be reduced or enhanced depending on the biological endpoint being investigated. The adaptive response was originally observed in human lymphocytes by Olivieri et al. (2) and since then reports have shown that many conditions can influence adaption of human lymphocytes (3,4,5). Occupational exposures to radiation have also been shown to induce an adaptive response in lymphocytes (6). Adaption, resulting from a small radiation dose, has been shown in other human cell types to protect against micronucleus formation and neoplastic transformation (7), and the inherent spontaneous risk of neoplastic transformation (8). Radiation has been shown to also induce the adaptive response in mouse germ cells (9).

Apoptosis is a form of cell death that has distinctive morphological and biochemical characteristics (10). Many extracellular and intracellular signals are known to elicit this genetically controlled and regulated response in a variety of cells (11). One of the biochemical events that occurs in apoptotic cells and distinguishes them from other cells and other modes of cell death, is the fragmentation of nuclear DNA (12). The time required for an apoptotic cell to fragment its DNA varies depending on the organism, cell type, and the type of inducing signal (13). Ionizing radiation is one agent known to induce apoptosis in human cells. When a cell's DNA is extensively or irreversibly damaged, the cell may undergo apoptosis to prevent further consequences to the organism. This mechanism is believed to be an important pathway to prevent potential cancer cells from becoming cancerous.

Many physical and biological factors influence the rate and magnitude of the adaptive and apoptotic responses. Radiation quality is one physical factor known to modulate both processes. In this report, we have studied the effects of tritium-beta radiation on these processes. It is known that gamma-radiation adapted yeast cells become thermal tolerant, radiation resistant, and less susceptible to mutation caused by a chemical agent MNNG (14). Here we have compared tritium-beta radiation to cobalt-60 gamma-radiation for inducing the adaptive response in yeast. Also, we have previously shown that human lymphocytes undergo apoptosis in a dose dependent manner when exposed to gamma-radiation and X-rays (15). Therefore, in this work we have compared tritium-beta exposure to gamma- and X-irradiation for induction of apoptosis.

MATERIALS AND METHODS

Tritium-Induced Adaptive Response in Yeast

Cell Growth. Diploid strain of the yeast *Saccharomyces cerevisiae* was used as the test organisms in these experiments. Cell cultures were grown at 23°C to mid-exponential phase (2-4 x 10^6 cells/mL) in liquid nutrient medium containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, and 2% glucose.

Radiation-Induced Thermal Tolerance. Thermal survival was determined by placing 2.5 mL cell aliquots ($2x10^6$ cells/mL in YES medium at 0°C) in 16 x 100 mm glass test tubes and submerging the glass test tubes into a shaking 52°C water bath. At specified time intervals glass test tubes were removed, rapidly cooled to 0°C, and the cells were plated for survival on nutrient agar plates. Thermal tolerance was induced with radiation by exposing yeast cells at 0°C to 200 Gy of either ^{60}Co-gamma-rays or tritium-beta rays. Tritium exposures were performed by adding tritiated water (stock 1 Ci/mL) to 0°C growth media containing cells. Cells were incubated in the tritiated media for various times until the desired dose was delivered to the cells. Cells were then washed three times in 0°C phosphate buffer, resuspended in fresh media, and incubated at 21°C for two hours. Thermal survival curves for the irradiated cells was performed as described above.

Radiation-Induced Radiation Resistance. Radiation resistance was determined in cells by irradiating cells with 200 Gy of either ^{60}Co-gamma rays or tritium-beta rays. Following radiation exposure, cells were

incubated under normal growth conditions for increasing periods of time. Radiation resistance was measured at each time interval by exposing cells to a 1.0 kGy dose of gamma rays delivered under oxic conditions. Survival was determined by plating cells on nutrient agar and scoring colony formation.

Radiation-Induced Mutation and Suppression of MNNG Mutation. Radiation-induced mutation was determined by measuring reversion from histidine dependence to histidine independence. Mutant cells able to form colonies were counted after incubation at 23°C for 6 days on nutrient agar lacking histidine. Total survival after the various treatments was measured by scoring colony formation on complete nutrient agar plates. Chemical mutagenesis was performed at 23°C in 20 mmol dm^{-3} (mM) phosphate buffer (pH 7.0) at a cell density of 1 x 10^7 cells/mL. Cells were treated with 20 mg/mL of MNNG (Aldrich Chemical Co.) for 30 minutes immediately after exposure to various doses of gamma-radiation or tritium-beta rays. After MNNG exposure, cells were washed once with 10% (w/v) sodium thiosulphate and twice with phosphate buffer at 0°C. Reversion to histidine independence and survival to treatments was determined as described above.

Tritium-Induced Apoptosis in Human Lymphocytes

Cell Culture. Blood samples from healthy male volunteers were collected in heparinized tubes and the mononuclear lymphocytes were isolated from the sample. The lymphocytes were washed twice at room in Hank's Balanced Salt Solution (Sigma, St. Louis, MO). The cells were incubated at 37°C in T25 culture flasks at a cell concentration of 4.0 x 10^5 cells /mL in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 20% fetal bovin serum, 2 mM L-glutamine, 10 U/mL penicillin and 10 mg/mL streptomycin sulphate. Cells were equilibrated to 37°C prior to radiation treatments. All flasks containing cells were incubated for 48 hours after treatments to allow lymphocytes undergoing apoptosis to fragment DNA prior to being assayed (Cregan et al. 1994, Boreham et al. 1996).

Tritium and X-Irradiation. Cells were exposed to tritium by adding the appropriate concentration of tritiated water (stock at 1 Ci/mL) to prewarmed complete growth media. The cells were incubated at 37°C in tritiated media for various times until the desired dose was delivered to the cells. Cells were then washed three times in fresh media and returned to the incubator for a 48-hour period. Paired lymphocyte samples in 37°C complete media, from the same donor, were also irradiated with 250 kVp X-rays at a dose rate which was equivalent to the tritium exposures. The cell samples were then washed three times, the same as the cells exposed to tritium, resuspended in fresh media, and returned to the incubator.

Apoptosis Measured by TdT and FADU Assays. Two assays were used to measure apoptosis. The terminal deoxynucleotidyl transferase (TdT) assay (ApopTag™, Oncor) and fluorescence analysis of DNA unwinding (FADU) assay (15,16). Briefly, the TdT assay fluorescently labels fragmented DNA within an apoptotic nucleus whereas the FADU assay measures DNA fragmentation by alkali unwinding of the DNA helix and subsequent labelling with ethidium bromide. For the TdT assay, apoptosis was reported as the proportion (%) of cells exhibiting fluoresence while values for the FADU assay were expressed as DNA fragmentation (Qd units) (16).

Data and figures. Each experiment was repeated at least three times from independent cultures. All figures shown represent the combined results of all the experiments. Standard error bars are shown except were the error is less than or equal to the symbol size. One-way analysis of variance and Student's T-tests were performed to determine the statistical significance between the various treatments. P-values are given where results are considered significant. When results were not significantly different no P-values are given.

RESULTS

Tritium-Induced Adaptive Response in Yeast

Radiation-Induced Thermal Tolerance. Yeast cells exposed to 52°C were killed in a time dependent manner (Figure 1A). When cells were exposed to a prior sublethal 200 Gy dose of gamma-radiation or tritium-beta radiation, they developed thermal tolerance. There was no difference in the levels of induced thermal tolerance between tritium-beta and cobalt-60 gamma-radiation ($p<0.001$).

Radiation-Induced Radiation Resistance. Yeast cells exposed to 200 Gy of either cobalt-60 gamma-radition or tritium-beta radiation developed radiation resistance with incubation time (Figure 1B). The level of induced radiation resistance was the same in cells induced with tritium-beta or cobalt-60 gamma-radiation ($p<0.001$).

Radiation-Induced Mutation and Suppression of MNNG Mutation. Increasing doses of cobalt-60 gamma-radiation or tritium-beta radiation induced similar levels of mutation in yeast cells (Figure 1C). When cells were exposed to MNNG, and then subsequently irradiated with either cobalt-60 gamma or tritium-beta radiation, the frequency of MNNG-generated mutation decreased. The suppression in mutation frequency was proportional to dose and not significantly different for both types of radiation (Figure 1D) ($p<0.01$).

Tritium-Induced Apoptosis in Human Lymphocytes

Initial DNA Damage at 0°C and 37°C. When human lymphocytes were exposed to 4 Gy of X-rays or tritium-beta radiation similar amounts of DNA damage were produced. When the exposures were performed at 37°C, under conditions of normal DNA repair, the initial damage was significantly reduced compared to exposures at 0°C when no DNA repair was possible (Figure 2A) ($p<0.001$). The level of apoptosis induced by both types of radiation at either temperature, however, was similar (Figure 2B) ($p<0.001$).

X-ray and Tritium-Induced Apoptosis at 0°C and 37°C. When lymphocytes were exposed to increasing doses of X-rays or tritium-beta radiation, delivered over four hours at 37°C, and apoptosis was measured using FADU, there was no difference in the levels of radiation-induced apoptosis (Figure 2C). When apoptosis was assessed using the TdT assay, a similar result was obtained except there was a higher level of apoptosis at 1.0 Gy for tritium-beta exposure (Figure 2D).

DISCUSSION

Two evolutionary distinct cell types, yeast and human lymphocytes, were used to test the RBE of tritium. Two biological endpoints were chosen, adaption to radiation and apoptosis, because these are cellular processes that respond to radiation at doses in the ranges relevant to occupationally exposed human cells.

Tritium-Induced Adaptive Response in Yeast. The adaptive response in yeast comparing tritium to cobalt-60 gamma-radiation and to 250 kVp X-rays showed no significant differences in the responses. The data strongly indicate that the RBE of tritium is not different from 1 for the adaptive response. The development of thermal tolerance and radiation resistance during cellular adaption in yeast occurs by two biologically separate mechanisms within the adaptive response. The two mechanisms are induced in response to DNA damage and therefore changes in the spectrum of DNA damage with changes in LET are known to change the magnitude of the responses (17). Since no differences were observed for tritium-beta and cobalt-60 gamma-radiation induced adaptive response, the microdosimetric differences between the two radiation qualities does not seem to be important with respect to the biological response of the cells for this endpoint. Enhanced radiation resistance to cell killing and suppression of chemically-induced mutation are believed to be a result of increased DNA repair capacity. Therefore, the data show that tritium-beta radiation induces DNA repair capacity as efficiently as cobalt-60 gamma-radiation.

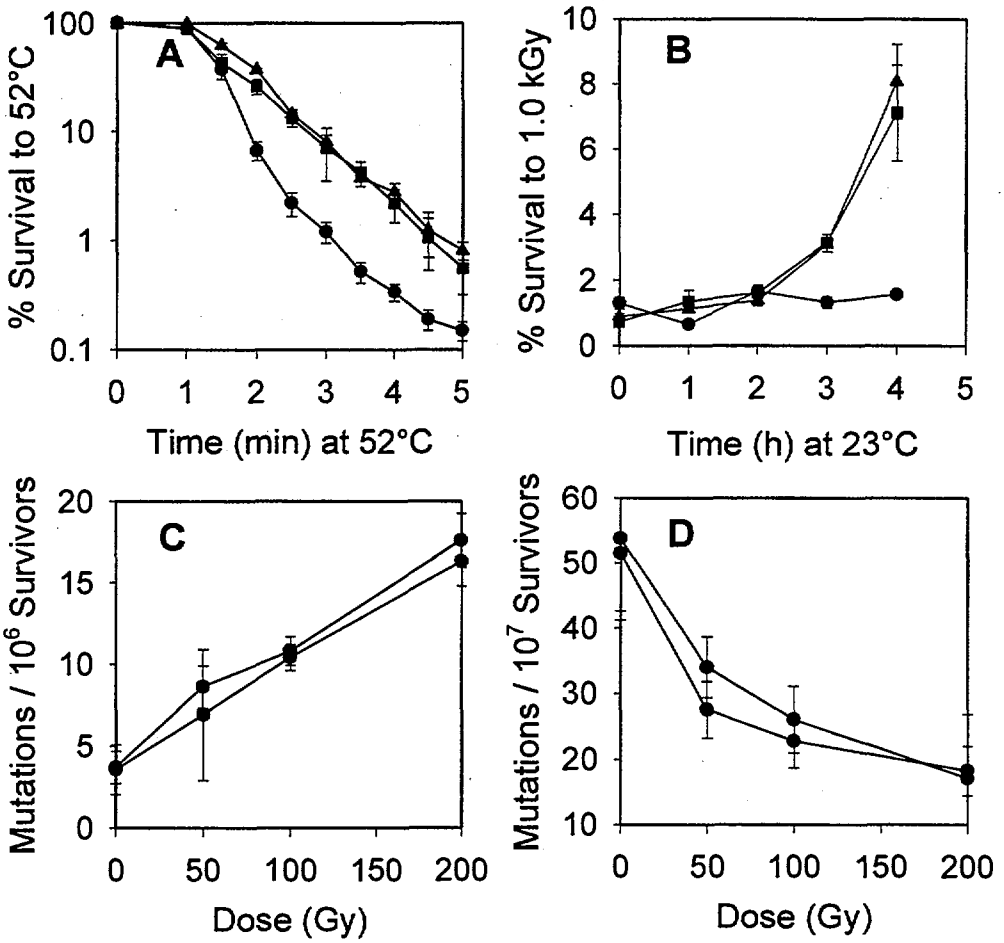

Figure 1. Radiation-Induced Adaptive Response in Yeast. (A) Thermal tolerance was induced by exposing cells to 200 Gy of cobalt-60 gamma-radiation (squares) or tritium beta-radiation (triangles), incubating cells for two hours, then heated at 52°C for various times. Unirradiated control cells are shown as circles. (B) Radiation resistance was induced by exposing cells to 200 Gy of cobalt-60 gamma-radiation (squares) or tritium beta-radiation (triangles), incubating cells for various times, and then testing survival to a 1 kGy gamma-radiation exposure delivered under oxic conditions. Unirradiated control cells are shown as circles. (C) Radiation-induced mutation was measured after exposing cells to increasing doses of cobalt-60 gamma-radiation (circles) or tritium-beta radiation (squares). (D) Suppression of MNNG-generated mutation was induced by exposing cells to increasing doses of cobalt-60 gamma-radiation (circles) or tritium-beta radiation (squares) before treating cells with 20 μg/mL of MNNG for 30 minutes.

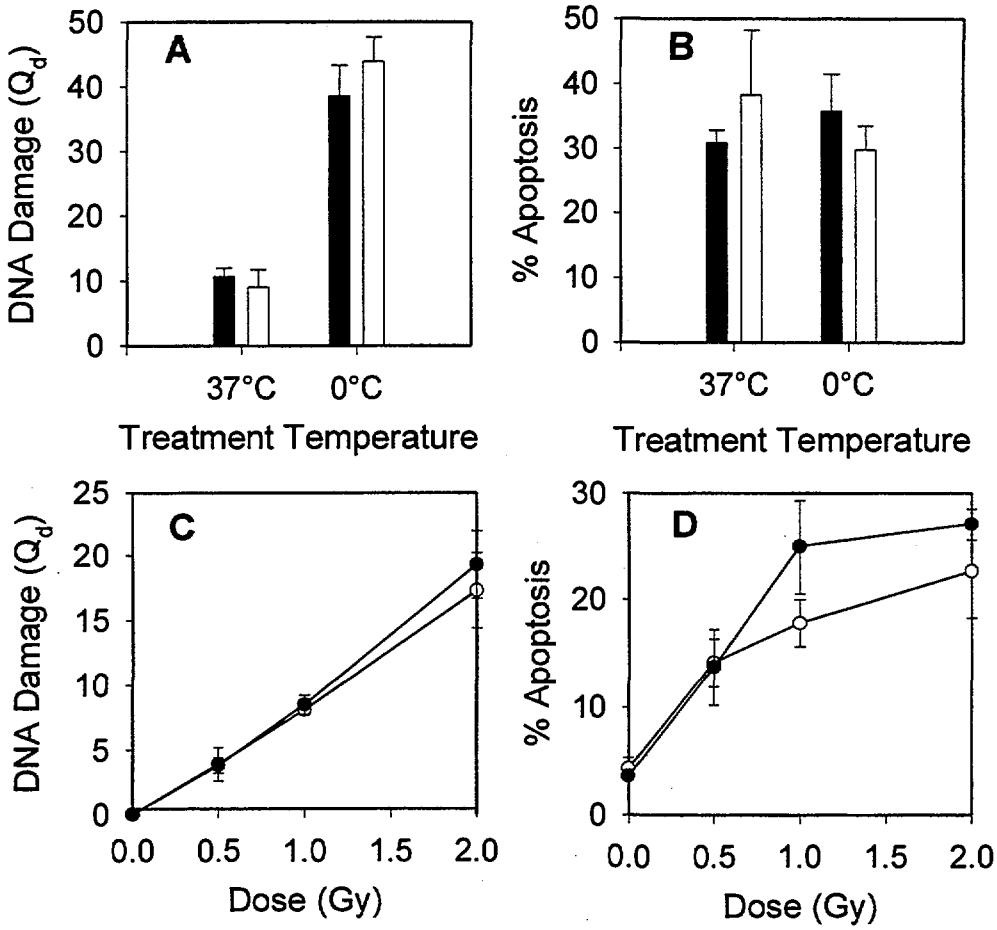

Figure 2. Radiation-Induced Apoptosis in Human Lymphocytes. (A) Initial DNA damage was measured immediately following exposure of lymphocytes to 4 Gy of tritium-beta radiation (closed bars) or 250 kVp X-rays (open bars), delivered over 4 hours at either 0°C or 37°C. (B) Radiation-induced apoptosis was measured using the TdT assay 48 hours after exposure of lymphocytes to 4 Gy of tritium-beta radiation (closed bars) or 250 kVp X-rays (open bars), delivered over 4 hours at either 0°C or 37°C. (C and D) Radiation-induced apoptosis was measured using the FADU assay (C) or the TdT assay (D) 48 hours after exposure of lymphocytes to increasing doses of tritium-beta radiation (closed cicles) or 250 kVp X-rays (open circles), delivered at 37°C at a dose rate of about 1 Gy/h.

Tritium-Induced Apoptosis in Human Lymphocytes. Radiation-induced apoptosis has been shown to be a sensitive indicator of radiation exposure (15). Here we have determined the relative effectiveness of tritium beta-radiation at inducing apoptosis. Overall the results showed that tritium was not different, per unit dose, than X-rays at inducing apoptosis. This further supports the contention that, although there may be microdosimetric differences between these two qualities of radiation, the biological responses to the damage are the same. Tritium and X-ray irradiations at 37°C produced less initial DNA damage than exposures at 0°C

(Figure 2A). This was expected since the exposure rate (about 1 Gy/h) was low enough to allow repair to remove some DNA damage during the irradiation period. Interestingly, this did not affect the overall level of radiation-induced apoptosis (Figure 2B). That is, the initial amounts of DNA damage at the end of the exposures were different when irradiations were done at 0°C of 37°C but the level of apoptosis resulting from the exposures were not different. This indicates that dose rate may not affect the induction of apoptosis. It is possible that it is not the rate of DNA damage but the total number of signalling lesions that induce the apoptotic response. Further investigation into the possible lack of a dose rate effect for radiation-induced apoptosis are in progress.

In conclusion, several tests using the adaptive response in yeast and apoptosis in lymphocytes comparing tritium-beta radiation to cobalt-60 gamma-radiation and X-rays showed no differences in the responses. The data show that the RBE of tritium for these endpoints is not significantly different from 1. The adaptive response and apoptosis are two cellular processes that are sensitive enough to respond to radiation doses in the occupational exposure range. Since both mechanisms do not have an unusual response to tritium exposures, it is further evidence that the risk associated with tritium exposure may not be extraordinary or unique.

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