

Cross-Sensitivity of X-Ray-Hypersensitive Cells Derived from LEC Strain Rats to DNA-Damaging Agents

Toyo OKUI, Daiji ENDOH¹⁾, Soichiro ARAI²⁾, Emiko ISOGAI³⁾, and Masanobu HAYASHI^{2)*}

Hokkaido Institute of Public Health, Sapporo 060, ¹⁾Laboratory of Radiation Biology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060, ²⁾Department of Veterinary Radiology, Faculty of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069, and ³⁾Department of Preventive Dentistry, Health Sciences University of Hokkaido, 1757 Ishikari-Tobetsu, Hokkaido 061-02, Japan

(Received 1 May 1996/Accepted 8 July 1996)

ABSTRACT. The cross-sensitivity of X-ray-hypersensitive lung fibroblasts from LEC strain (LEC) rats to other DNA-damaging agents was examined. The LEC cells were 2- to 3-fold more sensitive to bleomycin (BLM) that induces DNA double-strand breaks, and to a cross-linking agent, mitomycin C, than the cells from WKAH strain (WKAH) rats, while they were slightly sensitive to alkylating agents, ethyl nitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine, but not to UV-irradiation. Although no difference was observed in the initial yields of DNA double-strand breaks induced by BLM between LEC and WKAH cells, the repair process of DNA double-strand breaks was significantly slower in LEC cells than in WKAH cells. — **KEY WORDS:** alkylating agent, bleomycin, cross-sensitivity, LEC rat cell, mitomycin C.

J. Vet. Med. Sci. 58(11): 1067–1071, 1996

There are several syndromes such as ataxia telangiectasia (AT) with enhanced sensitivity to some chemical and physical mutagens, including ionizing radiation [17]. These disorders are frequently associated with an increase of spontaneous or induced chromosome aberrations [5, 10], as well as with a genetic predisposition to cancer [20, 27]. Studies on the enhanced sensitivity observed in AT patients have been clarified to be related to some biological processes involved in DNA metabolism, such as repair, recombination and replication.

The LEC rat has been established at the Center for Experimental Plants and Animals, Hokkaido University as a strain that suffered from spontaneous fulminant hepatitis associated with severe jaundice after 4 month-old or later [22]. Other characteristics of LEC rats include a high incidence of spontaneous liver cancer in long-surviving individuals [32] and an increased sensitivity to whole-body irradiation with X-rays [6, 7]. We have previously reported that the hypersensitivity of LEC rats to whole-body irradiation is controlled by a single autosomal recessive gene, *xhs* [9] and that the frequencies of all types of chromosome aberrations induced in the bone marrow cells of LEC rats by X-irradiation are approximately 2- to 3-fold higher than those of WKAH rats [15]. Animal models are useful for the understanding of the human disease such as AT. Furthermore, the study of the enhanced sensitivity of LEC cells to DNA-damaging agents has brought to light some of the biological processes, such as DNA repair. In the present study, we describe on the cross-sensitivity of cells from X-ray-hypersensitive LEC rat to different DNA-

damaging agents.

MATERIALS AND METHODS

Chemicals: Bleomycin (BLM) and mitomycin C (MMC) were purchased from Wako Chemical Co. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ethyl nitrosourea (ENU) were obtained from Aldrich Chemical Co. and Nakarai Chemical Co., respectively.

Cell cultures: LEC/Hkm (LEC) and WKAH/Hkm (WKAH) rats were maintained in conditions described previously [6]. Research was conducted according to the principles in the "Guide for the Care and Use of Laboratory Animals" prepared by Rakuno Gakuen University. The primary cultures of rat fibroblasts were initiated from lungs of LEC and WKAH rats at 2 weeks of age as described previously [9]. Cells were grown in monolayer culture in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Cell cultures were kept at ambient humidity and 37°C in an atmosphere containing 5% CO₂.

Far UV-irradiation: The medium was removed from the plates, and the cells were washed with phosphate-buffered saline (PBS), pH 7.2. The cells were UV-irradiated at fluence rate of 0.25 J/m²/sec by using a Toshiba Germicidal Mercury vapor lamp. Immediately after UV-irradiation, a fresh medium was added and the plates were returned to the incubator.

Treatment with reagents: All chemical reagents were dissolved usually in PBS immediately before use. Twelve hours after seeding, cells were pulsed for 1 hr with each reagent. Cell-culture dishes were then washed twice in PBS and growth medium was added.

Survival curves: Cell survival was determined using the conventional colony-forming assay. Propagated cells were

* CORRESPONDENCE TO: HAYASHI, M., Department of Veterinary Radiology, Faculty of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069, Japan.

collected by trypsinization and $2\text{--}50 \times 10^2$ viable cells were plated into 6-cm dishes. After treatments described above, the cells were incubated for 2 weeks. Dishes were methanol-fixed, stained with May-Grunwald and Giemsa and then colonies containing more than 50 cells were counted as survivors under a dissecting microscope. Survival curves were fitted to the data points by eye.

Repair of DNA double-strand breaks: Repair of DNA double-strand breaks (DSBs) was estimated by pulse-field gel electrophoresis according to the method of Waters and Lyons [31]. Logarithmically growing cells (5×10^4) were incubated at 37°C for 24–48 hr in MEM containing [^{14}C]thymidine (3.7 kBq/ml, Amersham Co.). After treatment with BLM at concentrations of 20, 40 and 100 $\mu\text{g/ml}$, cells were harvested at various incubation times, resuspended to 2×10^7 cells/ml in ice-cold lysis buffer (LB) (100 mM EDTA, 20 mM NaCl, 10 mM Tris-HCl, pH 8.0), and then mixed with an equal volume of low melting agarose (InCert agarose, Takara Co.) previously held at 50°C . The cell suspension was poured into 0.164-ml chambers and kept at 4°C to allow the agarose to solidify. The agarose cylinders were removed from moulds and treated with 1.0% N-rauroyl sarcosine and 1 mg/ml proteinase K in LB at 50°C for 24 hr. Following lysis and washing, the agarose gel cylinders were cut into 0.5-cm³ plugs containing approximately 5×10^5 total cell-equivalents of DNA and embedded into wells of 0.6% agarose gel (megarose, Beckman Co.). DNA was electrophoresed through the gel for 21 hr at 175 V and 10°C in a BioRad CHEF DRII gel apparatus. After electrophoresis, the individual gel lanes were cut into 10 slices and the DNA radioactivity present in each slice was determined by liquid scintillation counting [31]. The fraction of DNA radioactivity out of the plug was divided by the radioactivity in the agarose plug immediately after treatment with BLM, and plotted against incubation time.

RESULTS

Sensitivity of LEC rat cells to BLM: In the previous report [9], we have shown that lung and skin fibroblasts of LEC rats are 2- to 3- fold more sensitive to ionizing radiation than those of WKAH rats, and that the repair process of DNA double-strand breaks (DSBs) induced by X-irradiation is slower in LEC cells than in WKAH cells. As a concentration of BLM required to reduce cell survival to 37% (D_{37}) is an index used to compare the cellular sensitivity, the fibroblasts of LEC rats were approximately 2-fold more sensitive to treatment with BLM than those of WKAH rats on D_{37} values (Fig. 1a and Table 1). DNA DSBs induced by BLM were estimated by pulse-field gel electrophoresis. The percentage of DNA out of the plug reflects the fraction of DSBs remaining in the DNA of the agents-treated LEC and WKAH cells. No difference was observed in the initial yields of DSBs between LEC and WKAH cells after treatment with BLM (Table 2). However, after treatment with BLM at concentrations of 20, 40 and 100 $\mu\text{g/ml}$, the rejoining of DNA DSBs in LEC rat cells was significantly slower than that in WKAH cells (Fig. 1b and data not shown).

Cross-sensitivity to other genotoxic agents: To investigate the cross-sensitivity of LEC rat cells to different DNA-damaging agents, lung fibroblasts isolated from LEC and WKAH rats were irradiated with UV, or treated with alkylating agents (ENU and MNNG) and a cross-linking agent (MMC), and then cell survival was assayed by colony formation (Figs. 2a to d, and Table 1). The fibroblasts of LEC rats were 2- to 3-fold more sensitive on D_{37} values to treatment with MMC than those of WKAH rats. LEC cells were slightly sensitive (1.2- and 1.5-fold on D_{37} values, respectively) to ENU and MNNG than WKAH cells. On the contrary, after UV-irradiation, no differences in the survival curves and D_{37} values were observed between LEC

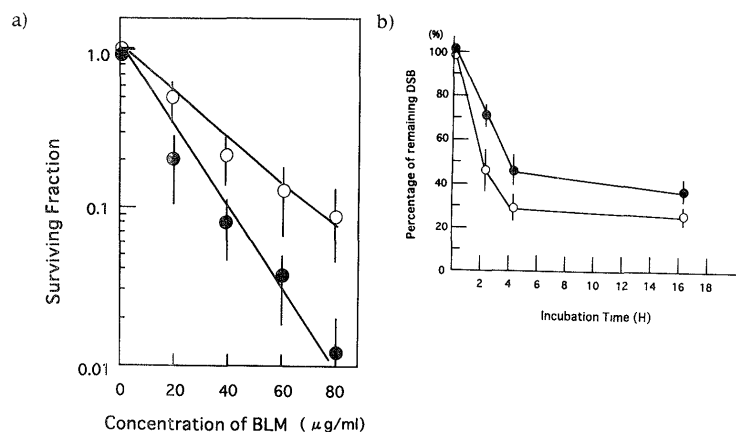


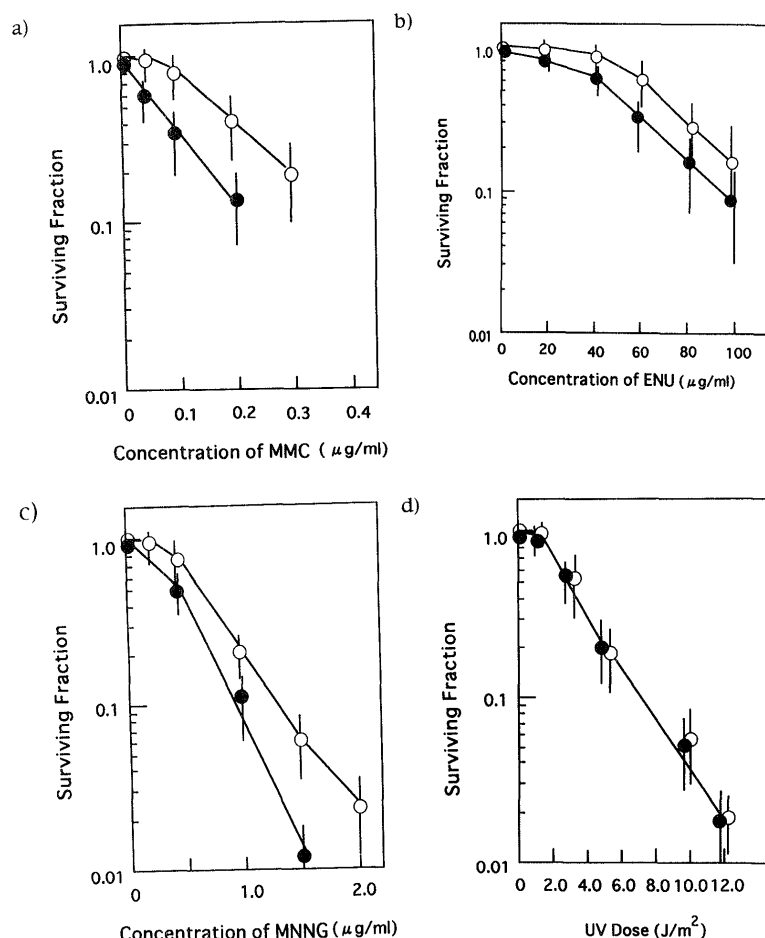
Fig. 1. (a) Survival curves of lung fibroblasts from LEC (●) and WKAH rats (○) after exposure to BLM. (b) DNA DSBs closure in the fibroblasts from LEC (●) and WKAH rats (○). The lung fibroblasts from LEC and WKAH rats were treated with BLM at 40 $\mu\text{g/ml}$, and their DNA was analyzed as described in the text. Points represent the average from four separate experiments. Error bars represent the standard deviation of the mean values ($n=4$).

Table 1. Sensitivity of LEC and WKAH cells to genotoxic agents

Agents	D_{37}	
	LEC cells	WKAH cells
BLM ($\mu\text{g/ml}$)	18.0	32.5
MMC ($\mu\text{g/ml}$)	0.086	0.196
ENU ($\mu\text{g/ml}$)	58.7	74.0
MNNG ($\mu\text{g/ml}$)	0.53	0.80
UV (J/m^2)	3.4	3.4

Table 2. Initial yields of DNA DSBs in LEC and WKAH cells treated with BLM

BLM ($\mu\text{g/ml}$)	Percentage of DNA out of plug (%)	
	LEC cells	WKAH cells
20	68.5 ± 3.2	68.3 ± 4.1
40	88.5 ± 5.0	86.2 ± 4.4
100	91.4 ± 7.3	92.1 ± 6.2

Fig. 2. Survival curves of LEC rat cells (●) and WKAH rat cells (○) after exposure to MMC (a), ENU (b), MNNG (c) and UV-rays (d). Error bars represent the standard deviation of the mean values ($n=4$).

and WKAH rat cells.

DISCUSSION

In this report, we characterized a cross-sensitivity of the X-ray-hypersensitive LEC rat cells to BLM, MMC, ENU and MNNG, except for UV irradiation which showed no significant differences in the sensitivity between LEC and WKAH cells. The cells derived from AT patients show hypersensitivity to X-rays and BLM [16, 17, 28, 29], whereas they have shown no hypersensitivity to far-UV

irradiation [13]. These findings indicate a resemblance of LEC rat cells to AT cells. AT cells are reported to be hypersensitive to ENU [18], but there are conflicting reports about their sensitivity to MNNG; some have shown hypersensitivity [17, 25], while others have reported no sensitivity [1, 2]. Although AT cells show no hypersensitivity to MMC [33], the LEC cells were 2- to 3-fold more sensitive to treatment with MMC than those of WKAH rats. Therefore, X-ray-hypersensitive LEC rat cells have more broad spectrum for cross-sensitivity to DNA-damaging agents as compared to human AT cells. Why the

differences occur in cross-sensitivity to DNA-damaging agents between AT and LEC cells remains unclear yet. Since BLM, MMC, ENU, MNNG and UV-irradiation induce the different types of DNA damages, repair processes of some types of DNA damages might be different between AT and LEC cells.

Among many types of DNA damages induced by ionizing radiation, radiation-induced DSBs cause major lethal damage to cells unless they were repaired [19, 24]. BLM acts by inducing DNA DSBs via a free radical attack on the deoxyribose moiety [26]. We have previously shown that the repair process of DNA DSBs in LEC cells induced by X-irradiation is slower than that in WKAH cells [9]. The present results showed that fibroblasts of LEC rats were approximately 2-fold more sensitive to treatment with BLM than those of WKAH rats, and that the rejoining of DNA DSBs induced by BLM in LEC cells was significantly slower than that in WKAH cells. Therefore, the hypersensitivity to BLM observed in LEC rat cells could be, at least partly, attributed to the impairment of repair processes to DNA DSBs produced by BLM, and the repair processes of DNA DSBs induce by ionizing radiation and BLM might be based on common factors.

Since AT cells can efficiently complete DNA DSBs rejoining [11, 24], LEC cells may be different to AT mutation with regard to the ability of DSBs rejoining. It is well known that DNA synthesis in AT cells is more radioresistant than that in normal cells [11, 16]. This is primarily due to the abnormality of many or all of the transient cell cycle arrests which also occur in normal cells following ionizing radiation [12, 21]. The abnormal cell cycle arrests of AT cells may affect the sensitivity to some DNA-damaging agents. We have recently reported a similar radioresistant DNA synthesis in LEC rat cells [8]. It has also been reported that a gene related to AT mutation is a human phosphatidylinositol-3'-kinase homologue [23]. A study concerning the gene of LEC rats is now in progress.

Although many mutant rodent cell lines with hypersensitivity to DNA-damaging agents, including ionizing radiation, have been established [4, 14, 30, 33], LEC rats exhibit a characteristic hypersensitivity to whole-body irradiation just like human AT mutation [16, 17] and scid mutation in mice [3]. Although there are some differences in the cross-sensitive profiles to DNA-damaging agents and ability of DSBs rejoining among LEC, AT and scid cells, the LEC rat provides a useful animal model for understanding the human diseases with enhanced sensitivity to some DNA-damaging agents and the repair processes of the damages.

ACKNOWLEDGEMENTS. This work was supported in part by grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

- Arlett, C. F. 1977. Lethal response to DNA damaging agents in a variety of human fibroblast cell strains. *Mutat. Res.* 46: 106.
- Barfknecht, T. B. and Little, J. B. 1982. Hypersensitivity of ataxia telangiectasia skin fibroblasts to DNA alkylating agents. *Mutat. Res.* 94: 369-380.
- Biederman, K. A., Sun, J., Giaccia, A. J., Tosto, L. M., and Brown, J. M. 1991. Scid mutation in mice confers hypersensitivity to ionizing radiation and deficiency in DNA double-strand break repair. *Proc. Natl. Acad. Sci. U.S.A.* 88: 1394-1397.
- Collins, A. R. 1993. Mutant rodent cell lines sensitive to ultraviolet, ionizing radiation and cross-linking agents: a comprehensive survey of genetic and biochemical characteristics. *Mutat. Res.* 293: 99-118.
- German, J. 1969. Chromosomal breakage syndromes. *Birth Defects* 5: 117-130.
- Hayashi, M., Endoh, D., Kon, Y., Yamashita, T., Hashimoto, N., Sato, F., Kasai, N., and Namioka, S. 1992. Higher sensitivity of LEC strain rats in radiation-induced acute intestinal death. *J. Vet. Med. Sci.* 54: 269-273.
- Hayashi, M., Endoh, D., Kon, Y., Yamashita, T., Sato, F., Kasai, N., and Namioka, S. 1993. Hypersensitivity of LEC strain rats in radiation-induced acute bone marrow death. *J. Vet. Med. Sci.* 55: 13-18.
- Hayashi, M., Ishimori, K., Maeda, A., Watanabe, T., Arai, S., and Okui, T. 1996. Radioresistant DNA synthesis in fibroblast cell lines derived from LEC strain rats. *Mutat. Res.* 352: 117-121.
- Hayashi, M., Okui, T., Endoh, D., Sato, F., Kasai, N., and Namioka, S. 1994. Radiation-hypersensitivity of LEC strain rats controlled by a single autosomal recessive gene. *Mutat. Res.* 314: 135-142.
- Hecht, F., Koeler, R. D., Rigas, D. A., Dahnke, G. S., Case, M. P., Eisdale, V., and Miller, R. W. 1966. Leukemia and lymphocytes in ataxia telangiectasia. *Lancet* 2: 1193.
- Jaspers, N. G. J., de Wit, J., Regulski, M. R., and Bootsma, D. 1982. Abnormal regulation of DNA replication and increased lethality in ataxia telangiectasia cells exposed to carcinogenic reagents. *Cancer Res.* 42: 335-341.
- Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, J. Jr. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 71: 587-597.
- Lehman, A. R., Kirk-Bell, S., Arlett, C. F., Harcourt, S. A., de Weerd-Kastelein, E. A., Keijzer, W., and Hall-Smith, P. 1977. Repair of ultraviolet light damage in a variety of human fibroblast cell strains. *Cancer Res.* 37: 904-910.
- Mckay, M. J., Mann, G. M., McDonald, D. A., Jones, S., and Kefford, R. F. 1994. Isolation and preliminary characterization of an X-ray-sensitive mammalian mutant cell line (WMXRS-1). *Mutat. Res.* 314: 261-271.
- Okui, T., Hayashi, M., Endoh, D., Sato, F., Kasai, N., Watanabe, T., and Namioka, S. 1994. A high frequency of induction of chromosome aberrations in the bone marrow cells of LEC strain rats by X-irradiation. *Mutat. Res. Lett.* 324: 165-169.
- Painter, R. B. and Young, B. R. 1980. Radiosensitivity in ataxia-telangiectasia: a new explanation. *Proc. Natl. Acad. Sci. U.S.A.* 77: 7315-7317.
- Paterson, M. C. and Smith, P. J. 1979. Ataxia-telangiectasia: an inherited human disorder involving hypersensitivity to ionizing radiation and related DNA-damaging chemicals. *Annu. Rev. Genet.* 13: 291-318.

18. Paterson, M. C., Smith, P. J., Middlestadt, M. V., Smith, B. P., and Bech-Hansen, N. T. 1979. Hypersensitivity to ethyl nitrosourea in cultured fibroblast strains from patients with ataxia telangiectasia. *Annu. Meet. Environ. Mutagen Soc.* 10th, 76.
19. Radford, I. R. 1986. Evidence for a general relationship between the induced level of DNA double-strand breakage and cell-killing after X-irradiation of mammalian cells. *Int. J. Radiat. Biol.* 49: 611-620.
20. Reed, E. B., Epstein, W. L., Border, W., and Sedgwick, R. 1966. Cutaneous manifestations of ataxia-telangiectasia. *J. A. M. A.* 195: 746-753.
21. Rudolph, N. S. and Latt, S. A. 1989. Flow cytometric analysis of X-ray sensitivity in ataxia telangiectasia. *Mutat. Res.* 211: 31-41.
22. Sasaki, M., Yoshida, M. C., Kagamori, K., Takeichi, N., Kobayashi, H., Dempo, K., and Mori, M. 1985. Spontaneous hepatitis in an inbred strain of Long-Evans rats. *Rat News Lett.* 14: 4-6.
23. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Satiel, A., Gatti, R. A., Cheesa, L., Sanal, O., Lavin, M. F., Jaspers, N. G. J., Malcolm, A., Taylor, R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 278: 1749-1753.
24. Schans, G. P., van der Centen, H. B., and Loman, P. H. M. 1982. DNA lesions induced by ionizing radiation. *Progress in Mutation Res.* 4: 285-299.
25. Scudiero, D. A. 1980. Decreased DNA repair synthesis and defective colony-forming ability of ataxia telangiectasia fibroblast cell strains treated with N-methyl-N'-nitro-N-nitrosoguanidine. *Cancer Res.* 40: 984-990.
26. Siloh, Y., Tabor, E., and Becker, Y. 1983. Abnormal response of ataxia telangiectasia cells to agents that the deoxyribose moiety of DNA via a targeted free radical mechanism. *Carcinogenesis* 4: 1317-1322.
27. Swift, M. R., Reitnauer, P. J., Morrell, D., and Chase, C. L. 1987. Breast and other cancers in families with ataxia-telangiectasia. *New. Engl. J. Med.* 316: 1289-1294.
28. Taylor, A. R. M., Harnden, D. G., Arlett, C. F., Harcourt, S. A., Lehman, A. R., Stevens, S., and Bridges, B. A. 1979. Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity. *Nature (Lond.)* 258: 427-429.
29. Taylor, A. R. M., Rosney, C. M., and Campbell, J. B. 1979. Unusual sensitivity of ataxia telangiectasia to bleomycin. *Cancer Res.* 39: 1046-1050.
30. Verhaegh, G. W. C. T., Jongmans, W., Morolli, B., Jaspers, N. G. J., van der Schans, G. P., Loman, P. H. M., and Zdzienicka, M. Z. 1995. A novel type of X-ray-sensitive Chinese hamster cell mutant with radioresistant DNA synthesis and hampered DNA double-strand break repair. *Mutat. Res.* 337: 119-129.
31. Waters, R. L. and Lyons, B. W. 1990. Detection of ionizing radiation-induced DNA double-strand breaks by filter elution is affected by nuclear chromatin structure. *Radiat. Res.* 124: 309-316.
32. Yoshida, M. C., Masuda, R., Sasaki, M., Takeichi, N., Kobayashi, H., Dempo, K., and Mori, M. 1987. New mutation causing hereditary hepatitis in the laboratory rat. *J. Hered.* 78: 361-365.
33. Zszenicka, M. Z., Tran, Q., van der Schans, G. P., and Simons, J. W. I. M. 1988. Characterization of an X-ray-hypersensitive mutant of V79 Chinese hamster cells. *Mutat. Res.* 194: 239-249.