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# LETHAL AND MUTAGENIC EFFECTS OF CALIFORNIUM-252 RADIATION IN CULTURED HUMAN CELLS

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SADAYUKI BAN, Ph.D. 伴 貞幸
SHOZO IIDA 飯田昭三
AKIO A. AWA, D.Sc. 阿波章夫
SHOZO SAWADA, Ph.D. 澤田昭三

放影研
RERF

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財団法人 放 射 線 影 響 研 究 所

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財団法人 放射線影響研究所

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# LETHAL AND MUTAGENIC EFFECTS OF CALIFORNIUM-252 RADIATION IN CULTURED HUMAN CELLS

## 培養されたヒト細胞における californium-252 放射線の致死及び突然変異誘発効果

SADAYUKI BAN, Ph.D. ( 伴 貞幸 ); SHOZO IIDA ( 飯田昭三 );
AKIO A. AWA, D.Sc. ( 阿波章夫 ); SHOZO SAWADA, Ph.D. ( 澤田昭三 )*

*Department of Genetics*
遺伝学部

## SUMMARY

HeLa MR cells were exposed to radiation emitted from a man-made spontaneously fissioning isotope, californium-252. The neutron (n) to gamma-ray ratio in the radiation dose was measured to be 2.0. The extrapolation number (n-value) of the dose-survival curve was 1.3, and the exponential slope ($D_0$) was 200 cGy. A dose-dependent increase in mutation to 6-TG$^r$ was observed. The relative biological effectiveness (RBE) for cell killing of the neutrons from $^{252}$Cf, calculated relative to high dose rate X rays, was 2.6 at 50% survival. The RBE for mutation induction was 2.7 at a mutation frequency of $5 \times 10^{-5}$ per surviving cell.

## INTRODUCTION

The neutron doses to the A-bomb survivors are being substantially modified in the process of reassessment of atomic bomb radiation dosimetry in Hiroshima and Nagasaki.[1] It is very important and of interest to assess the biological effects of neutrons and to obtain basic biological data resulting from mixed radiation of $\gamma$ rays and neutrons. The availability of a californium-252 ($^{252}$Cf) source[2] has made such an assessment possible. The mean energy of the fissionlike neutron spectrum of $^{252}$Cf is 2.3 MeV which is in the energy range of A-bomb neutrons. Approximate energy of prompt fission neutrons from the A-bomb was 0-20 MeV.[3] In this paper we describe the lethal and mutagenic effects of $^{252}$Cf radiation on HeLa MR cells. High dose rate X rays (110 cGy/min) were used as the reference radiation to calculate the RBE.

## 要 約

人造核種 californium-252 の核分裂によって生じる放射線を Hela MR 細胞に照射した．放射線量の中，$\gamma$線に対する中性子(n)の割合は2.0であった．線量-生存率曲線の外挿値(n値)は1.3で，対数的傾斜($D_0$)は 200 cGy であった．6-TG$^r$ への突然変異率は線量に依存して上昇した．高線量率X線に対する $^{252}$Cf 中性子の細胞致死における生物学的効果比(RBE)は，50%生存率において2.6であった．突然変異誘発における RBE は，1生存細胞当たりの突然変異率 $5 \times 10^{-5}$ において2.7であった．

## 緒 言

広島・長崎での原爆放射線量の見直し作業の過程において，生存者への中性子線量が修正されつつある．[1] 中性子の生物学的影響を評価すること，及び$\gamma$線と中性子との混合放射線の基礎的な生物学的データを得ることは非常に重要であり，また興味あるところである．カルフォルニウム-252 ($^{252}$Cf) 線源[2] を利用できるようになって，このような評価が可能になった．$^{252}$Cf の核分裂中性子スペクトルの平均エネルギーは 2.3 MeV であり，これは原爆中性子のエネルギー範囲の内にある．原爆からの一次核分裂中性子のおおよそのエネルギーは 0～20 MeV であった．[3] 本報告では，HeLa MR 細胞における $^{252}$Cf 放射線の致死効果及び突然変異効果について述べる．高線量率 (110 cGy/分) のX線を対照線源として用い，RBE を求めた．

*RERF Consultant and Research Institute for Nuclear Medicine and Biology, Hiroshima University
放影研顧問及び広島大学原爆放射能医学研究所

## MATERIALS AND METHODS

**Cells and Culture Medium.** HeLa MR cells [HeLa S3(Mer$^-$)][4] cultured in αMEM (GIBCO, New York) supplemented with 10% fetal calf serum were used. MR cells were suspended in the culture medium during neutron irradiations for nearly seven hours. Throughout this period few cells became attached to the glass wall. After irradiation, the cells were simply resuspended by shaking gently. MR cells have a high colony forming ability in liquid growth medium and soft agar medium.

**Irradiations.** Actively growing cells were harvested with 0.25% trypsin and 0.01% EDTA solution and suspended in growth medium. Cells, $5 \times 10^6$ (1 ml), were put into a test tube (pyrex glass, inner diameter 15 mm, length 125 mm). Test tubes were transported to the Research Institute for Nuclear Medicine and Biology of Hiroshima University about 1 km from our laboratory. Each test tube was set up in one of eight aluminum tubes (inner diameter 18 mm, length 120 mm, 1 mm thick) standing on 8 cm from the center of continuously rotating table. Neutron irradiations were carried out at room temperature using a stainless encapsulated $^{252}$Cf source (length of 17 mm, diameter of 9.5 mm, Type X-35, Amersham International plc, England) loaded into the central axis of radiation. The dose rate, as determined using Three-Terminal Ionization Chamber (Types IC-17 and IC-17G, Far-West Technology, Inc., USA) and Fricke-dosimeter, was 1.50-1.53 cGy/min. The ratio of neutrons to γ rays in dose was 2.0. X rays (8 mA, 220 kVp, 0.3 Cu plus 0.5 Al filter, 110 cGy/min) were used as the reference radiation.

**Percent Survival.** Immediately after irradiation, an appropriate number of cells was seeded on each of six plastic dishes (Corning Co., New York, Product #25010) per dose and incubated in 95% air and 5% $CO_2$ at 37°C for nine days. Colonies were then fixed and stained with Giemsa. Colonies consisting of 50 or more cells were counted, and percent survival was determined.

**Induction of 6-TG$^r$ Mutants.** Cells were kept in growth with subculturing every two days in the period after irradiation until they were given a 6-TG(6-thioguanine: Wako Pure Chemicals, Tokyo) challenge. For each dose, $1 \times 10^6$ cells

## 材料及び方法

**細胞と培養液.** 10%胎牛血清を添加したαMEM (GIBCO 社, ニューヨーク) 中で培養された HeLa MR 細胞〔HeLa S3 (Mer$^-$)〕[4] を用いた. MR 細胞は培養液中に懸濁して, 約7時間に及ぶ中性子照射を受けた. その間にほとんどの細胞はガラス壁に付着しなかった. 照射後, 軽い振とうによって簡単に細胞浮遊液になる. MR 細胞は液体培地及び軟寒天培地中で高いコロニー形成能を有する.

**放射線照射.** 増殖の良い細胞を0.25%トリプシンと0.01% EDTA 溶液で剝離し, 増殖培養液に懸濁する. $5 \times 10^6$ 個の細胞 (1 ml) を試験管 (パイレックスガラス, 内径 15 mm, 長さ 125 mm) に入れる. 試験管を我々の研究室から約 1 km 離れている広島大学原爆放射能医学研究所へ移送する. 各試験管は, 連続回転台の中心から 8 cmの位置に立っている8本のアルミニウム管 (内径 18mm, 長さ 120 mm, 厚さ 1 mm) の1本ずつに入れる. 中性子照射は, 放射線の中心軸に位置するステンレス密封の $^{252}$Cf 線源 (長さ17mm, 直径 9.5mm, X-35型, Amersham 社, 英国) を用いて, 室温で行った. 線量率は, スリーターミナル電離箱 (IC-17 型と IC-17G 型, Far-West Technology 社, 米国) とフリッケ線量計とで測定し, 1.50～1.53 cGy/分であった. γ線量に対する中性子量の比は2.0であった. X 線 (8 mA, 220 kVp, 0.3 Cu＋0.5 Al フィルター, 110 cGy/分) を対照線源として用いた.

**生存率.** 放射線照射後, 適当数の細胞を1線量当たり6枚のプラスチック皿 (Corning 社, ニューヨーク. 製品番号 25010) に播種し, 95% 空気＋5% $CO_2$, 37°C で9日間培養した. コロニーを固定し, ギムザ溶液で染色した. 50以上の細胞から成るコロニーを計数し, 生存率を求めた.

**6-TG$^r$ 突然変異体の誘発.** 照射後, 6-TG (6-チオグアニン, 和光純薬, 東京) 処理までの間, 2日ごとに継代培養を行い, 細胞の増殖を維持した. 各線量

were seeded in each of six flasks (Corning Co., Product #25100) and 100 cells in each of six dishes. Growth medium was added to the dishes and cell survival was assayed. A 0.2% agar medium (40 ml) containing 10$\mu$g/ml of 6-TG was added to the flasks and incubated at 37°C for 3-4 weeks. After incubation, the visible 6-TG resistant (6-TG$^r$) colonies in each flask were scored. The number of mutant colonies was corrected for the decrease in survival of cells incubated for various periods following irradiation; and the mutation frequencies were expressed as the number of 6-TG$^r$ colonies per $10^6$ surviving cells.

当たり，6本のフラスコ（Corning社，製品番号25100）に各 $1\times10^6$ 細胞を，6枚のディッシュに各100細胞を播種した．ディッシュには増殖培養液を添加し，生存率を求めた．フラスコには，10 $\mu$g/ml 6-TG を含む0.2%寒天培地（40 ml）を添加して，37°Cで3-4週間培養する．培養後，各フラスコ中に観察される6-TG抵抗性（6-TG$^r$）コロニーを数える．突然変異コロニー数を照射後種々の時間培養した細胞の生存率の減少で補正し，$1\times10^6$ 生存細胞当たりの6-TG$^r$ コロニー数で突然変異率を表した．

**RBE$_n$.** If the assumption is made that the $\gamma$-ray component and the neutron component act independently, the relative biological effectiveness of neutrons (RBE$_n$) can be calculated as follows:

**RBE$_n$**: 混合放射線中の $\gamma$線と中性子が独立して作用すると仮定するならば，中性子の生物学的効果比（RBE$_n$）は次式で計算される．

$$RBE_n = \frac{RBE_{(n+\gamma)} - f_\gamma}{f_n}$$

Here, f represents fraction of dose due to n and $\gamma$.

ここで，f は中性子又は $\gamma$線による線量の割合を示す．

## RESULTS

Dose-survival responses of HeLa MR cells after exposure to $^{252}$Cf radiation and X rays are shown in Figure 1. After X irradiation the survival curve has an n-value of 3.6 and an exponential slope ($D_0$) of 195 cGy. After $^{252}$Cf irradiation, n-value of 1.3 and $D_0$ of 200 cGy were obtained. At 50% survival, RBE$_{(n+\gamma)}$ was 2.05 and RBE$_n$, 2.6. At 10% survival, RBE$_{(n+\gamma)}$ was 1.34 and RBE$_n$, 1.5.

## 結　果

$^{252}$Cf 放射線及びX線照射後の HeLa MR 細胞の線量-生存率を図1に示す．X線に対する生存率曲線ではn値3.6，対数的傾斜（$D_0$値）195 cGy であるのに対し，$^{252}$Cf 放射線ではn値1.3，$D_0$値 200 cGy であった．50%生存率において RBE$_{(n+\gamma)}$ は2.05，RBE$_n$ は2.6であった．10%生存率では RBE$_{(n+\gamma)}$ は1.34，RBE$_n$ は1.5であった．

It is well known that the number of mutations expressed is markedly affected by the length of time that cells are cultured after radiation exposure. The optimal expression time (defined as the time required after irradiation prior to challenge for the maximum number of mutant colonies to be detected) was determined for 200 and 400 cGy of X rays (Figure 2).

照射後の細胞培養時間の長さが，表現される突然変異数に大きく影響することはよく知られている．200 cGy 及び 400 cGy のX線照射後の最適表現時間（最も多くの突然変異コロニーを検出するための，照射後から選択前の時間として決定される）を求めた（図2）．

Mutation frequency increased with time up to the 7th day and remained stable until the 11th day after irradiation. Cell survival became 100% from the seventh day after exposure to 200 cGy and from the eighth day after exposure to 400 cGy. From these findings, the frequency of mutant colonies was determined on the ninth day following irradiation in all experiments.

突然変異率は放射線照射後7日目まで上昇し，11日目まで安定に保たれていた．細胞生存率は 200 cGy 照射後7日目から，400 cGy 照射後8日目から100%になった．これらの結果から，次のすべての実験には，照射後9日目の突然変異コロニーの頻度を決定した．

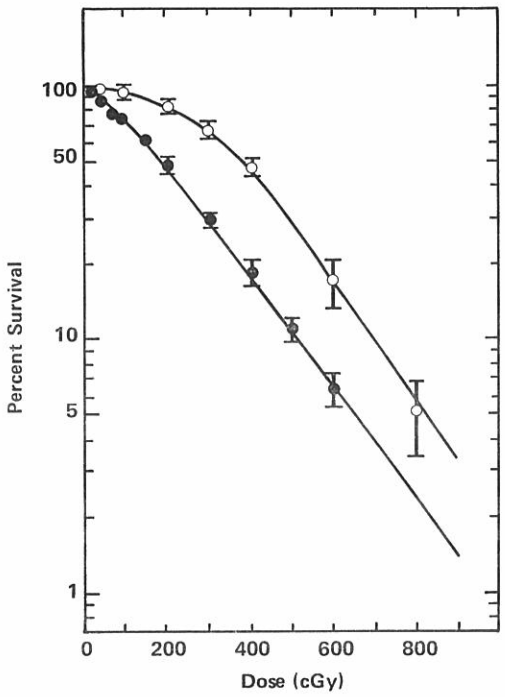


*Figure 1. Dose-survival of HeLa MR cells against doses of $^{252}Cf$ radiation (●) and X rays (○). Bars indicate standard deviations of the mean for three independent experiments. Plating efficiencies of nonirradiated cells were 95% or higher. Dose rates were 1.50-1.53 cGy/min for $^{252}Cf$ radiation and 110 cGy/min for X rays.*

図1　種々の線量の$^{252}$Cf 放射線(●)及びX線(○)照射後のHeLa MR細胞の線量-生存率．棒線は三回の個々の実験の平均と標準偏差を示す．非照射細胞のコロニー形成率は95%以上であった．線量率は，$^{252}$Cf 放射線が1.50～1.53 cGy/分で，X線が110 cGy/分であった．

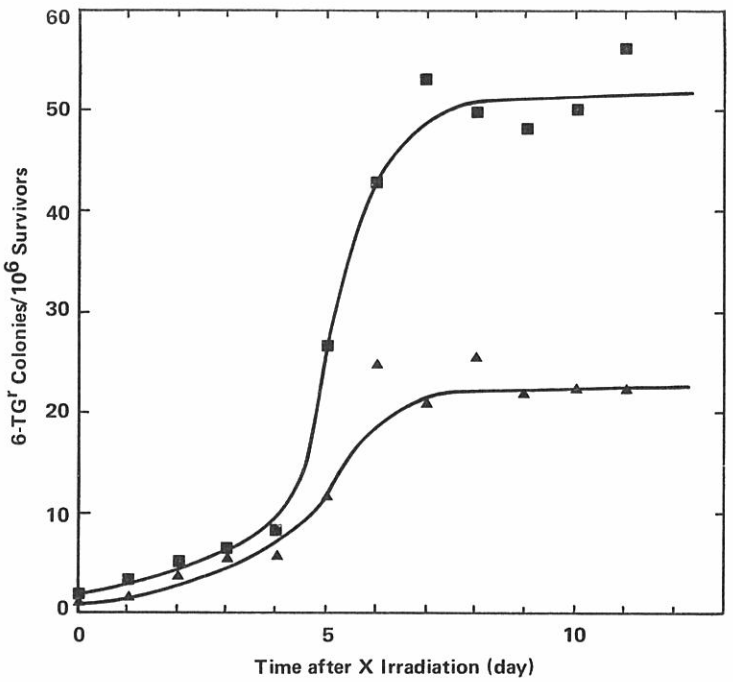


*Figure 2. The effect of the mutation expression time on the mutation frequency of HeLa MR cells following irradiation with 200 cGy (▲) or 400 cGy (■) of X rays. Curves fitted by eye to data.*

図2　200 cGy(▲)又は400 cGy(■)のX線を照射されたHeLa MR細胞の突然変異頻度に及ぼす突然変異発現時間の効果．曲線は目測で一致させた結果である．

The mutation frequencies per $10^6$ surviving HeLa MR cells as a function of radiation dose are shown in Figure 3. Because the lethal effect is large with irradiation in the high dose range, mutation frequencies were investigated in a dose range of 400 cGy or less for X ray and 200 cGy or less for $^{252}$Cf radiation. At these maximum doses, the percent survival was approximately 45% or higher.

Spontaneous mutation frequencies were 0.7-5.1 $\times 10^{-6}$. Mutation frequencies following X irradiation showed a significant nonlinear increase with increase of dose. Mutation frequencies following irradiation with $^{252}$Cf radiation, however, increased linearly with dose. Mutation frequencies were low in the shoulder part of the X-ray-survival response curve. A clear increase of mutation frequency was observed in the low dose range which corresponded to the almost negligible shoulder of the $^{252}$Cf-radiation-survival response curve. Each radiation had an inverse correlation between percent survival and mutation frequency. $RBE_{(n+\gamma)}$ was 2.1 and $RBE_n$, 2.7, at a mutation frequency of $5 \times 10^{-5}$ per survivor.

Figure 4 shows a comparison of mutation frequencies (from Figure 3) per percent survival (from Figure 1). The amount of genetic damage (mutation) per lethal damage was indistinguishable for both types of radiation.

## DISCUSSION

The RBE values of radiation emitted from $^{252}$Cf sources reported to date vary widely depending on endpoints.[5] Broadly classified, the RBE for the following endpoints have been accurately quantified.

1) Tissue-weight-reducing effect. Sensitivity to neutrons differs greatly according to the exposed tissue of the experimental animal. For example, neutrons were highly effective on mouse testis, with the RBE being as high as 3.4-5.1,[6] but their RBE was no more than 1.0 on the mouse spleen.[7] The RBE was 1.7 on mouse thymus.[8]

2) Lethal effect on cells under hypoxic conditions in vitro. The RBE for cell killing under hypoxic conditions is very high. Todd et al[9] have reported the RBE to be 7.0 at 10% survival of human kidney T1 cells. Other reports give an RBE of 4.1-5.0 at 10% survival of HeLa cells.[10-12]

照射線量当たりの HeLa MR 細胞の $10^6$ 生存細胞当たりの突然変異率を図3に示す．高線量域においては致死効果が大き過ぎるので，X線で 400 cGy 以下，$^{252}$Cf 放射線で 200 cGy 以下の線量域での突然変異率を調べた．これらの最大線量で，生存率は約45%以上であった．

自然突然変異率は $0.7 \sim 5.1 \times 10^{-6}$ であった．X線照射後の突然変異率は，線量の増加に伴い有意な非線形増加を示す．しかし，$^{252}$Cf 放射線照射後の突然変異率は，線量の増加に伴い直線的増加を示す．X線-生存率曲線の肩の部分において突然変異率は低い．$^{252}$Cf 放射線-生存率曲線では肩がほとんどないのに対応して $^{252}$Cf 放射線の低線量域においても突然変異率の明らかな上昇がみられた．両放射線において，生存率と突然変異誘発率との間には逆の相関がみられた．突然変異率 $5 \times 10^5$/生存細胞での $RBE_{(n+\gamma)}$ は2.1，$RBE_n$ は2.7であった．

生存率(図1)当たりの突然変異率(図3)を比較したのが図4である．両放射線の，細胞致死損傷当たりの遺伝的損傷(突然変異)はほぼ完全に一致した．

## 考　察

今までに発表されている $^{252}$Cf 放射線の RBE値は，指標によって非常に大きな幅をもっている．[5] 大きく分類して次の三系において定量性が優れている．

1) 組織重量の減少効果．実験動物の被曝される組織によって中性子に対する感受性は非常に異なる．例えば，マウス睾丸では中性子の効果は大きく，その RBE は3.4～5.1であった．[6] ところがマウス脾臓では中性子の RBE は1.0にすぎない．[7] マウス胸腺における RBE は1.7であった．[8]

2) 試験管内における低酸素下での細胞致死効果．低酸素下での細胞致死の RBE は非常に高い．Todd ら[9] は，ヒト腎由来の T1 細胞での10%生存率における RBE は7.0と報告している．HeLa 細胞の10%生存率における RBE は4.1～5.0の報告もある．[10-12]

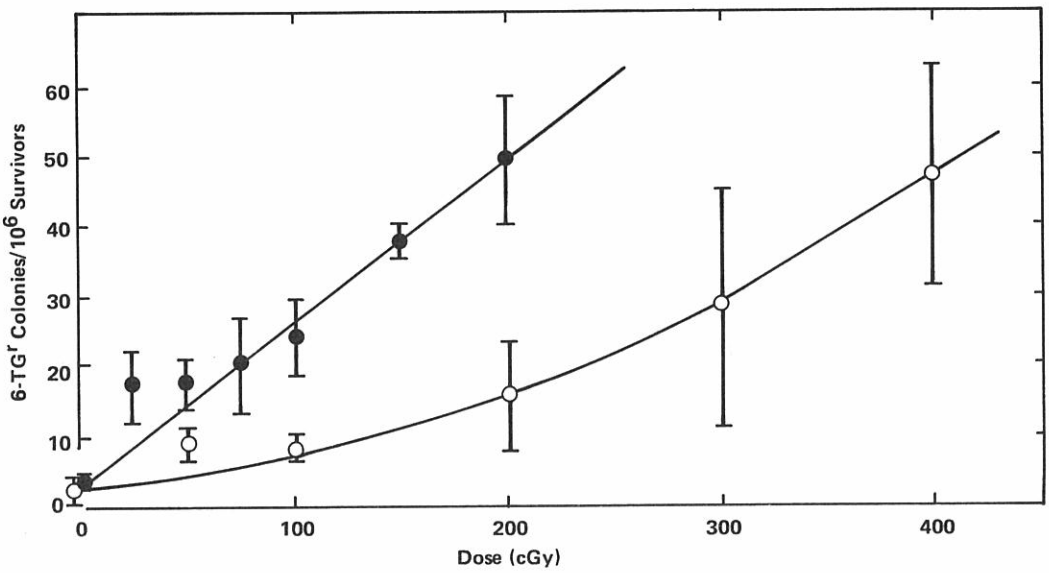


*Figure 3. Induction of 6-TG resistant mutants following irradiation with $^{252}Cf$ radiation (●) or X rays (○). Spontaneous mutation frequencies were $0.7\text{-}5.1 \times 10^{-6}$. Bars indicate standard deviations of the mean for three independent experiments.*

図3　$^{252}$Cf 放射線(●)又はX線(○)照射後の6-TG抵抗性突然変異体の誘発．自然突然変異頻度は$0.7\sim5.1\times10^{-6}$であった．棒線は3回の個々の実験の平均と標準偏差を示す．

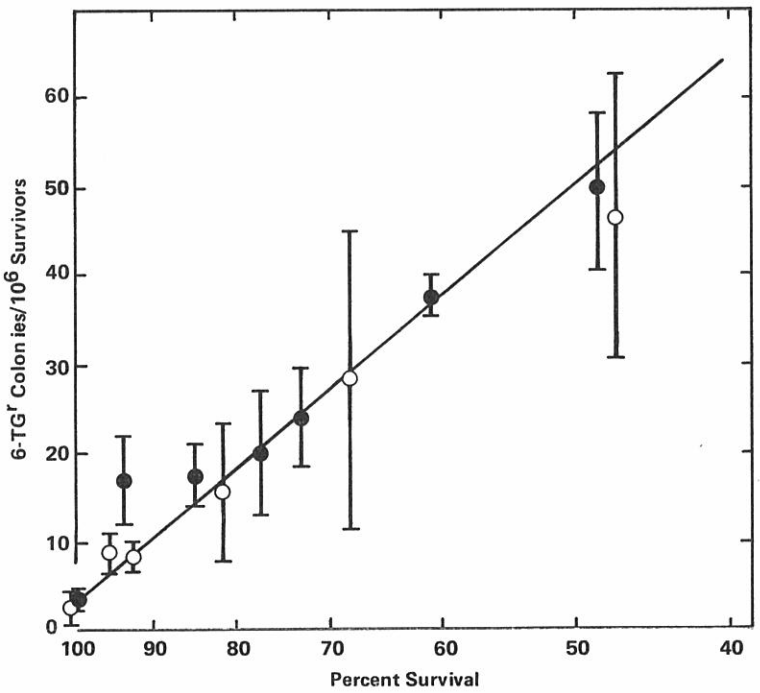


*Figure 4. Radiation-induced mutation frequencies per percent survival. Prepared on the basis of Figures 1 and 3.*

図4　生存百分率に対する放射線誘発突然変異頻度．図1と3を基に作成．

3) Lethal effect on cells under oxic conditions in vitro. The oxygen enhancement ratio for cell killing by neutrons is smaller than that for low LET radiation.[10,11] Results of various investigations have indicated the RBE in oxic systems was 4.3 or less.[10-12]

This is the first report of mutagenicity of low-dose-rate $^{252}$Cf radiation, compared with that of high-dose-rate X rays. Lowering of dose rate of $\gamma$ rays has resulted in a reduction of lethal effect.[13] Mutagenic effect of a given $\gamma$ doses was also reduced with chronic exposure, compared to acute irradiation.[14,15] The simplest explanation of reduction in the lethal and mutagenic effects might be that a fraction of radiation damage was repaired by the error-free processes during a prolonged period of low-dose-rate irradiation. The reduction in effectiveness was almost similar for both cell killing and mutation induction. Thacker and Stretch[15] found the linear relations between surviving fraction and induced mutant frequency at the low and high dose rates were almost completely same.

In the present paper, the RBE of $^{252}$Cf radiations to X rays was compared for lethal and genetic damage to cells irradiated under oxic conditions. Since the dose rates of both radiations were extremely different, the relation between percent survival and mutation frequency was compared. $RBE_n$ at 50% survival was 2.6. $RBE_n$ was 2.5-2.8 at a mutation frequency of $4.6\text{-}5.2 \times 10^{-5}$ equivalent to 40%-50% survival for the two types of radiation. For example, $RBE_n$ was 2.7 at the mutation frequency $5 \times 10^{-5}$. The lethal damage and the genetic damage from the two types of radiation were almost completely proportional (Figure 4). Nakamura et al[16] reported that fast neutrons generated from cyclotron induced more damage that led to 6-TG$^r$ mutations than did $\gamma$ rays. In their irradiation protocols, mouse L5178Y cells were exposed to high-dose-rate (20 rad/min) neutrons which include a contaminanting $\gamma$ ray of only 8% of the total dose. The different results may be due to the differences in the irradiation protocols, quality of neutrons, and kind of cells used.

A number of reports published recently are clarifying the structural changes occurring in the HPRT gene of 6-TG$^r$ (HPRT-deficient)

3) 試験管内における酸素存在下での細胞致死効果. 中性子の細胞致死に及ぼす酸素効果率は，低 LET 放射線のそれに比べて非常に小さい.[10,11] したがって，酸素存在下での RBE は多くの報告では4.3以下である.[10-12]

本報は，低線量率 $^{252}$Cf 放射線の突然変異能を，高線量率X線のそれと比較して調べた最初の報告である. ガンマ線の線量率を下げると，致死効果が減少する.[13] 急性照射に比べて緩照射の場合に，ガンマ線の照射線量当たりの突然変異効果もまた減少する.[14,15] 致死及び突然変異効果の減少は，長い低線量率照射期間中に放射線障害の一部が誤りを起こさない過程で修復されると，最も簡単に説明できる. 効果の減少は，細胞致死及び突然変異誘発の両方においてほとんど類似している. Thacker 及び Stretch[15] は，低線量率及び高線量率の誘発突然変異率と生存率間の線形関係はほとんど完全に等しいことを見付けた.

本報告では，酸素存在下で放射線を照射した細胞での致死及び遺伝的損傷について，X線に対する $^{252}$Cf 放射線の RBE を比較した. 両放射線の線量率が非常に異なるので，生存百分率と突然変異率との関係について比較した. 50％生存率での $RBE_n$ は2.6であった. 両放射線の40％～50％生存率を与える線量域での突然変異率 $4.6\sim5.2\times10^{-5}$ での $RBE_n$ は2.5～2.8であった. 例えば，突然変異率 $5\times10^{-5}$ での $RBE_n$ は2.7であった. 両放射線の致死損傷と遺伝的損傷はほぼ完全に比例している（図４）. 中村ら[16] は，サイクロトロンより発生する速中性子は，$\gamma$線よりも 6-TG$^r$ 突然変異に導く損傷をより多く誘発することを報告している. 彼らの照射実験法では，総線量中わずかに８％の $\gamma$線の混在しか含まない高線量率（20 rad/分）中性子をマウス L5178Y 細胞に照射した. 結果が異なるのは，照射実験法，中性子の質，そして使用細胞の種類の違いによるのであろう.

最近の多くの報告によって，物理的又は化学的変異原処理後の 6-TG$^r$ （HPRT 欠損）突然変異細胞の

mutant cells after treatment with physical or chemical mutagens. Numerous partial and complete deletions have been observed in X ray-induced mutant HPRT genes.[17-19] Similarly many large genetic changes were induced by ionizing particles (α rays).[18] We have not made any structural analysis of the HPRT genes of mutants induced by $^{252}$Cf radiation and X rays. However, the same amount of genetic damage per lethal damage for the two types of radiation may suggest that both induce similar type of mutations.

HPRT 遺伝子の構造的変化が明らかになりつつある．X線誘発突然変異 HPRT 遺伝子には多くの部分欠損又は完全欠損がみられた．[17-19] 同様に電離粒子線（α線）も遺伝子欠損を多く誘発する．[18] 我々は，$^{252}$Cf 放射線及びX線誘発突然変異体の HPRT 遺伝子の構造解析はしていない．しかし，両放射線の致死損傷当たりの遺伝的損傷が等しいことから，両放射線が類似の型の突然変異を誘起していることが示唆される．

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