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# Growth Retardation of Paramecium and Mouse Cells by Shielding Them from Background Radiation

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**Impaired growth/Paramecium/Mouse L5178-Y/Background radiation.**

In the 1970s and 1980s, Planel *et al*. reported that the growth of paramecia was decreased by shielding them from background radiation. In the 1990s, Takizawa *et al*. found that mouse cells displayed a decreased growth rate under shielded conditions. The purpose of the present study was to confirm that growth is impaired in organisms that have been shielded from background radiation. Radioprotection was produced with a shielding chamber surrounded by a 15 cm thick iron wall and a 10 cm thick paraffin wall that reduced the γ ray and neutron levels in the chamber to 2% and 25% of the background levels, respectively. Although the growth of *Paramecium tetraurelia* was not impaired by short-term radioprotection (around 10 days), which disagreed with the findings of Planel *et al*., decreased growth was observed after long-term (40–50 days) radiation shielding. When mouse lymphoma L5178Y cells were incubated inside or outside of the shielding chamber for 7 days, the number of cells present on the 6th and 7th days under the shielding conditions was significantly lower than that present under the non-shielding conditions. These inhibitory effects on cell growth were abrogated by the addition of a $^{137}$Cs γ-ray source disk to the chamber. Furthermore, no growth retardation was observed in XRCC4-deficient mouse M10 cells, which display impaired DNA double strand break repair.

## INTRODUCTION

Worldwide, humans are exposed to a mean background radiation dose of about 2.4 mSv per year.[1] Although there have been many studies on the biological effects of high dose radiation, there is no consensus about the biological effects of low-dose ionizing radiation such as background radiation. The International Commission on Radiological Protection (ICRP) employs a linear no-threshold (LNT) relationship between dose and health effects, and the LNT model has also been applied to low-level exposure.[2,3] However, this extrapolation might be too simplistic to accurately represent the biological effects of low dose or low dose-rate radiation, such as their effects on cancer and genetic disorders.[4]

According to the LNT model, exposure to extremely low dose or low dose-rate radiation such as natural background radiation should induce adverse biological effects. However, in the 1970s and 1980s Planel *et al*. reported that the growth of paramecia was decreased by shielding them from background radiation.[5–10] In their experiments, they used a lead chamber that reduced the background γ radiation to 1/6 of its normal level. The paramecia cultured in the chamber showed a low growth rate, and their growth rate was recovered by placing a low dose-rate γ-ray source in the lead chamber. In the 1990s, Takizawa *et al*. reported that mouse L5178Y cells displayed a decreased growth rate when the cells were cultured in a lead shielding chamber that reduced the background γ radiation level to 1/3 of the normal level.[11] However, few reports have attempted to confirm these phenomena. The lack of evidence to support these previous findings has prevented the molecular mechanisms responsible for them from being elucidated.

The purpose of the present study is to confirm that organisms shielded from background radiation display impaired growth. In this study, radioprotection was performed with a shielding chamber surrounded by a 15 cm thick iron wall and a 10 cm thick paraffin wall. Using this shielding device, the levels of γ rays and neutrons were reduced to 2% and 25% of their normal background levels, respectively. We

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cultured paramecium and mouse cells in a shielding chamber and investigated the effects of low-level background radiation on cell proliferation.

## MATERIALS AND METHODS

### *Shielding apparatus and dosimetry*

As shown in Fig. 1, radioprotection was achieved with a shielding chamber surrounded by a 15 cm thick iron wall (external size: 1.2 m wide × 1.2 m height × 0.8 m depth). We used old iron (more than 70 years after its refinement) as a shield material rather than lead in order to reduce secondary radiation. The iron wall was further surrounded by a 10 cm thick paraffin wall. The paraffin contained lithium to absorb neutrons. Gamma spectrophotometry was performed using a $\phi$ 2 cm × 2 cm NaI detector (MSP-20, Ohyo Koken Kogyo Co., Ltd., Tokyo, Japan) and an INSPECTOR-2000 multi-channel analyzer (Canberra Co., Meriden, Connecticut, U.S.A.). The spectra were converted to dose values using the G(E) function method.[12] Neutron dosimetry was performed using a $BF_3$ detector (ND-8534-60, Mitsubishi Electric, Co. Ltd., Tokyo, Japan) and a $^3$He neutron dose rate meter (TPS-451BS, Aloka, Co. Ltd., Tokyo, Japan).

In the latter part of the present study, a small disk containing 1 μCi ($3.7 \times 10^4$ Bq) of $^{137}$Cs was placed above the cells in the shielded chamber to compensate for the γ-radiation shielding. The dose rate of $^{137}$Cs γ-rays at the cell culture was calculated geometrically.

### *Paramecium experiments*

*Paramecium tetraurelia* (mating type VII, stock 51) and *Klebsiella pneumoniae* were kindly provided by Prof. Y. Takagi (Nara Women's University). WGP (Wheat Glass Powder) was purchased from Pines International Inc. (Lawrence, KS, U.S.A.). A 0.5% WGP infusion supplemented with stigmasterol (0.5 mg/L) and $Na_2HPO_4 \cdot 12H_2O$ (0.5 g/L) was used as the culture medium.[13] *Paramecium* feeds on microorganisms like bacteria, algae, and yeasts. The medium was inoculated with *Klebsiella pneumoniae* 2 days before use.[13] To examine the effect of catalase and hydrogen peroxide in the medium, *Escherichia coli* strain DSH67[14] that lacks catalase and superoxide dismutase (SOD) was inoculated instead of *Klebsiella pneumoniae*. A single *Paramecium* cell was isolated with a micropipette under a dissecting microscope and inoculated into a culture dish. The cell was then cultured in polystyrene 96-well microtiter plates containing 200 μL/well of the medium or in polystyrene $\phi$ 60 mm dishes containing 5 mL/dish of the medium. For incubation, we prepared two identical polystyrene foam boxes. One box was covered with aluminum film and then put in the shield chamber. The other was placed in a wooden box next to the shield chamber and used as a negative control culture. In these boxes, culture vessels were placed on a warmed copper plate (36 cm × 36 cm), beneath which temperature-controlled water was circulating. A water circulator (NCB3200, Tokyo Rikakikai Co, Ltd., Tokyo, Japan) controlled the water temperature and circulated the water beneath the copper plate in both boxes. The temperature of the copper plates was monitored using a BAT-12 thermocouple thermometer (Neuroscience Inc., Tokyo, Japan). The temperature difference between the copper plates in the two boxes was maintained within 0.1°C. The incubation temperature fluctuated within ± 0.1°C of the desired temperature. We placed a beaker of water on each plate to ensure that the ambient air remained moist. The number of cells was counted under a dissecting microscope, after appropriate dilution.

Autogamy in *paramecium* is a form of sexual reproduction in a single cell, and induced by natural starvation. Unless the medium is changed, *Paramecia* eat up the bacteria in the medium within several days, and then food-exhausted culture begins. To measure the percentage of

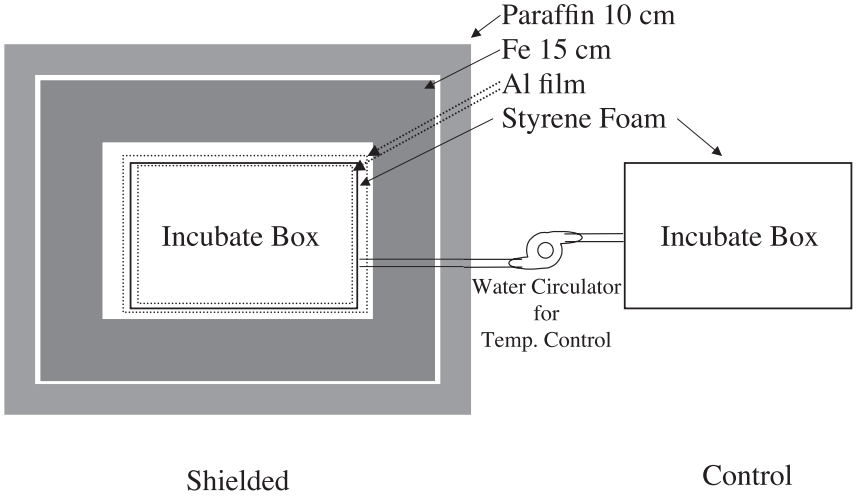


**Fig. 1.** Shielding device.

autogamy, about 100 cells were sampled from the food-exhausted culture and stained with Dippell's staining method,[15] and the percentage of cells displaying macronuclear fragments was examined under a microscope.[16] If all sampled cells were autogamous, one cell was isolated from the food-exhausted culture and used as an autogamous cell for the subsequent cell culture experiment.

*Mammalian cell experiments*

Mouse L5178Y and M10 cells were purchased from RIKEN BioResource (Ibaraki, Japan). The cells were maintained in RPMI-1640 medium (Sigma-Aldrich Co., Ayrshire, U.K.) supplemented with 10% FCS (JRH Biosciences, Lenexa, Kansas, U.S.A.) at 37°C under 5% $CO_2$. Airtight cell culture was performed in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 10% FCS (JRH Biosciences) and 25 mM HEPES using a 25 $cm^2$ polystyrene suspension culture flask with a double seal cap (Asahi Techno Glass Co., Tokyo, Japan). Cells in the logarithmic growth phase were inoculated into the culture flasks at a concentration of $5.0 \times 10^3$ cell/mL. The flasks were then placed in one of two incubators (SLI170D, Tokyo Rikakikai Co, Ltd., Tokyo, Japan). One incubator was placed in the shield chamber, and the other was placed next to the shielded chamber as a negative control culture. To measure the cell growth rate, the number of living cells was counted using the trypan blue staining method on the 5th, 6th, and 7th days after the initial cell inoculation. Cell counting was performed with a single-blind method.

## RESULTS

*Radioprotection*

Gamma spectrograms acquired inside or outside of the shielding chamber are shown in Fig. 2A. The spectrography was performed for 168 hours. Two peaks for $^{40}K$ (1,461 keV) and $^{208}Tl$ (2,615 keV) were detected during the spectrometry outside of the chamber, while only one peak for $^{40}K$ was detected inside the chamber. γ-rays with energy levels higher than 2,000 keV were almost completely shielded by the chamber. The dose rates of γ-ray were 2.1 and 109 nGy/hour inside and outside the chamber, respectively; *i.e.*, the dose rate was reduced to about 2% of the background level by the shielding apparatus.

The neutron spectrograms obtained inside or outside of the shielding chamber are shown in Fig. 2B. The spectrography was performed for 200 hours in both conditions. The peak detected around 160 ch was due to thermal neutrons. The neutron dose rates were 2 and 8 nGy/hour inside and outside the chamber, respectively. The neutron dose rate was reduced to about one quarter of its normal value by the shielding chamber.

*Effect of background radiation blocking on Paramecium cell growth*

After autogamy induction in food-exhausted culture, a single *Paramecium* was isolated in a dish and cultured at 25°C for 4 days. Then, a single cell was collected and placed into a ϕ 6 cm plastic culture dish containing 5 mL medium. Thirty dishes, each of which contained a single cell, were placed into culture boxes located inside or outside of the shielding chamber, and the *Paramecia* were grown at 25°C for 10 days. We removed 3 dishes from each culture box everyday and counted the number of *Paramecia* until the 5th day. After the 5th day, we sampled a small aliquot of culture medium everyday instead of removing the dishes and counted the number of *Paramecia* after appropriate dilution. The resultant growth curves are shown in Fig. 3A. One *Paramecium* proliferated at a growth rate of 3.7–3.9 divisions per day, and the number of cells reached $4 \times 10^4$ cells in 5 mL culture medium on the 4th day in both conditions. No difference in cell growth was observed between the two conditions.

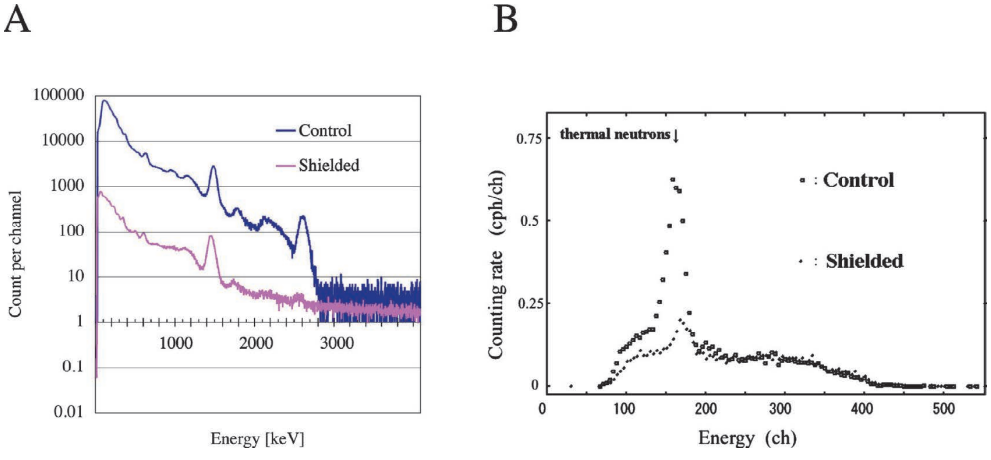


**Fig. 2.** Dosimetry. A: Gamma spectrograms obtained inside (red) and outside (purple) of the shielding chamber. B: Neutron spectrograms obtained inside (square) and outside (diamond) of the shielding chamber.

Since the proliferation of *Paramecia* was much faster in the present study than in previous studies,[10] we diluted culture media 2, 4, or 16-fold with sodium phosphate buffer (0.5 g/L of $Na_2HPO_4 \cdot 12H_2O$) to reduce their proliferation rate. The growth curves of the *Paramecia* cultured in the 4-fold diluted media are shown in Fig. 3B. Although the saturation cell number was reduced, neither the 2 nor 4-fold dilution affected the proliferation rate of the cells (data not shown). In the culture subjected to 16-fold dilution, the proliferation rate decreased, and growth saturation had not occurred by the 10th day; however, no difference in cell growth was observed between the two conditions (data not shown). To reduce the proliferation rate, we also cultured *Paramecia* at 17°C (Fig. 3C) and 20°C (data not shown), instead of 25°C. Although the proliferation rate was reduced by lowering the temperature, the saturation cell number was not affected, and no difference in cell growth was observed between the two conditions.

Croute *et al*. observed a radiation shielding effect on paramecium cell proliferation on the 11th day after autogamy.[6] In our experiments, no growth retardation of the cells in the shielding chamber was observed in cell cultures started from a single 11-day-old cell (*i.e.* cell on the 11th day after autogamy) (Fig. 3D).

We therefore conducted experiments to examine the effect of radiation shielding on the lifespan of paramecia. After inducing autogamy by food-exhausted culture, a single *Paramecium* was isolated and cultured at 25°C for 4 days. Then, a single cell was collected and inoculated into each well of the first row of a 96-well plastic microtiter plate (each well contained 200 µL medium). The microtiter plates were placed in culture boxes located inside or outside of the

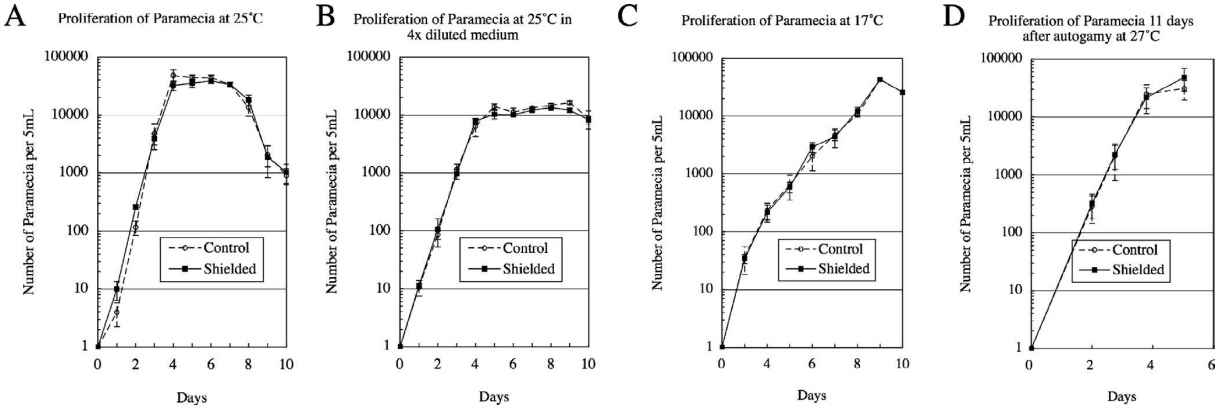


**Fig. 3.** The growth curves of *Paramecia*. Each plot shows the mean ± S.D. of three independent cultures. A: The growth curve of *Paramecia* in the standard culture conditions. B: The growth curve of *Paramecia* in 4-fold diluted medium. C: The growth curve of *Paramecia* at 17°C. D: The growth curve of *Paramecia* 11 days after autogamy.

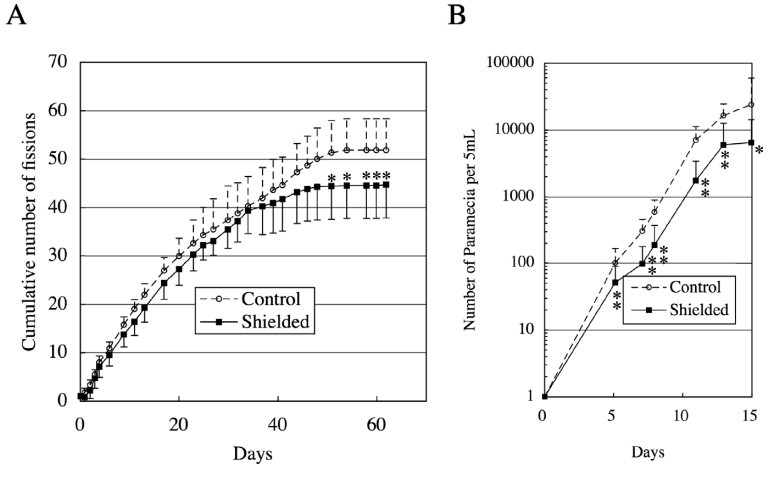


**Fig. 4.** The growth curves of the *Paramecia* in the long-term shielding experiment. A: The Y-axis represents the cumulative fission number. Each plot shows the mean ± S.D. of six independent passage lines. Statistical differences ($P < 0.05$) between the shielded and the non-shielded conditions were found on 50th, 53rd, 57th, 59th and 61st days (indicated with asterisks). B: The growth retardation after long-term shielding. The growth curves were acquired using the *Paramecia* that had been cultured inside or outside the shielding chamber for 40 days. * indicates $P < 0.05$, ** indicates $P < 0.01$ (the shielded *vs* the non-shielded conditions).

shielding chamber. The temperature of the culture box was kept at 18°C throughout the experiment. We counted the number of *Paramecia* in each well and moved a single cell to a new well of the next row in the plate successively with 200 μL medium every 2 or 3 days. The cumulative number of fissions during the culture is shown in Fig. 4. The experiment was performed twice in the same conditions to examine the reproducibility of the results (data not shown). The *Paramecia* inside the shielding chamber stopped proliferating around day 40, although the cells outside the chamber continued proliferating until the 50th day. The cumulative fission numbers of the *Paramecia* cultured under the shielding conditions were significantly lower than those of the *Paramecia* cultured under the non-shielded conditions ($P < 0.05$) after the 50th days in the experiment.

To confirm that the shielding induced growth retardation, we performed a short-term proliferation experiment using *Paramecia* that had been cultured inside or outside the shielding chamber for 40 days. On the 40th day, single cells were isolated and inoculated into culture dishes, which were then placed inside or outside of the shielding chamber, and the growth rates of the cells at 18°C were then measured for an extra 15 days. Therefore, total shielded duration of the *Paramecia* under the shielded condition was 55 days. The growth rate of the cells cultured inside the shielding chamber was significantly lower than that of the cells cultured outside the chamber (on the 5th–13th days, $p < 0.01$; on the day 15th day, $p < 0.05$) (Fig. 4B).

To confirm the effect of radiation shielding on *Paramecium* growth, we compensated for the background radiation shielding by adding a $^{137}$Cs γ-ray source to the long-term shielding experiments. The *Paramecium* cells were cultured in a shielding chamber in which a 1 μCi ($3.7\times10^4$ Bq) $^{137}$Cs source disk had been attached to the ceiling. The dose rate of $^{137}$Cs γ-rays on the surface of the *Paramecium* culture was 99 nGy/hour. As mentioned above, the background dose rate outside the chamber was 109 nGy/hour. Figure 5 shows that the inhibitory effect of the radiation shielding on *Paramecium* growth disappeared when the cells in the shielding chamber were exposed to low dose-rate radiation at levels similar to the background level. The experiment was performed twice in the same conditions to examine the reproducibility of the results (data not shown).

### *Effect of radiation shielding on mouse cell growth*

Mouse lymphoma L5178Y cells were inoculated into 30 culture flasks at a concentration of $5.0 \times 10^3$ cells/mL and incubated in culture boxes placed inside or outside of the shielding chamber (15 flasks in each condition) at 37°C for

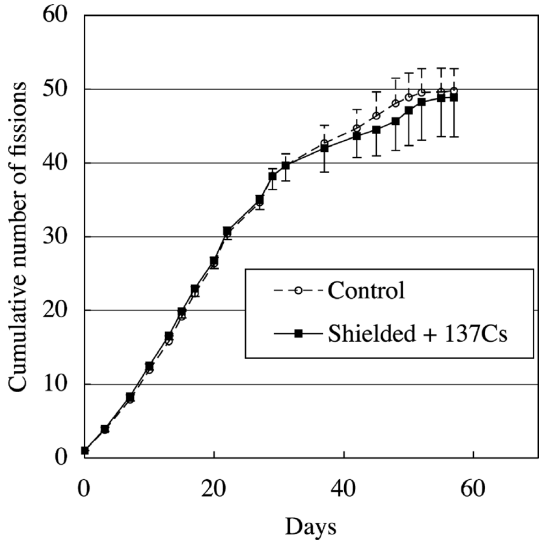


**Fig. 5.** The growth curves of the *Paramecia* in the long-term shielding experiment in the presence of a $^{137}$Cs radiation source. The radiation source compensated for the shielded radiation. The Y-axis represents cumulative fission number. Each plot shows the mean ± S.D. of six independent passage lines.

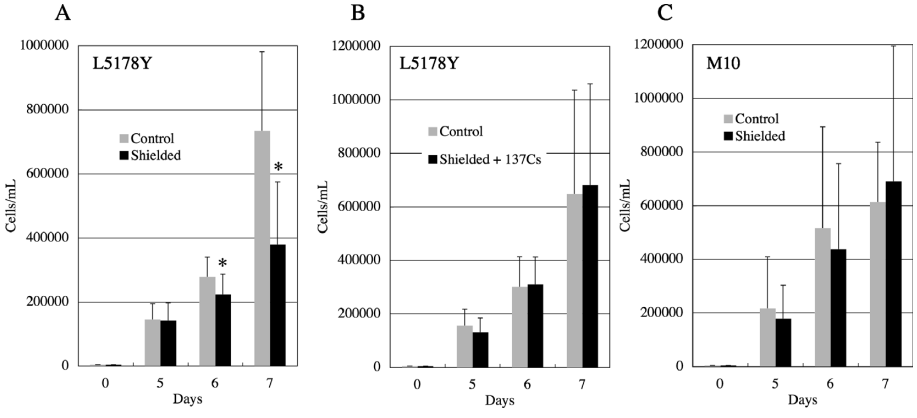


**Fig. 6.** The growth of mouse cell lines. The mean ± S.D. of five independent cultures is shown. A: The growth of lymphoma L5178Y cells. * indicates $P < 0.05$ (the shielded *vs* the non-shielded conditions). B: The growth of L5178Y cells in the presence of a $^{137}$Cs radiation source. C: The growth of M10 cells, a radiation sensitive derivative of the L5178Y cell line that lacks XRCC4.

7 days. We removed 5 flasks from each culture box on the 5th, 6th, and 7th days and counted the number of cells they contained. Figure 6A shows the growth of L5178Y cells in the shielded and non-shielded conditions. In the non-shielded cultures, the cell concentration was $1.45 \times 10^5$ cells/mL, $2.78 \times 10^5$ cells/mL, and $7.35 \times 10^5$ cells/mL on the 5th, 6th, and 7th days, respectively. On the other hand, in the shielded cultures the cell concentration was $1.42 \times 10^5$ cells/mL, $2.23 \times 10^5$ cells/mL, and $3.79 \times 10^5$ cells/mL on the 5th, 6th, and 7th days, respectively. The numbers of cells present under the shielding conditions on the 6th and 7th days were significantly lower than those present under the non-shielding conditions ($P < 0.05$).

The L5178Y cells were then cultured in the presence of a $^{137}$Cs γ-ray source disk to compensate for the shielded background radiation. Figure 6B shows that the inhibitory effect of the radiation shielding on cell growth disappeared when the cells were exposed to low dose-rate radiation (99 nGy/h) at levels similar to the background level.

We also performed shielding culture experiments with M10 cells, a radiation sensitive derivative of mouse L5178Y cells that display deficient XRCC4 expression. The M10 cells were inoculated into 30 culture flasks at a concentration of $5.0 \times 10^3$ cells/mL and incubated in culture boxes located inside or outside of the shielding chamber (12 flasks in each condition) at 37°C for 7 days. We removed 4 flasks from each culture box on the 5th, 6th, and 7th days and counted the number of the cells present. Figure 6C shows the growth of M10 cells in the shielding and non-shielding conditions and indicates that there was no significant difference in the cell growth rate between the two conditions.

## DISCUSSION

In the present study, the growth of *Paramecia* was retarded when they were shielded from background radiation throughout their whole life; however, we could not confirm the growth impairing effects of short-term radioprotection on *Paramecia* reported by Planel *et al*.[5] However, the present study did not rigorously reproduce the aforementioned group's experiments in terms of apparatus, shielding, and culture conditions. In our experiment, the γ-ray dose rates inside and outside of the chamber were 2.1 and 109 nGy/hour, respectively, whereas those in the previous study were 34 nGy/hour and 200 nGy/hour, respectively.[5] Furthermore, the *Paramecia* were cultured in a commercially available and sophisticated WGP medium in the present study; however, they were cultured in Scotch grass or straw medium by Planel *et al*. These differences might have produced the discrepancies between the previous and present studies. In addition, we used a medium inoculated with an *Escherichia coli* strain that lacks catalase and SOD instead of *Klebsiella pneumoniae* in the short-term radioprotection experiment since Croute *et al*. reported that the presence of catalase and hydrogen peroxide in the medium might play an important role in low-dose radio-induced growth stimulation.[7] However, the effect of radioprotection on *Paramecium* growth did not differ between the cultures containing the mutant and wild-type *E. coli* strains (data not shown).

In the present study, the induction of *Paramecium* growth retardation by radioprotection was only detected in senescent cells. In addition, in a previous study, no radioprotective effect was observed in *Paramecia* that were very close to autogamy.[5] If reactive oxygen species (ROS) are produced by background radiation, then *Paramecium* cells are exposed to ROS throughout their whole life. Therefore, an increase in ROS-induced DNA damage due to a decline in ROS-scavenging activity in the senescent cells might have caused their growth retardation. In fact, Croute *et al*. reported that the presence of hydrogen peroxide without catalase in the medium led a growth inhibition of *paramecium*.[7] If DNA repair-deficient or ROS-scavenging enzyme-deficient *Paramecium* mutants were available, the mechanism of this phenomenon could be clarified.

In this study, mouse L5178Y cells displayed a decreased growth rate when they were shielded from radiation, which is concordant with the previous observation by Takizawa *et al*.[11] No such growth retardation was noted in XRCC4-deficient M10 cells, which display impaired DNA double strand break (DSB) repair. The gene product of XRCC4 functions together with DNA ligase IV and DNA-dependent protein kinase in the repair of DSB.[17–19] The induction of this DSB repair pathway or DSB itself by background radiation might be involved in the growth retardation induced by radioprotection.

After the first report of impaired growth during radioprotection, few studies have attempted to confirm these phenomena. The difficulty of preparing a shielding apparatus is one of the obstacles for researchers in this field. In the present study, we used a shielding apparatus that displayed a greater protection efficiency than those used in previous studies and found that the growth of organisms was impaired during radioprotection. With our shielding apparatus, the growth of other species and mammalian cell lines should be studied in order to discover whether the growth retardation induced by background radiation shielding is a general phenomenon that affects all organisms.

Radiation hormesis induced by chronic low-dose radiation has been extensively documented.[20–22] In these studies, irradiation with chronic low-dose radiation above natural background doses induced beneficial effects in organisms; *i.e.*, the absence of low-dose radiation had unfavorable effects on the organisms. If background radiation acts in the same manner as chronic low-dose radiation on organisms, the growth retardation reported in the present study might be one aspect of radiation hormesis. If low-dose ionizing radiation including background radiation confers a growth advantage on

organisms, determination of the threshold between harmful and beneficial doses (or dose rates) is important.

## ACKNOWLEDGEMENTS

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