extensive use worldwide and common production (Donaldson et al., 2002), are organophosphorus pesticides (OPs). Present in agricultural runoff, OPs may enter the aquatic environment and reach high concentrations downstream from discharge sites, potentially producing adverse effects in humans and wildlife (Haywood and Karalliedde, 2000; Fleischli et al., 2004).

Acetylcholinesterase (AChE; E.C. 3.1.1.7), the target enzyme of most OPs and their active metabolites, is a key neuroregulatory enzyme that is common to many species from insects to reptiles, birds and mammals. In addition, pseudocholinesterase (PChE), an enzyme present in serum, is also targeted and inhibited by OPs (Ma and Chambers, 1995, 1994; Sultatos, 1987; Sultatos et al., 1985). Toxicity of OPs is a composite effect that depends on concentration, duration of exposure, and organism size. Different organisms display different degrees of sensitivity to cholinesterase (ChE) inhibitors as OPs act as suicide substrates for AChE and PChE, irreversibly phosphorylating critical serine residues in the enzyme active site, leading to irreversible inactivation of the ChE in cholinergic synapses and neuromuscular junctions (Betancourt and Carr, 2004; Liu et al., 2002; Amitai et al., 1998; Mileson et al., 1998). To attain an acceptable daily intake (ADI) of OPs, a single pesticide is administered using a suitable animal model to assess toxicity. Unfortunately, OPs are rarely present in environmental samples as single chemical compounds, but commonly coexist as mixtures of different pesticides; thus, it is important to evaluate the total toxicity of multiple OPs in a sample using an indicator of overall potential toxicity.

While it is expensive and time consuming to evaluate the combined effects of multiple pesticides in laboratory animals, there are few efficient alternative test methods. The limited investigation of acute interactive toxicity of mixtures containing two OPs has previously been done (Karanth et al., 2004, 2001; Hazarika et al., 2003; Richardson et al., 2001). The multiple OPs we chose to evaluate can be grouped into two categories according to their structures: (1) nine chemical compounds containing a P=S moiety: butamifos, chlorpyrifos, diazinon, EPN, fenitrothion (MEP), isofenphos, isoxathion, prothiofos, and tolclofos-methyl; and (2) six chemical compounds containing a P=O moiety: acephate, dichlorvos (DDVP), edifenphos (EDDP), fosetyl, iprobenfos (IBP), and trichlorfon (DEP). In this paper, we establish a simple, inexpensive, and robust in vitro evaluation system suitable for hazard assessment of combinations of multiple OPs. Using 5-methyl -2-thenoylthiocholine-iodide (MTTC) as an indicator of ChE inhibition, the overall toxicity of OP mixtures, representative of those typically encountered in environmental water samples, was examined.

2. Materials and methods

2.1. Chemicals

Butamifos oxon, chlorpyrifos oxon and tolclofos-methyl oxon were obtained from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) and chlorpyrifos was obtained from GL Sciences Inc. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Laboratory water was purified using a Milli-Q gradient A10 Elix with an EDS polisher system (Millipore, Bedford, MA, USA).

2.2. Standard solutions

Stock solutions of PChE (1250 IU/L) and MTTC (2.0 mM) were prepared and stored at 4 °C until further needed. A 0.25 mM chromogen solution of 5, 5′-dithiobisnitrobenzoic acid (DTNB) was prepared using 0.1 mol/L phosphate buffer (pH 7.4).

Individual standard solutions of pesticides were prepared in dimethyl sulfoxide, and each experiment was performed using seven dilutions of each standard solution. Evaluation of a single pesticide was performed using final concentrations of 10, 5, 2, 1, 0.5, 0.2, and 0.1 μg/mL. Combinations of two pesticides were evaluated by fixing the concentration of one pesticide at 0.1 μg/mL and varying the concentration of the second pesticide to 5, 2.5, 1, and 0.5, 0.25, 0.1, and 0.05 μg/mL. The pesticides that showed strong inhibition were diluted 100 fold. Combinations of three pesticides were evaluated by fixing the concentrations of two compounds at 0.05 μg/mL each, and varying the concentration of the third pesticide to 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 μg/mL. The pesticides that showed strong inhibition were diluted 5000 fold.

2.3. The evaluation of ChE activity

The active ChE enzymatically cleaves the substrate MTTC to release thiocholine. The released thiocholine reacts with the chromogen DTNB to generate a yellow product (Fig. 1), quantifiable at 405 nm by UV absorption that is impeded when ChE activity is inhibited by pesticide (Karahasanoglu and Ozand, 1967).

All experiments were done in triplicate. A 7 mIU solution of ChE and each appropriate pesticide sample were uniformly mixed in a ratio of 4:1. MTTC substrate solution (63 μL) was added to 7 μL of the mixed solution of ChE and OP in a 96 microwell plate and then 280 μL of the DTNB chromogen solution was added. The plate was incubated at 37 °C for 7 min, and the absorbance at 405 nm was measured using an

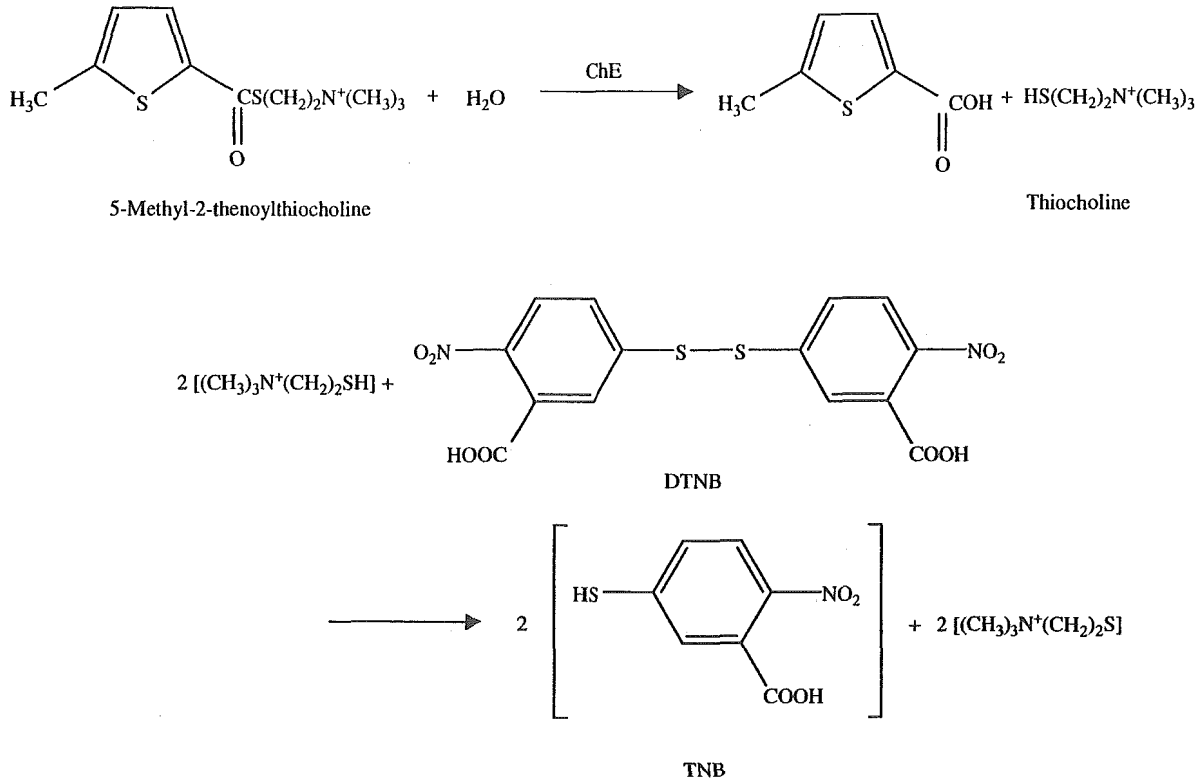

Fig. 1. The chemical reaction for generation of thiocholine and subsequent measurement of ChE activity.

Table 1
Intended use and concentration of 20% inhibition for the oxon form of nine pesticides

Group	Pesticides	Intended use	IC_{20} (μg/mL)
A	Chlorpyrifos oxon	I	0.0011
	Isoxathion oxon	I	0.0013
	Diazinon oxon	I	0.0089
B	Prothiofos oxon	I	0.13
	EPN oxon	I	0.14
	MEP oxon	I	0.33
C	Butamifos oxon	H	1.04
	Tolclofos-methyl oxon	G	5.09
	Isofenphos oxon	I	—

I: insecticide, G: germicide, H: herbicide.

Table 2
Intended use and concentration of 20% inhibition for six pesticides

Group	Pesticides	Intended use	IC_{20} (μg/mL)
A	DDVP	I	0.048
	EDDP	G	0.08
B	DEP	I	0.49
C	IBP	G	2.81
	Fosetyl	G	0.3
	Acephate	I	—

I: insecticide, G: germicide.

Ultrospec Visible Plate Reader II 96 (Amersham Biosciences, Tokyo, Japan).

The method employed typically yields a 40% background at 405 nm and IC_{20} results (Tables 1 and 2) are calculated and corrected by setting the maximal inhibition (Figs. 3 and 4) to 100%.

3. Results and discussion

3.1. Inhibition of ChE activity by 15 kinds of OPs

Parent compounds containing a P=S moiety exhibited limited inhibition (Fig. 2); however, these species undergo desulfuration to form active oxon metabolites, containing a P=O moiety. This desulfuration can occur

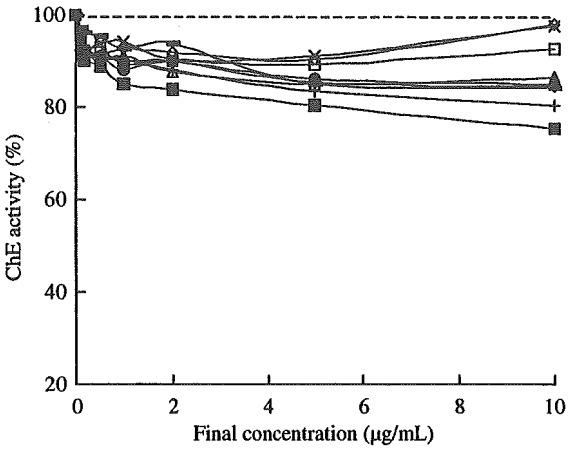

Fig. 2. ChE activity for the parent compounds of nine pesticides: —■—; isofenphos, —●—; tolclofos-methyl, —▲—; butamifos, —□—; MEP, —◊—; EPN, —■—; prothiofos, —+—; diazinon, —×—; isoxathion, —♦—; chlorpyrifos.

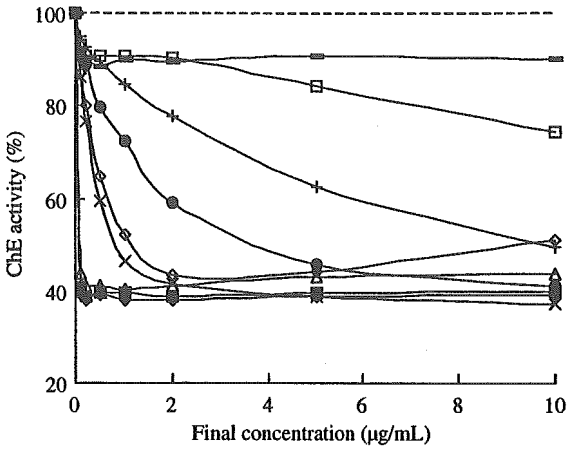

Fig. 3. ChE activity for the oxon form of nine pesticides: —■—; isofenphos oxon, —●—; tolclofos-methyl oxon, —▲—; butamifos oxon, —□—; MEP oxon, —◊—; EPN oxon, —■—; prothiofos oxon, —+—; diazinon oxon, —×—; isoxathion oxon, —♦—; chlorpyrifos oxon.

through metabolic activation by organisms or by nonbiological oxidation in the environment (Ma and Chambers, 1995, 1994; Sultatos, 1987; Sultatos et al., 1985; Butler and Murray, 1997). Evaluation of the inhibitory activity of the oxon metabolites of these nine pesticides confirmed that the degree of inhibition was stronger than that of the parent compounds (Fig. 3). OPs were classified into three groups according to the level of ChE inhibition (Table 1). Group A showed strong inhibition of ChE with 20% inhibition (IC_{20}) at 100 ng/mL or less. Group B showed slight inhibition with IC_{20} between 0.1 and 1 μg/mL. Group C showed limited inhibition with IC_{20} exceeding 0.1 μg/mL. The degree of ChE inhibition for six pesticides containing an original P=O moiety is shown in Fig. 4, and these pesticides were similarly classified into three groups (Table 2). Of the pesticides, fosetyl inhibited ChE activity at low concentrations but the degree of inhibition did not increase with increasing concentrations (Fig. 4). Accordingly, we classified fosetyl into group C even though the IC_{20} was calculated to be 0.3 μg/mL. Consequently, the results suggest that the toxicity of OPs is due to the presence of the P=O moiety that makes the P=O pesticide more similar in structure to acetylcholine, the substrate of ChE, than the comparable P=S pesticide. However, there was no obvious relationship found between the degree of inhibition and intended use (i.e. insecticide, germicide, herbicide) of the pesticide (Tables 1 and 2).

3.2. The relation between ChE activity inhibition and the chemical structure

Strong inhibitors such as chlorpyrifos oxon, isoxathion oxon and diazinon oxon (Fig. 5) in group A

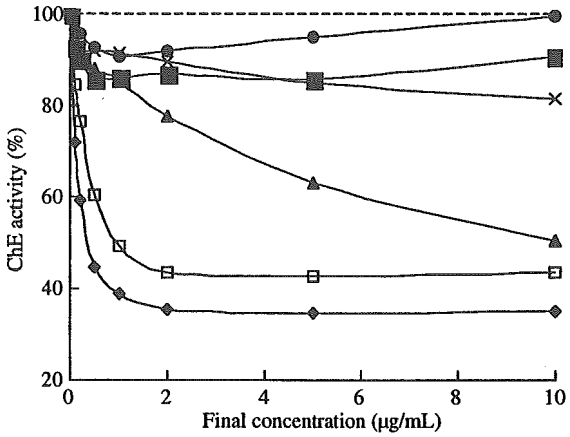

Fig. 4. The degree of ChE activity of six pesticides: —□—; acephate, —×—; fosetyl, —■—; IBP, —▲—; DEP, —●—; EDDP, —♦—; DDVP.

are classified as nitrogenous heterocyclic ester thionophosphate insecticides, and this –P–O–C=N– structure may be critical to the pesticide's inhibitory activity. ChE inhibition produced by isofenphos oxon, acephate and butamifos oxon was weak; thus these compounds belong to group C (Fig. 6). They are classified as ester amidophosphates, and this structure provides selective toxicity against insects compared to mammals. Many OPs developed after the 1960s typically are of this class and present relatively low risks of toxicity for mammals. While there seems to be a relation between the structure and the degree of ChE inhibition, the precise role of the

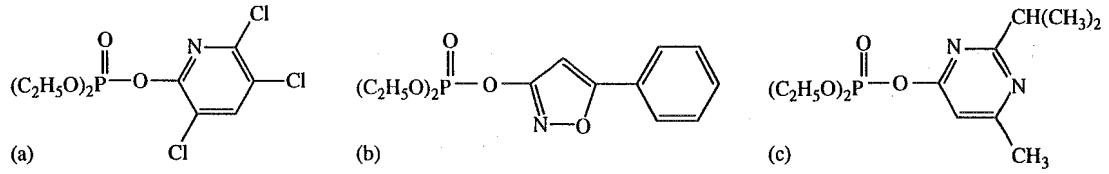

Fig. 5. Chemical structures of (a) chlorpyrifos oxon, (b) isoxathion oxon and (c) diazinon oxon.

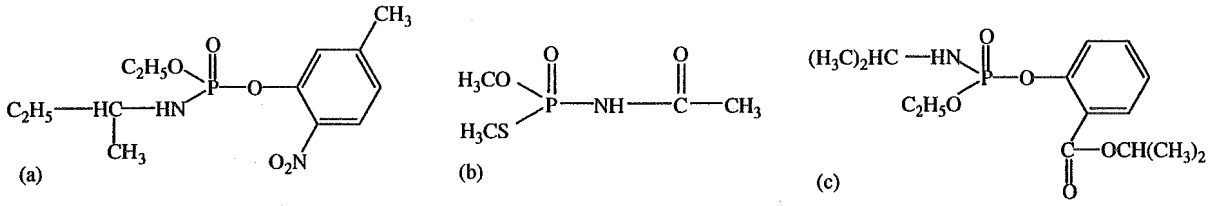

Fig. 6. Chemical structures (a) butamifos oxon, (b) acephate, and (c) isofenphos oxon.

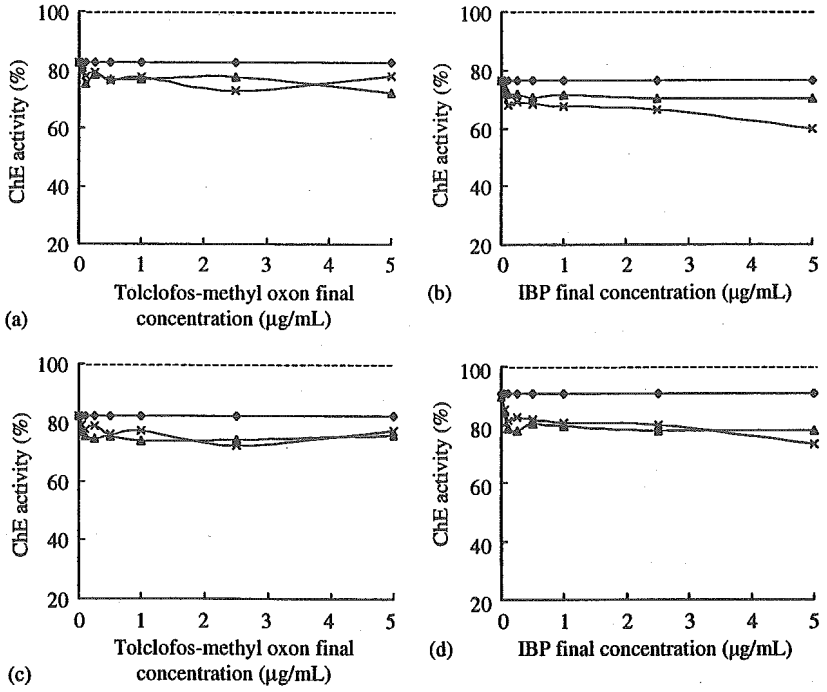

Fig. 7. The combined influence of two pesticides is shown: (a) chlorpyrifos oxon and tolclofos-methyl oxon, (b) DDVP and IBP, (c) isofenphos oxon and tolclofos-methyl oxon, (d) dcephate and IBP, —◆—; X were added into the reaction at the fixed concentration, respectively, —▲—; combined influence under the condition of the fixed concentration of X and eight concentrations of Y, —■—; predicted value of the combined influence, X: (a) chlorpyrifos oxon, (b) DDVP, (c) isofenphos oxon, and (d) acephate Y: (a) tolclofos-methyl oxon, (b) IBP, (c) tolclofos-methyl oxon, and (d) IBP.

structure of the pesticide and the mechanism of ChE inhibition is unknown.

3.3. The evaluation of OPs combinations

We examined the inhibition activity of the following two-compound combinations: chlorpyrifos oxon + isoxathion oxon, DDVP + EDDP, chlorpyrifos oxon + tolclofos-methyl oxon, DDVP + IBP, isofenphos oxon + tolclofos-methyl oxon, and IBP + acephate. No synergistic effects were observed; all combinations were additive with respect to ChE inhibition. Chlorpyrifos oxon + tolclofos-methyl oxon, DDVP + IBP, isofenphos oxon + tolclofos-methyl oxon and IBP + acephate

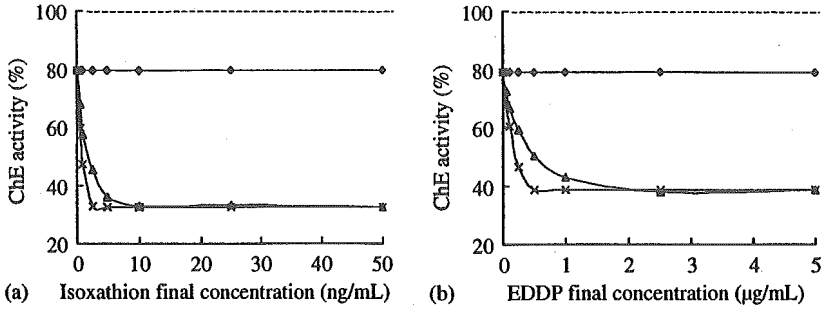

Fig. 8. The competing influence of two pesticides, including one strong inhibitor found in group A is shown: (a) chlorpyrifos oxon and Isoxathion oxon, (b) DDVP and EDDP, —◆—; X were added into the reaction at the fixed concentration, respectively, —▲—; combined influence under the condition of the fixed concentration of X and eight concentrations of Y, —■—; Predicted value of the combined influence X: (a) chlorpyrifos oxon, (b) DDVP, Y: (a) isoxathion oxon, (b) EDDP.

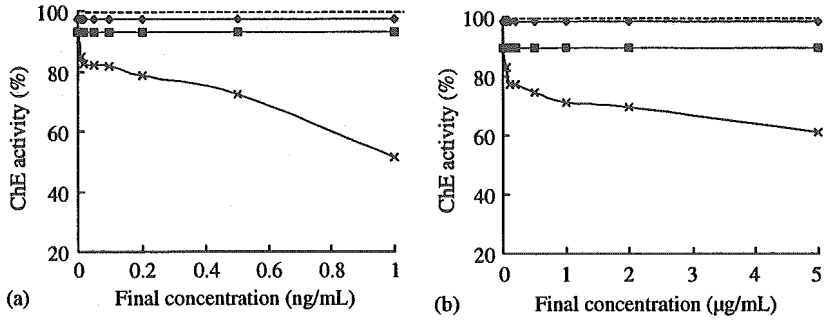

Fig. 9. The combined influence of three pesticides is shown: (a) chlorpyrifos oxon, isoxathion oxon and diazinon oxon (b) isofenphos oxon, tolclofos-methyl oxon and butamifos oxon, —◆—; X were added into the reaction at the fixed concentration, respectively, —×—; X and Y were added into the reaction at the fixed concentration, respectively, —■—; combined influence under the condition of the fixed concentration of X, Y and eight concentrations of Z. X: (a) chlorpyrifos oxon, (b) isofenphos oxon, Y: (a) isoxathion oxon, (b) tolclofos-methyl Oxon, Z : (a) diazinon oxon, (b) butamifos oxon.

showed additive effects approximately corresponding to the sum of the predicted values for individual OPs (Fig. 7). However, the total activity of chlorpyrifos oxon + isoxathion oxon and DDVP + EDDP (combinations of members from group A) showed less additive activity than that predicted from the activity of the individual components (Fig. 8).

Previously, Richardson et al. (2001) evaluated in vitro interaction between two OPs on ChE activity. When chlorpyrifos oxon and azinphos methyl oxon were added simultaneously to brain tissue in vitro, an additive effect was noted. Sequential exposure led to an additive effect at low concentrations but greater than additive inhibition at higher concentration. This study did not include the effect of three-compound combination exposures. There is no paper that examined the effect of three-compound combination exposure otherwise.

In our study, three-compound combinations, chlorpyrifos oxon + isoxathion oxon + diazinon oxon and isofenphos oxon + tolclofos-methyl oxon + butamifos oxon, were examined (Fig. 9). An additional inhibitory effect was observed upon addition of the third compound when compared to the previously observed two-compound results. This suggests that exposure to multiple OPs might have adverse influences on human and wildlife due to an additive effect, even if each pesticide is present at concentrations under recognized NOAEL.

4. Conclusions

Our results indicate that the comprehensive evaluation of multiple pesticides is essential to assess the actual toxicity risks posed by exposure to mixtures of environmental pollutants having common biomolecular targets. In the present work, we demonstrate the utility of our simple, inexpensive, and robust in vitro evaluation system to examine the combined influence of OPs commonly encountered in the aquatic environment. Of note, the relative strength of ChE inhibition observed for these pesticides in our model system are well correlated with recognized values for ADI. Of course,

the adverse effect of environmental pollutants, including multiple compounds, should be evaluated in appropriate animal models. Future in vivo results could be compared to the results obtained using the in vitro method in order to gain a greater understanding of how to measure an apparent environmental exposure and establish daily toxicity monitoring. However, the reported in vitro method is useful for evaluating the toxicity of OPs and OP mixtures, and can be easily applied to risk assessment of complex OPs mixtures commonly observed as environmental pollutants. Consequently we suggest that this method be applied as a monitoring technique in order to preserve water quality and reduce the risk of pesticides upon the ecosystem.

Acknowledgements

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References

Amitai, G., Moorad, D., Adani, R., Doctor, B.P., 1998. Inhibition of acetylcholinesterase and butyrylcholinesterase by chlorpyrifos-oxon. Biochem. Pharmacol. 56, 293–299.

Betancourt, A.M., Carr, R.L., 2004. The effect of chlorpyrifos and chlorpyrifos-oxon on brain cholinesterase, muscarinic receptor binding, and neurotrophin levels in rats following early postnatal exposure. Toxicol. Sci. 77 (1), 63–71.

Butler, A.M., Murray, M., 1997. Biotransformation of parathion in human liver: participation of CYP3A4 and its inactivation during microsomal parathion oxidation. J. Pharmacol. Exp. Ther. 280 (2), 966–973.

Donaldson, D., Kiely, T., Grube, A., 2002. Pesticides industry sales and usage 1998 and 1999 market estimates. US Environmental Protection Agency http://www.epa.gov/oppbead1/pestsales/99pestsales/market_estimates1999.pdf.

Fleischli, M.A., Franson, J.C., Thomas, N.J., Finley, D.L., Riley Jr., W., 2004. Avian mortality events in the United States caused by anticholinesterase pesticides: a retrospective summary of National Wildlife Health Center records from 1980 to 2000. Arch. Environ. Contam. Toxicol. 46 (4), 542–550.

Haywood, P.T., Karalliedde, L., 2000. Management of poisoning due to organophosphorus compounds. Curr. Anaesth. Crit. Care 11 (6), 331–337.

Hazarika, A., Sarkar, S.N., Hajare, S., Kataria, M., Malik, J.K., 2003. Influence of malathion pretreatment on the toxicity of anilofos in male rats: a biochemical interaction study. Toxicology 185, 1–8.

Karahasanoglu, A.M., Ozand, P.T., 1967. Rapid screening test for serum cholinesterase. J. Lab. Clin. Med. 70 (2), 343–351.

Karanth, S., Olivier Jr., K., Liu, J., Pope, C., 2001. *In vivo* interaction between chlorpyrifos and parathion in adult rats: sequence of administration can markedly influence toxic outcome. Toxicol. Appl. Pharmacol. 177 (3), 247–255.

Karanth, S., Liu, J., Olivier Jr., K., Pope, C., 2004. Interactive toxicity of the organophosphorus insecticides chlorpyrifos and methyl parathion in adult rats. Toxicol. Appl. Pharmacol. 196 (2), 183–190.

Liu, J., Chakraborti, T., Pope, C., 2002. *In vitro* effects of organophosphorus anticholinesterases on muscarinic receptor-mediated inhibition of acetylcholine release in rat striatum. Toxicol. Appl. Pharmacol. 178, 102–108.

Ma, T., Chambers, J.E., 1994. Kinetic parameters of desulfuration and dearylation of parathion and chlorpyrifos by rat liver microsomes. Food Chem. Toxicol: Int. J. Br. Ind. Biol. Res. Assoc. 32 (8), 763–767.

Ma, T., Chambers, J.E., 1995. A kinetic analysis of hepatic microsomal activation of parathion and chlorpyrifos in control and phenobarbital-treated rats. J. Biochem. Toxicol. 10 (2), 63–68.

Mileson, B.E., Chambers, J.E., Chen, W.L., Dettbarn, W., Ehrich, M., Eldefrawi, A.T., Gaylor, D.W., Hamernik, K., Hodgson, E., Karczmar, A.G., Padilla, S., Pope, C.N., Richardson, R.J., Saunders, D.R., Sheets, L.P., Sultatos, L.G., Wallace, K.B., 1998. Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol. Sci. 41 (1), 8–20.

Richardson, J.R., Chambers, H.W., Chambers, J.E., 2001. Analysis of the additivity of in vitro inhibition of cholinesterase by mixtures of chlorpyrifos-oxon and azinphos-methyl-oxon. Toxicol. Appl. Pharmacol. 172, 128–139.

Sultatos, L.G., 1987. The role of the liver in mediating the acute toxicity of the pesticide methyl parathion in the mouse. Drug Metab. Dispos: Biol. Fate Chem. 15 (5), 613–617.

Sultatos, L.G., Minor, L.D., Murphy, S.D., 1985. Metabolic activation of phosphorothioate pesticides: role of the liver. J. Pharmacol. Exp. Ther. 232 (3), 624–628.

ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology ■ (2006) ■

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

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[Q3] 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 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.

Materials and methods

Mice

C3H/He mice, which are prone to radiation-induced myeloid leukemia, were used in the present study. Incidence of spontaneous

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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.

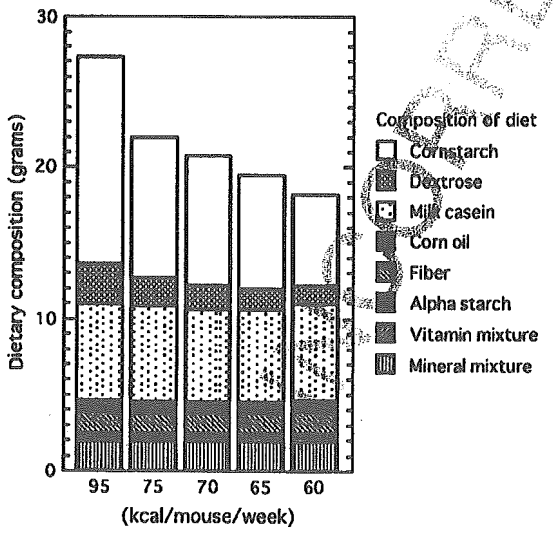

Figure 1. Five dietary regimens based on diets of different caloric contents (see text). The total, in grams, fed to each mouse per week is indicated in the bar graph for each dietary regimen. For caloric restriction, 60-, 65-, 70-, 75-, and 95-kcal dietary regimens, were used to maintain the body weight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

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- t- 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 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

Table 1. ■

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	40	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; 9 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) ($p<0.01$).

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 hematopoietic 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 hematopoietic malignancies and tumor-free death were evaluated by Fisher's exact test (Table 1).

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

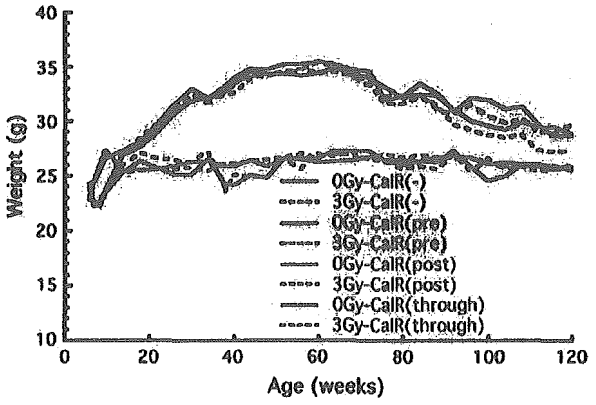

Figure 2. Changes in mean body weight vs age in weeks for all experimental groups, CalR(–), black; CalR(pre), red; CalR(post), green; and CalR(through), blue; with or without 3-Gy irradiation. Note the body weights of the 0Gy-CalR(pre) and 3Gy-CalR(pre) groups immediately returned to the non-CalR level 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).

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

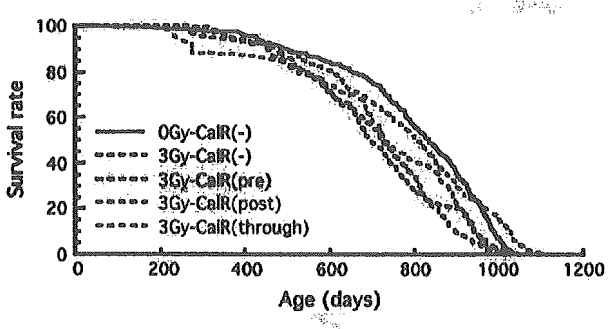

Figure 3. Survival curves for irradiated groups compared with nonirradiated control group; namely, the 3Gy-CalR(pre), 3Gy-CalR(post), and 3Gy-CalR(through) groups indicated by red, green, and blue dotted lines, respectively, are shown with those of the CalR(–) groups with or without irradiation; namely, 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(–) ($p < 0.0001$).

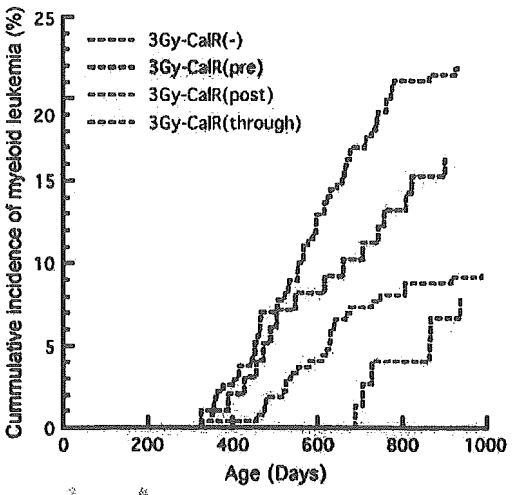

Figure 4. Cumulative incidence of myeloid leukemias. Incidences of myeloid leukemias in all the groups with caloric restriction, 3Gy-CalR(post), 3Gy-CalR(through) and 3Gy-CalR(pre) are lower than that in 3Gy-CalR(–) (see Table 1). The 3Gy-CalR(through) group shows the lowest incidence, whereas the 3Gy-CalR(post) 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.

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-CalR(pre), namely, 3Gy-CalR(post), and 3Gy-CalR(though). 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. [Q4]

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, hematopoietic neoplasms other than myeloid leukemias were not focused on in our examinations. Results show that there was no significant decrease in incidence due to CalR in any of the irradiated groups except for the nonirradiated groups, namely, the [Q5] 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 hematopoietic 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 hematopoietic 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 ($136.7/10^6$ 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. Dday-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 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 [Q6] marrow showed any significant differences among the [Q7] groups.

Changes in cell-cycling fraction of the hematopoietic 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

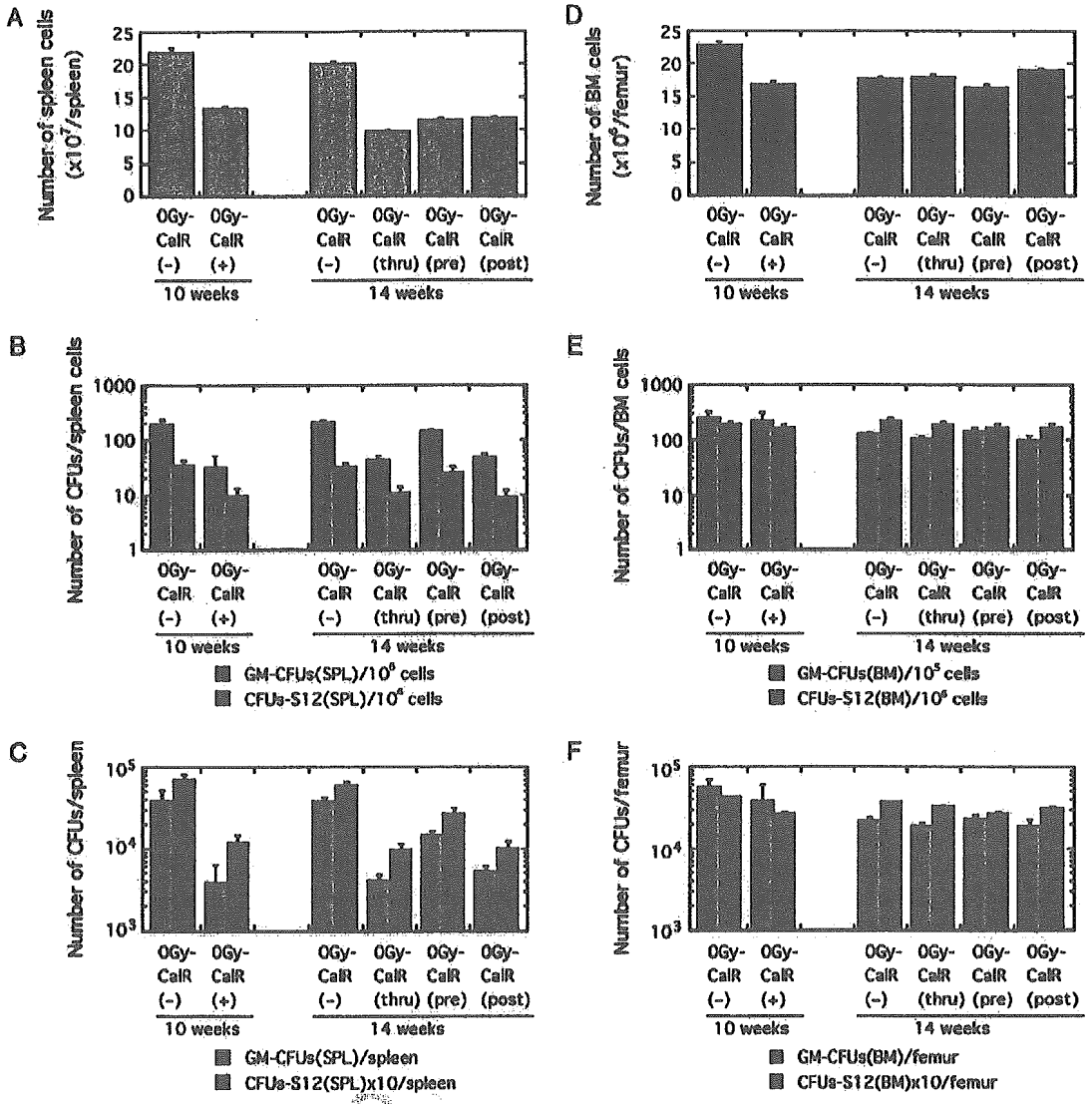

Figure 5. Number of hemopoietic cells (A,D), number of stem/progenitor cells per unit number of cells (B,E), and number of stem/progenitor cells per organ and/or tissue (C,F) are shown for the spleen (A–C) and femur (D–F). Each figure shows data at 10 weeks old, that is, 4 weeks after restriction started (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.

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

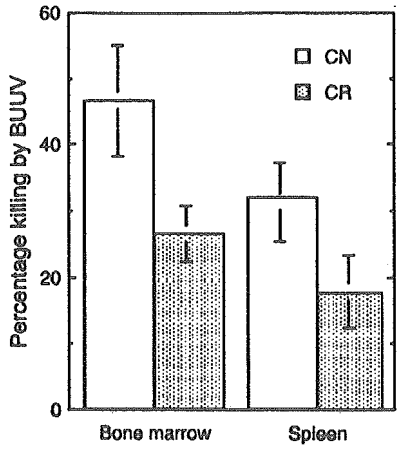

Figure 6. Percent cycling fraction (percentage killing by bromodeoxyuridine ultraviolet [BUUV] method) of hematopoietic progenitor cells, splenic colony-forming units (CFU-S) in bone marrow and spleen of mice with or without caloric restriction. BUUV assay was utilized [16], see Materials and Methods 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).

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 hematopoietic 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.

Uncited References

The following references were uncited: [56,57]

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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, Caderni 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.
36. 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.
37. 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.
38. 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.
39. 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.
40. 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.
42. 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.
43. 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.
44. Feuers RJ, Weindruch R, Hart RW. Caloric restriction, aging, and antioxidant enzymes. Mutat Res. 1993;295:191–200.
45. 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.
46. 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.
47. 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.
48. 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.
49. 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.
50. 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.
51. McMurray RW, Bradsher RW, Steele RW, Pilkington NS. Effect of prolonged modified fasting in obese persons on in vitro markers of immunity: lymphocyte function and serum effects on normal neutrophils. Am J Med Sci. 1990;299:379–385.
52. Scanga CB, Verde TJ, Paolone AM, Andersen RE, Wadden TA. Effects of weight loss and exercise training on natural killer cell activity in obese women. Med Sci Sports Exerc. 1998;30:1666–1671.
53. 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.
54. 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.
55. 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.
56. 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.
57. 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.

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Original

Electron Microscopical Evidence of the Protective Function of Thioredoxin (TRX/ADF) Transgene against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced Cellular Toxicity in the Liver and Brain

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Abstract: The present study was performed to assess the protective role of thioredoxin/adult T-cell leukemia-derived factor (TRX/ADF) on the liver and brain cell damages induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in ADF wild-type (WT) and transgenic (Tg) mice. The ADF WT and Tg mice were intraperitoneally injected with a single dose of TCDD (150 μg/kg body weight). One day after the treatment, the liver and brain tissues were examined electron microscopically to evaluate the cellular toxicity. In the ADF WT mice, marked reduction of subcellular components, such as mitochondria, rough endoplasmic reticula, and glycogen granules, as well as swelling of the remaining mitochondria, were evident in the liver cells. However, attenuation of these changes was evident in TCDD-treated TRX/ADF mice. Similar subcellular changes noted in the neuronal cells of TCDD-treated WT mice were also attenuated in Tg mice. The results suggest that oxidative cellular damage contributes to the acute toxicity induced by TCDD and that TRX/ADF protects against it. (J Toxicol Pathol 2005; **18**: 41–46)

Key words: Ah receptor, brain, liver, 2,3,7,8-tetrachlrodibenzo-*p*-dioxin (TCDD), thioredoxin/adult T-cell leukemia-derived factor (TRX/ADF), transgenic (Tg) mouse

Introduction

As one of the aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a widely spread environmental pollutant that has a broad spectrum of toxic effects on a variety of tissues such as the thymus, liver, testes and central nervous system in mammals[1–6]. Although a number of studies have shown that the toxic effects of TCDD are mediated by intracytoplasmic aromatic hydrocarbon receptor (AhR)[7–9], the toxic mechanism of TCDD on the target organs is still not fully understood. Among the toxic events, oxidative stress is considered to play a major role in the toxic mechanism of TCDD, as characterized by marked increases of lipid peroxidation, the formation of reactive oxygen species, and DNA single-strand break[9–14].

Exogenous xenobiotics, such as aromatic hydrocarbons, result in profound induction of cytochrome P450 enzymes in the liver, resulting in the generation of reactive oxygen species[15,16]. On the other hand, the brain is rich in peroxidizable fatty acids and has relatively low catalase activity[17]. Therefore, these organs are considered to be highly susceptible to oxidative stresses[18]. In fact, the contribution of oxidative stress in TCDD-induced cellular damage of the liver and brain has been suggested in previous studies[13,18–22].

Adult T-cell leukemia-derived factor (ADF) is a human thioredoxin (TRX) associated with the reduction/oxidation (redox) regulation of the cellular environment[23]. TRX/ADF is a stress-inducible protein and its expression is up-regulated after viral infection as well as in cellular stress conditions induced by oxidative agents such as hydrogen peroxide or diamide, irradiation with X-rays and ultraviolet

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light, or ischemic reperfusion[23]. Previous studies have shown that TRX/ADF plays a role in the cellular defense mechanism against oxidative cellular damage via the regulation of intracellular redox status, since exogenously administered TRX/ADF protected cells from oxidative cellular injury[24,25].

We recently reported for the first time the protective function of TRX/ADF against TCDD-induced hematotoxicity in ADF transgenic (Tg) mice, indicating oxidative stress contributes to the hematotoxic mechanism of TCDD[26]. We hypothesized in the present study that overexpression of TRX/ADF might also be effective for protection against the toxic effects of TCDD on the liver and brain tissues in which oxidative stress has also been implicated in the toxic mechanism. For this purpose, we injected TCDD with a dosage capable of inducing oxidative stress in the liver following acute exposure[21], to ADF wild-type (WT) and transgenic (Tg) mice, and then compared subcellular changes electron microscopically in the liver and brain tissues.

Materials and Methods

Animals

TRX/ADF overexpressed mice (ADF Tg mice), originally produced by Dr. A. Mitsui[27], were maintained in a laboratory facility with a 12:12-hour light-dark cycle at an ambient temperature of 21 ± 2°C at the National Institute of Health Sciences (NIHS) of Japan by breeding ADF WT and Tg mice. Animals were screened by PCR of their tail DNA to determine their genotypes. At 8 weeks of age, male ADF WT and Tg mice (23.5–24.8 g) were transferred to a vinyl isolator established in a hazard room designed to prevent contamination from the outside environment and randomly allocated within the same genotype to housing with 6 animals per cage. A pelleted basal diet (CRF-1; Funabashi Farm, Funabashi, Japan) and tap water were provided *ad libitum* throughout the study.

Chemical

TCDD was obtained from Radian International, Cambridge Isotope Laboratories, Inc. (Andover, MA, USA; purity: 98 %). TCDD was initially dissolved in a small volume of acetone and subsequently adjusted to the concentration of 10 μg/ml in olive oil.

Experimental design

ADF WT and Tg mice were divided into vehicle controls and TCDD treatment groups, each consisting of 6 animals. After one week of acclimation, TCDD at 150 μg/kg was intraperitoneally injected once to animals of treatment groups, and the corresponding volume of olive oil was similarly injected to vehicle controls. The dosage of TCDD was selected based on previous study results that showed oxidative stress in the liver was induced by a single bolus injection to mice[21]. One day after the treatment, the animals were sacrificed by decapitation and then examined grossly. The liver and brain were then excised and their weights were measured.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the NIHS, Japan.

Morphological assessment

For histological examination, liver tissues in all animals were fixed in 10% neutral buffered formalin (pH 7.4). After routine processing, the paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope.

For electron microscopical examination, tissue specimens from the liver and cerebral cortex were respectively prepared from three animals each of the control and treatment groups of ADF WT and Tg mice. Small tissue blocks, sized 1 mm^3, were fixed with 2.5% glutaraldehyde in 0.2 M Sorenson's sodium phosphate buffer, pH 7.2, for 8 hours at 4°C. After washing with 0.1 M PBS (pH 7.4), the tissues were post-fixed with 1% osmium tetroxide for 90 minutes. After washing in 0.1 M PBS, the tissues were dehydrated with ethanol and propylene oxide and then embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate. The sections were examined with JEOL-1200 EX II electron microscope (JEOL, Tokyo, Japan).

Results

After one day of TCDD treatment, absolute liver weight had decreased to 71.4% of the vehicle control group in ADF WT mice and 83.2% in ADF Tg mice (data not shown).

Histologically, apoptotic liver cell debris and also focal liver cell necrosis were sparsely observed in the centrilobular areas of both TCDD-treated WT and ADF Tg mice, without showing apparent difference in the severity between genotypes (data not shown). Vehicle control animals did not show such liver cell changes in either genotype.

Electron microscopically, liver cells of the WT mice treated with TCDD exhibited a prominent decrease of cytoplasmic glycogen granules and rough endoplasmic reticula (RERs) and an increase of smooth endoplasmic reticula (SERs) (Fig. 1B). The number of mitochondria was also decreased and the remaining mitochondria showed swelling with disorganized cristae and lucent matrix. Increased fat droplets were also evident in the cytoplasm of less affected hepatocytes. On the other hand, transgene of Trx/ADF notably attenuated these morphological changes following TCDD treatment (Fig. 1C). In the cerebral cortex, neuronal cells showed a decrease in the number of RERs, ribosomes and mitochondria in WT mice treated with TCDD (Fig. 2B) but not in ADF Tg mice treated similarly with TCDD (Fig. 2C). Vehicle control animals did not show such neuronal cell changes in either genotype.

Discussion

In the present study, acute treatment with TCDD

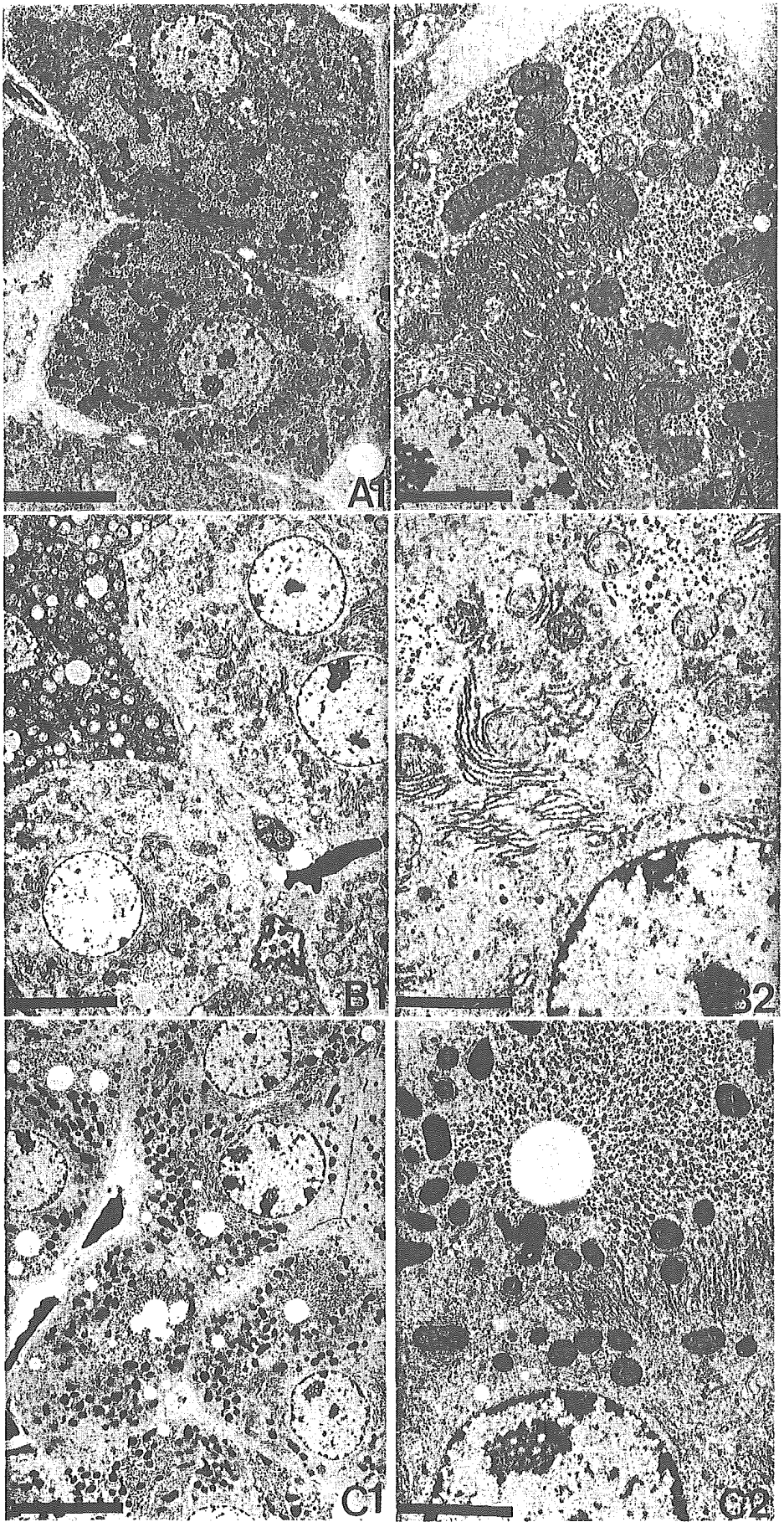

Fig. 1. Electron micrographs of liver cells from ADF WT and Tg mice treated with vehicle or TCDD. (A) Vehicle-treated ADF WT mouse, (B) TCDD-treated ADF WT mouse, and (C) TCDD-treated ADF Tg mouse. Note cytoplasmic swelling associated with a profound decrease of glycogen granules, RERs and mitochondria in the liver cells of the TCDD-treated ADF WT mouse (B). Swelling of the remaining mitochondria with disorganized cristae and lucent matrix is also evident (B). Attenuation of these morphological changes is evident in the TCDD-treated ADF Tg mouse (C). Uranyl acetate and lead citrate. Bar=10 μm (A1, B1, C1), Bar=3 μm (A2, B2, C2).

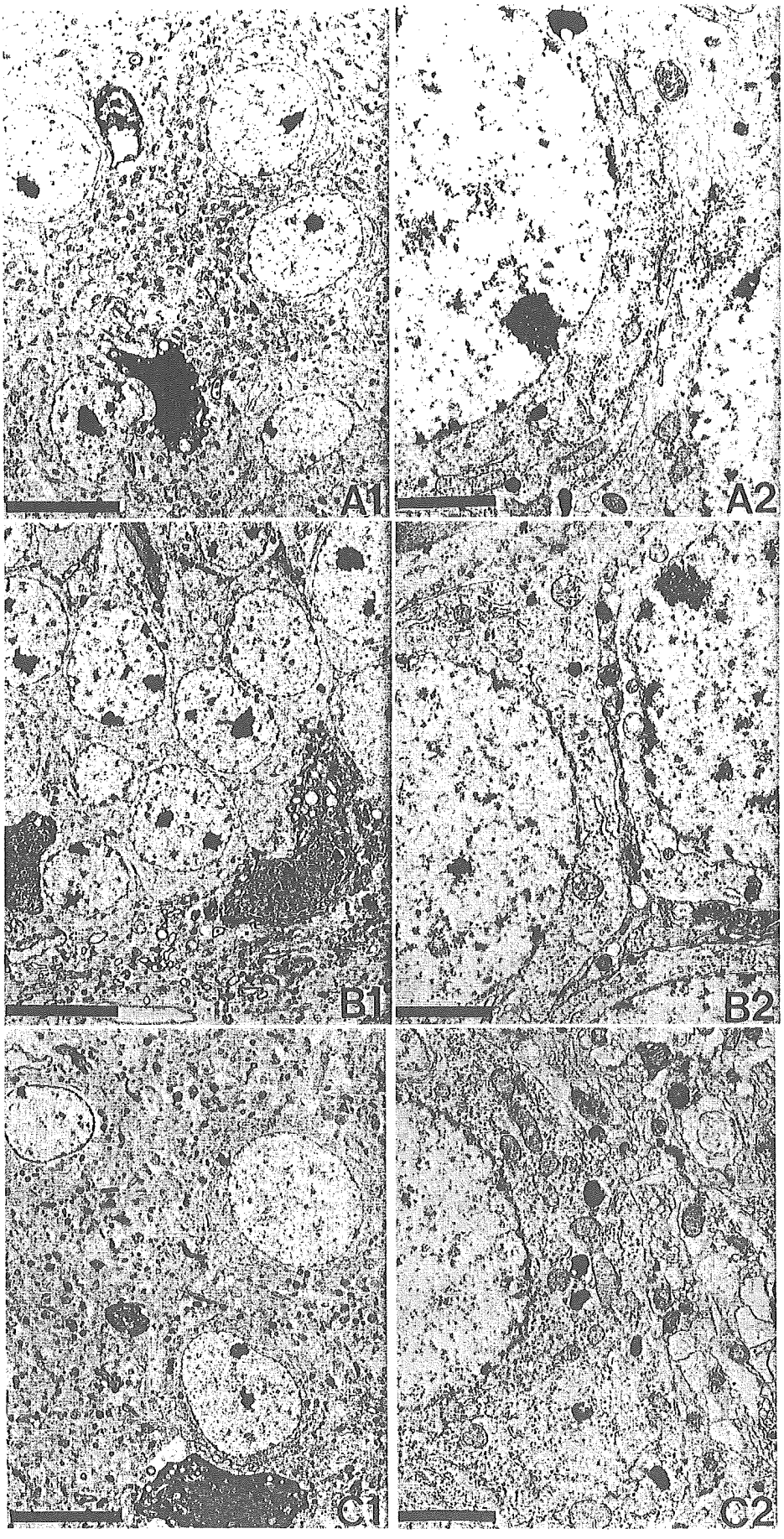

Fig. 2. Electron micrographs of neuronal cells in the cerebral cortex from ADF WT and Tg mice treated with vehicle or TCDD. (A) Vehicle-treated ADF WT mouse, (B) TCDD-treated ADF WT mouse, and (C) TCDD-treated ADF Tg mouse. Note the decrease of RER, ribosome and mitochondria in the cytoplasm of neuronal cells of the TCDD-treated ADF WT mouse (B). In the TCDD-treated ADF Tg mouse, mitochondrial swelling is also evident, but attenuation of the morphological changes can be seen, too. (C). Uranyl acetate and lead citrate. Bar=10 μm (A1, B1, C1), Bar=2 μm (A2, B2, C2).