shown). The UGT1A1 protein level in hepatic microsomes, which is associated with bilirubin glucuronidating activity, showed a variation factor of approximately 10. Although the expression levels of UGT1A3 and UGT1A4 did not vary between the individuals examined in this study, recent findings have demonstrated that human PXR variants is implicated in the expression of UGT1A3 and UGT1A4.[19] The UGT1A9 protein level in hepatic microsomes, associated with propofol glucuronidating activity, showed a variation factor of approximately 4. Yamanaka *et al.*[20] recently identified sequence variations in the UGT1A9 promoter that may affect the level of enzyme expression.

In human, UGT1A isoform-dependent glucuronidation plays an important role in the metabolism of drugs as xenobiotic compounds. The expression of UGT1A isoforms varies during development and with different dietary intake. Polymorphism in the flanking promoter regions of the *UGT1A* gene cluster may affect the transcriptional efficiency. It is therefore important to define the level of hepatic UGT protein for each individual to estimate the glucuronidating efficiency *in vivo*. The antipeptide antibodies developed in this study allow us to analyze the expression of the human UGT1A isoforms and assess the relative contribution of each isoform for the glucuronidation of various drugs.

References

1) Wells, P. G., Mackenzie, P. I., Chowdhury, J. R., Guillemette, C., Gregory, P. A., Ishii, Y., Hansen, A. J., Kessler, F. K., Kim, P. M., Chowdhury, N. R. and Ritter, J. K.: Glucuronidation and the UDP-glucuronosyltransferases in health and disease. *Drug Metab. Dispos.*, **32**: 281–290 (2004).
2) Mackenzie, P. I., Bock, K. W., Burchell, B., Guillemette, C., Ikushiro, SI., Iyanagi, T., Miners, J. O., Owens, I. S. and Nebert, D. W.: Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet. Genomics.*, **10**: 677–685 (2005).
3) Radominska-Pandya, A., Bratton, S. and Little, J. M.: A historical overview of the heterologous expression of mammalian UDP-glucuronosyltransferase isoforms over the past twenty years. *Curr. Drug Metab.*, **6**: 141–160 (2005).
4) Tukey, R. H. and Strassburg, C. P.: Genetic multiplicity of the human UDP-glucuronosyltransferases and regulation in the gastrointestinal tract. *Mol. Pharmacol.*, **59**: 405–414 (2001).
5) Ikushiro, S., Emi, Y. and Iyanagi, T.: Identification and analysis of drug-responsive expression of UDP-glucuronosyltransferase family 1 (UGT1) isozymes in rat hepatic microsomes using anti-peptide antibodies. *Arch. Biochem. Biophys.*, **324**: 267–272 (1995).
6) Catania, V. A., Luquita, M. G., Sánchez Pozzi, E. J., Pellegrino, J. M., Ikushiro, S., Emi, Y., Iyanagi, T. and Mottino, A. D.: Effect of spironolactone on the expression of rat hepatic UDP-glucuronosyltransferase. *Biochemical Pharmacology*, **66**: 171–177 (2003).
7) Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T. and Degawa, M.: A possible mechanism for decrease in serum thyroxine level by polychlorinated biphenyls in wistar and gunn rat. *Toxicol. Sci.*, **81**: 309–315 (2004).
8) Nishimura, N., Yonemoto, J., Yokoi, C., Takeuchi, Y., Ikushiro, S., Nishimura, H. and Tohyama, C.: Disruption of thyroid hormone homeostasis at weaning of holtzman rats by lactational but not in utero exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Sci.*, **85**: 607–614 (2005).
9) Emi, Y., Ueda, K., Ohnishi, A., Ikushiro, S. and Iyanagi, T.: Transcriptional enhancement of UDP-glucuronosyltransferase form 1A2 (UGT1A2) by nuclear factor I-A (NFI-A) in rat hepatocytes. *J. Biochem.*, **138**: 313–325 (2005).
10) Iyanagi, T.: Molecular basis of multiple UDP-glucuronosyltransferase isoenzyme deficiencies in the hyperbilirubinemic rat (Gunn rat). *J. Biol. Chem.*, **266**: 24048–24052 (1991).
11) Iwai, M., Maruo, Y., Ito, M., Yamamoto, K., Sato, H. and Takeuchi, Y.: Six novel UDP-glucuronosyltransferase (UGT1A3) polymorphisms with varying activity. *J. Hum. Genet.*, **49**: 123–128 (2004).
12) Girard, H., Court, M. H., Bernard, O., Fortier, L. C., Villeneuve, L., Hao, Q., Greenblatt, D. J., von Moltke, L. L., Perussed, L. and Guillemette, C.: Identification of common polymorphisms in the promoter of the UGT1A9 gene: evidence that UGT1A9 protein and activity levels are strongly genetically controlled in the liver. *Pharmacogenetics.*, **14**: 501–515 (2004).
13) Miles, K. K., Stern, S. T., Smith, P. C., Kessler, F. K., Ali, S. and Ritter, J. K.: An investigation of human and rat liver microsomal mycophenolic acid glucuronidation: evidence for a principal role of UGT1A enzymes and species differences in UGT1A specificity. *Drug Metab. Dispos.*, **33**: 1513–1520 (2005).
14) Maruo, Y., Iwai, M., Mori, A., Sato, H. and Takeuchi, Y.: Polymorphism of UDP-glucuronosyltransferase and drug metabolism.: *Curr. Drug Metab.*, **6**: 91–99 (2005).
15) Sugatani, J., Kojima, H., Ueda, A., Kakizaki, S., Yoshinari, K., Gong, Q. H., Owens, I. S., Negishi, M. and Sueyoshi, T.: The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. *Hepatology.*, **33**: 1232–1238 (2003).
16) Yueh, M. F., Huang, Y. H., Hiller, A., Chen, S., Nguyen, N. and Tukey, R. H.: Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. *J. Biol. Chem.*, **278**: 15001–15006 (2003).
17) Xie, W., Yeuh, M. F., Radominska-Pandya, A., Saini, S. P., Negishi, Y., Bottroff, B. S., Cabrera, G. Y., Tukey, R. H. and Evans, R. M.: Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proc. Natl. Acad. Sci. USA.*, **100**: 4150–4155 (2003).
18) Sugatani, J., Nishitani, S., Yamakawa, K., Yoshinari,

K., Sueyoshi, T., Negishi, M. and Miwa, M.: Transcriptional regulation of human UGT1A1 gene expression: activated glucocorticoid receptor enhances constitutive androstane receptor/pregnane X receptor-mediated UDP-glucuronosyltransferase 1A1 regulation with glucocorticoid receptor-interacting protein 1. *Mol. Pharmacol.*, **67**: 845–855 (2005).

19) Gardner-Stephen, D., Heydel, J. M., Goyal, A., Lu, Y., Xie, W., Lindblom, T., Mackenzie, P. I. and Radominska-Pandya, A.: Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. *Drug. Metab. Dispos.*, **32**: 340–347 (2004).

20) Yamanaka, H., Nakajima, M., Katoh, M., Hara, Y., Tachibana, O., Yamashita, J., McLeod, H. L. and Yokoi, T.: A novel polymorphism in the promoter region of human UGT1A9 gene (UGT1A9*22) and its effects on the transcriptional activity. *Pharmacogenetics.*, **14**: 329–332 (2004).

ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 34 (2006) 274–283

Caloric restriction prevents radiation-induced myeloid leukemia in C3H/HeMs mice and inversely increases incidence of tumor-free death: implications in changes in number of hemopoietic progenitor cells

Kazuko Yoshida[a], Yoko Hirabayashi[b], Fumiko Watanabe[a], Toshihiko Sado[a], and Tohru Inoue[c]

[a]*Radiation Hazards Research Group, National Institute of Radiological Sciences, Chiba, Japan;* [b]*Division of Cellular and Molecular Toxicology and* [c]*Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo, Japan*

(Received 9 June 2005; revised 25 October 2005; accepted 30 November 2005)

Objectives. **Previously, we found a clear decrease in the incidence of radiation-induced myeloid leukemia in C3H/HeMs mouse caused by caloric restriction (CalR). In this report, CalR before and after irradiation was examined to determine whether they exert different effects on the prevention of radiation-induced myeloid leukemogenesis and the consequent extension of life span by CalR.**

Methods. **The C3H/HeMS strain, which is prone to radiation-induced myeloid leukemia, was used. Groups subjected to different CalR timings, pre- and postirradiation, were compared with groups not subjected to CalR during their lifetime for the incidences of neoplasms, specifically that of myeloid leukemia, and the incidence of tumor-free death. A single dose of 3Gy X-ray was administered to mice at 10 weeks old. Results of colonization assay before and after CalR were compared with the incidence of leukemogenesis among the groups.**

Results. **Irrespective of the CalR timing in terms of irradiation, there was a significant difference in the prevention of myeloid leukemogenesis, and a consequent difference in longevity (731 ~ 805 days for CalR groups vs. 697 days for the group without CalR; Log rank, $P<0.03$). During CalR, the number of hemopoietic progenitor cells (HPCs), potential leukemogenic targets, significantly decreased (0.4×10^4 vs. 4.2×10^4 of granulomacrophage colonyforming units per spleen; 1.3×10^4 vs. 7.6×10^4 of the splenic colony forming units per spleen), but this decreased number of HPCs returned to that of the non-CalR control group, when the CalR group was returned to nonrestricted diet (returned to 1.5×10^4 granulomacrophage colony-forming units per spleen; returned to 2.8×10^4 splenic colony-forming units per spleen). Although preirradiation CalR followed by a conventional non-CalR diet negates the potential preventive effect, prevention conferred by pre-and postirradiation CalR suggests different underlying mechanisms; preirradiation CalR prevents the initiation of direct genotoxic leukemogenesis, while postirradiation CalR the indirect, epigenetic, leukemogenesis.**

Conclusion. **The incidences of tumor-free death significantly increased in all the groups undergoing CalR except for the group subjected to preirradiation CalR, which contributed to the longevity of the groups undergoing CalR.** **© 2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

Radiation-induced leukemia was noted as the highest risk factor for mortality among atomic bomb survivors in Hiroshima and Nagasaki [1,2]. Relative risk of leukemia has been estimated to be approximately 6.5, whereas that for other tumors is 1.2 [2]. Experimentally, caloric restriction (CalR) has been found to be only a preventive factor for the risk comparable to epidemiological relevancy in atomic bomb survivors. Thus, timing of restriction seems to be an additional factor that should be taken into account when trying to understand not only the underlying mechanism, but also the epidemiological relevancy of CalR.

Our previous study of CalR using C3H/He mice, which are prone to radiation-induced myeloid leukemia [3], in relation to radiation-induced leukemogenesis showed that CalR led to a significant decrease in the incidence of

Offprint requests to: Kazuko Yoshida, Ph.D., Radiation Hazards Research Group, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan; E-mail: yosida@nirs.go.jp

0301-472X/06 $–see front matter.
doi: 10.1016/j.exphem.2005.11.016

myeloid leukemias [4]. Furthermore, when timing of CalR between lifetime CalR and postirradiation CalR were compared, onset of myeloid leukemia was significantly delayed in the former compared with the latter, although both resulted in a significant decrease in total incidence of myeloid leukemias. Thus, the present study was designed to elucidate the role of different CalR timings, including preirradiation CalR, in leukemogenic prevention. Possible target cells for radiation leukemogenesis are hemopoietic stem cells, that is, long-term repopulating stem cells [5] and the population of such hemopoietic stem cells changes proportionally in response to different types of progenitor cell [6], such as granulocyte macrophage colony-forming units (CFU-GM) and other progenitors, including splenic colony-forming units (CFU-S) [7,8]. In relation to these, the number of hemopoietic progenitor cells (HPCs), and the kinetics of HPCs, i.e., cell-cycle parameters, were evaluated and compared among the CalR groups as possible markers predict leukemogenesis.

CalR induces a notable decrease in splenic weight and, consequently, in the number of HPCs, which may respond proportionally to the number of hemopoietic stem cells, the potential target cells for myeloid leukemogenesis [9]. In our previous experiments, we observed the effect of CalR throughout the lifespan of mice, which raised the question as to whether risk of leukemogenesis is a function of the number of potential target cells and, consequently, a function of the number of HPCs at the time of irradiation. In the present study, to answer this question, CalR in mice was started at 6 weeks old for the first group until the time of irradiation, at 10 weeks old, and mice were then returned to a regular non-CalR diet. In the other group, restriction was started at 10 weeks old and continued throughout their lifespan. The former treatment was designed to modify the stage of leukemogenesis before irradiation, and the latter to determine the effect of diet on the stage of leukemogenesis after irradiation. We refer to the former treatment as modification of the "initiation stage" of leukemogenesis, because this treatment modifies the number of possible target cells for leukemic initiation; and the latter stage as modification of the "promotion stage" of leukemogenesis, because this treatment modifies proliferation and differentiation of potentially initiated cells after irradiation.

CalR neither more significantly prevented radiation-induced development of neoplasms other than myeloid leukemias nor inversely increased the incidence of any neoplasm. Consequently, because of decreased incidence of myeloid leukemias, incidence of tumor-free death increased.

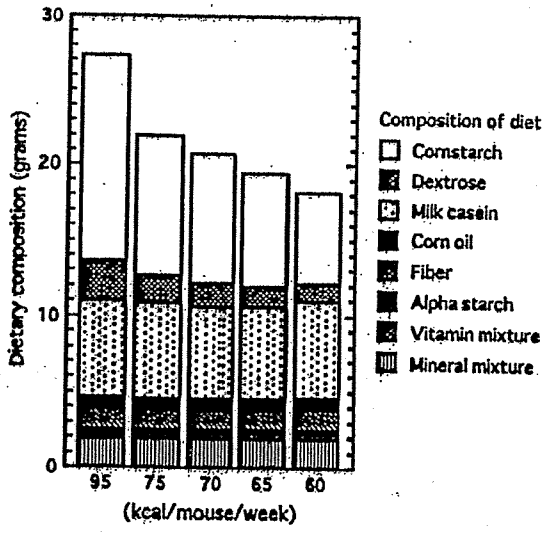


Figure 1. Five dietary regimens based on diets of different caloric contents (see text). The total, in grams, fed to each mouse per week is indicated in the bar graph for each dietary regimen. For caloric restriction, 60-, 65-, 70-, 75-, and 95-kcal dietary regimens, were used to maintain the body weight of each mouse within 25–27 g.

Materials and methods

Mice

C3H/He mice, which are prone to radiation-induced myeloid leukemia, were used in the present study. Incidence of spontaneous myeloid leukemia in C3H/He male mice is 1%, which increased to 23.3% after 3-Gy whole-body x-ray irradiation [3]. Six-week-old male C3H/HeNirsMs mice bred at our institute and released as cohort were used. Mice were housed individually, but were housed in groups if their weights were within 6% to 8% of each other, in environmentally controlled clean conventional rooms supplied with high-efficiency particulate air under a 12-hour light to 12-hour dark cycle in an authorized animal facility of the Laboratory Animal Research Center at the National Institute of Radiological Sciences. Mice were monitored weekly for maintenance of body weight, and their health status was assessed twice daily [4]. All equipment and supplies, including cages, water bottles, and wooden chips used for bedding, were sterilized.

Diets

Diets of different caloric contents, 3.31, 3.35, 3.38, 3.42, and 3.48 kcal/g, were used. Caloric intake was adjusted by varying amounts of carbohydrate and dextrose, while providing constant amounts of other nutrients, such as proteins, lipids, vitamins, and minerals (Fig. 1). Noncaloric restricted (control) mice were provided 95 kcal/week, per mouse, based on the 3.48 kcal/g diet. For CalR, according to the body weight monitored three times a week, diets were of different calorie-controlled regimens, i.e., 60, 65, 70, 75, and 95 kcal/week, per mouse (see section, Calorie Restriction Procedure).

Irradiation

Mice were exposed to 3-Gy whole-body x-ray irradiation at a 200-kV/20-mA pulse using a therapeutic x-ray irradiator (Simadzu, Kyoto, Japan) with 0.5-mm Al and 0.5-mm Cu filters, at a dose rate of 0.614 Gy/minute and a 56-cm focus surface distance. All mice in the treatment group were irradiated at 10 weeks old.

Table 1. Incidence of myeloid leukemia, other neoplasms and tumor-free mice, and mean survival days

Experimental groups	No. of mice[a]	Median survival time in days[b] (range)	Myeloid leukemia		Other tumor		Tumor- free mice	
			No. of case	(%)[c]	No. of case	(%)[d]	No. of case	(%)[c]
0Gy-CalR(–)	258	839 (805–865)	3	1.2	299	115.9	26	10.1[g,h]
3Gy-CalR(–)	270	697 (678–730)	60	22.2[e,f]	308	114.1	20	7.4[i,j]
0Gy-CalR(pre)	93	885 (846–924)	2	2.2	111	119.4	10	10.8
3Gy-CalR(pre)	98	722 (679–772)	16	16.3	119	121.4	7	7.1
0Gy-CalR(post)	263	896 (874–925)	0	0	213	81.0	94	35.7[g]
3Gy-CalR(post)	274	805 (768–833)	26	9.5[e]	315	115.0	48	17.5[i]
0Gy-CalR(through)	69	874 (798–898)	0	0	49	71.0	32	46.4[h]
3Gy-CalR(through)	75	731 (690–845)	6	8.0[f]	76	101.3	15	20.0[j]

[a]No. of mice refers to number of effective mice. Accidental deaths occurred due to the leakage of water bottles; 5 in 0Gy-CalR(–), 1 in 0Gy-CalR(pre), 2 in 3Gy-CalR(pre), 4 in 0Gy-CalR(post), and 8 in 3Gy-CalR(post). [b]Median survival time and the upper and lower 95% probability ranges estimated by the Kaplan-Meier method [17] (see Materials and Methods). [c]Fisher exact test for the incidence of myeloid leukemia and tumor-free mice was performed. [d]Percentages > 100% are due to multiplicity of tumor incidences. [e]3Gy-CalR(–) vs 3Gy-CalR(post) ($p<0.0001$). [f]3Gy-CalR(–) vs 3Gy-CalR(thru) ($p<0.01$). [g]0Gy-CalR(–) vs 0Gy-CalR(post) ($p<0.001$). [h]0Gy-CalR(–) vs 0Gy-CalR(thru) ($p<0.0001$). [i]3Gy-CalR(–) vs 3Gy-CalR(post) ($p<0.001$). [j]3Gy-CalR(–) vs 3Gy-CalR(thru) ($p<0.01$).

Calorie restriction procedure

Mice were subjected to four dietary conditions on the basis of the timing of CalR and thus divided into four groups: i.e., no restriction, CalR(–); preirradiation restriction (6–10 weeks old), CalR(pre); postirradiation restriction (from 10 weeks old to death), CalR(post); and a group subjected to lifetime CalR [from 6 weeks old to death, CalR(through)]. All of these groups were subdivided into two groups at 10 weeks old: those receiving 3-Gy irradiation or no irradiation (3 or 0Gy-) (see Irradiation section). Namely, there were eight groups; 3Gy-CalR(–) and 0Gy-CalR(–) groups, 3Gy-CalR(pre), and 0Gy-CalR(pre) groups, 3Gy-CalR(post) and 0Gy-CalR(post) groups, and 3Gy-CalR(through) and 0Gy-CalR (through) groups. The number of animals in each group is shown in Table 1. Identically designed cohort studies were combined; thus, animal numbers shown in Table 1 are different among the experimental groups.

Noncaloric restricted groups were fed a 95-kcal diet from 6 weeks old until death. Mice in the CalR(pre) groups were fed a 65-kcal diet from the start of the experiment, i.e., from 6 to 10 weeks old; thereafter they were fed a 95-kcal diet. Mice in the CalR(post) groups were fed a 95-kcal diet for the first 4 weeks old, i.e., from 6 to 10 weeks old, after which their body weights were controlled between 25 and 27 g with a 60- to 95-kcal dietary regimen. Caloric intake of the CalR(post) groups, however, exceeded their body weight by about 2 g, thus, it was fixed at 65 kcal from 10 to 12 weeks old until body weight decreased to 25 to 27 g. Mice in the CalR(through) groups were fed a 65-kcal diet for the first 4 weeks, i.e., from 6 to 10 weeks old, after which their body weights were controlled throughout their lifetime from 25 to 27 g with a 60–95-kcal dietary regimen. Average caloric intake from 10 weeks old calculated was 77 kcal/week, per mouse, in the CalR(post) and the CalR(through) groups.

As in our previous study, all mice were observed throughout their lifespan. All mice—except for 8% that succumbed to leukemic sudden death—exhibiting or developing anemia, or having palpable spleens, were sacrificed during the agonal period. All sacrificed mice were confirmed to have been myelogenous and had been transplantable by transplantation assay [3]. This leukemogenicity was maintained also in p53-deficient C3H/He mice as determined by fluorescein-activated cell sorting, using c-kit, Mac-1, Gr-1, B220, sIgM, Thy1.2, and CD3, among others [10]. Conventional histological examinations were routinely performed at our laboratory [11,12]. Complete necropsies were performed and organs were examined both grossly and histologically. Tissues were fixed with 4% formaldehyde in phosphate-buffered saline, embedded in paraffin, sectioned at 4-μm thickness, and routinely stained with hematoxylin and eosin. Cause of death was identified in each case. Hepatomas observed in the present study have been described elsewhere [13].

Assay of HPCs

To monitor the number of HPCs, the number of progenitor cells per spleen and that per bone marrow were evaluated by in vivo and/or in vitro colonization assay at 10 and 14 weeks (see section, Calorie Restriction Procedure). Day-12 CFU-S were assayed by

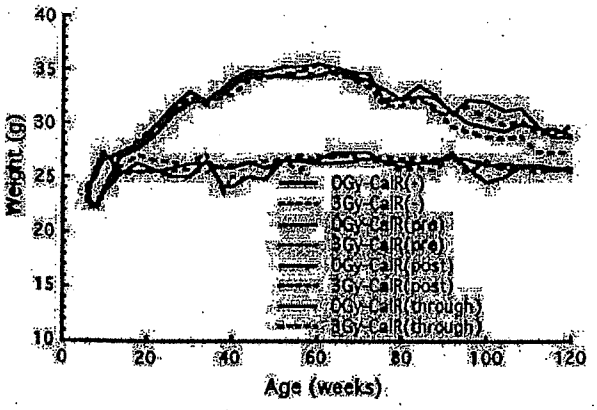


Figure 2. Changes in mean body weight vs age in weeks for all experimental groups, CalR(–), black; CalR(pre), red; CalR(post), green; and CalR(through), blue; with or without 3-Gy irradiation. Note the body weights of the 0Gy-CalR(pre) and 3Gy-CalR(pre) groups immediately returned to the non-CalR level after the dietary change at 10 weeks old, and their body weight profiles are similar to those of the controls, that is, the 0Gy-CalR(–) and 3Gy-CalR(–) groups (see text).

spleen colonization assay in accordance with the method of Till and McCulloch [14]. Mice irradiated with a lethal dose were injected intravenously with bone marrow cells or spleen cells from donor mice. Three femurs or three spleens from three donor mice of each group were pooled and assayed. Recipient mice were sacrificed on day 12 (day-12 CFU-S) after cell transfusion. Spleens with or without colonies were fixed with Bouin's solution, and surface colonies were counted.

CFU-GM were also assayed by the methylcellulose method in semisolid culture [15]. Bone marrow cells and spleen cells were cultured in alpha medium supplemented with 20% fetal bovine serum and pokeweed-mitogen–stimulated spleen-cell–conditioned medium [15]. After 7-day incubation, all CFU-GM containing more than 50 cells were enumerated.

Assay of stem cell kinetics

The bromodeoxyuridine ultraviolet (BUUV) method was used, so designated on the basis of the incorporation of bromodeoxyuridine (BrdUrd) using an osmotic minipump, followed by the specific purging of BrdUrd-incorporated cells by exposure to ultraviolet light (UV) with a peak at 365 nm (UVA), and then followed by assaying the ratio of the number of hemopoietic colonies (CFU-S, in the present study) of the purged group to that of the control group. The CFU-S–specific parameters for cell kinetics, such as doubling time, size of cell cycling (undergoing DNA synthesis) or quiescent fractions, and also size of cell-cycling fraction during a unit time interval [16] were determined. Three mice each from the 0Gy-CalR(–) and CalR groups were examined at 50 weeks old, i.e., 44 weeks after caloric restriction for the CalR groups and generally close to the time that leukemogenesis is about to become overt.

Statistical analyses

Data were stored in a computer and processed for statistical analyses using the Kaplan-Meier method for survival curves and the log-rank test [17] for statistical significance. Median survival period and the upper and lower 95% probability ranges were calculated (Table 1). Incidences of hemopoietic malignancies and tumor-free death were evaluated by Fisher's exact test (Table 1).

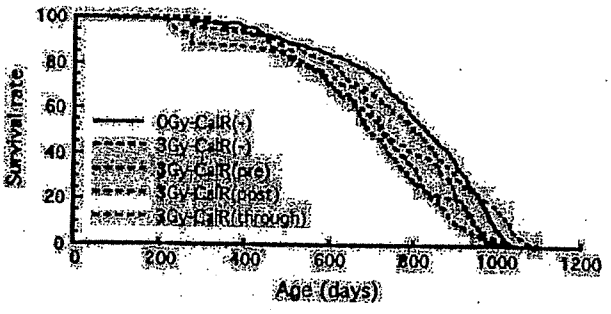


Figure 3. Survival curves for irradiated groups compared with nonirradiated control group; namely, the 3Gy-CalR(pre), 3Gy-CalR(post), and 3Gy-CalR(through) groups indicated by red, green, and blue dotted lines, respectively, are shown with those of the CalR(–) groups with or without irradiation; namely, the 3Gy-CalR(–) group indicated by a black dotted line and the 0Gy-CalR(–) group by a black solid line. For survival data for CalR groups, refer to Experimental Procedure section and Materials and Methods section. Note that the groups fed the calorie-restricted diet after 10 weeks of age without irradiation exhibit prolonged longevity. Log-rank test for mean survival curves; 3Gy-CalR(–) vs 3Gy-CalR(post) ($p < 0.0001$), 3Gy-CalR(–) vs 3Gy-CalR(through) ($p < 0.03$), 0Gy-CalR(–) vs 3Gy-CalR(–) ($p < 0.0001$).

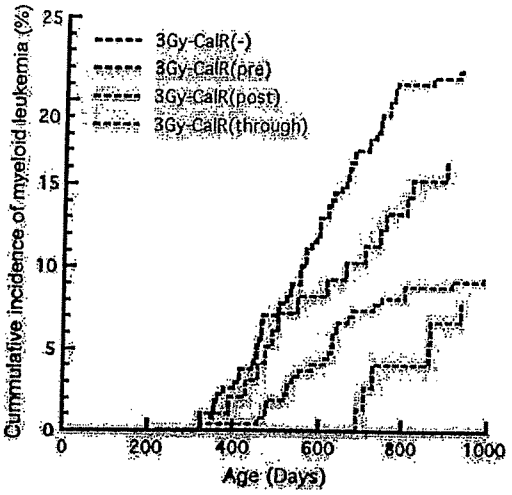


Figure 4. Cumulative incidence of myeloid leukemias. Incidences of myeloid leukemias in all the groups with caloric restriction, 3Gy-CalR(post), 3Gy-CalR(through) and 3Gy-CalR(pre) are lower than that in 3Gy-CalR(–) (see Table 1). The 3Gy-CalR(through) group shows the lowest incidence, whereas the 3Gy-CalR(post) group shows the second lowest. The 3Gy-CalR(pre) group shows a lower incidence than the 3Gy-CalR(–) group but with no statistical significance. The latency periods of the myeloid leukemias in the 3Gy-CalR(post) and 3Gy-CalR(through) groups were significantly prolonged as compared with that in the 3Gy-CalR(–) group.

Results

Effect of CalR diets on growth curves and survival

Body-weight changes in the experimental groups obtained in the present study are shown in Figure 2. There was no apparent difference in weight between unirradiated and irradiated mice in the same dietary group.

Body weights of the CalR groups given a 65-kcal diet for 6 to 10 weeks decreased to a mean weight of 22 g. Mice in these groups had lower body weights than those in the other experimental groups. Moreover, animals assigned to undergo a dietary regimen designed to maintain their weight between 25 and 27 g successfully after they reached 10 weeks old; indeed, achieved weights in this range. Changes in the body weight of the groups without caloric restriction are shown in Figure 2.

Survival curves for 3-Gy–irradiated groups with and without caloric restriction, and the 0Gy-CalR(–)group as a control are shown in Figure 3, and the comparable median survival periods (days) are listed in Table 1. Irrespective of the dietary regimen, there was a significant decrease in the lifespan of mice in all 3-Gy–irradiated groups compared with the 0-Gy groups (see, significances in legend to Fig. 3), and also in the median survival periods of mice

in the 3-Gy–irradiated groups compared with the nonirradiated 0-Gy groups (697–805 days vs 839–896 days, in Table 1). Irrespective of dietary regimen, there was a significant difference in longevity among all the irradiated groups, except for the 3Gy-CalR(pre) group, compared with that of the irradiated group without caloric restriction.

CalR prevents radiation-induced myeloid leukemias

All four irradiated groups [3Gy-CalR(–), 3Gy-CalR(pre), 3Gy-CalR(post), and 3Gy-CalR(through)] demonstrated increased incidences of myeloid leukemias as compared with the corresponding nonirradiated groups [0Gy-CalR(–), 0Gy-CalR(pre), 0Gy-CalR(post) and 0Gy-CalR(through]; 1.2% to 22.2% ; 2.2% to 16.3%; 0.0% to 9.5%; and 0.0% to 8.0%, respectively) (Table 1).

As shown in Figure 4, onset of radiation-induced myeloid leukemias was markedly delayed by CalR, specifically in the 3Gy-CalR(through) group. Total incidence of myeloid leukemias in the 3Gy-CalR(through) group was the lowest ($p < 0.01$; Fisher's exact test). The increased rate of incidence and the total incidence of radiation-induced leukemias in the 3Gy-CalR(post) group were lower than for those in the 3Gy-CalR(–) group (Fig. 4, $p < 0.0001$, Kaplan-Meier method; Table 1, 9.5% vs 22.2%; $p < 0.0001$, by Fisher's exact test). In the 3Gy-CalR(pre) group, neither onset delay, nor a significant decrease in the incidence of myeloid leukemias was observed, as compared with the 3Gy-CalR(–) group (Fig. 4, 325 days vs 321 days; Table 1; 16.3% vs 22.2%, resp.; $p = 0.217$, Fisher's exact test). However, there was no significant difference in the incidence of leukemia among the three caloric restriction groups, 3Gy-CalRs, namely, 3Gy-CalR(pre), 3Gy-CalR(post) and 3Gy-CalR (through). When the changes in the incidence of myeloid leukemias for all of the CalR groups, except that for the 3Gy-CalR(pre) group, were examined, the increase in the incidence of myeloid leukemias noted in 3Gy-CalR(–) was prevented markedly.

Because our primary aim was to examine radiation-induced myeloid leukemias and because we used strain C3H/He, a less-inducible strain for thymic lymphomas and lymphoid leukemias, hemopoietic neoplasms other than myeloid leukemias were not focused on in our examinations. Results show that there was no significant decrease in incidence by CalR in any of the irradiated groups, 3Gy-CalRs, except for the nonirradiated groups, namely, the 0Gy-CalR (post) and 0Gy-CalR(through) groups (data not shown).

Total incidence of nonhematopoietic neoplasms showed a limited decrease in only the 0Gy groups, i.e., the 0Gy-CalR(post) (81.0%) and 0Gy-CalR(through) groups (71.0%) as compared with 115.9% in the 0Gy-CalR(–) group (see Table 1 section, Other Tumors). These neoplasms include hepatomas/hepatocellular carcinomas, pulmonary tumors, tumors in the alimentary tract, genitourinary tumors, endocrine tumors, soft-tissue tumors, and dermal and skin-appendage tumors, among others.

Changes in number of hemopoietic stem/progenitor cells during or after caloric restriction

Because hemopoietic stem cells are assumed to be possible targets for radiation-induced leukemogenesis, and the number of hemopoietic stem/progenitor cells correlates proportionally to the number of CFU in vivo (CFU-S) and/or in vitro (CFU-GM), the numbers of CFU-S and CFU-GM were evaluated. A previous preliminary evaluation revealed that the number of hemopoietic stem/progenitor cells in the CalR groups decreases at the time of irradiation (10 weeks old) compared with that in the CalR(–) groups [9]. In this study, the number of HPCs at the time of irradiation (10 weeks old) and that 4 weeks after the dietary change (14 weeks old) were solely focused on and compared with those in the bone marrow and spleen (Fig. 5).

The 0Gy-CalR mice were fed a 65-kcal diet between the 6th week and 10th week. Thereafter, the 0Gy-CalR(pre) group was fed a 95-kcal diet, whereas the other 0Gy-CalR (through) group was fed the 65-kcal diet continuously. At 10 weeks old, as shown in Figure 5A (top left), the number of spleen cells in the CalR group markedly decreased as compared with that in the 0Gy-CalR(–) control group (1.32×10^8 vs 2.17×10^8 cells per spleen, respectively, second from the left vs far left). Although at 14 weeks old, in another CalR group, 0Gy-CalR(pre), the number of spleen cells originally assumed to be the same as that in the 0Gy-CalR(through) group did not decrease but rather increased as compared with the 0Gy-CalR(through) group (1.13×10^8 and 0.97×10^8 cells per spleen, respectively) due to the dietary change from a 65-kcal to a 95-kcal dietary regimen from 10 weeks old until 14 weeks old. In the 0Gy-CalR(post) group, CalR was not implemented until the 10th week; thereafter, in this particular experiment, the group was fed a 65-kcal diet until the 14th week. The number of splenic cells in the 0Gy-CalR(post) group had already significantly decreased by 14 weeks old, i.e., 4 weeks after the dietary change, as compared with the 0Gy-CalR(–) group (1.17×10^8 vs 2.00×10^8 cells per spleen).

In Figure 5B (middle, left), the numbers of progenitor cells (CFU-GM and day-12 CFU-S) per unit number of spleen cells are shown (from left to right). The number of colonies in vitro (CFU-GM) per 10^6 spleen cells for the 0Gy-CalR group markedly decreased as compared with that for the 0Gy-CalR(–) control group at 10 weeks old [lighter columns; $30.0/10^6$ cells for the 0Gy-CalR group, second from the left vs $191.7/10^6$ cells for the 0Gy-CalR(–) group, farthest left]. At 14 weeks old, the number of CFU-GM for the corresponding group, i.e., the 0Gy-CalR(through) group, showed a similar significant decrease as compared with the 0Gy-CalR(–) control group ($43.3/10^6$ cells vs $201.7/10^6$ cells). The 0Gy-CalR(pre) groups, whose number of CFU-GM was similarly assumed to be the same as that for the 0Gy-CalR(through) group, did not show any decrease as compared with the 0Gy-CalR(through) group

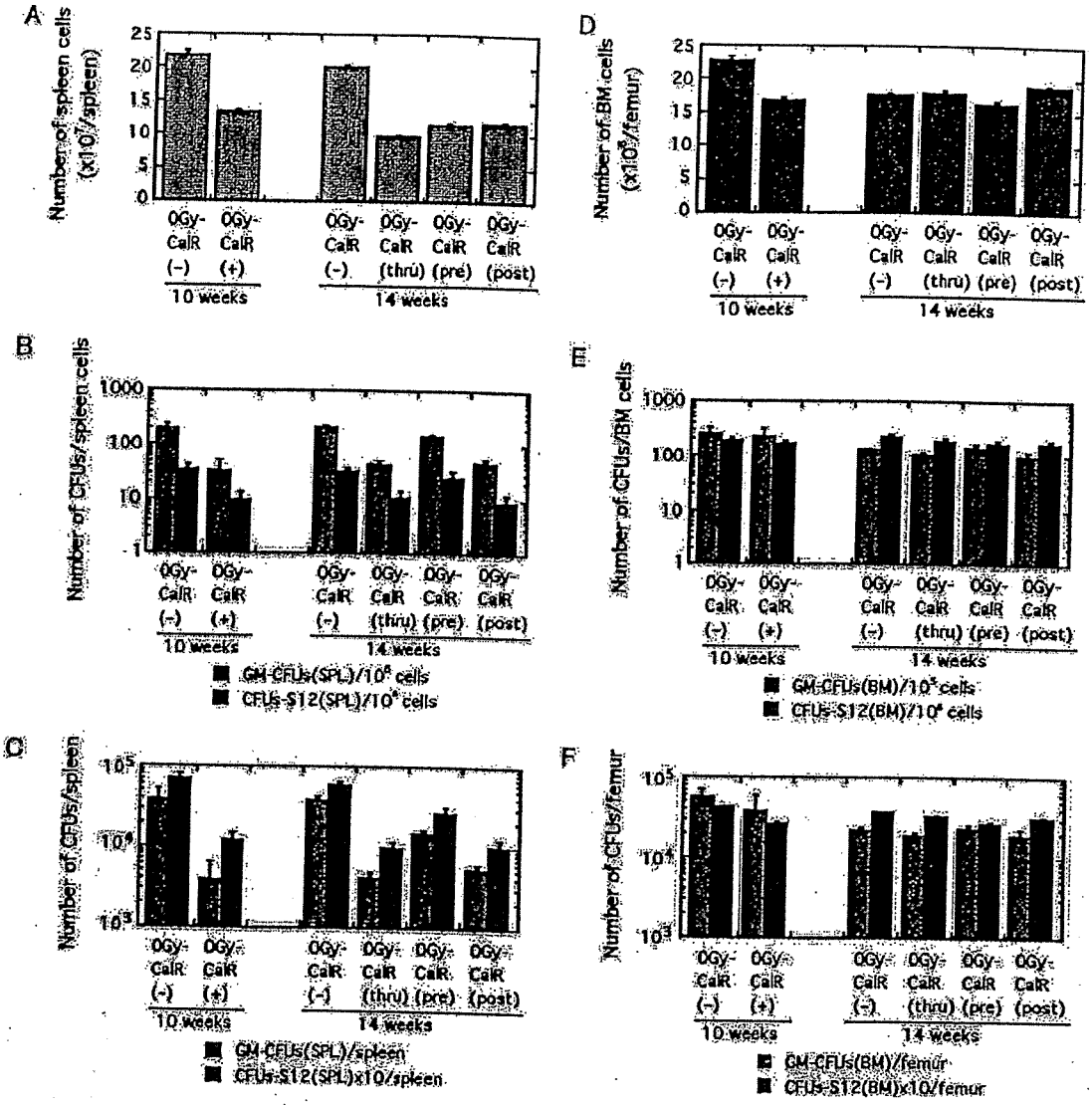


Figure 5. Number of hemopoietic cells (A,D), number of stem/progenitor cells per unit number of cells (B,E), and number of stem/progenitor cells per organ and/or tissue (C,F) are shown for the spleen (A–C) and femur (D–F). Each figure shows data at 10 weeks old, that is, 4 weeks after restriction started (left); and data at 14 weeks old, that is, 4 weeks after the dietary change (right). The right four columns represent the 0Gy-CalR(–), 0Gy-CalR(through), 0Gy-CalR(pre), and 0Gy-CalR(post) groups. For the two types of progenitor cell, the number of colony-forming units in the spleen (CFU-S) for day-12 (12D) granulocyte macrophage-colony-forming units (GM-CFU) determined by the in vitro assay was examined. Mice irradiated with a lethal dose of x-rays (810 cGy) were injected intravenously with spleen cells or femoral bone marrow cells from donor mice. For donor cells, three spleens or three femoral bone shafts from three donor mice of each group were pooled and assayed. Recipient mice were sacrificed on 12D (CFUs-S) after spleen cell transfusion. GM-CFU were assayed by methylcellulose method in semi-solid culture [15]. Spleen cells or femoral bone marrow cells were cultured in alpha medium supplemented with 20% fetal bovine serum and the pokeweed-mitogen–stimulated spleen-cell–conditioned medium (see Materials and Methods section in text). 0Gy-CalR(–) = mice fed a 95-kcal diet from 6 weeks old. CalR(thru) [CalR(through) in the text] = mice fed a 65-kcal diet from 6 weeks old. 0Gy-CalR(pre) = mice fed a 65-kcal diet from 6 to 10 weeks old, and thereafter a 95-kcal diet. 0Gy-CalR(post) = mice fed a 95-kcal diet from 6 to 10 weeks old, and thereafter a 75-kcal diet.

($136.7/10^6$ cells, $43.3/10^6$ cells, respectively) due to the dietary change from a 65-kcal to a 95-kcal dietary regimen from 10 weeks old to 14 weeks old. For the 0Gy-CalR(post) group, the number of CFU-GM significantly decreased as compared with the 0Gy-CalR(–) group ($46.7/10^6$ vs. $201.7/10^6$ cells) due to caloric restriction that started from 10 weeks old. Day 12 CFU-S (Fig. 5B, darker columns; second column of each group) also showed a trend similar to that of CFU-GM. Thus, the numbers of progenitor cells per spleen, calculated from these values, are shown in Figure 5C (bottom; CFU-GM in lighter columns and day-12 CFU-S in darker columns). When Figure 5C is

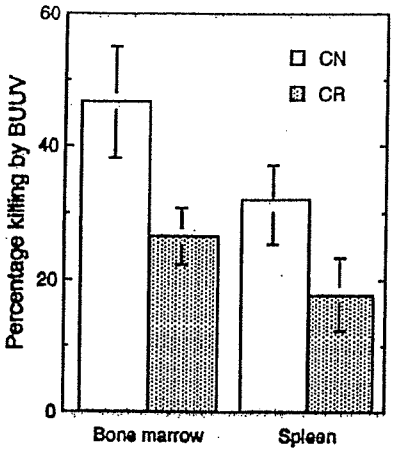


Figure 6. Percent cycling fraction (percentage killing by bromodeoxyuridine ultraviolet [BUUV] method) of hemopoietic progenitor cells, splenic colony-forming units (CFU-S) in bone marrow and spleen of mice with or without caloric restriction. BUUV assay was utilized [16], see Materials and Methods section in text. Cells from three mice each from the 0Gy-CalR(–) and CalR(through) groups were pooled and examined at 50 weeks old, that is, 44 weeks after caloric restriction. Data shown are the mean of three experiments for the spleen and of four experiments for the bone marrow. 0Gy-CalR(–) = mice fed a 95-kcal diet from 6 weeks old. 0Gy-CalR(thru) [CalR(through) in the text] = mice fed a 65-kcal diet from 6 weeks old; after 10 weeks old, the mice were fed a different diet to maintain the body weight of each mouse within 25–27 g ($p < 0.01$, the bone marrow; $p < 0.01$, the spleen).

compared with Figure 5B, all values in the figure show a similar trend but are markedly higher than those shown in Figure 5B.

The number of HPCs in each group seems to correlate with the incidence of leukemia in each group. This may be due to differences in the numbers of stem/progenitor cells between the 0Gy-CalR(–) vs 0Gy-CalR(post) groups and between the 0Gy-CalR(through) vs 0Gy-CalR(pre) groups, induced by the dietary change at 10 weeks old and its subsequent consequences. For the readers' reference, three sets of data (Fig. 5D–F) comparable to those shown in Figure 5A to C but obtained from the bone marrow are presented. None of the data for groups for the bone marrow showed any significant differences among the groups.

Changes in cell-cycling fraction of the hemopoietic stem/progenitor cells during or after caloric restriction

Effect of caloric restriction on the cell-cycle kinetics was evaluated by BUUV assay [16]. In Figure 6, the cycling fraction of hemopoietic stem/progenitor cells is represented by the percentage killing of CFU-S. In this assay, only cycling CFU-S that incorporated BrdUrd were specifically killed by UVA (365-nm peak wavelength), causing a decrease in total number of colonies assayed in the irradiated spleen. The assayed bone marrow cells, as well as spleen cells, showed a significant decrease in percentage killing in the groups subjected to caloric restriction compared with the groups not subjected to caloric restriction [46.0% in the 0Gy-CalR(–) group vs 26.0% in the 0Gy-CalR group for the bone marrow, and 31.4% in the 0Gy-CalR(–) group vs 17.7% in the 0Gy-CalR group for the spleen; at 50 weeks old]. Because the fraction that incorporated BrdUrd and was killed by UV exposure refers to that which shows a reversal of the quiescent fraction, dormant fraction; caloric restriction restored the number of stem/progenitor cells in the quiescent state, which may also contribute to the prevention of leukemogenesis.

Tumor-free death with extension of lifespan by caloric restriction

On the basis of the observation that the percentage of mice that died free of any tumor increased significantly under the regimen of caloric restriction, the following question remains to be answered. Does suppression of tumor development contribute to changes in the spectrum of diseases other than tumors, and to the extension of lifespan, or to changes in the spectrum of disease attributable to tumor-free deaths?

The percentage of mice that died free of tumors was determined by anatomic and pathological examinations at death. In the nonrestricted dietary groups, the percentage of tumor-free mice decreased from 10.1 for the 0Gy-CalR(–) group to 7.4 for the 3Gy-CalR(–) group, following 3-Gy irradiation. When caloric intake was restricted from 6 weeks until death [0Gy-CalR(through)], the percentage of tumor-free deaths increased to 46.4%, the highest, and when it was restricted from 10 weeks (0Gy-CalR(post), the percentage of tumor-free deaths was 35.7%, the second highest, among the nonirradiated diet-restricted groups [0Gy-CalR(through) and 0Gy-CalR(post); Table 1]. Following irradiation (3 Gy), the percentage of tumor-free deaths in the 3Gy-CalR(through) group was 20.0% and that in the 3Gy-CalR(post) group was 17.5%.

Although the 0Gy-CalR(through) group was expected to show the longest survival period, the median survival period and maximum lifespan in this group was limited to 874 days and 1115 days, respectively [vs 896 days and 1145 days, respectively, for the 0Gy-CalR(post) group], the reason for this is as yet unknown; presumably, CalR in the developmental stage of life may not be completely beneficial for health, but it may be beneficial for extending lifespan. Extension of lifespan was caused by changes in the spectrum of diseases attributable to tumor-free death. Cause of tumor-free death is either glomerulosclerotic renal failure, subsequent auricular thromboses with or without pulmonary edemas and increased pulmonary effusions, or cardiac failure due to progressive myocardial fibrosis and calcinosis associated or not associated with coronary sclerosis.

Discussion

Dietary restriction, particularly caloric restriction, is a major carcinogenic modifier observed during experimental carcinogenesis and significantly decreases incidence of

spontaneous tumors and tumors induced by chemical and radiation exposure [18–23]. In our previous study, incidence of myeloid leukemia was experimentally decreased by caloric restriction [4]. In the present study, particular attention was paid to the timing of caloric restriction, that is, pre- or postirradiation, to determine which would be more effective in preventing development of radiation-induced leukemias, and to determine the underlying mechanism that would play a role in this prevention by caloric restriction. In addition, we speculated that decreased incidence of leukemias may correlate with number of hemopoietic stem/progenitor cells as target cells for leukemic transformation, because caloric restriction in our previous preliminary study decreases the number of HPCs when the restriction was implemented throughout the experimental period [9]. When caloric restriction was implemented from 6 weeks to 10 weeks old only before 3-Gy irradiation [3Gy-CalR(pre)], the incidence of radiation-induced myeloid leukemias was lower than that in the nonrestricted control-diet group [3Gy-CalR(–)] (22.2% vs 16.3% in Table 1). However, there were no statistically significant differences in the incidence of leukemias (Table 1) and in the number of progenitor cells between the 0Gy-CalR(pre) and control diet [0Gy-CalR(–)] groups, evaluated 4 weeks after the dietary change (Fig. 5). On the other hand, the incidence of myeloid leukemias significantly decreased (9.5%) in mice when caloric restriction was started after irradiation and continued throughout their lifespan [3Gy-CalR(post), in Table 1]. This is essentially the same result as that obtained in our previous study [4], although the number of progenitor cells in the 3Gy-CalR(post) group at the time of irradiation was expected to be the same as those in the nonrestricted control diet 0Gy-CalR(–) and 3Gy-CalR(–) groups. On the other hand, 4 weeks of caloric restriction after the age of 10 weeks decreased the number of progenitor cells in the 0Gy-CalR(post) group, which is close to that of hemopoietic progenitor cells in the 0Gy-CalR(through) group [see 0Gy-CalR(through) and 0Gy-CalR(post) groups for reference in Fig. 5].

In the case of preirradiation CalR, 3Gy-CalR(pre) mice were returned to the nonrestricted diet immediately after irradiation. Thus, the body weight of these mice increased rapidly to the non-CalR level after irradiation as compared with that of mice in the restricted diet group [CalR(post) group]. Moreover, the number of progenitor cells in the CalR(pre) group was approximately the same as that in the control diet [0Gy-CalR(–)] group 4 weeks after the dietary change (Fig. 5). After return to the regular non-CalR diet, the HPCs, with or without potential lethal damage caused by the 3-Gy irradiation, may have received strong growth stimulation signals. Consequently, despite return to non-CalR level of HPCs and the negated prevention of myeloid leukemogenesis, the results imply that preirradiation CalR potentially prevented leukemia, which was negated by return to the regular non-CalR diet. Thus, the results also imply that the effect of CalR during the initiation stage of leukemogenesis may be canceled out by return to non-CalR during the promotion stage of leukemogenesis. Presumably due to the characteristics of the bone marrow function in mice, it was noted that there was no significant difference in the number of CFU-S observed in the femoral bone marrow of mice among experimental treatments in any experimental treatment [24,25]. The reason spleen colonies were assayed was that the contribution of the spleen to radiation-induced leukemias was reported to be more highly significant than that of the bone marrow [24–29]. The bone marrow showed no significant difference in number of progenitor cells (Fig. 5D–F), because external impacts (caloric restriction in the present study) on mice generally cannot change the function of bone marrow significantly, as bone marrow maintains the minimal essential steady-state hematopoiesis fully functional [29]. Our present study of nonlymphoid leukemias specifically focused on myeloid leukemias, which have been presumed to be hemopoietic stem cell diseases [30].

Tessitore and colleagues reported that complete fasting followed by refeeding is responsible for induction of hepatocarcinogenesis in rats by a subnecrogenic dose of carcinogen, possibly due to an enhancement of the rat's growth and that of the growth of aberrant crypt foci in the rat colon and rectum by the carcinogen [31,32]. Repeated fasting/refeeding and caloric restriction in the present study may not have induced comparable growth stimulation; however, caloric restriction and fasting may share possible biological effects on the cell-cycling rate [33–36].

In the present study, the number of target progenitor cells for leukemic transformation may have been lower in the CalR groups than in the non-CalR groups at the time of irradiation. However, the number of such progenitor cells with potential lethal damage may have increased afterward, when the dietary regimen was changed. Consequently, the number of target progenitor cells may have decreased, followed by a rapid increase as observed by Tessitore and colleagues [31,32], which may explain why the 3Gy-CalR(pre) group did not exhibit any significant decrease in the incidence of leukemias. Target cells for radiation-induced leukemogenesis may not be identical to conventionally assayable progenitor cells or CFU-S. However, the number of such target cells may be proportional to the number of progenitor cells and/or CFU-S [37,38]. Thus, we conclude that caloric restriction contributes to the decrease in the incidence of radiation-induced leukemias on the basis of two mechanisms. First, suppression during the initiation stage of direct genotoxic leukemogenesis, i.e., caloric restriction started before irradiation and continued until irradiation. Second, suppression during the promotion stage of indirect epigenetic leukemogenesis, i.e., restriction started after irradiation and continued until death. Furthermore, cell-cycle kinetics in progenitor cells, CFU-S, evaluated by BUUV assay showed a qualitative suppression during the promotion

stage, during which CFU-S in the CalR groups were more quiescent than those in the non-CalR control (74.0% vs 54.0% in quiescence in the bone marrow, and 82.3% vs 68.6% in quiescence in the spleen; opposite to the case of percentage killing), resulting in a lower risk of epigenetic leukemogenesis for these cells. The 0Gy-CalR(–) and CalR(through) groups were evaluated at 50 weeks old, which is the time leukemogenesis generally becomes overt. These findings are compatible with those of Hursting et al. [35], who described that caloric restriction delays spontaneous tumorigenesis in p53 knockout mice due to a decreased amount of cell-cycling fractions in splenocytes and their precursors.

Our results demonstrate that caloric restriction is effective in suppressing the nongenotoxic promotion stage as well as the genotoxic initiation stage of radiation-induced leukemogenesis. Furthermore, from the results of the present study, the mechanisms underlying effects of caloric restriction on suppression of leukemogenesis, can be speculated to have many different aspects. Our present study focused on the number and cell cycling of hemopoietic stem/progenitor cells, despite other preventive factors that may also contribute, such as oncogene expression [39–41], DNA methylation [41], free-radical formation [42] , induction of apoptosis [43–46] , and activation of immunity [47–51], among others. Several studies of the pathway of insulin signaling and the mechanism underlying the effects of caloric restriction on suppression of tumorigenesis have been reported. Dunn et al. reported that dietary restriction decreased the level of insulin-like growth factor 1 (IGF-1) and the extents of apoptosis, cell proliferation, and tumor progression in p53-deficient mice [52]. The administration of recombinant IGF-1 to CalR mice led to improvement of physiological factors in mice fed a nonrestricted diet [52]. In *Caenorhabditis elegans*, mutation of the *daf-2* gene, in association with that of the *daf-16* gene encoding a member of the insulin receptor signaling molecules, extends the lifespan of the microorganism and confers oxidative stress resistance [53,54]. The *daf-2* gene network also controls longevity by regulating the Mn-SOD (superoxide dismutase)-associated antioxidant defense system [55]. The signal transduction of IGF in *C. elegans* is homologous to that of IGF-1/insulin in mammals. Therefore, the signal-transduction pathway of IGF-1/insulin may be partly involved in the decrease in the incidence of radiation-induced myeloid leukemias following caloric restriction.

In the present study, the incidence of tumors other than myeloid leukemias also decreased statistically significantly in the group in which caloric restriction was started at post-irradiation (Table 1). Caloric restriction limited to the pre-irradiation period, from 6 to 10 weeks old caused a limited decrease in the incidence of myeloid leukemogenesis, implying that for other tumors, caloric restriction suppresses the promotion stage rather than the initiation stage of radiation-induced tumorigenesis. Results of the present study may contribute to identifying the potential preventive factor comparable to the epidemiological relevancy in atomic bomb survivors. A significant increase in the incidence of tumor-free death to 46.4%, the highest, in the nonirradiated restricted group was observed when caloric intake was restricted from 6 weeks until death, which is the first evidence of an increase in the incidence of tumor-free death with an increase in that of cardiovascular diseases by caloric restriction after irradiation, as confirmed by laborious observations of mice throughout their lifetime.

Acknowledgments

We thank Ms. S. Wada and Ms. K. Nojima of the National Institute of Radiological Sciences and Ms. C. Aoyagi and Ms. H. Aihara of the National Institute of Health Sciences for technical assistance. This work was supported by a special project grant for experimental studies on Radiation Health Detriment and Its Modifying Factors from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Beebe GW, Kato H, Land CE. Studies of the mortality of A-bomb survivors: 6. Mortality and radiation dose, 1950–1974. Radiat Res. 1978; 75:138–201.
2. The effects on populations of exposure to low levels of ionizing radiation. BEIR. Vol. III. Washington, DC: National Academy Press; 1980. p. 524.
3. Seki M, Yoshida K, Nishimura M, Nemoto K. Radiation-induced myeloid leukemia in C3H/He mice and the effect of prednisolone acetate on leukemogenesis. Radiat Res. 1991;127:146–149.
4. Yoshida K, Inoue T, Nojima K, Hirabayashi Y, Sado T. Calorie restriction reduces the incidence of myeloid leukemia induced by a single whole-body radiation in C3H/He mice. Proc Natl Acad Sci U S A. 1997;94:2615–2619.
5. Osawa M, Nakamura K, Nishi N, et al. In vivo self-renewal of c-Kit+ Sca-1+ Lin(low/-) hemopoietic stem cells. J Immunol. 1996;156: 3207–3214.
6. Cronkite EP, Bond VP, Carsten AL, Inoue T, Miller ME, Bullis JE. Effects of low level radiation upon the hematopoietic stem cell: implications for leukemogenesis. Radiat Environ Biophys. 1987;26:103–114.
7. Cronkite EP, Inoue T, Bullis JE. Influence of radiation fractionation on survival of mice and spleen colony-forming units. Radiat Res. 1994; 138:266–271.
8. Cronkite EP, Inoue T, Hirabayashi Y, Bullis J. Are stem cells exposed to ionizing radiation in vivo as effective as nonirradiated transfused stem cells in restoring hematopoiesis? Exp Hematol. 1993;21:823–825.
9. Yoshida K, Inoue T, Hirabayashi Y, Matsumura T, Nemoto K, Sado T. Radiation-induced myeloid leukemia in mice under calorie restriction. Leukemia. 1997;11(suppl 3):410–412.
10. Yoshida K, Seki M, Hayata I, Niwa O, Tadokoro K, Tada N. Myeloproliferative disorder due to abnormal production of hematopoietic stimulators. Leuk Res. 1987;11:621–627.
11. Frith CH, Ward JM, Harleman JH, et al. Hematopoietic system. In: Mohr U, ed. International Classification of Rodent Tumors: The Mouse. Berlin/New York/Heidelberg: Springer-Verlag; 2001. p. 417–451.
12. Seki M, Inoue T. Granulocytic leukemia, mouse. In: Jones TC, Ward JM, Mohr U, Hunt RD, eds. Hemopoietic System. Berlin/New York/ Heidelberg: Springer-Verlag; 1990. p. 46–50.
13. Yoshida K, Inoue T, Hirabayashi Y, Nojima K, Sado T. Calorie restriction and spontaneous hepatic tumors in C3H/He mice. J Nutr Health Aging. 1999;3:121–126.

14. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961;14: 213–222.
15. Yoshida K, Seki M, Hayata I, Niwa O, Tadokoro K, Tada N. Myeloproliferative disorder due to abnormal production of hematopoietic stimulators. Leuk Res. 1987;11:621–627.
16. Hirabayashi Y, Matsumura T, Matsuda M, et al. Cell kinetics of hemopoietic colony-forming units in spleen (CFU-S) in young and old mice. Mech Ageing Dev. 1998;101:221–231.
17. Pagano M, Gauvreau K. Principles of Biostatistics. 2nd ed. Boston: Duxbury Press; 2000.
18. Visscher MB, Ball ZB, Barnes RH, Sivertsen I. The influence of caloric restriction upon the incidence of spontaneous mammary carcinoma in mice. Surgery. 1942;11:48–55.
19. Tannenbaum A. The dependence of the genesis of induced skin tumors on the caloric intake during different stages of carcinogenesis. Cancer Res. 1944;4:673–677.
20. Rusch HP, Kline BE, Baumann CA. The influence of caloric restriction and of dietary fat on tumor formation with ultraviolet radiation. Cancer Res. 1945;5:431–435.
21. Saxton LA Jr, Boon MC, Furth J. Observations on the inhibition of development of spontaneous leukemia in mice by underfeeding. Cancer Res. 1944;4:401–409.
22. Gross L, Dreyfuss Y. Reduction in the incidence of radiation-induced tumors in rats after restriction of food intake. Proc Natl Acad Sci U S A. 1984;81:7596–7598.
23. Gross L, Dreyfuss Y. Prevention of spontaneous and radiation-induced tumors in rats by reduction of food intake. Proc Natl Acad Sci U S A. 1990;87:6795–6797.
24. Upton AC, Wolff FF, Furth J, Kimball AW. A comparison of the induction of myeloid and lymphoid leukemias in x-radiated RF mice. Cancer Res. 1958;18:842–848.
25. Yoshida K, Nojima K, Seki M, Inoue T. Radiation-induced myeloid leukemia following splenectomy in C3H/He mice. J Radiat Res. 1996;37:380.
26. Kalpaktsoglou PK, Yunis EJ, Good RA. Early splenectomy and survival of inbred mice. Nature. 1967;215:633–634.
27. Moloney WC, King VP. Reduction of leukemia incidence following splenectomy in the rat. Cancer Res. 1973;33:573–574.
28. Inoue T, Bullis JE, Cronkite EP, Hubner GE. Relationship between number of spleen colonies and 125IdUrd incorporation into spleen and femur. Proc Natl Acad Sci U S A. 1983;80:435–438.
29. Inoue T, Bullis JE, Cronkite EP, Kubo S. Stem cell proliferation and 125IdUrd incorporation into spleen and whole skeletal tissue. Ann N Y Acad Sci. 1985;459:162–178.
30. Kogan SC, Ward JM, Anver MR, et al. Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. Blood. 2002;100:238–245.
31. Tessitore L, Tomasi C, Greco M, et al. A subnecrogenic dose of diethylnitrosamine is able to initiate hepatocarcinogenesis in the rat when coupled with fasting/refeeding. Carcinogenesis. 1996;17:289–292.
32. Premoselli F, Sesca E, Binasco V, Cademi G, Tessitore L. Fasting/re-feeding before initiation enhances the growth of aberrant crypt foci induced by azoxymethane in rat colon and rectum. Int J Cancer. 1998;77:286–294.
33. Hikita H, Vaughan J, Pitot HC. The effect of two periods of short-term fasting during the promotion stage of hepatocarcinogenesis in rats: the role of apoptosis and cell proliferation. Carcinogenesis. 1997;18: 159–166.
34. Lok E, Nera EA, Iverson F, Scott F, So Y, Clayson DB. Dietary restriction, cell proliferation and carcinogenesis: a preliminary study. Cancer Lett. 1988;38:249–255.
35. Hursting SD, Perkins SN, Phang JM. Calorie restriction delays spontaneous tumorigenesis in p53-knockout transgenic mice. Proc Natl Acad Sci U S A. 1994;91:7036–7040.
36. James SJ, Muskhelishvili L. Rates of apoptosis and proliferation vary with caloric intake and may influence incidence of spontaneous hepatoma in C57BL/6 x C3H F1 mice. Cancer Res. 1994;54:5508–5510.
37. Hirabayashi Y, Inoue T, Yoshida K, Inayama Y, Kanisawa M. The detection of normal hidden stem cells during the development of leukemia: assays with PGK isozyme. Exp Hematol. 1990;18:7–10.
38. Iwasawa T, Hirabayashi Y, Kubota N, Inoue T, Kakehi M, Matsui K. Hyperthermic purging in vitro of murine leukemia cells (MK-8057): surviving fractions of normal and leukemic stem cells and the long-term survival of mice injected with the post-hyperthermic leukemia cells. Exp Hematol. 1991;19:332–337.
39. Nakamura KD, Duffy PH, Lu MH, Turturro A, Hart RW. The effect of dietary restriction on myc protooncogene expression in mice: a preliminary study. Mech Ageing Dev. 1989;48:199–205.
40. Himeno Y, Engelman RW, Good RA. Influence of calorie restriction on oncogene expression and DNA synthesis during liver regeneration. Proc Natl Acad Sci U S A. 1992;89:5497–5501.
41. Hass BS, Hart RW, Lu MH, Lyn-Cook BD. Effects of caloric restriction in animals on cellular function, oncogene expression, and DNA methylation in vitro. Mutat Res. 1993;295:281–289.
42. Feuers RJ, Weindruch R, Hart RW. Caloric restriction, aging, and antioxidant enzymes. Mutat Res. 1993;295:191–200.
43. James SJ, Muskhelishvili L. Rates of apoptosis and proliferation vary with caloric intake and may influence incidence of spontaneous hepatoma in C57BL/6 x C3H F1 mice. Cancer Res. 1994;54:5508–5510.
44. Grasl-Kraupp B, Bursch W, Ruttkay-Nedecky B, Wagner A, Lauer B, Schulte-Hermann R. Food restriction eliminates preneoplastic cells through apoptosis and antagonizes carcinogenesis in rat liver. Proc Natl Acad Sci U S A. 1994;91:9995–9999.
45. Lee JH, Jung KJ, Kim JW, Kim HJ, Yu BP, Chung HY. Suppression of apoptosis by calorie restriction in aged kidney. Exp Gerontol. 2004;39: 1361–1368.
46. Thompson HJ, Zhu Z, Jiang W. Identification of the apoptosis activation cascade induced in mammary carcinomas by energy restriction. Cancer Res. 2004;64:1541–1545.
47. Kharazi AL, James SJ, Taylor JM, Lubinski JM, Naramura LT, Makinodan T. Combined chronic low dose radiation –caloric restriction: a model for regression of spontaneous mammary tumor. Int J Radiat Oncol Bio Phys. 1994;28:641–647.
48. Kubo C, Day NK, Good RA. Influence of early or late dietary restriction on life span and immunological parameters in MRL/Mp-lpr/lpr mice. Proc Natl Acad Sci U S A. 1984;81:5831–5835.
49. Grossmann A, Maggio-Price L, Jinneman JC, Wolf NS, Rabinovitch PS. The effect of long-term caloric restriction on function of T-cell subsets in old mice. Cell Immunol. 1990;131:191–204.
50. Sun D, Krishnan A, Su J, Lawrence R, Zaman K, Fernandes G. Regulation of immune function by calorie restriction and cyclophosphamide treatment in lupus-prone NZB/NZW F1 mice. Cell Immunol. 2004;228:54–65.
51. Spaulding CC, Walford RL, Effors RB. The accumulation of non-replicative, non-functional, senescent T cells with age is avoided in calorically restricted mice by an enhancement of T cell apoptosis. Mech. Ageing dev. 1997;93:25–33.
52. Dunn SE, Kari FW, French J, et al. Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53-deficient mice. Cancer Res. 1997; 57:4667–4672.
53. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A C. elegans mutant that lives twice as long as wild type. Nature. 1993;366:461–464.
54. Ogg S, Paradis S, Gottlieb S, et al. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature. 1997;389:994–999.
55. Honda Y, Honda S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J. 1999;13:1385–1393.

Environmental and Molecular Mutagenesis 47:48–55 (2006)

MX, a By-Product of Water Chlorination, Lacks In Vivo Genotoxicity in *gpt* Delta Mice but Inhibits Gap Junctional Intercellular Communication in Rat WB Cells

Akiyoshi Nishikawa,[1]* Kimie Sai,[2] Kazushi Okazaki,[1] Hwa-Young Son,[1] Keita Kanki,[1] Madoka Nakajima,[3] Naohide Kinae,[3] Takehiko Nohmi,[4] James E. Trosko,[5] Tohru Inoue,[6] and Masao Hirose[1]

[1]*Division of Pathology, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[2]*Division of Biosignaling, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[3]*Laboratory of Food Hygiene, School of Food and Nutritional Science, University of Shizuoka, Shizuoka 422-8526, Japan*
[4]*Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[5]*National Food Safety Toxicology Center, Department of Pediatrics and Human Development, Michigan State University, East Lansing, MI 48824, U.S.A.*
[6]*Division of the Biological Safety Research Center, National Institute of Health Sciences, Tokyo 158-8501, Japan*

3-Chloro-4-(dichloromethyl)–5-hydroxy-2(*5H*)-furanone (MX), a by-product of water chlorination, is a potent bacterial mutagen and rat carcinogen. In the present study, the in vivo mutagenicity, cell proliferative activity, and carcinogenicity of MX were investigated in *gpt* delta mice. Groups of 5 male and female 7-week-old *gpt* delta C57BL/6J transgenic mice were given MX at doses of 0, 10, 30, or 100 ppm in their drinking water for 12 weeks, and then killed to assess in vivo mutagenicity using 6-thioguanine and Spi$^-$ selection, and cell proliferative activity using immunohistochemistry for proliferating cell nuclear antigen (PCNA). Further groups of 10 male and female *gpt* delta mice were given 0 or 100 ppm MX for 78 weeks, and a full necropsy with histopathological examination of all organs was conducted to detect neoplastic lesions. The 12-week MX treatment did not result in mutagenicity in the livers or lungs or cell proliferative activity in several organs of the mice, and the 78-week treatment did not cause carcinogenicity. Additional investigations were conducted to evaluate the potential of MX to inhibit gap junctional intercellular communication (GJIC) in rat liver epithelial cells (WB cells) by the scrape loading/dye transfer method. Inhibition of GJIC was detected within 2 hr with a noncytotoxic dose of MX (4 μg/ml), followed by partial restoration after 5 hr. A second phase of inhibition occurred after 10 hr and then the lowered level persisted for the 24 hr-incubation period. Dose-dependent inhibition was evident at both 2 hr and 24 hr, with much stronger effects at the former time. These findings indicate that MX is not mutagenic, mitogenic or carcinogenic in mice, and suggest that the compound exerts epigenetic actions leading to GJIC inhibition. Environ. Mol. Mutagen. 47:48–55, 2006.

Key words: MX; in vivo genotoxicity; gap junction

INTRODUCTION

3-Chloro-4-(dichloromethyl)–5-hydroxy-2(*5H*)-furanone (MX) (illustrated in Fig. 1), a by-product resulting from organic acid reactions during chlorine disinfection, has been isolated and identified as a nonvolatile compound in drinking water from several countries, including Finland [Hemming et al., 1986], the United States [Meier et al., 1987 a,b], England [Horth et al., 1989], and Japan [Suzuki and Nakanishi, 1990]. MX is strongly mutagenic in *Salmonella typhimurium* without metabolic activation

This article contains supplementary material available via the Internet at http:www.interscience.wiley.com/jpages/0893-6692/suppmat.

*Correspondence to: Akiyoshi Nishikawa, Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. E-mail: nishikaw@nihs.go.jp

Received 27 April 2005; provisionally accepted 12 May 2005; and in final form 10 June 2005

DOI 10.1002/em.20167

Published online 16 August 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE I. Body Weight and Relative Organ Weights for Mice Treated Orally with MX for 12 Weeks

Treatment	No. of mice	Body weight (g)	Liver (%)	Kidneys (%)	Lungs (%)	Spleen (%)	Testes (%)
Male							
Control	5	31.8 ± 2.7[a]	4.39 ± 0.43	1.09 ± 0.13	0.24 ± 0.02	0.21 ± 0.04	0.66 ± 0.05
MX 10 ppm	5	30.7 ± 1.7	4.25 ± 0.35	1.15 ± 0.10	0.25 ± 0.02	0.25 ± 0.04	0.67 ± 0.05
MX 30 ppm	5	31.3 ± 2.4	4.44 ± 0.18	1.09 ± 0.05	0.25 ± 0.02	0.20 ± 0.02	0.70 ± 0.08
MX 100 ppm	5	29.8 ± 2.6	4.21 ± 0.33	1.07 ± 0.04	0.25 ± 0.03	0.22 ± 0.03	0.69 ± 0.05
Female							
Control	5	21.8 ± 0.8	4.24 ± 0.13	1.10 ± 0.09	0.29 ± 0.02	0.30 ± 0.06	
MX 10 ppm	5	21.5 ± 0.5	4.13 ± 0.12	1.10 ± 0.06	0.29 ± 0.02	0.32 ± 0.03	
MX 30 ppm	5	22.5 ± 1.1	4.27 ± 0.33	1.09 ± 0.05	0.29 ± 0.03	0.32 ± 0.03	
MX 100 ppm	5	22.6 ± 1.5	4.06 ± 0.09	1.07 ± 0.04	0.30 ± 0.03	0.32 ± 0.07	

[a]Data represent means ± SD.

plasmid. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG). The plates were incubated at 37°C for the selection of colonies harboring plasmids carrying the chloramphenicol acetyltransferase gene and a mutated *gpt* gene. The rescued phages were also infected into YG6020, and poured on plates containing Cm without 6-TG to determine the number of rescued plasmids. The mutant frequency (MF) was calculated as described previously [Nohmi et al., 2000].

Spi⁻ Selection

Spi⁻ selection was performed as described previously [Nohmi et al., 2000]. Briefly, the packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection, respectively. Infected cells were mixed with molten lambda-trypticase soft agar and poured onto lambda-trypticase agar plates. On the following day, plaques (Spi⁻ candidates) were punched out using sterilized glass pipettes and suspended in SM buffer. To confirm the Spi⁻ phenotype of candidate mutant plaques, suspensions were spotted on three types of plates, with XL-1 Blue MRA, XL-1 Blue MRA P2, and WL95 P2 strains spread with soft agar. Confirmed Spi⁻ mutants making clear plaques on every strain were collected and stored as lysed phages at 4°C [Nohmi et al., 1999].

Cell Culture and Treatment with MX

MX was dissolved in 100% ethanol at a dose of 10 mg/ml and kept as a stock solution at −20°C until use. The stock solution was diluted in D-medium (Formula No. 78-5470EF, GIBCO, Grand Island, NY) without serum for treatment. WB-F344 rat liver epithelial cells (WB cells) of oval cell origin [Tsao et al., 1984] were obtained from Drs. J. W. Grisham and M. S. Tsao of the University of North Carolina (Chapel Hill, NC) and plated onto 35-mm dishes in 2 ml of D-medium, supplemented with 5% fetal bovine serum (GIBCO) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Confluent cells were washed with phosphate-buffered saline (PBS) and incubated with serum-free D-medium containing MX for up to 24 hr.

GJIC Assay

Before GJIC assay, the noncytotoxic range of MX was determined spectrophotometrically by measuring the absorbance of neutral red (Wako Chemical, Osaka, Japan) taken up into the cells at a wavelength of 540 nm, according to the method of Borenfeund and Puerna [1985]. The WB cells were treated with MX at concentrations ranging from 0.03 to 10 μg/ml for 24 hr, and viability was expressed as a fraction of the control value. GJIC was assessed in the noncytotoxic range of MX (0–4 μg/ml) by the scrape-loading and dye transfer method originally described by El-Fouly et al. [1987] with the slight modifications described in a previous report [Sai et al., 1998]. Briefly, after treatment with MX, the cells were washed with PBS and 0.05% Lucifer Yellow (LY) (Molecular Probes, Eugene, OR), and a fluorescent dye, which travels through gap junctions, dissolved in PBS, was added to the culture dish. For loading LY into cells, incisions were made on the cell monolayer by scraping with a blade. After incubation at room temperature for 3 min, the cells were washed with PBS and then fixed with 4% formalin. The distance of LY-transfer was viewed with a confocal microscope (Fluoview; Olympus, Tokyo, Japan) and measured by an analytical program in Fluoview. The value for GJIC was expressed as a fraction of the control value, as described previously [Sai et al., 1998].

Statistical Analysis

Data were evaluated by the Student's t-test or analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test or the Kruskal-Wallis test.

RESULTS

Lack of Mutagenicity, Cell Proliferative Activity or Carcinogenicity

No *gpt* delta mice died during the 12-week treatment. There were no differences in water consumption (data not shown) or in body or organ weights between the groups, in either male or female mice, killed at 12 weeks (Table I). PCNA-labeling indices for various organs, including the liver, lung, kidney, and thyroid did not differ among the groups (data not shown), nor did they differ in the gastrointestinal tract (Table II). As shown in Table III, the MFs for the *gpt* gene in the livers and lungs of male mice were, respectively, 2.17, 2.32, 1.76 and 1.50 ($\times 10^{-6}$), and 1.88, 2.50, 1.82 and 2.05 ($\times 10^{-6}$) in the groups receiving 0, 10, 30, and 100 ppm MX for 12 weeks. The respective MFs in the livers of female mice were 2.03, 2.17, 4.19 and 2.40 ($\times 10^{-6}$) (Table III). As shown in Table IV, the MFs for the *red/gam* genes assessed by Spi⁻ selection in male livers and lungs were, respectively, 1.55, 1.74, 1.99 and 1.68, and 1.10, 0.84, 0.81 and 1.25 ($\times 10^{-6}$) in the groups treated with 0, 10,

TABLE II. PCNA-Labeling Indices (%) for the Gastrointestinal Tract of Mice Treated Orally with MX for 12 Weeks

Treatment	No. of mice	Fore-stomach	Glandular stomach	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum
Male									
Control	5	46.1 ± 5.3[a]	32.0 ± 2.5	42.1 ± 2.6	44.1 ± 4.2	43.2 ± 2.5	41.0 ± 2.8	36.3 ± 6.0	42.8 ± 2.0
MX 10 ppm	5	46.0 ± 3.0	32.9 ± 3.9	43.8 ± 4.9	45.0 ± 3.3	44.9 ± 4.0	39.5 ± 2.1	38.2 ± 3.6	39.1 ± 6.1
MX 30 ppm	5	48.6 ± 3.5	33.5 ± 2.9	44.2 ± 5.1	44.6 ± 3.3	42.1 ± 2.2	40.7 ± 1.4	37.7 ± 4.2	40.1 ± 3.1
MX 100 ppm	5	47.9 ± 3.1	33.6 ± 1.6	43.9 ± 3.6	45.7 ± 5.1	45.0 ± 4.0	39.2 ± 3.8	36.2 ± 2.5	38.3 ± 5.1
Female									
Control	5	43.5 ± 4.2	34.5 ± 5.0	44.4 ± 7.1	45.5 ± 4.6	41.0 ± 3.7	42.0 ± 3.5	33.4 ± 3.6	38.9 ± 4.3
MX 10 ppm	5	42.1 ± 2.9	33.6 ± 5.0	43.8 ± 4.7	43.9 ± 3.7	41.0 ± 4.6	41.9 ± 2.8	34.4 ± 4.1	36.9 ± 2.9
MX 30 ppm	5	43.6 ± 4.8	32.5 ± 3.6	44.9 ± 3.9	43.7 ± 2.3	44.2 ± 5.6	41.0 ± 2.8	35.6 ± 5.1	37.9 ± 3.7
MX 100 ppm	5	44.0 ± 3.3	33.4 ± 4.1	44.5 ± 4.5	44.5 ± 3.5	43.4 ± 3.5	40.7 ± 1.7	35.0 ± 5.0	39.5 ± 4.1

[a]Data represent means ± SD.

TABLE III. *Gpt* Mutant Frequency (MF) in Livers and Lungs of Mice Treated Orally with MX for 12 Weeks[a]

Treatment	No. of animals	No. of mutations	No. of colonies screened	MF ($\times 10^{-6}$)
Liver (Male)				
Control	5	7	3,675,000	2.17 ± 1.43[b]
MX 10 ppm	5	10	4,396,500	2.32 ± 1.61
MX 30 ppm	5	6	3,472,500	1.76 ± 2.46
MX 100 ppm	5	8	5,811,000	1.50 ± 0.97
Liver (Female)				
Control	5	7	3,159,000	2.03 ± 1.87
MX 10 ppm	5	4	2,191,500	2.17 ± 3.02
MX 30 ppm	5	9	2,479,500	4.19 ± 3.13
MX 100 ppm	5	6	2,239,500	2.40 ± 2.65
Lung (Male)				
Control	5	6	3,027,000	1.88 ± 1.48
MX 10 ppm	5	10	3,907,500	2.50 ± 1.76
MX 30 ppm	5	9	4,510,500	1.82 ± 1.19
MX 100 ppm	5	8	4,051,500	2.05 ± 1.43

[a]See web-based Supplementary Material for animal-by-animal data.
[b]Data represent means ± SD.

TABLE IV. Spi$^-$ Mutant Frequency (MF) in Livers and Lungs of Male Mice Treated Orally with MX for 12 Weeks[a]

Treatment	No. of animals	No. of mutations	No. of plaques screened	MF ($\times 10^{-6}$)
Liver				
Control	5	11	6,645,000	1.55 ± 1.24[b]
MX 10 ppm	5	16	9,267,000	1.74 ± 0.94
MX 30 ppm	5	17	9,774,000	1.99 ± 1.10
MX 100 ppm	5	17	10,245,000	1.68 ± 0.77
Lung				
Control	5	6	4,617,000	1.10 ± 0.80
MX 10 ppm	5	3	2,202,000	0.84 ± 1.11
MX 30 ppm	5	3	5,154,000	0.81 ± 1.14
MX 100 ppm	5	6	4,926,000	1.25 ± 1.27

[a]See web-based Supplementary Material for animal-by-animal data.
[b]Data represent means ± SD.

30 and 100 ppm MX for 12 weeks. Thus there were no differences in MFs between groups following the 12-week MX treatment. Similarly, the MFs for the *gpt* genes in nontumorous livers receiving 100 ppm MX for 78 weeks were comparable with those in corresponding control livers, in both male and female mice (Table V).

Although 2 out of 10 control males and 3 of 10 100-ppm MX-treated females were killed in a moribund condition or found dead before 78 weeks, four of these mice survived more than 52 weeks. Including these animals, only four neoplastic lesions were detected in total: lymphomas in one each of the treated and control males, and one histiocytic sarcoma, and one osteosarcoma in treated females. Thus, there was no evidence that MX was carcinogenic in this preliminary carcinogenicity study in which 10 male and female mice per group were tested.

Inhibition of GJIC

To select appropriate doses of MX for the GJIC assay (noncytotoxic range), cell viability was determined after MX-treatment. As shown in Figure 2, the highest dose of 10 μg/ml significantly ($P < 0.001$) reduced the viability of WB cells after MX treatment for 24 hr, and the second highest dose of 5 μg/ml produced a small reduction in viability. On the basis of this result, the maximum dose of MX was set to 4 μg/ml. After treatment of WB cells with a noncytotoxic dose of MX (4 μg/ml), GJIC was significantly ($P < 0.05$-0.001) inhibited throughout the 24 hr period (Fig. 3). Especially, marked inhibition of GJIC was observed within 2 hr, followed by a partial restoration after 5 hr (Fig. 4). A second phase of inhibition occurred at 10 hr and a slightly lower level of inhibition was detected in the 24-hr incubation sample (Fig. 4). A clear dose-dependent inhibition of GJIC in WB cells treated with MX at doses of 1, 2, and 4 μg/ml was observed for both the 2-hr and 24-hr incubations, with much stronger inhibition at 2 hr (Fig. 5).

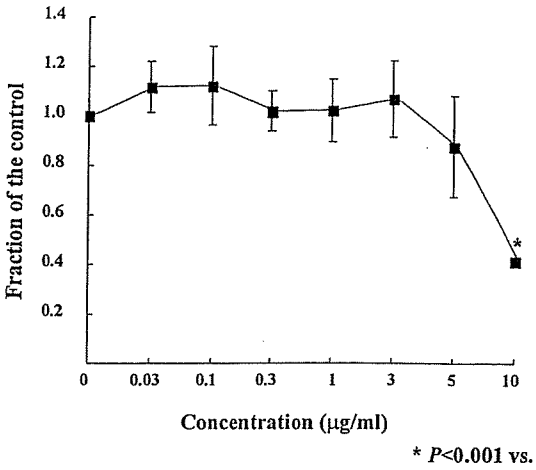
DISCUSSION

In a previous carcinogenicity study using rats, MX induced tumors when given in drinking water at doses of 5.9–70.0 ppm for 104 weeks, with evidence of statistically significant increases of intrahepatic cholangiomas, lung

TABLE V. *Gpt* Mutant Frequency (MF) in Livers of Mice Treated Orally with 100 ppm MX for 78 Weeks[a]

Treatment	No. of animals	No. of mutations	No. of colonies screened	MF ($\times 10^{-6}$)
Male				
Control	5	8	2,068,500	3.35 ± 2.29[b]
100 ppm MX	5	16	4,302,000	3.63 ± 1.13
Female				
Control	5	7	3,205,500	2.47 ± 1.46
100 ppm MX	5	11	3,901,500	2.65 ± 2.16

[a]See web-based Supplementary Material for animal-by-animal data.
[b]Data represent means ± SD.



* $P<0.001$ vs. control

Fig. 2. Viability of WB cells after MX-treatment for 24 hr. Values are means ± SD from data of assays performed in triplicate.

adenomas, and some other tumors [Komulainen et al., 1997]. In the present study, however, MX, a strong in vitro mutagen, failed to exert in vivo mutagenicity or carcinogenicity in *gpt* delta mice. These results are consistent with a recent report of negative mutagenicity in *cII* transgenic medaka [Geter et al., 2004]. Our data provide additional information for assessing the human risk of MX because the species used, genes analyzed, and neoplastic lesions examined are more appropriate than those of the fish model. Transgenic medaka are possible resources for assessing ecological risks [Muir and Howard, 1999], but transgenic rodents have been shown to be more useful for assessing human risks of environmental chemicals [Gorelick and Mirsalis, 1996; Nohmi et al., 1996; Nohmi et al., 1999 ; Nohmi et al., 2000; Horiguchi et al., 2001; Masumura et al., 2002; Takeiri et al., 2003]. In addition, among reporter gene-transgenic rodent models, *gpt* delta mice and rats have an advantage in that not only point mutations but also deletions can be detected [Nohmi et al., 1996; Nohmi et al., 1999; Nohmi et al., 2000]. In the present study, the lack of mutagenicity was linked with an absence of carcinogenicity.

MX was reported to induce 2800–13000 revertants/nmol in the *Salmonella typhimurium* TA100 without metabolic activation [Ishiguro et al., 1988; LaLonde et al., 1991], indicating that the compound is one of the most potent mutagens in TA100. The contribution of MX to the total mutagenicity associated with chlorine-treated tap water was estimated to be 15–57% in Finland [Kronberg and Vertiainen, 1988], 15–34% in the United States [Meier et al., 1987a], and 7–21% in Japan [Furihata et al., 1992]. MX is also a mammalian cell clastogen [Meier et al., 1987b] and gives rise to specific DNA adducts [Franzen et al., 1998; Munter et al., 1998]. Despite its strong in vitro mutagenicity, MX was only marginally active at inducing nuclear anomalies in the small intestine of mice when given as a single oral dose of 0.37 mmol/kg [Daniel et al., 1991]. Thus, it has been hypothesized that mammalian cells may effectively detoxify chlorohydroxyfuranones, including MX [Daniel et al., 1991; Meier et al., 1996]. In fact, it has been shown that MX is efficiently inactivated by endogenous defensive systems such as those involving glutathione and cysteine [LaLonde and Xie, 1993; Watanabe et al., 1994]. The results of the present study support the conclusion that such defensive systems could be sufficient to detoxify MX ingested in drinking water at concentrations well above environmental levels [Melnick et al., 1997]. A recent study [Huuskonen et al., 2003] showing no teratogenicity in rats receiving up to 60 ppm MX also supports this hypothesis.

Potentially relevant to our findings is Thilly's [2003] challenge to the hypothesis that environmental chemicals induce a substantial fraction of human point mutations. Thilly's interpretation, based on direct measurements of the kinds and numbers of point mutations in human tissues, rests on the fact that no clear relationship has been established between mutation and exposure to environmental chemical and physical agents, except for sunlight–induced mutations in the skin. He suggested an alternative hypothesis that point mutations arise in tumors of exposed organisms as errors in replication of undamaged DNA during exposure to chemicals that select these initiated cells rather than induce onco-mutations. In other words, these "carcinogens" are actually tumor promoters acting epigenetically, rather than direct mutagens [Trosko, 1997].

In the present study, it was demonstrated that MX inhibited cell–cell communication in WB cells, which is in line with a previous report of weak inhibitory effects on metabolic cooperation in cultured Chinese hamster lung V79 cells [Matsumura et al., 1994]. Our results suggest that MX may inhibit intercellular communication in both liver and bile duct cells of rats, because rat WB cells of oval cell origin, with the potential to differentiate into either cell type, were employed here. Regarding the time course, the peaks at 2 and 10 hr suggest that there is transient inhibition due to post-translational mechanisms, but then a later reduction of expression of connexin genes, such as the gene encoding Cx43, occurs. Most tumor promoters, both complete carcinogens and pure tumor pro-

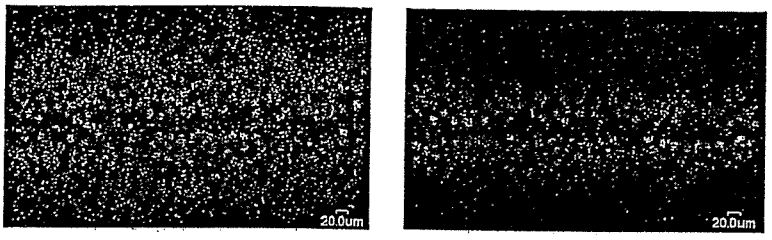


Fig. 3. Inhibition of GJIC in WB cells after MX treatment.

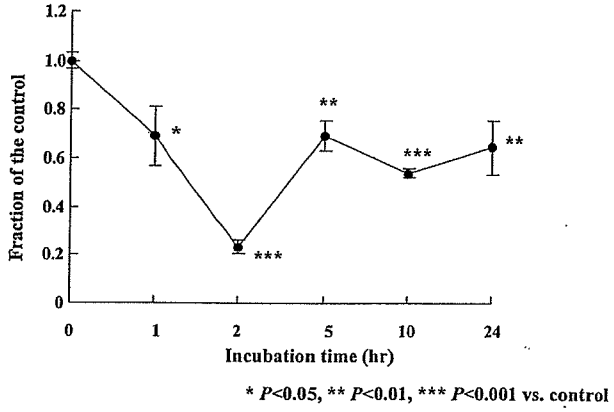


Fig. 4. Time course of GJIC in WB cells after MX treatment. Values are means ± SD of data from assays performed in triplicate.

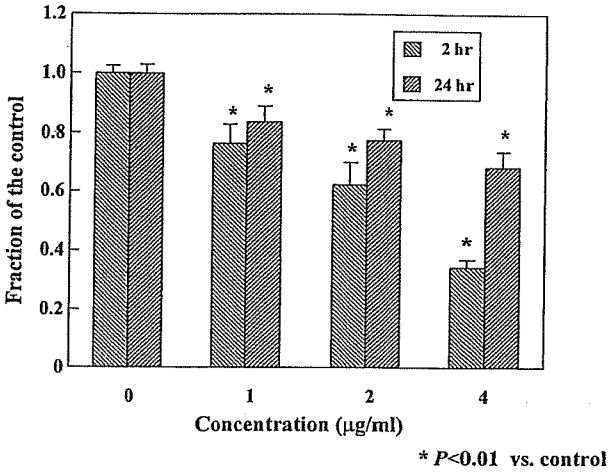


Fig. 5. Dose-dependent inhibition of GJIC in WB cells after MX treatment. Values are means ± SD of data from assays performed in triplicate.

moters including non-liver tumor promoters like TPA, have the ability to inhibit cell–cell communication in WB cells [Loewenstein, 1979; Trosko et al., 1983; Trosko and Goodman, 1994; Evans and Martin, 2002].

It has been suggested that MX might primarily affect the mucosa of the alimentary tract, when exposure levels are sufficient to overwhelm the defense capacity relying on glutathione or other factors, or when defense systems are impaired [Nishikawa et al., 1994; Meier et al., 1996]. Previously, we reported that MX induces cell proliferation in the gastric mucosa of rats even at low, nontoxic doses such as 25 ppm [Nishikawa et al., 1994] as well as at higher doses [Furihata et al., 1992]. In this context, mitogenic potential, in addition to possible regenerative responses, have been suggested to be involved in the mechanisms underlying stomach tumor-promoting activity [Nishikawa et al., 1999]. Nevertheless, in the present study, cell proliferation was not increased by the MX treatments in any of the organs examined, including the gastrointestinal tract, suggesting the possible existence of species differences. It is conceivable that tumor-promoting processes are species-specific in some cases, but except for limited in vivo mutagenicity assays [Yoshikawa et al., 1982; Nohmi et al., 1983; Madle et al., 1986 Degawa et al., 1990], there is little evidence that tumor-initiating processes might similarly differ with the animal species.

In conclusion, the results of the present study indicate that MX inhibits GJIC in cultured rat WB cells but is not mitogenic, mutagenic, or carcinogenic in *gpt* delta mice. Thus the carcinogenicity of MX in rats may be, at least partially, associated with tumor-promoting effects. It remains to be determined whether MX is mutagenic in transgenic rats, such as the Big Blue or *gpt* delta rat.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Health Sciences from the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

Borenfeund E, Puerna JA. 1985. Toxicity determined in vitro by morphological alteration and neutral red absorption. Toxicol Lett 24: 119–124.

Daniel FB, Olson GR, Stober JA. 1991. Induction of gastrointestinal tract nuclear anomalies in B6C3F1 mice by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone and 3,4-(dichloro)-5-hydroxy-2[5*H*]-furanone mutagenic byproducts of chlorine disinfection. Environ Mol Mutagen 17:32–39.

Degawa M, Agatsuma T, Hashimoto Y. 1990. Species difference among experimental rodents in the activity and induction of cytochrome

P-450 isozymes for mutagenic activation of carcinogenic aromatic amines. Jpn J Cancer Res 81:1253–1258.

El-Fouly MH, Trosko JE, Chang CC. 1987. Scrape loading and dye transfer: a rapid and simple technique to study gap junctional intercellular communication. Exp Cell Res 168:422–430.

Evans WH, Martin PEM. 2002. Gap junctions: structure and function. Mol Membr Biol 19:121–136.

Franzen R, Tanabe K, Morita M. 1998. Isolation of a MX-guanine adduct formed at physiological conditions. Chemosphere 36: 2803–2808.

Furihata C, Yamashita M, Kinae N, Matsushima T. 1992. Genotoxicity and cell proliferative activity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone [MX] in rat glandular stomach. Wat Sci Tech 25:341–345.

Geter DR, Winn RN, Fournie JW, Norris MB, DeAngelo AB, Hawkins WE. 2004. MX [3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone], a drinking-water carcinogen, does not induce mutations in the liver of *cII* transgenic medaka (*Oryzias latipes*). J Toxicol Environ Health A 67:373–83.

Gorelick NJ, Mirsalis JC. 1996. A strategy for the application of transgenic rodent mutagenesis assays. Environ Mol Mutagen 28:434–442.

Grassilli E, Carcereri de Prati A, Monti D, Troiano L, Menegazzi M, Barbieri D, Franceschi C, Suzuki H. 1992. Studies of the relationship between cell proliferation and cell death: II. Early gene expression during concanavalin A-induced proliferation or dexamethasone-induced apoptosis of rat thymocytes. Biochem Biophys Res Commun 188:1261–1266.

Hemming J, Holmbolm B, Reunanen M, Kronberg L. 1986. Determination of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in chlorinated drinking and humic water. Chemosphere 15:549–556.

Horiguchi M, Masumura K, Ikehata H, Ono T, Kanke Y, Nohmi T. 2001. Molecular nature of UVB-induced deletions in the murine epidermis. Cancer Res 61:3913–3918.

Horth H, Fielding M, Gibson T, James HA, Ross H. 1989. Identification of mutagens in drinking water. Water Research Centre Technical Report PRD 2038-M, Medmenham, England.

Huuskonen H, Venalainen R, Komulainen H. 2003. Developmental toxicity evaluation of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) in Wistar rats. Birth Defects Res B 68:172–179.

Ishiguro Y, Santodonato J, Neal MW. 1988. Mutagenic potency of chlorofuranones and related compounds in Salmonella. Environ Mol Mutagen 11:225–234.

Kinae N, Sugiyama C, Nasuda MY, Goto K, Tokumoto K, Furugori M, Shimoi K. 1992. Seasonal variation and stability of chlorinated organic mutagens in drinking water. Wat Sci Tech 25:333–340.

Komulainen H, Kosma V-M, Vaittinen S-L, Vartiainen T, Kaliste-Korhonen E, Lötjönen S, Tuominen RK, Tuomisto J. 1997. Carcinogenicity of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in the rat. J Natl Cancer Inst 89:848–856.

Kronberg L, Vertiainen T. 1988. Ames mutagenicity and concentration of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone and its geometric isomer E-2-chloro-3-(dichloromethyl)-4-oxo-butenoic acid in chlorine-treated tap water. Mutat Res 206:177–182.

LaLonde RT, Cook GP, Perakyla H, Dence CW, Babish JG. 1991. *Salmonella typhimurium* (TA100) mutagenicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone and its open- and closed-ring analogs. Environ Mol Mutagen 17:40–48.

LaLonde RT, Xie S. 1993. Glutathione and *N*-acetylcysteine inactivation of mutagenic 2(5H)-furanones from the chlorination of humics in water. Chem Res Toxicol 6:445–451.

Loewenstein WR. 1979. Junctional intercellular communication and the control of growth. Biochim Biophys Acta 560:1–65.

Madle E, Korte A, Beek B. 1986. Species differences in mutagenicity testing: I. Micronucleus and SCE tests in rats, mice, and Chinese hamsters with aflatoxin B_1. Teratog Carcinog Mutagen 6:1–13.

Masumura K, Kuniya K, Kurobe T, Fukuoka M, Yatagai F, Nohmi T. 2002. Heavy-ion-induced mutations in the *gpt* delta transgenic mouse: Comparison of mutation spectra induced by heavy-ion, X-ray and γ-ray radiation. Environ Mol Mutagen 40:207–215.

Matsumura H, Watanabe M, Matsumoto K, Ohta T. 1994. 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) induces gene mutations and inhibits metabolic cooperation in cultured Chinese hamster cells. J Toxicol Environ Health 43:65–72.

Meier JR, Knohl RB, Coleman WE, Ringhand HP, Munch JW, Kaylor WH, Streicher RP, Kopfler FC. 1987a. Studies on the potent bacterial mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone: aqueous stability, XAD recovery and analytical determination in drinking water and in chlorinated humic acid solution. Mutat Res 189:363–373.

Meier JR, Blazak WF, Knohl RB. 1987b. Mutagenic and clastogenic properties of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone: a potent bacterial mutagen in drinking water. Environ Mol Mutagen 10:411–424.

Meier JR, Monarca S, Patterson KS, Villarini M, Daniel FB, Moretti M, Pasquini R. 1996. Urine mutagenicity and biochemical effects of the drinking water mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), following repeated oral administration to mice and rats. Toxicology 110:59–70.

Melnick RL, Boorman GA, Dellarco V. 1997. Water chlorination, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), and potential cancer risk. J Natl Cancer Res 89:832–833.

Muir WM, Howard RD. 1999. Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the Trojan gene hypothesis. Proc Natl Acad Sci USA 96:13853–13856.

Munter T, Le Curieux F, Sjoholm R, Kronberg L. 1998. Reaction of the potent bacterial mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) with 2′-deoxyadenosine and calf thymus DNA: identification of fluorescent propenoformyl derivatives. Chem Res Toxicol 11:226–233.

Nishikawa A, Kinae N, Furukawa F, Mitsui M, Enami T, Hasegawa T, Takahashi M. 1994. Enhancing effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone on cell proliferation and lipid peroxidation in the rat gastric mucosa. Cancer Lett 85:151–157.

Nishikawa A, Furukawa F, Lee I-S, Kasahara K, Tanakamaru Z, Nakamura H, Miyauchi M, Kinae N, Hirose M. 1999. Promoting effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone on rat glandular stomach carcinogenesis initiated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Cancer Res 59:2045–2049.

Nohmi T, Yoshikawa K, Nakadate M, Ishidate M Jr. 1983. Species difference in the metabolic activation of phenacetin by rat and hamster liver microsomes. Biochem Biophys Res Commun 110:746–752.

Nohmi T, Katoh M, Suzuki M, Matsui M, Yamada M, Watanabe M, Suzuki H, Horiya N, Ueda O, Shibuya T, Ikeda H, Sofuni T. 1996. A new transgenic mouse mutagenesis test system using Spi$^-$ and 6-thioguanine selections. Environ Mol Mutagen 28:465–470.

Nohmi T, Suzuki M, Masumura K, Yamada M, Matsui K, Ueda O, Suzuki H, Katoh M, Ikeda H, Sofuni T. 1999. Spi$^-$ selection: an efficient method to detect γ-ray-induced deletions in transgenic mice. Environ Mol Mutagen 34:9–15.

Nohmi T, Suzuki T, Masumura K. 2000. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res 455:191–215.

Padmapria AA, Just G, Lewis NG. 1985. Synthesis of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone, a potent mutagen. Can J Chem 63:828–832.

Sai K, Upham BL, Kang K-S, Hasegawa R, Inoue T, Trosko JE. 1998. Inhibitory effect of pentachlorophenol on gap junctional intercellular communication in rat liver epithelial cells in vitro. Cancer Lett 130:9–17.

Suzuki N, Nakanishi J. 1990. The determination of strong mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in Japan. Chemosphere 21:387–392.

Takeiri A, Mishima M, Tanaka K, Shioda A, Ueda O, Suzuki H, Inoue M, Masumura K, Nohmi T. 2003. Molecular characterization of mitomycin C-induced large deletions and tandem-base substitutions in the bone marrow of *gpt* delta transgenic mice. Chem Res Toxicol 16:171–179.

Thilly WG. 2003. Have environmental mutagens caused oncomutations in people? Nat Genet 34:255–259.

Trosko JE. 1997. Challenge to the simple paradigm that 'carcinogens' are 'mutagens' and to the in vitro and in vivo assays used to test the paradigm. Mutat Res 373:245–249.

Trosko JE, Chang CC. 1988. Nongenotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication. In: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects. NY:Cold Spring Harbor. Cold Spring Harbor Laboratory Banbury Report 31. pp 139–170.

Trosko JE, Goodman JI. 1994. Intercellular communication may facilitate apoptosis: implications for tumor promotion. Mol Carcinog 11:8–12.

Trosko JE, Ruch RJ. 1998. Cell-cell communication in carcinogenesis. Front Biosci 3:208–236.

Trosko JE, Ruch RJ. 2003. Gap junctions as targets for cancer chemoprevention and chemotherapy. Curr Drug Targets 3:465–482.

Trosko JE, Chang CC, Medcalf A. 1983. Mechanisms of tumor promotion: potential role of intercellular communication. Cancer Invest 1:511–526.

Tsao MS, Smith JD, Nelson KG, Grisham JW. 1984. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. Exp Cell Res 154:38–52.

Watanabe M, Kobayashi H, Ohta T. 1994. Rapid inactivation of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), a potent mutagen in chlorinated drinking water, by sulfhydryl compounds. Mutat Res 312:131–138.

Wilson MR, Close TW, Trosko JE. 2000. Cell population dynamics (apoptosis, mitosis, and cell-cell communication) during disruption of homeostasis. Exp Cell Res 254:257–268.

Yoshikawa K, Nohmi T, Miyata R, Ishidate M Jr., Ozawa N, Isobe M, Watabe T, Kada T, Kawachi T. 1982. Difference in liver homogenates from Donryu, Fischer, Sprague-Dawley and Wistar strains of rat in the drug-metabolizing enzyme assay and the *Salmonella*/ hepatic S9 activation test. Mutat Res 96:167–186.

Yotti LP, Chang CC, Trosko JE. 1979. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. Science 206:1089–1091.

Meeting Report: Validation of Toxicogenomics-Based Test Systems: ECVAM–ICCVAM/NICEATM Considerations for Regulatory Use

Raffaella Corvi,[1] Hans-Jürgen Ahr,[2] Silvio Albertini,[3] David H. Blakey,[4] Libero Clerici,[5] Sandra Coecke,[1] George R. Douglas,[4] Laura Gribaldo,[1] John P. Groten,[6] Bernd Haase,[7] Karen Hamernik,[8] Thomas Hartung,[1] Tohru Inoue,[9] Ian Indans,[10] Daniela Maurici,[1] George Orphanides,[11] Diana Rembges,[5] Susanna-Assunta Sansone,[12] Jason R. Snape,[13] Eisaku Toda,[14] Weida Tong,[15] Joost H. van Delft,[16] Brenda Weis,[17] and Leonard M. Schechtman[18,19]

[1]European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection (IHCP), Joint Research Centre of the European Commission (JRC), Ispra, Italy; [2]Bayer HealthCare AG, Wuppertal, Germany; [3]Hoffmann-La Roche, Basel, Switzerland; [4]Environmental Health Centre, Health Canada, Ottawa, Ontario, Canada; [5]Physico-Chemical Exposure, IHCP, JRC, Ispra, Italy; [6]TNO, Utrecht, the Netherlands; [7]QIAGEN, Hilden, Germany; [8]U.S. Environmental Protection Agency, Washington, DC, USA; [9]National Institute of Health Sciences, Tokyo, Japan; [10]Health Safety Executive, London, United Kingdom; [11]Syngenta, Macclesfield, United Kingdom; [12]European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom; [13]AstraZeneca, Brixham, United Kingdom; [14]Organisation for Economic Co-operation and Development, Paris, France; [15]Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas, USA; [16]University of Maastricht, Maastricht, the Netherlands; [17]National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina, USA; [18]U.S. Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, NC, USA; [19]U.S. Food and Drug Administration, National Center for Toxicological Research, Rockville, Maryland, USA

This is the report of the first workshop "Validation of Toxicogenomics-Based Test Systems" held 11–12 December 2003 in Ispra, Italy. The workshop was hosted by the European Centre for the Validation of Alternative Methods (ECVAM) and organized jointly by ECVAM, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The primary aim of the workshop was for participants to discuss and define principles applicable to the validation of toxicogenomics platforms as well as validation of specific toxicologic test methods that incorporate toxicogenomics technologies. The workshop was viewed as an opportunity for initiating a dialogue between technologic experts, regulators, and the principal validation bodies and for identifying those factors to which the validation process would be applicable. It was felt that to do so now, as the technology is evolving and associated challenges are identified, would be a basis for the future validation of the technology when it reaches the appropriate stage. Because of the complexity of the issue, different aspects of the validation of toxicogenomics-based test methods were covered. The three focus areas include *a)* biologic validation of toxicogenomics-based test methods for regulatory decision making, *b)* technical and bioinformatics aspects related to validation, and *c)* validation issues as they relate to regulatory acceptance and use of toxicogenomics-based test methods. In this report we summarize the discussions and describe in detail the recommendations for future direction and priorities. *Key words:* acceptance, alternatives, biomarker, predictive test, regulatory use, standardization, toxicogenomics, toxicology, validation. *Environ Health Perspect* 114:420–429 (2006). doi:10.1289/ehp.8247 available via *http://dx.doi.org/* [Online 17 August 2005]

Toxicogenomics, an emerging field in molecular toxicology, offers the promise of new approaches to identify and characterize such factors as the biologic activity of new and existing chemicals and drugs and could play an important role in hazard assessment for human health. This revolutionary field can potentially affect many scientific and medical areas, including the development of a new generation of alternative predictive testing and screening methods that could lend themselves to the reduction, refinement, and replacement of animals used for such purposes.

The European Centre for the Validation of Alternative Methods (ECVAM), the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) are currently investigating the specific considerations necessary for adequate validation of toxicogenomics-based test methods. The primary objective of ECVAM and ICCVAM/NICEATM is to facilitate development, validation, and regulatory acceptance of new, revised, and alternative test methods that reduce, refine, and replace the use of animals (referred to as the three Rs; Russell and Burch 1959) in testing while maintaining and promoting scientific quality and the protection of human health, animal health, and the environment. The efforts of such organizations as ICCVAM/NICEATM and ECVAM have helped foster the principles of the three R's and have contributed to progress in the use of alternative methods for regulatory, research, and educational purposes.

Experience in the validation of conventional alternative test methods has led to an understanding that new and innovative approaches likely will be necessary to standardize test methods based on toxicogenomics and to evaluate the scientific validity and regulatory applicability of such test methods. It is envisioned that the entire validation process will be more complex and challenging than that typically encountered thus far for other alternative test methods. This is because not only will the technology itself need to be standardized and validated, but the methods that are based upon the technology and their predictive aspects will also need to undergo validation if they are to be employed in regulatory decision-making processes. In addition the validation process must be able to accommodate the anticipated rapid changes in technology that could affect the performance of the test method and its reliability for a specific purpose.

Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Because data are already being generated using these technologies, it is both timely and important to address the subject of validation now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically validated

Address correspondence to R. Corvi, European Centre for the Validation of Alternative Methods, IHCP, Joint Research Centre of the European Commission, Via E. Fermi 1, 21120 Ispra, Italy. Telephone: 39-0332-785266. Fax: 39-0332-785845. E-mail: raffaella.corvi@jrc.it

Supplemental Material is available online (http://ehp.niehs.nih.gov/members/2005/8247/suppl.pdf).

We gratefully acknowledge W.S. Stokes, Director of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, for contributions to the planning and organization of the workshop as well as insightful and constructive comments.

This document represents the consensus of the participants' views expressed as individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

The authors declare they have no competing financial interests.

Received 22 April 2005; accepted 17 August 2005.