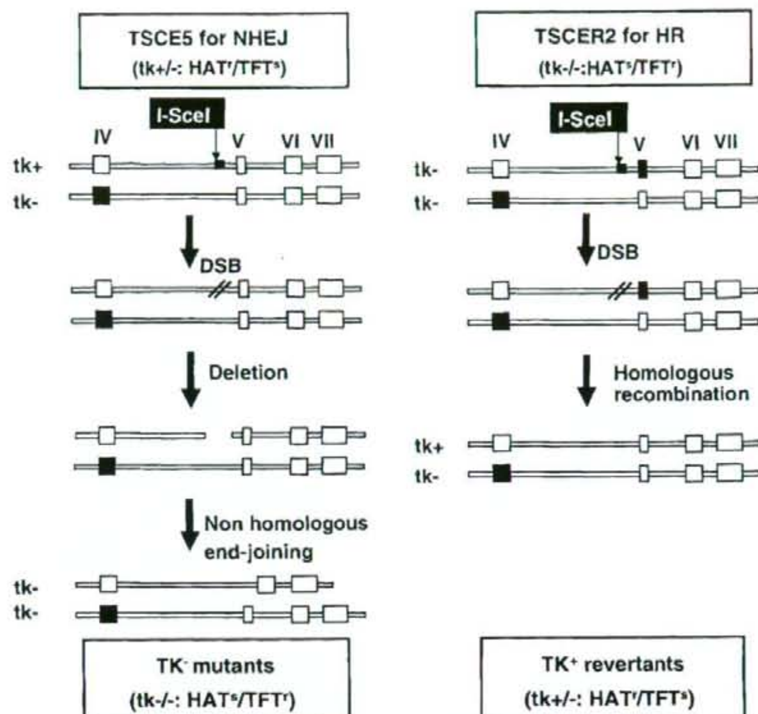

Fig. 1 Strategy of DSB repair assays for I-SceI double strand breaks. The two constructed cell lines—the original TSCE5 line containing the I-SceI recognition insert and its derived compound heterozygote TSCER2—are shown together with the selectable phenotypes generated by repair of double strand breaks (DSBs) through non-homologous end-joining (NHEJ) in TSCE5 cells or homologous repair (HR) in TSCER2 cells (see text)

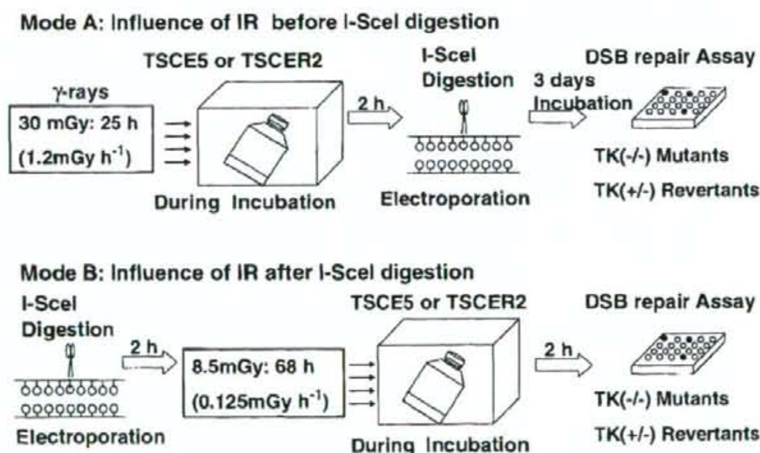

Fig. 2 Experimental scheme of radiation exposure and I-SceI expression. In mode A, cells were exposed to low-dose, low-dose-rate γ-irradiation and then transfected with the I-SceI vector by electroporation (see text). In *mode B* cells were transfected with the I-SceI vector and then exposed to γ-irradiation at a much lower dose and dose-rate (see text)

Determination of TK^- mutants and TK^+ revertants

In mode A, immediately after transfection the cells were suspended in 50 ml of fresh RPMI1640 medium, incubated for 3 days, and then seeded into 96-microwell plates. In mode B, the cell seeding was done at 2 h after the irradiation/incubation following the I-SceI digestion as described above. TSCE5 cells were seeded at 100 cells per well and incubated in the presence of 2.0 μg ml^{-1} trifluorothymidine (TFT) for the detection of TK-deficient

mutants. TSCER2 cells were seeded at 2,000 cells per well in the presence of HAT (200 μM hypoxanthine, 0.1 μM aminopterin, 17.5 μM thymidine) for the detection of TK-proficient revertants.

Results

TK mutant frequency after γ-irradiation

Table 1a shows frequencies of TK^- mutants in TSCE5 cells not expressing I-SceI without irradiation (control) and after exposure to 30 mGy γ-irradiation at 1.2 mGy h^{-1} (mode A). The mean TK^- mutant frequency (MF) values were found to be 2.7×10^{-6} and 4.7×10^{-6} for control and γ-irradiated sample, respectively, and both the level of MFs and the increase caused by γ-irradiation were consistent with those of a previous investigation [3]. Table 2a shows TK^- mutant frequency in unirradiated control TSCE5 cells and cells that were exposed to 8.5 mGy γ-irradiation at 0.125 mGy h^{-1} (mode B). The mean TK^- MF values for un- and γ-irradiated cells in the mode B experiment (3.0×10^{-6} and 2.1×10^{-6}, respectively) were very similar to values obtained after the 12 mGy exposure (at the same dose-rate as in mode B), namely 3.2×10^{-6} and 2.0×10^{-6} for un- and γ-irradiated sample, respectively. These results suggest that the lower doses (8.5 and 12 mGy) delivered at a lower does rate (0.125 mGy h^{-1}) did not enhance the TK^- mutant frequency but rather reduced it. We did not determine the frequency of TK^+ revertants for unirradiated control and γ-irradiated TSCER2 cells because we expected those frequencies to be too low for us to accurately estimate the effect of IR exposure. In fact, the spontaneous revertant frequency (RF) in TSCER2 is in the range of 10^{-8} [4, 5].

Effect of radiation exposure prior to I-SceI transfection on DSB repair (mode A)

In the mode A experiment, the low-dose, low-dose-rate γ-irradiation was performed prior to transfection with the I-SceI vector to estimate the influence of pre-IR exposure on repair of I-SceI introduced DSB. We calculated the relative TK^- mutant frequency MF_{rel} = (MF (γ-rays + I-SceI)/MF (I-SceI)) for each experiment because the transfection efficiencies varied. The mean MF_{rel} in TSCE5 cells exposed to IR prior to transfection was 1.0 (Table 1a), indicating that irradiation had no effect on NHEJ repair of I-SceI-induced DSBs. The relative TK^+ revertant frequency, RF_{rel} in TSCER2 cells was determined in an analogous manner. Exposure to irradiation prior to transfection consistently enhanced RF_{rel}, and the mean RF_{rel} was 1.5 (Table 1b), indicating that irradiation enhanced the HR repair of DSBs by 50%. This 50% increase was found to be statistically significant by *t*-test ($P = 0.021$, if taken as paired data).

Table 1 Effect on DSB repair of exposure to 30 mGy IR at 1.2 mGy h^{-1} prior to transfection with I-SceI expression vector (mode A)

a) NHEJ efficiency in TSCE5 cells

Exp.	TK^- Mutant Frequency, MF ($\times 10^{-6}$)				Effect of IR (MF_{rel}[a])
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	3.5	6.1	8,600	8,500	0.99
2	1.8	3.2	2,900	3,200	1.1
Mean	2.7	4.7	5,800	5,900	1.0 ($P = 0.82$)[c]

b) HR efficiency in TSCER2 cells

Exp.	TK^+ Revertant Frequency, RF ($\times 10^{-6}$)				Effect of IR (RF_{rel}[b])
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	–	–	90	114	1.3
2	–	–	62	96	1.5
3	–	–	25	45	1.8
Mean	–	–	59	85	1.5 ($P = 0.021$)[c]

[a] MF_{rel} was calculated as MF (γ-rays + I-SceI)/MF (I-SceI)

[b] RF_{rel} was calculated as RF (γ-rays + I-SceI)/RF (I-SceI)

[c] Assuming that they were paired data, *P* value was calculated by *t*-test

Table 2 Effect on DSB repair of exposure to 8.5 mGy IR at 0.125 mGy h^{-1} following transfection with I-SceI expression vector (mode B)

a) NHEJ efficiency in TSCE5 cells

Exp.	TK^- Mutant Frequency, MF ($\times 10^{-6}$)				Effect of IR (MF_{rel}[a])
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	2.8	1.3	3,400	4,500	1.3
2	3.1	2.8	12,000	17,000	1.4
3	–	–	11,000	11,000	1.0
Mean	3.0	2.1	8,800	10,800	1.2 ($P = 0.12$)[c]

b) HR efficiency in TSCER2 cells

Exp.	TK^+ Revertant Frequency, RF ($\times 10^{-6}$)				Effect of IR (RF_{rel}[b])
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	–	–	82	160	2.0
2	–	–	160	270	1.7
3	–	–	110	190	1.7
Mean	–	–	120	210	1.8 ($P = 0.0013$)[c]

[a] MF_{rel} was calculated as MF (γ-rays + I-SceI)/MF (I-SceI)

[b] RF_{rel} was calculated as RF (γ-rays + I-SceI)/RF (I-SceI)

[c] Assuming that they were paired data, *P* value was calculated by *t*-test

Effect of radiation exposure after I-SceI transfection on DSB repair (Mode B)

In the mode B experiment, the γ-irradiation was performed after transfection with the I-SceI vector to estimate the post-IR exposure effects on DSB repair. The mean MF_{rel} in TSCE5 cells exposed to IR following transfection was 1.2 (Table 2a) and the difference between unirradiated and irradiated cells was not statistically significant, indicating that post-transfection γ-irradiation had hardly any effect on NHEJ repair of DSBs. The mean RF_{rel} in TSCER2 cells under the same conditions, however, was 1.8 (Table 2b), indicating that exposure to γ-irradiation following transfection with I-SceI enhanced the HR repair of DSBs by 80%. This 80% increase was also found to be statistically significant by t-test ($P = 0.0013$, if taken as paired data).

Discussion

The efficiency of transfection using the amaxa nucleofection system was estimated to be about 40-fold higher than that using BioRad electroporation system, and this higher efficiency enabled us to more accurately estimate the repair of a single DSB at the specific I-SceI recognition site. As in our previous studies [4, 5], we observed that the frequencies of TK^+ revertants after the I-SceI vector transfection were lower than those of TK^- mutants. That finding seems to be consistent with the notion that NHEJ is the major repair pathway in mammalian cells [9]. Because our I-SceI system does not cover all NHEJ and HR events, it is however difficult to estimate the extent of DSB repair by HR. For example, our system does not cover sister-chromatid HR, which is probably the major HR pathway in mammalian cells. Small gene conversion events, not expanding to the exon 5 region, also cannot be detected by this system. Furthermore, there might be unkown factors, specific to this I-SceI site, which reduce the occurrences of the gene conversion type of events. Although the I-SceI system might over-estimate the repair efficiency of NHEJ compared with HR, it is a good model for elucidating the DSB repair associated with low-dose IR exposure.

Although transfection efficiencies varied from experiment to experiment, the relative TK^- mutant frequency and TK^+ revertant frequency were sufficient for evaluating the influence of IR on DSB repair. Both modes (A and B) of delivering low-dose, low dose-rate γ-irradiation were found to hardly influence NHEJ at the I-SceI site. Since an adaptive mutagenic response, a reduction of TK^- mutation frequency, was observed in TK6 cells exposed to X-rays (5 cGy of priming dose and 2 Gy of challenge dose) [10], we also measured DSB repair in cells in which the challenging X-ray exposure was replaced by I-SceI digestion. In those measurements, similarly, NHEJ was barely influenced by the priming X-ray radiation (unpublished data), suggesting that an acute low-dose IR exposure also might provide the same tendency of "no influence" as that observed with the low-dose, low-dose-rate γ-irradiation. In contrast to NHEJ, both modes of γ-irradiation in the present experiments were found to considerably enhance HR at the I-SceI sites. This enhanced HR was not due to radiation-induced S/G2 arrest, because the low-dose IR did not affect the cell cycle (data not shown). Similar results were obtained when using a priming X-radiation (5 cGy; unpublished data).

The above similarities suggest that the enhancement of HR repair observed in the present study is a manifestation of an adaptive response where the low-dose, low-dose-rate γ-irradiation was the priming exposure. The inefficient effect of γ-irradiation on NHEJ does not seem to be consistent with a higher efficiency of DSB repair in radioadapted cells [11], as was shown by the reduction of genetic alterations at the chromosome level [12–14]. Since IR-induced DSBs were the major targets for adaptation in those studies, their DSBs might differ in some way from the I-SceI-induced DSBs we report on here. In other words, the fate of site-specific I-SceI breaks might reflect repair of spontaneous DSBs more faithfully than that of DSBs induced directly by relatively high dose exposure. At the present stage, it is very difficult to speculate plausible mechanisms responsible for the apparent adaptive response of DSB repair. We believe that the characteristics of I-SceI breaks and their continuous generation after the transfection are related to the observed repair characteristics. The enhanced repair by HR upon low-dose, low-dose-rate γ-irradiation is obviously not due to an enhanced cleavage of the I-SceI site after irradiation, since we have not observed such enhanced repair by NHEJ.

It remains to be tested whether NHEJ is really not enhanced by low-dose, low-dose-rate IR or whether it apparently remained stable because of limitations of the methodology. Recently, the fates of I-SceI breaks located in TSCE5 cells were determined in randomly isolated clones using non-phenotypic selection [5]. About 97% of the clones showed perfect rejoining, and deletions corresponding to the events detectable by the present selection method (i.e. large enough to affect the adjacent exon) were found in only 0.54% of the clones. Thus, if perfect NHEJ events or small deletion events were enhanced by low-dose, low-dose-rate γ-irradiation, we would not detect them by the present methodology.

In addition, the mechanisms responsible for HR repair, which is active in S/G2 phase cells, remains to be elucidated. In our previous studies using genetic analyses, we observed small homozygous LOH events in primed cells in the X-ray plus X-ray radioadaptive experiment mentioned

above [10]. We observed the same pattern of LOH mutants after low-dose, low-dose-rate γ-ray exposures [3], although the frequency was low. These results can be explained by the enhanced contribution of HR observed in the I-SceI digestion system, because this system could recover the non-crossing over gene conversion events very efficiently among the TK^+ revertants. In near future we need to elucidate HR pathway leading to gene conversion, where a central core of protein, most likely the RecA homolog RAD51, plays a key role [15].

DSBs arise from endogenous sources including reactive oxygen species generated during cellular metabolisms. The DSB generation process mediated by reactive oxygen is suggested to be also involved in the indirect effects of the ionizing radiation exposure. As already described, the site-specific I-SceI break in our system can be considered as a good model for endogeneous DSBs. Thus, enhanced HR repair activity induced by low-dose, low-dose-rate IR, might be regarded as defense machinery against DNA damage, whether occurring spontaneously and/or after low-dose, low-dose rate IR. At present, we are making an effort to apply the I-SceI digestion system for estimating DSB repair in bystander cells.

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Meeting report

International Symposium on Genotoxic and Carcinogenic Thresholds

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Rodent toxicity assays are usually conducted at high doses based on the maximum tolerable doses. Since the doses used for the assays are sometime 1,000 or 10,000 times higher than the levels at which humans are actually exposed, it is questioned whether carcinogenicity observed at high doses can be observed at low doses. In regulatory sciences, a default assumption for chemical carcinogens is that carcinogenicity observed at high doses can be linearly extrapolated to low doses without thresholds when genotoxicity (or DNA reactivity) is involved in the mechanisms of carcinogenesis. This means that genotoxic carcinogens impose cancer risk to humans even at very low doses. Genotoxicity is a property of chemicals that can interact with DNA, thereby inducing mutations and chromosome aberrations. These genetic alterations are generally thought as molecular basis of carcinogenesis. Recently, the assumption, i.e., the linear non-threshold (LNT) model for chemical carcinogens, has been challenged by several lines of experimental evidence where a large scale of rodents is employed to generate dose-response curves that suggest the presence of practical thresholds. In addition, the LNT assumption appears counterintuitive because it is well known that humans possess a variety of defense mechanisms against genotoxic and carcinogenic insults. The defense mechanisms include detoxication metabolism, error-free DNA repair and translesion DNA synthesis, apoptosis and so on. These mechanisms may effectively suppress genotoxic and carcinogenic activities of chemicals, thereby constituting "practical" thresholds for genotoxic and carcinogenic chemicals. To discuss the low dose effects of genotoxic and carcinogenic compounds and the implication in regulatory sciences, International Symposium on Genotoxic and Carcinogenic Thresholds has been held in Tokyo on July 22 and 23, 2008. Since the topic is related to multi expert areas, 21 scientists including five oversea speakers were invited from various scientific fields such as genotoxicology, chemical pathology, radiation biology, analytical chemistry, statistics and drug metabolism. An administrative official and a representative of consumers were also invited. Here, we summarize the presentations of the symposium to discuss future perspectives in the threshold issue of genotoxic and carcinogenic compounds.

Session 1 (chaired by Makoto Hayashi and Shoji Fukushima)

Opening Address

Takehiko Nohmi (National Institute of Health Sciences, Japan)

Nohmi declared the opening of the symposium and introduced basic concepts related to thresholds for genotoxic and carcinogenic compounds. Currently, carcinogens are classified into "genotoxic" and "non-genotoxic". The genotoxic carcinogens are DNA reactive and induce cancer in multiple organs in trans-species of rodents. They are usually positive in some of *in vitro* and *in vivo* tests of genotoxicity. In contrast, non-genotoxic carcinogens induce tumors in a variety of mechanisms other than DNA damage. The mechanisms include hormonal effects, cytotoxicity and inflammation and so on. The classification, i.e., genotoxic or non-genotoxic, has relevance in administrative regulation of chemicals because it is assumed that genotoxic carcinogens have no thresholds in cancer risk and therefore no ADI (acceptable daily intake) can be set for genotoxic carcinogens. Nohmi questioned the scientific basis for the regulatory policy since it is well known that humans possess multiple defense mechanisms to detoxify genotoxic carcinogens. He stressed the importance of mechanistic understanding of genotoxic carcinogens at low doses to solve the issue of genotoxic and carcinogenic thresholds.

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1. Possible Mechanisms of Genotoxic Thresholds

Takehiko Nohmi (National Institute of Health Sciences, Japan)

Genomic DNA is continuously exposed to endogenous and exogenous genotoxic compounds and thus mutations and chromosome aberrations are inevitably induced at some extent even without external treatments to damage DNA. Nohmi pointed out that spontaneous mutations play important roles in carcinogenesis and also that endogenous DNA damage is a critical factor for estimation of biological and statistical significance of small increases in mutations at low doses. Nohmi showed experimental evidence that error-free DNA repair constitutes "practical thresholds" for genotoxicity of chemicals using mutants of Ames tester strains that are deficient in repair capacity to DNA damage. The mutants include derivatives of *Salmonella typhimurium* deficient in O^6-methylguanine DNA methyl transferase (Δ*ada*Δ*ogt*), 8-oxo-guanine DNA glycosylase (Δ*mutM*) or endonuclease III and VIII (Δ*nth*Δ*nei*). These strains exhibit hypersensitivity to mutagenicity of alkylating agents (Δ*ada*Δ*ogt*), oxidizing agents that damage purine bases (Δ*mutM*) or pyrimidines bases (Δ*nth*Δ*nei*) in DNA. He mentioned that error-free translesion DNA synthesis catalyzed by specialized DNA polymerases may play important roles in constitution of the practical thresholds. It is now known that humans possess more than 14 DNA polymerases per cell and about half are involved in DNA repair and translesion DNA synthesis. Finally, he introduced *gpt* delta transgenic mouse/rat models for *in vivo* genotoxicity. In particular, *gpt* delta transgenic rat may be important to identify genotoxicity (or mutations) in target organs in carcinogenicity.

2. Evidence of Thresholds in Genotoxic Carcinogens: Evidence Based on Carcinogenic Mechanism

Shoji Fukushima (Japan Bioassay Research Center)

Fukushima reported low dose carcinogenicity and genotoxicity of heterocyclic amines, i.e., 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and *N*-nitroso compounds, i.e., dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) in rats. When Fischer 344 rats were fed diets containing MeIQx at doses of 0.001 to 100 ppm in a large scale, i.e., 1,200 rats, lowest effective doses were found to be different depending on the biomarkers, i.e., DNA adducts, 0.01 ppm; 8-hydroxyguanine in DNA, 1 ppm; *lacI* mutations, 10 ppm; glutathione *S*-transferase placental form (GST-P) positive foci formation, 100 ppm; cancer in liver, >100 ppm. The results indicate the existence of practical thresholds for the carcinogenicity. Similarly, there were doses below which no tumor formation was observable for IQ, PhIP, DMN and DEM. Since these carcinogens are all genotoxic, it can be concluded that practical thresholds exist at least for some of genotoxic carcinogens. Fukushima also reported that potassium bromate and 1,4-dioxane, which induced kidney and liver tumors in rats via indirect oxidative damage and cytotoxicity, respectively, exhibited perfect thresholds for the carcinogenicity. It is desirable to regulate genotoxic and carcinogenic compounds based on the view point that there are practical thresholds for genotoxic carcinogens.

3. Strategy of the Scientific Committee on Occupational Exposure Limits (SCOEL) of the European Union in the Derivation of Occupational Exposure Limits (OEL) for Carcinogens

Herman M. Bolt (Institut fur Arbeitsphysiologie an der Universitat Dortmund, Germany)

Bolt introduced recommendations by SCOEL for regulation of carcinogenic compounds. According to them, carcinogens can be categorized into four classes. (A) Non-threshold genotoxic carcinogens such as vinyl chloride and dimethyl sulfate. For these compounds, the LNT model can be applied for the low-dose risk evaluation and the regulations may be based on the principle of "as low as reasonably achievable (ALARA)". (B) Genotoxic carcinogens, for which the existence of thresholds cannot be supported by experimental evidence yet. Acrylamide is one of the compounds in this class, and the LNT model may be used as a default assumption. (C) Genotoxic carcinogens with practical thresholds. The examples are formaldehyde, vinyl acetate and trichloroethylene and their OELs are 0.2 ppm, 5 ppm and 10 ppm, respectively. (D) Non-genotoxic or non DNA-reactive carcinogens, for which true (or perfect) thresholds and no observed adverse effect level (NOAEL) can be set. Tumor promoters, spindle poisons, topoisomerase II inhibitors and hormones are typical examples in this class. He stressed the importance to incorporate mechanistic information into regulation of carcinogenic compounds.

4. Threshold of Genotoxicity

Makoto Hayashi (Biosafety Research Center, Foods, Drugs and Pesticides, Japan)

Hayashi reported that statistical power of mouse peripheral blood micronucleus (MN) assay increased when one million cells per animal were analyzed by flow cytometry in comparison to 2,000 cells by manual analysis. Hayashi and his colleagues examined the sensitivity of mouse MN assays with five clastogens, i.e., mitomy-

cin C, Ara-C, colchicine, acrylamide and potassium bromate. Although there were no significant differences in MN induction among mice when 2,000 cells were analyzed, clear differences became apparent when one million cells were analyzed. It indicates that larger sample sizes give higher power of statistics and also that the sensitivity of MN assay can be improved when cells but not animals are considered as evaluation units. However, lowest doses for MN induction by potassium bromate or acrylamide were not changed even after the sample sizes were increased to one million cells per mouse. He also introduced current topics in International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) where test batteries for genotoxicity were being reorganized.

Session 2 (chaired by Samuel M. Cohen and Akiyoshi Nishikawa)

5. *in vivo* Approaches to Study Mechanism of Action of Genotoxic Carcinogens

Akiyoshi Nishikawa (National Institute of Health Sciences, Japan)

Nishikawa reported *in vivo* approaches to study mechanism of action of genotoxic carcinogens. Currently, genotoxicity and carcinogenicity of chemicals are assessed separately by genotoxicity assays, i.e., Ames test, *in vitro* chromosome aberration test (or mouse lymphoma gene mutation test) and mouse MN test, and by long-term rodent carcinogenicity test, respectively. It is uncertain, therefore, to what extent the detected genotoxic potential can contribute to the carcinogenicity. To solve the issue, he utilized *gpt* delta transgenic rats and mice carrying lambda phage EG10 as a reporter for mutations and showed these animals were powerful tools for the evaluation of both genotoxicity and carcinogenicity in the same organs. Interestingly, MX, which is a genotoxic chlorinated water by-product in Ames test, failed to exert genotoxicity or carcinogenicity *in vivo*. On the other hand, dicyclanil, a known non-genotoxic carcinogen, was genotoxic in the liver of female *gpt* delta mouse. He reported these animal models might have great potential to apply for risk assessment of genotoxic carcinogens. Understanding of the detailed mechanism of carcinogenic action would be crucial for more precise risk assessment of genotoxic carcinogens at low doses.

6. Possible Involvement of Adaptation Mechanisms in the Achievement of an Ineffective Dose Range for the Carcinogenicity of Genotoxic Carcinogens

Dai Nakae (Tokyo Metropolitan Institute of Public Health, Japan, Tokyo University of Agriculture, Japan)

Nakae reported that genotoxic carcinogens had ineffective doses for the carcinogenicity and some adaptation mechanisms might contribute to this phenomenon. To demonstrate this postulate, he and his colleagues performed large scale studies using male Fischer 344 Big Blue rats given a 16-week chronic feeding administration of 0.0001 to 1 ppm of genotoxic carcinogen, i.e., DEN. The number and area of GST-P positive foci in liver were significantly increased only at the highest dose of 1 ppm while mutant frequencies were elevated at a dose of 0.001 ppm and the above. Levels of 8-hydroxyguanine were not changed at all doses used. He suggested these findings might indicate the existence of a practical threshold or an ineffective dose range for the carcinogenicity of genotoxic carcinogens. To utilize the DNA adduct as a marker to determine a practical threshold, he concluded it needs validation of large bodies of data.

7. Possible Dose Threshold for Liver Carcinogenesis by Mutagenic Liver Carcinogens

Hiroyuki Tsuda (Nagoya City University Graduate School of Medical Sciences, Japan)

Generally, under industrial exploitation procedure, the development of new chemicals is immediately stopped when their genotoxicity is clarified. However, Tsuda and his colleagues claimed that some genotoxic substances had non-effective doses in long-term animal experiments. He proposed the existence of the biological threshold level for genotoxic liver carcinogens. Tsuda had examined seven chemicals, i.e., 1,4-dioxan, 2,4-diaminotoluene, *N*-nitrosomorpholine, 1,2-dimethylhydrazine, quinoline, 2-nitropropane and carbon tetrachloride, which were produced during manufacturing process of petroleum-related products, with two individual medium-term carcinogenesis assays (Ito-model for promotion assay and Tsuda-model for initiation assay). In these models, GST-P positive foci were used as a marker to detect preneoplastic lesions. Tsuda suggested that biological threshold levels might exist around NOAEL, but interactions between the threshold and biological defense responses were not clear, and concluded that more extensive research is required to clarify the definitive biological threshold level.

8. Thresholds in Genotoxicity and Carcinogenicity: Urinary Bladder Carcinogens

Samuel M. Cohen (University of Nebraska Medical Center, U.S.A.)

Thresholds for carcinogenic risk are a profound theme that requires extensive discussion on the mechanisms from DNA-damage to carcinogenesis. Cohen showed three bladder carcinogens in rodent models and discussed relationships among genotoxicity, cytotoxicity and carcinogenicity. A non-genotoxic substance uracil formed urinary solids which induced cytotoxicity and cancer in a threshold manner. Genotoxic substance 2-acetylaminofluorene (AAF) is DNA reactive and forms bladder DNA adducts in a dose-responsive linear manner. However, the tumor response is non-linear because cytotoxicity at high doses increases cell proliferation, a necessary component for the carcinogenesis. Arsenic is genotoxic and induces bladder cancer in animal models and humans. However, the genotoxicity occurs by indirect mechanisms, not by direct DNA reactivity. Therefore, the genotoxicity may have a threshold, occurring only at high doses. In discussion, he proposed that "DNA reactivity" was a more definitive term than "genotoxicity", because genotoxicity included mechanisms other than DNA reactivity, such as spindle poison or topoisomerase II inhibition. He claimed that it needs careful considerations to use the term of "threshold". He concluded that true threshold should be defined as the levels where zero cancer risks are expected although practical threshold may be valuable for practical purposes.

Session 3 (chaired by Kirk Kitchin and Masao Hirose)

9. Roles of the Food Safety Commission

Masao Hirose (Food Safety Commission, Japan)

Hirose introduced the Food Safety Commission (FSC) in Japan, which was established in 2003, and its major roles, i.e., risk assessment of the hazards contained in foods, risk communication and responses to emergency situations. FSC includes three risk assessment groups for chemical substances, biological materials and emerging foods. FSC has received requests related to risk assessment for more than 1000 items from the risk management organizations such as Ministry of Health, Labour and Welfare, since establishment. The assessments have been completed for about 550 items including 154 pesticides and 165 veterinary medicines. FSC conducts assessment on its own initiative (self-tasks) when the Commission considers issues needed to be evaluated from the analyses of food safety information, public opinion and similar information. FSC follows the classical concept that genotoxic carcinogens do not have threshold levels and thus ADI cannot be applied to genotoxic and carcinogenic compounds added to foods such as food additives and pesticides. From the FSC's point of view, it would not proper to establish ADI to genotoxic compounds.

10. Thresholds for Genotoxic Carcinogens: View from the National Food Safety

Takashi Kunieda (Ministry of Health, Labour, and Welfare, Japan)

Kunieda reported that regulatory approaches to genotoxic carcinogens in food have not been well established yet and thus global consensus-building in this field is needed. Regulation of carcinogens in food is one of major issues in the national food safety program, because cancer deaths account for 30 percent of all deaths in Japan and most consumers have special concerns about the carcinogenicity of substances in food. Non-genotoxic substances do not directly damage DNA, and carcinogenic thresholds are considered to exist. It is possible to ensure the safety of these substances by establishing applicable standards based on the ADI or tolerable dairy intake (TDI). On the other hand, genotoxic substances directly damage DNA, and no carcinogenic thresholds are considered to exist. As the ADI or TDI cannot be established, it is required to individually respond to ensure the food safety from these substances, according to characteristics of them. Risk management is carried out for unavoidable chemicals based on the following risk assessments approaches to reduce human exposure to ALARA: carcinogenic risk calculated by low-dose extrapolation and margin of exposure using the benchmark dose.

11. Consumers View

Kazuo Onitake (Japanese Consumers' Co-operative Union)

Onitake reported the opinion from the consumers' view as a representative of Japanese Consumers' Co-operative Union (JCCU), whose major objective is to protect the health of consumers. JCCU has been addressing many issues related to food safety, such as food additives and residues of agricultural chemicals, for a long period of time. JCCU is of the opinion that when managing risk associated with the use of chemicals or with the presence of chemicals as contaminants from the environment, risk assessments should be performed before any action is taken and other legitimate factors should be taken into consideration. JCCU agrees with the principle that genotoxic carcinogens do not have biological threshold and ADI cannot be applied to those chemicals intentionally added to foods such as food additives, pesticides and veterinary drugs. JCCU believes that this position is responding to the expectations of

consumers who are concerned about any possible risks from genotoxic carcinogens in food.

12. Theoretical and Experimental Approaches to Possible Thresholds of Response in Carcinogenicity

Kirk T. Kitchin (Environmental Protection Agency, U.S.A.)

Kitchin reported that no convincing examples of carcinogenic thresholds in humans are known, except for one theoretical approach, the two-stage clonal growth model by the Moolgavkar group. In animals, at least four good examples of carcinogenic thresholds have been observed. DNA adducts data for the five well studied chemicals were fairly linear while the foci and tumor data show supralinear, linear and threshold curves, making it difficult to generalize. Currently there is no good scientific and regulatory understanding of chemicals that act simultaneously or sequentially via both linear and nonlinear carcinogenic pathways (genotoxic and nongenotoxic). In order to elucidate the dose-response of chemicals of dual carcinogenic dose-response properties (linear and non linear), Kitchin proposes the studies for two or more such chemicals in a large scale coordinated fashion employing at least 1,000 animals, five different treatment groups, six different study parameters and 8 different scientific disciplines.

Session 4 (chaired by David Lovell and Yoshiya Shimada)

13. Modification of Threshold Dose in Radiation-induced Mouse Lymphoma Development

Yoshiya Shimada (National Institute of Radiological Sciences, Japan)

Shimada reported the studies of radiation-induced mouse thymic lymphoma focusing on dose response of lymphoma induction and the effects of genetic factors, i.e., DNA repair capacity of mouse, and environmental factors, i.e., alkylating agents. The dose limit for radiation protection is based on the LNT hypothesis, where the carcinogenic risk is proportional to radiation dose, even at low doses. However, the results showed that the dose response relationship for mouse thymic lymphomagenesis after repeated X-irradiation has an apparent threshold at dose of around 400 mGy per fraction. DNA repair capacity for double strand breaks or mismatch of nucleotides is a critical determinant for manifestation of threshold.

14. The Progress of Trace Analytical Technique for Measurement of Chemicals in Foods

Munetomo Nakamura (Japan Food Research Laboratories)

Nakamura reported the recent progress of analytical methods using gas chromatograph/mass spectrometer (GC/MS(/MS)) and liquid chromatograph/mass spectrometer (LC/MS(/MS)). In 2006, the positive list system for agricultural chemicals was introduced in Japan. At the same time, many maximum residue limits have been established. Therefore, a lot of analytical methods for residual chemical substances had to be developed. GC/MS(/MS) or LC/MS(/MS) technique can analyze many substances at one time with good selectivity and sensitivity. Those benefits simplify purification steps too. Those methods are adopted as official methods for analysis of pesticide residues in foods, veterinary medicines and carcinogenic and genotoxic mycotoxins.

15. Statistical Consideration on the Identification of Threshold through Toxicological Experiments

Isao Yoshimura (Tokyo University of Science, Japan)

Yoshimura argued that, in principle, it is impossible to identify the threshold via hypothesis testing in the case of toxicological experiments because the probability of false negative decisions cannot be managed in this context. When a mechanism for producing a threshold is hypothesized from a toxicological (or biological) perspective and is mathematically formulated as a dose-response relationship, statistics may be helpful in evaluating the existence (or non-existence) of the threshold. It is important to select a model from a particular set of mathematical dose-response functions. The determination of a practical threshold using *in vitro* experiments may be an alternative to the identification of a "true" threshold, if an appropriate *in vitro* assay affords a large scale experiment at low doses.

16. Statistical Perspective on the Threshold Problem in Toxicological Experiments

David P. Lovell (University of Surrey, U.K.)

Lovell reported mathematical and statistical approaches which do or do not include thresholds and statistical methods which try to identify no observed effect levels (NOELs). There is an increasing appreciation of the potential to identify 'pragmatic' thresholds using experimental systems with a range of biomarkers. The accurate characterization and estimation of these dose-response relationships require careful experimental design which can improve the accuracy of the estimates of the response while avoiding the introduction of ar-

tefactual effects. Statistical approach such as Design of Experiment (DoE) methodology, which builds on the traditional factorial design, can provide efficient approaches for the description and estimation of dose-response relationships of both individual and combinations of agents.

Session 5 (chaired by Minako Nagao and Hansruedi Glatt)

17. *Cells* Genetically Engineered for Xenobiotic-metabolizing Enzymes: Detection of Genotoxic Effects at Extremely Low Substrate Concentrations

Hansruedi Glatt (German Institute of Human Nutrition, Germany)

Glatt developed Chinese hamster V79 cell lines expressing various human phase-I and phase-II enzymes. Using the transgenic cell lines, he investigated the genotoxicity of a lot of pro-genotoxicants. Human CYP1B1 expressed in the target cell (V79-hCYP1B1) exhibited the genotoxicity of benzo[a]pyrene (BP) at less than 10 nM, while rat liver S9-mediated assay required 7 μM to induce gene mutations. BP induced sister chromatid exchange (SCE) from 10 pM in the cells. The concentration-response curve [$y=f(x)$] for SCE–unlike for gene mutations–strongly deviated from linearity. Other promutagens required expression of CYP forms different from CYP1B1 and/or non-CYP enzymes (such as sulfotransferases or acetyltransferases) for their activation at low substrate concentrations. In general, compounds requiring expression of non-CYP enzymes in recombinant cells remained inactive in the standard V79/S9 gene mutation assay.

18. Genotoxic consequences of a single double strand break in human cells

Masamitsu Honma (National Institute of Health Sciences, Japan)

Honma mentioned that "threshold of genotoxicity" can not be established, because genotoxicity is generally recognized by experimental assays. Experimentally, thresholds are inferred from dose-reduction experiments in which dosages are decreased to the level at which adverse effects are no longer observed. This strategy demonstrates not a threshold, but rather a detection limit. Ultimately, the most straightforward evidence for a genotoxic threshold would come from examining the effect of a single DNA damage. If this causes mutation, no threshold will exist. If it does not, there will be a threshold for genotoxicity. He developed a novel system to introduce a unique double-strand break (DSB) into the genomic DNA of human cells by restriction enzyme digestion, and demonstrated that 99 % of DSB are repaired by error-prone repair resulting deletion mutations. This result suggested that there is no threshold for genotoxic compounds which cause DSB.

19. Additive Mutagenic Effects of DNA Damages Formed by Multiple Mutagens at Virtually Non-mutagenic Dose Level of Each

Toshihiro Ohta (Tokyo University of Pharmacy and Life Sciences, Japan)

Ohta reported additive mutagenic effects induced by multiple mutagens in which each mutagen did not show mutagenicity at low levels. Six mutagens (furylframide, MX, 4-nitroquinoline *N*-oxide, sodium azide, 1-nitropyrene and captan) induced base-substitution mutations much more efficiently in *Salmonella typhimurium* TA100 (*hisG46, rfa, uvrB*/pKM101), a strain deficient in nucleotide excision repair, than in TA1975P (*hisG46, rfa*/pKM101), a repair proficient strain. Virtually non-mutagenic dose levels were selected by looking for the doses where the chemical was apparently mutagenic to strain TA100 but not to strain TA1975P. The six mutagens were mixed at the virtually non-mutagenic dose level of each and a possible combined mutagenic effect was investigated with strain TA1975P. A significant and reproducible increase in the number of revertants in TA1975P was observed with combined mutagens. Similar investigations were performed using six heterocyclic amines.

20. Consideration on Extension of the Threshold Concept in Animals to Humans

Minako Nagao (Keio University, Japan)

Nagao reported the history of toxicology to reevaluate the presence or absence of threshold in genotoxicity or carcinogenicity. In standard animal carcinogenesis studies, its detection limit is about 10%. In *in vivo* genotoxicity studies, on the other hand, detection limits are about 2-fold of the background. Even if a significant increase in mutation frequency is not observed, mutation spectrum analyses sometimes demonstrate induction of genetic changes. Thus, impacts of the biological responses occurring under the detection limit of an assay system need to be extensively investigated. She also suggested the presence of thresholds in neoplasm induction by PhIP in the colon but not in the breast or hematopoietic system. The presence or the absence of thresholds for a particular carcinogen might be different depending on the target organs. She concluded that clarification of underlying mechanisms would be necessary to confirm presence of threshold.

21. Scientific Implications and Social Impact of Threshold Concept for Genotoxic Carcinogens

Yuzo Hayashi (Japan Health Food & Nutrition Food Association, Japan)

Hayashi discussed the classification of genotoxic and non-genotoxic carcinogens. This classification, however, can not be applied to all instances due to insufficiencies in necessary information. Therefore, the non-threshold concept was introduced exclusively for genotoxic carcinogens and has been adopted in Japan as a basis for regulatory risk assessment. Dose-response studies recently conducted with various genotoxic agents suggest the existence of a threshold. It should be emphasized, however, that a threshold is not a value which can be determined directly from dose-response data. In this context, scientific efforts in support of the adoption of a threshold should be focused on the development of appropriate mathematical models, and the establishment of toxicological concepts. A realistic step towards a paradigm shift from the non-threshold concept is to seek general consensus on the introduction of an appropriate "virtually safe dose" instead of a threshold.

22. Closing Remarks

Shoji Fukushima (Japan Bioassay Research Center)

Fukushima emphasized that evaluation of threshold in carcinogenicity of genotoxic carcinogens is a very important problem in cancer risk assessment and management. Furthermore, various services as well as consumers and industrial workers mutually desire the fast solution of this problem. In the present Symposium, the speakers did the presentations on the matter of risk assessment, risk management and risk communication for free and active discussion as well as exchanging ideas and opinions. Compared to the Symposium organized in two and half years before by Dr. M. Hayashi (NIHS, formerly) and he, in this time more people were gathered and a deeper and mutual comprehension was achieved. It is very important to evaluate the benefit and risk of chemicals on the basis of our latest scientific results and to continue discussion and argumentation on carcinogenic threshold. Furthermore, together with overall look on the problem of threshold, more and more understanding is continuously desired.

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Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats

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Abstract

Male and female rats were fed a diet containing flame retardant hexabromocyclododecane (HBCD) at 0, 150, 1500 or 15,000 ppm throughout the study beginning at the onset of a 10-week pre-mating period and continuing through the mating, gestation and lactation periods for two generations. The mean daily intakes of HBCD during the whole period of administration were 10.2, 101 and 1008 mg/kg bw in F0 males, 14.0, 141 and 1363 mg/kg bw in F0 females, 11.4, 115 and 1142 mg/kg bw in F1 males, and 14.3, 138 and 1363 mg/kg bw in F1 females for 150, 1500 and 15,000 ppm, respectively. The incidence of rats with decreased thyroid follicles size was increased in F0 and F1 males and females at 1500 ppm and higher. Serum TSH levels were increased in F0 and F1 females at 1500 ppm and higher, and serum T4 levels were decreased in F0 males and females at 15,000 ppm. The number of the primordial follicles in the ovary of F1 females was reduced at 1500 ppm and higher. There were increases in the absolute and relative weights of the liver in male adults and male and female weanlings at 1500 ppm and higher, and in female adults at 15,000 ppm, and of the thyroid in male and female adults at 15,000 ppm. Decreased body weight and body weight gain associated with reduced food consumption were found in F1 males and females at 15,000 ppm. Decreases were found in the viability index of F2 pups and the body weight of male F1 and F2 pups and female F2 pups at 15,000 ppm. In F2 pups, there were low incidences of the completion of eye opening in males at 15,000 ppm and in females at 1500 ppm and higher, and of completed mid-air righting in females at 15,000 ppm. The data indicate that the NOAEL of HBCD in this study was 150 ppm (10.2 mg/kg bw/day). The estimated human intake of HBCD is well below the NOAEL in the present study.

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Keywords: Hexabromocyclododecane; Brominated flame retardant; Two-generation reproductive toxicity; Developmental toxicity; Rat

1. Introduction

Although about 80 different brominated organic flame retardants are registered, tetrabromobisphenol A, the polybrominated diphenyl ethers and hexabromocyclododecane (HBCD) account for most of the total volume [1]. HBCD is a nonaromatic, brominated cyclic alkane used as an additive flame retardant. Total market demand for HBCD in 2001 was estimated as 2800 tons in America, 9500 tons in Europe, 3900 tons in Asia and 500 tons in the rest of the world [2]. The commercial product is a mixture of three stereoisomers, alpha, beta and gamma, which are typically present at approximately 6, 8 and 80%, respectively [3]. Its primary application is in extruded (XPS) and expanded (EPS) polystyrene foam that is used as thermal insulation in the building industry. HBCD is the only suitable flame retardant for these applications. A secondary, although important, application of HBCD is as a flame retardant for upholstery textiles [3,4]. The partition coefficient (Log Kow) value of 5.6 suggests that this chemical is suspected to have high bioaccumulation potential [4]. HBCD has been used for about 20 years, and is detected in practically all environmental media [5]. HBCD was identified in sediment from several places along the River Viskan in Sweden [6] and the River Cinca in Spain [7]. HBCD was detected in fishes, pike (*Esox lucius*) [6] and barbel (*Barbus graellsi*) [7], indicating that it is bioavailable and bioaccumulates. The bioconcentration factor of this compound is reported to be 18,100 in fathead minnow (*Pimephales promelas*) [8]. HBCD was also detected from common whelk (*Buccinium undatum*), sea star (*Asterias rubens*), hermit crab (*Pagurus bernhardus*), gadoid fish species whiting (*Merlangius merlangus*), cod (*Gadus morhua*),

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harbor seal (*Phoca vitulina*) and harbor porpoise (*Phocoena phocoena*) from the North Sea [9]. These findings show evidence of HBCD bioaccumulation at the trophic level and biomagnification in the ascending aquatic food chain [9]. As a result of widespread use and the physical and chemical properties, HBCD is now considered to be a ubiquitous contaminant in the environment and humans [5,10]. It could be hypothesized that food intake is the largest single source of human exposure to HBCD [11].

HBCD was detected at ranging from 0.3 to 20 μg/g lipid in 49 samples of the 85 human breast milk samples collected from Norway between 1993 and 2001 [12]. The concentration of HBCD in the Stockholm human milk showed a fluctuating increase over time, and from 1980 the concentration increased from 0.13 pmol/g lipid to 0.60 pmol/g lipid in 2004 [13]. The HBCD concentration of human milks collected in 2002 to 2003 from North America was ranging from 0.3 to 10 μg/g lipid [14]. The presence of such a chemical compound in biological systems has aroused great concern about its toxicological potential. The biological effects produced by chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity are relevant to humans [15]. The toxic effects of HBCD are briefly summarized by NRC [4], American Chemical Council [3], de Wit [16], Darnerud [11], Birnbaum and Staskal [17]. However, information on the effects of HBCD is insufficient to assess the overall toxicity of this compound. Following oral administration to male rats, HBCD was rapidly absorbed from the gastrointestinal tract, distributed primarily to the body fat, and eliminated rapidly, primarily in the feces [4]. In a 28-day repeated dose toxicity study, no toxic effects were noted in male and female SD rats at any dose of HBCD given by gavage at up to 1000 mg/kg bw/day [18]. In a 90-day repeated dose toxicity study in SD rats given HBCD at 0, 100, 300, or 1000 mg/kg bw/day by gavage, increased weights of the liver and prostate, and γ-glutamyltransferase, and decreased weight of the thyroid/parathyroid were found [19]. The author of this study concluded that these changes were probably of limited, if any, toxicological significance, because they were reversible, and not associated with specific target organ damage or diminished function. The dose-related effects of HBCD on the thyroid hormone axis were observed in a recent 28-day repeated dose study (OECD407) enhanced for endocrine and immune parameters using Wistar rats dosed by gavage at 0–200 mg/kg bw/day [20]. After a single dose of HBCD by gavage at 0.9 or 13.5 mg/kg bw by gavage on postnatal day (PND) 10, spontaneous activity and learning and memory in the water maze were altered when tested at the age of 3 months in NMRI mice [21]. As for the developmental toxicity of HBCD, two studies are available. There was no maternal or developmental toxicity in SD rats given HBCD by gavage on days 6–19 of pregnancy at any doses up to 1000 mg/kg bw/day [22]. No maternal or developmental toxicity was noted in Wistar rats given HBCD in diet at up to 1% (equivalent to 600 mg/kg bw/day) on days 0–20 of pregnancy [23]. No reproductive difficulties in dams or postnatal development in offspring were found even at the highest dose.

Although the testing for reproductive toxicity in an animal model is an important part of the overall toxicology, no information is available for the reproductive toxicity of HBCD at the present time; therefore, a two-generation reproductive toxicity study was conducted.

2. Materials and methods

This study was performed in 2005–2006 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) in compliance with the OECD guideline 416 Two-generation Reproduction Toxicity Study [24]. This study was conducted in accordance with the principles for Good Laboratory Practice [25], "Law for the Humane Treatment and Management of Animals" [Law No. 105, October 1, 1973, revised December 22, 1999, Revised Law No. 221; revised June 22, 2005, Revised Law No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006] and "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006].

2.1. Chemical and dosing

Hexabromocyclododecane (HBCD; 1,2,5,6,9,10-hexabromocyclododecane; CAS No. 3194-55-6) was obtained from Wildlife International, Ltd. (Easton, MD). The test substance was a composite of HBCD commercial products from Albemarle Corporation (Baton Rouge, LA), Great Lakes Chemical Corporation (West Lafayette, IN) and Ameribrom Inc. (New York, NY), and Wildlife International, Ltd. prepared the composite. The preparation of HBCD was a mixture of three enantiomers. HBCD-α, HBCD-β and HBCD-γ, and their respective proportions in the used batch were 8.5, 7.9 and 83.7%. The HBCD (test substance number # 7086) used in this study was 99.7% pure, and was kept in a sealed container under cool (2–7 °C) and dark conditions. The purity and stability of the chemical were verified by analysis using liquid chromatography before and after the study.

Rats were given dietary HBCD at a concentration of 0 (control), 150, 1500 or 15,000 ppm. The dosage levels were determined based on the results of a previous 90-day oral repeated dose toxicity study [19] in male and female Crl:CD(SD)IGS BR rats given HBCD at 0, 100, 300 or 1000 mg/kg bw/day for 90 days. The author concluded that all test article-related changes, even at 1000 mg/kg bw/day, were reversible, not associated with specific target organ damage or diminished function (data not shown).

Dosed diet preparations were formulated by mixing HBCD into an appropriate amount of a powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) for each dietary concentration. The control rats were fed a basal diet only. Analysis showed that the HBCD was homogeneous in the diet and stable for at least 21 days at room temperature, and was administered at the desired feed concentrations throughout the study.

2.2. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies, and historical control data are available. Male and female rats at 4 weeks of age were purchased from Tsukuba Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. One hundred and ninety-two rats were randomly assigned 24/sex/group as F0 animals, and all animals were assigned a unique number and ear tattooed prior to the start of the experiment. Animals were housed individually in suspended aluminum/stainless steel cages, except during the acclimation, mating and nursing periods. From day 17 of pregnancy to the day of weaning, individual dams and litters were reared using wood chips as bedding (White Flake, Charles River Laboratories Japan, Inc.).

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Animals were reared on a basal diet or diet containing HBCD and filtered tap water *ad libitum* and maintained in an air-conditioned room at 22 ± 3 °C, with humidity of 50 ± 20%, a 12-h light/dark (20:00–08:00) cycle and ventilation at 10–15 times/h.

2.3. Experimental design

Twenty-four F0 rats (5-week-old males and females)/sex/group were fed a diet containing HBCD at 0, 150, 1500 or 15,000 ppm for 10 weeks prior to the mating period. Administration of HBCD was continued throughout the mating, gestation and lactation periods. Twenty-four male and 24 female F1 weanlings (1 male and 1 female in each litter) in each group were selected as F1 parents on PNDs 21–25 to equalize the body weights among groups. The day on which F1 parental animals were selected was designated as 0 week of dosing for the F1 generation. The administration of HBCD in the diet was not suspended during PNDs 21–25. F1 selected rats were administered HBCD in the diet of their respective formulations in the same manner as described for F0 rats. Administration of HBCD in the diet was continued throughout the mating, gestation and lactation periods. On PND 26, unselected F1 weanlings and all F2 weanlings were necropsied.

2.4. Mating procedures

Each female was mated with a single male of the same dosage group until copulation occurred or the mating period had elapsed. The mating periods for F0 and F1 animals were 3 weeks. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. F0 females that did not mate during the 3-week mating period were cohabited with another male from the same group who had been proven to copulate. For F1 matings, cohabitation of siblings was avoided.

2.5. Parental data

All adult rats were observed twice a day for clinical signs of toxicity, and body weights and food consumption were recorded weekly. For females exhibiting evidence of successful mating, body weight and food consumption of dams were recorded on days 0, 7, 14 and 20 of pregnancy and days 0, 4, 7, 14 and 21 of lactation. Daily vaginal lavage samples of each F0 and F1 female were evaluated for estrous cyclicity throughout the 2-week pre-cohabitation period and during cohabitation until evidence of copulation was detected. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles. After weaning their pups, parental female rats were necropsied at the proestrous stage of the estrous cycle. For each female, the number of uterine implantation sites was recorded.

2.6. Litter data

Once insemination was confirmed, female rats were checked at least three times daily on days 21–25 of pregnancy to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21 (the day of weaning). The day on which parturition was completed by 13:00 was designated as PND 0. Total litter size and the numbers of live and dead pups were recorded, and live pups were counted, sexed, examined grossly, and individually weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, litters were randomly adjusted to eight pups comprising of four males and four females. No adjustment was made for litters of fewer than eight pups. Pups were assigned a unique number and limb tattooed on PND 4.

2.7. Developmental landmarks

All F1 and F2 pups were observed for pinna unfolding on PND 3, incisor eruption on PND 11, and eye opening on PND 14. One male and one female F1 and F2 pup selected from each dam were evaluated for the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18 [26]. All F1 offspring selected as F1 parents were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. Body weight of the respective F1 rats was recorded on the day of preputial separation or vaginal opening. The anogenital distance (AGD) was measured using calipers on PND 4 in all F1 and F2 pups, and the normalized value of AGD to body weight, AGD per cube root of body weight ratio, was calculated [27].

2.8. Behavioral tests

Spontaneous locomotor activity was measured with a multi-channel activity monitoring system (Supermex; Muromachi Kikai Co., Ltd., Tokyo, Japan) in 10 male and 10 female F1 rats selected from each group at 4 weeks of age. Rats were placed individually in transparent polycarbonate cages (27.6 W × 44.5 D × 20.4 H cm, CL-0108-1, CLEA Japan Inc., Tokyo, Japan), which were placed under an infrared sensor that detects thermal radiation from animals. Spontaneous motor activity was determined for 10 min intervals and for a total of 60 min.

A test in a water-filled multiple T-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The apparatus was similar to that described by Biel [28]. The water temperature of the maze was kept 21–22 °C. As a preliminary swimming ability test, each rat was allowed to swim three times in a straight channel on the day before the maze trial, and then tested in the maze with three trials per day for the next three consecutive days. The elapsed time between entry into the water at the starting point and touching the goal ramp and number of errors were recorded. To prevent the exhaustion of the rats, no animal was allowed to remain in the water for more than 3 min in any trial.

2.9. Termination/necropsy adults

Parental rats were necropsied: males after the parturition of paired females, females after weaning of their pups. The proestrous stage of the estrous cycle was characterized by examination of the vaginal smears of female rats on the day of necropsy. A complete necropsy was performed on all rats found dead and those killed at the scheduled sacrifice. Live rats were euthanized by exsanguination under ether anesthesia. The external surfaces of the rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. Weights of the brain, pituitary, thyroid, thymus, liver, kidney, spleen, adrenal, testis, epididymis, seminal vesicle (with coagulating glands and their fluids), ventral prostate, uterus and ovary were recorded. Weights of the thyroid and seminal vesicle were measured after fixation. Major organs were stored in 10% neutral-buffered formalin. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol.

Histopathological evaluation of F0 and F1 adults was performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin: the pituitary, liver, thymus, kidney, spleen, adrenal, bone marrow, mesenteric lymph node, Peyer's patches, testis, epididymis, seminal vesicle, coagulating gland, ventral prostate, ovary, uterus, vagina and mammary gland of all males and females in the control and highest dose (15,000 ppm) groups and of females with abnormal estrous cycles, males and females without evidence of copulation or insemination and females with abnormal delivery or totally dead pups in all groups. Any organs or tissues of F0 and F1 adults showing gross alterations were evaluated histopathologically. The thyroid in all rats in all groups was examined histopathologically. In ten F1 females of each group, the number of primordial follicles was counted [29]. The right ovary was fixed in 10% neutral-buffered formalin and then dehydrated and embedded in paraffin in a longitudinal orientation by routine procedures. Sections were cut serially at 5 μm and every 20th section was serially mounted on a slide and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.10. Termination/necropsy pups

Following the adjustment of litter size on PND 4, culled pups were euthanized by inhalation of carbon dioxide and subjected to a gross external and internal necropsy. No tissues from these pups were collected.

The weanlings not selected to become parents were euthanized and necropsied as described for the adults. Organ weights of one male and one female F1 and F2 weanling selected from each dam were measured as described above for adults. The weights of the pituitary, thyroid and seminal vesicle were not determined. All pups found dead before weaning were also necropsied.

In all male and female F1 and F2 weanlings whose organs were collected, histopathological evaluations of the liver, in the control and 15,000 ppm groups, and thyroid, in all groups, were performed after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin.

2.11. Hematological and blood biochemical parameters

On the day of the scheduled sacrifice, blood samples were collected from the abdominal aorta of adult rats under ether anesthesia.

Hematological examinations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Blood samples were analyzed for the following hematological parameters, using 2K-EDTA as an anticoagulant: white blood cell (WBC) count and differential leukocyte count.

Blood biochemical evaluations were performed in 10 males and 10 females of F0 and F1 rats randomly selected from each group. Serum samples obtained from centrifuged whole blood were analyzed for biochemistry parameters such as total protein, albumin and globulin.

2.12. Serum hormone levels

On the day of the scheduled sacrifice, blood samples were collected from the abdominal aorta of adult rats. Eight males and eight proestrous females of F0 and F1 generations from each group were selected randomly for blood collection. Hormone levels were determined by Panapharm Laboratories Co., Ltd. (Uto, Japan). Serum levels of testosterone, 5α-dihydrotestosterone (DHT), luteinizing hormone (LH) and follicle stimulating hormone (FSH), thyroxine (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH) in males, and estradiol, progesterone, LH, FSH, T3, T4 and TSH in females were measured with a radioimmunoassay kit. Double antibody kits were used for measurement of testosterone, estradiol, progesterone, T3 and T4 concentration (Diagnostic Products Corp., Los Angeles, CA) and DHT concentration (Diagnostic Systems Laboratories Inc., Webster TX). Serum concentrations of LH, FSH and TSH were measured using (rat LH)[^{125}I], (rat FSH)[^{125}I] and (rat TSH)[^{125}I]assay systems (Amersham Biosciences Ltd., Little Chalfont, Buckinghamshire, UK), respectively.

2.13. Sperm parameters

Sperm parameters were determined for all F0 and F1 male adults on the day of the scheduled sacrifice. The right testis was used to count testicular homogenization-resistant spermatid heads. The right cauda epididymis was weighed and used for sperm analysis. Sperm motility was analyzed using a computer-assisted cell motion analyzer (TOX IVOS, Hamilton Thorne Biosciences, Beverly, MA). The percentage of motile sperm and progressively motile sperm, and the swimming speed and pattern were determined. After recording sperm motion, the cauda epididymal fluid was diluted and the sperm were enumerated using a hemacytometer under a light microscope. Sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. Sperm were stained with eosin and mounted on a slide glass. Two hundred sperm in each sample were examined under a light microscope, and the percentage of morphologically abnormal sperm was calculated.

2.14. Statistical analysis

Statistical analysis was performed according to the methods of Gad [30]. Data on offspring before weaning were statistically analyzed using the litter as the experimental unit.

Body weight, body weight gain, food consumption, length of estrous cycle, pre-coital interval, gestation length, numbers of implantations and pups delivered, delivery index, sperm parameters, hematological and blood biochemical parameters, hormone levels, organ weight, organ/body weight ratio (relative organ weight), number of primordial follicles, reflex response time, age and body weight at sexual maturation, parameters of behavioral tests, AGD, AGD/cube root of body weight ratio, and viability of pups were analyzed for statistical significance using the following method. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances. If the variances were equivalent, the groups were compared by one-way analysis of variance (ANOVA). If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalent variances, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pairwise comparisons were made by the Mann–Whitney U test.

The incidence of pups with changes in clinical and gross internal observations, and completion rate of developmental landmarks and reflexes were analyzed by the Wilcoxon rank sum test.

The incidence of parent animals with changes in clinical, gross internal and histopathological findings, the incidence of weanlings with changes in histopathological findings, the incidence of females with normal estrous cycles, the copulation index, fertility index, gestation index, neonatal sex ratio and completion rate of the reflex response test were analyzed by Fisher's exact test.

The 0.05 level of probability was significant. The probability was designated as the cut-off for statistical significance.

3. Results

3.1. Clinical observations, body weight and food consumption during the pre-mating, mating, gestation and lactation periods (F0 and F1)

One F0 male at 15,000 ppm was euthanized at 13 weeks of dosing because of a moribund condition resulting from accidental injury in the home cage. One F1 male at 1500 ppm was dead from accidental injury in the home cage. One F0 male at 15,000 ppm and one F1 male at 1500 ppm died without any apparent clinical signs of toxicity at 5 and 7 weeks of dosing, respectively. In F0 females at 15,000 ppm, one was euthanized during the pre-mating period because of a moribund condition, and one died on day 22 of pregnancy due to dystocia. No significant difference was seen between control and HBCD-treated groups in the incidence of clinical signs of toxicity in either male or female F0 and F1 rats during the pre-mating, mating, gestation, or lactation period (data not shown).

Fig. 1 shows the body weights of F0 males and females during dosing. In F0 males, the mean body weight and/or body weight gain were significantly higher than those of controls almost throughout the dosing period at 1500 ppm and in the first 5 weeks of dosing at 15,000 ppm. In F0 females, the mean body weight gain was significantly increased on days 0–4 of lactation at 150 ppm and during weeks 0–3 of dosing at 15,000 ppm compared to controls, and the mean body weight was significantly increased on week 2 of dosing at 15,000 ppm. The body weight gain was significantly decreased on days 0–14 of pregnancy at 15,000 ppm compared to controls.

Fig. 2 presents the body weights of F1 males and females during dosing. Significant decreases compared to controls were observed in the body weight during weeks 3–6 of dosing and body weight gain during the first 6 weeks of dosing in F1 males at 15,000 ppm. Compared with control group, a significantly lowered mean body weight was observed during weeks 3 and 6–10 of dosing, the whole period of gestation and days 0–14

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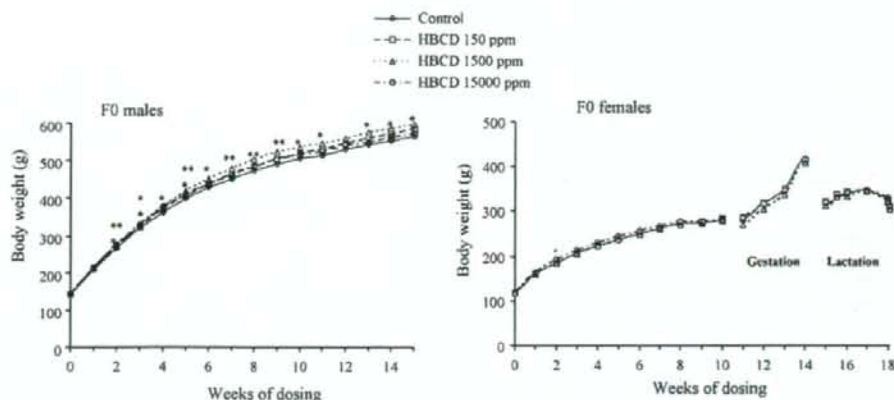

Fig. 1. Body weights of F0 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

of lactation, and a significantly reduced mean body weight gain was observed during weeks 0–10 of dosing at 15,000 ppm in F1 females.

Food consumption was generally paralleled to the body weights/body weight gains during most of the study (data not shown).

The mean daily intakes of HBCD were 12.5, 125 and 1238 mg/kg bw during the pre-mating period, 9.6, 96 and 941 mg/kg bw during the gestation period, and 23.4, 240 and 2200 mg/kg bw during the lactation period in F0 females for 150, 1500 and 15,000 ppm, respectively. The mean daily intakes of HBCD were 14.0, 138 and 1365 mg/kg bw during the pre-mating period, 9.7, 100 and 995 mg/kg bw during the gestation period, and 19.6, 179 and 1724 mg/kg bw during the lactation period in F1 females for 150, 1500 and 15,000 ppm, respectively. The mean daily intakes of HBCD during the whole period were 10.2, 101 and 1008 mg/kg bw in F0 males, 14.0, 141 and 1363 mg/kg bw in F0 females, 11.4, 115 and 1142 mg/kg bw in F1 males, and 14.3, 138 and 1363 mg/kg bw in F1 females for 150, 1500 and 15,000 ppm, respectively.

3.2. Reproductive effects (F0 parents/F1 offspring and F1 parents/F2 offspring)

Table 1

presents the reproductive and developmental parameters for F0 parent/F1 offspring. HBCD produced no significant deviations in estrous cycles, although a few control and HBCD-treated rats had extended estrus or diestrus. Copulation was not observed in two males and two females at 1500 ppm and two males and one female at 15,000 ppm. Two females each at 150 and 1500 ppm did not become pregnant and three females at 15,000 ppm neither. One pregnant female each at 150 and 15,000 ppm did not deliver live pups. There were significantly longer gestation length and lower sex ratio of live pups at 1500 ppm compared

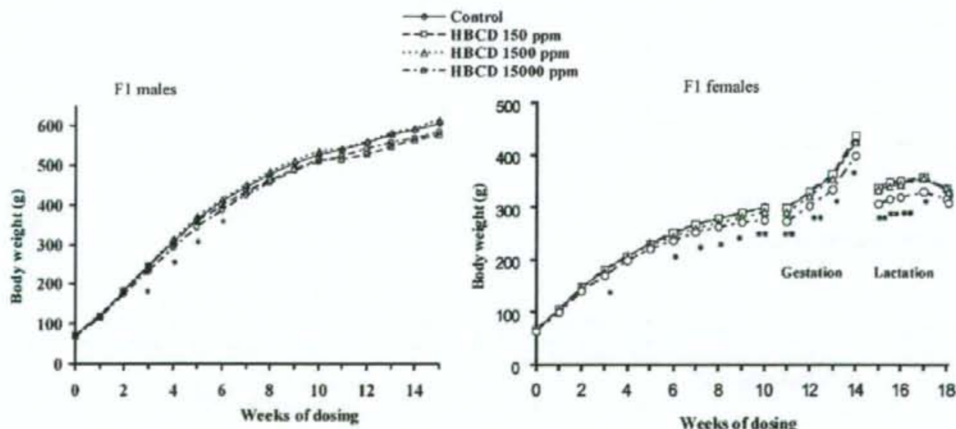

Fig. 2. Body weights of F1 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

Table 1
Reproductive and developmental findings in F0 parents/F1 offspring and F1 parents/F2 offspring

HBCD (ppm)	0 (control)	150	1500	15,000
F0 parents/F1 offspring				
No. of rats (male/female)	24/24	24/24	24/24	23/23
Females with normal estrous cycles (%)[a]	91.7	95.8	87.5	87.0
Copulation index (male/female) (%)[b]	100/100	100/100	91.7/91.7	91.3/95.7
Fertility index (male/female) (%)[c]	100/100	91.7/91.7	90.9/90.9	85.7/86.4
No. of pregnant females	24	22	20	19
Pre-coital interval (days)[d]	3.4 ± 3.9	3.1 ± 3.3	2.7 ± 1.4	3.5 ± 4.3
No. of implantations[d]	14.2 ± 2.1	13.7 ± 3.3	14.5 ± 1.4	14.5 ± 2.7
Gestation index (%)[e]	100	95.5	100	94.7
Delivery index (%)[f]	92.0	89.3	90.7	93.6
Gestation length (days)[d]	22.1 ± 0.3	22.3 ± 0.5	22.6 ± 0.5**	22.2 ± 0.4
No. of pups delivered[d]	13.0 ± 2.3	13.3 ± 1.7	13.3 ± 2.6	13.5 ± 2.8
No. of litters	24	21	20	18
Sex ratio of F1 pups[g]	0.524	0.471	0.426*	0.572
No. of litters totally lost	0	0	0	1
Viability index during lactation (%)[h,i,j]				
Day 0	99.6	97.5	98.8	99.2
Day 4	95.6	98.7	98.7	95.8
Day 21	93.2	99.4	98.1	93.8
Male pup weight during lactation (g)[d]				
Day 0	6.8 ± 0.5	6.9 ± 0.6	7.2 ± 0.7	6.8 ± 0.6
Day 4	10.2 ± 1.7	10.7 ± 1.8	10.8 ± 1.6	9.5 ± 1.8
Day 7	16.4 ± 3.1	17.5 ± 2.4	16.9 ± 2.2	15.6 ± 2.0 (17)[k]
Day 14	36.1 ± 4.8 (23)[k]	36.3 ± 3.6	36.1 ± 3.9	33.5 ± 2.6 (17)[k]
Day 21	61.1 ± 7.1 (23)[k]	62.3 ± 6.5	61.9 ± 6.5	55.4 ± 4.0 (17)[k],*
Female pup weight during lactation (g)[d]				
Day 0	6.3 ± 0.5 (23)[k]	6.6 ± 0.7	6.8 ± 0.6*	6.5 ± 0.7
Day 4	9.6 ± 1.4 (23)[k]	10.3 ± 1.8	10.4 ± 1.5	9.2 ± 1.6
Day 7	15.4 ± 2.8 (23)[k]	17.0 ± 2.5	16.9 ± 2.3	15.1 ± 1.6 (17)[k]
Day 14	33.5 ± 5.3 (23)[k]	35.5 ± 3.6	35.7 ± 3.6	32.6 ± 3.0 (17)[k]
Day 21	56.5 ± 8.0 (23)[k]	59.9 ± 6.4	60.5 ± 5.9	53.2 ± 4.7 (17)[k]
F1 parents/F2 offspring				
No. of rats (male/female)	24/24	24/24	23/24	24/24
Females with normal estrous cycles (%)[a]	95.8	91.7	91.7	91.7
Copulation index (male/female) (%)[b]	100/100	100/100	100/100	100/100
Fertility index (male/female) (%)[c]	95.8/95.8	95.8/95.8	87.0/87.5	87.5/87.5
No. of pregnant females	23	23	21	21
Pre-coital interval (days)[d]	2.6 ± 1.6	3.4 ± 4.1	3.3 ± 3.7	2.3 ± 1.3
No. of implantations[d]	14.3 ± 2.5	14.7 ± 3.4	14.0 ± 3.2	14.3 ± 2.8
Gestation index (%)[e]	100	100	95.2	100
Delivery index (%)[f]	91.4	94.8	88.1	92.6
Gestation length (days)[d]	22.5 ± 0.5	22.4 ± 0.6	22.4 ± 0.5	22.4 ± 0.5
No. of pups delivered[d]	13.2 ± 3.4	13.9 ± 3.3	13.4 ± 2.4	13.1 ± 2.4
No. of litters	23	23	20	21
Sex ratio of F2 pups[g]	0.523	0.492	0.517	0.486
No. of litters totally lost	1	1	0	8**
Viability index during lactation (%)[h,i,j]				
Day 0	98.6	97.7	96.0	97.8
Day 4	86.9	87.3	92.1	68.4*
Day 21	85.0 (22)[k]	89.6 (22)[k]	71.3	49.7 (20)[k],**
Male pup weight during lactation (g)[d]				
Day 0	6.8 ± 0.8	6.7 ± 0.7 (22)[k]	7.1 ± 0.6	6.6 ± 0.6
Day 4	9.1 ± 2.3 (22)[k]	9.3 ± 1.3 (22)[k]	9.0 ± 1.8	8.0 ± 1.3 (19)[k]
Day 7	14.7 ± 3.9 (22)[k]	15.4 ± 2.8 (22)[k]	14.3 ± 3.6 (19)[k]	11.5 ± 2.9 (17)[k],*
Day 14	31.4 ± 8.0 (22)[k]	33.8 ± 5.0 (22)[k]	31.0 ± 7.2 (18)[k]	24.2 ± 6.6 (14)[k],**
Day 21	53.0 ± 12.6 (22)[k]	56.2 ± 6.7 (22)[k]	54.1 ± 10.1 (18)[k]	42.6 ± 8.3 (13)[k],**
Female pup weight during lactation (g)[d]				
Day 0	6.5 ± 0.8	6.3 ± 0.6	6.7 ± 0.6	6.2 ± 0.6

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Table 1 (*Continued*)

HBCD (ppm)	0 (control)	150	1500	15,000
Day 4	8.9 ± 2.3 (22)[k]	8.5 ± 1.3 (22)[k]	8.8 ± 1.8	7.3 ± 1.3 (20)[k,**]
Day 7	14.3 ± 3.5 (21)[k]	14.2 ± 2.8 (22)[k]	13.5 ± 3.9	10.7 ± 2.6 (17)[k,**]
Day 14	31.2 ± 6.5 (21)[k]	31.3 ± 5.1 (22)[k]	29.3 ± 7.3	23.9 ± 5.9 (13)[k,**]
Day 21	52.0 ± 10.0 (21)[k]	52.8 ± 6.6 (22)[k]	51.2 ± 10.8	41.6 ± 8.4 (13)[k,**]

[a] Incidence of females with normal estrous cycles (%) = (no. of females with normal estrous cycles/no. of females examined) × 100.
[b] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[c] Fertility index (%) = (no. of animals that impregnated a female or were pregnant/no. of animals with successful copulation) × 100.
[d] Values are given as the mean ± S.D.
[e] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[f] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[g] Sex ratio = total no. of male pups/total no. of pups.
[h] Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[i] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.
[j] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 4 after cull) × 100.
[k] Data were obtained from the numbers of litters in parentheses because females that had no male and/or female pups and/or experienced total male and/or female pup loss during lactation were excluded.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

to controls. One dam experienced total litter loss by day 5 of lactation at 15,000 ppm; however, there were no significant differences in the copulation index, fertility index, gestation index, pre-coital interval, number of implantations, delivery index, number of F1 pups delivered, or viability of F1 pups during lactation between the control and HBCD-treated groups. Mean body weight of female F1 pups on PND 0 was significantly higher at 1500 ppm, and that of male F1 pups on PND 21 was significantly lowered at 15,000 ppm, compared to controls.

Table 1 also shows the reproductive and developmental parameters for F1 parent/F2 offspring. In F1 females, there were extended diestrus vaginal smears in a few control and HBCD-treated rats, but no significant effect of HBCD was found on the incidence of females with normal estrous cycles. All pairs in all groups copulated. One female each in the control and 150 ppm groups, and three females each at 1500 and 15,000 ppm were not impregnated. One pregnant female did not deliver live pups at 1500 ppm. One dam experienced total litter loss by day 4 of lactation in the control group and by day 2 of lactation at 150 ppm. At 15,000 ppm, eight dams experienced total litter loss by days 4, 5, 7, 9, 11, 13 or 18 of lactation, and a significantly increased incidence of dams with total litter loss was noted. No clear clinical signs of toxicity were noted in these dams with total litter loss. No significant changes were observed in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F2 pups delivered or the sex ratio of F2 pups. A significantly decreased viability index was noted in F2 pups on PNDs 4 and 21 at 15,000 ppm. Mean body weights were significantly lowered compared to controls in male F2 pups on PNDs 7, 14 and 21 and in female F2 pups on PNDs 4, 7, 14 and 21 at 15,000 ppm.

3.3. *Developmental landmarks (F1 and F2)*

Table 2 presents physical development of F1 and F2 pups. There was no significant difference in the incidence of male and female F1 and F2 pups that displayed pinna unfolding, or incisor eruption between the control and HBCD-treated groups. The incidence of male and female F1 pups showing completion of eye opening was increased compared to controls at 1500 ppm. In F2 pups, the incidence of pups showing eye opening was lowered compared to controls in males at 15,000 ppm and in females at 1500 and 15,000 ppm. The AGD and AGD per cube root of body weight ratio were not significantly different between control and HBCD-treated groups in male and female F1 and F2 pups.

Table 3 shows reflex ontogeny in F1 and F2 pups. All male and female F1 pups in all groups completed the surface righting reflex, negative geotaxis reflex and mid-air righting reflex. No significant changes were observed in reflex response time, except for faster response in the surface righting in males at 15,000 ppm, in F1 pups of both sexes in HBCD-treated groups. In F2 pups, a few pups failed to complete the reflex response in HBCD-treated groups, and a significantly low incidence of females completed mid-air righting was noted at 15,000 ppm; however, there was no significant difference in the incidence of male and female pups with completed response in other reflexes and in the reflex response time between control and HBCD-treated groups.

Table 4 presents data on sexual development in F1 rats. No significant differences between control and HBCD-treated groups were noted in the age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening.

3.4. *Behavioral effects (F1)*

Spontaneous locomotor activity for 10 min intervals and for a total of 60 min was not significantly different between control and HBCD-treated groups in male and females F1 rats (data not shown).

On the first day of the T-maze test, the pre-test swimming trials in the straight channel revealed that all male and female F1 rats in each group could swim satisfactorily, and no sig-

Table 2
Physical development in F1 and F2 pups

HBCD (ppm)	0 (control)	150	1500	15,000
F1 pups				
No. of litters examined	24	21	20	18
Pinna unfolding (%)[a,b]				
Male	86.0 ± 26.5	92.5 ± 16.5	93.6 ± 15.7	81.3 ± 27.9
Female	85.8 ± 29.5 (23)[c]	94.7 ± 14.7	97.3 ± 7.5	86.4 ± 23.8
Incisor eruption (%)[a,b]				
Male	91.6 ± 17.6 (23)[c]	96.4 ± 12.0	92.1 ± 17.0	89.7 ± 19.9 (17)[c]
Female	94.9 ± 11.4 (23)[c]	95.2 ± 10.1	92.5 ± 20.0	92.2 ± 15.4 (17)[c]
Eye opening (%)[a,b]				
Male	48.2 ± 41.5 (23)[c]	56.7 ± 37.9	77.1 ± 36.3*	45.8 ± 34.6 (17)[c]
Female	49.3 ± 37.8 (23)[c]	66.7 ± 41.3	82.9 ± 33.5**	54.9 ± 41.4 (17)[c]
AGD[a]				
Male pup AGD (mm)	5.37 ± 0.41	5.44 ± 0.36	5.38 ± 0.32	5.20 ± 0.51
Male pup AGD/($bw^{1/3}$)	2.49 ± 0.11	2.48 ± 0.10	2.44 ± 0.12	2.46 ± 0.14
Female pup AGD (mm)	2.60 ± 0.23 (23)[c]	2.67 ± 0.16	2.62 ± 0.18	2.57 ± 0.23
Female pup AGD/($bw^{1/3}$)	1.22 ± 0.09 (23)[c]	1.23 ± 0.06	1.20 ± 0.06	1.23 ± 0.06
F2 pups				
No. of litters examined	23	22	20	21
Pinna unfolding (%)[a,b]				
Male	79.9 ± 36.4 (22)[c]	90.5 ± 22.8	82.1 ± 29.8	70.1 ± 39.2 (20)[c]
Female	73.6 ± 39.6	90.6 ± 22.8	81.5 ± 31.1	66.8 ± 40.9
Incisor eruption (%)[a,b]				
Male	86.4 ± 25.3 (22)[c]	92.8 ± 19.6	97.2 ± 11.8 (18)[c]	86.3 ± 27.7 (14)[c]
Female	85.7 ± 26.9 (21)[c]	90.9 ± 26.2	97.5 ± 11.2	90.0 ± 28.0 (15)[c]
Eye opening (%)[a,b]				
Male	72.7 ± 40.0 (22)[c]	62.5 ± 40.6	47.2 ± 44.8 (18)[c]	33.9 ± 34.7 (14)[c,**]
Female	82.9 ± 26.8 (21)[c]	72.7 ± 37.7	53.8 ± 40.3*	48.1 ± 42.0 (13)[c,*]
AGD[a]				
Male pup AGD (mm)	5.12 ± 0.54 (22)[c]	5.12 ± 0.41	5.04 ± 0.42	4.84 ± 0.39 (19)[c]
Male pup AGD/($bw^{1/3}$)	2.46 ± 0.12 (22)[c]	2.44 ± 0.13	2.43 ± 0.08	2.42 ± 0.12 (19)[c]
Female pup AGD (mm)	2.69 ± 0.30 (22)[c]	2.71 ± 0.24	2.71 ± 0.29	2.54 ± 0.21 (20)[c]
Female pup AGD/($bw^{1/3}$)	1.30 ± 0.07 (22)[c]	1.33 ± 0.09	1.32 ± 0.09	1.32 ± 0.06 (20)[c]

[a] Values are given as the mean ± S.D.
[b] Incidence of animals that displayed pinna unfolding, incisor eruption or eye opening (%).
[c] Data were obtained from the numbers of litters in parentheses because females that had no male and/or female pups and/or experienced total male and/or female pup loss during lactation were excluded.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

nificant changes were observed in the elapsed time to traverse the straight channel. In males, there were a significantly shorter elapsed time at 1500 and 15,000 ppm and fewer number of errors at 15,000 ppm on day 3 of the T-maze. In females, there was no significant difference in the elapsed time or number of errors of the T-maze between control and HBCD-treated groups (data not shown).

3.5. Necropsy and histopathology (F0, F1 and F2)

No compound-related gross lesions or microscopic alterations were observed in reproductive organs in male and female F0 and F1 adults showing reproductive difficulties, in male and female F0 and F1 adults of the highest dose group and in dead animals before scheduled sacrifice. There were no compound-related gross lesions or remarkable microscopic alterations in other tissues and organs, except for the thyroid, in male and female F0 and F1 adults.

Table 5 presents the histopathological findings in the thyroid of male and female F0 and F1 adults. Decreased size of follicles in the thyroid was found in F0 and F1 adults at 1500 ppm and higher, and in F1 females at 150 ppm as well. A significant increased incidence of rats with decreased follicle size was noted in F0 males (25%) and females (21%) and F1 females (21%) at 1500 ppm and F0 males (87%) and females (48%) and F1 males (46%) and females (54%) at 15,000 ppm, compared to controls (0%). Background incidence of decreased follicle size in the laboratory performed current study was 0% in a total of 56 males and 56 females in 6 studies (5–12/sex/study) form 1998 to 2004. Hypertrophy of the follicular cells in the thyroid was

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Table 3
Reflex ontogeny in F1 and F2 pups

HBCD (ppm)	0 (control)	150	1500	15,000
F1 pups				
No. of pups examined (male/female)	24/23	21/21	20/20	17/17
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Surface righting reflex response time (s)[a]				
Male	2.3 ± 1.1	2.0 ± 0.6	1.8 ± 0.5	1.6 ± 0.3[**]
Female	3.1 ± 1.8	2.4 ± 1.5	2.9 ± 2.6	2.6 ± 2.6
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Negative geotaxis reflex response time (s)[a]				
Male	17.7 ± 7.1	16.8 ± 8.0	15.2 ± 7.8	19.4 ± 5.9
Female	13.9 ± 6.2	11.5 ± 6.2	12.7 ± 6.3	17.0 ± 6.9
Mid-air righting reflex completion rate (%)				
Male/female	100 (23)[b]/100	100/100	100/100	100/100
F2 pups				
No. of pups examined (male/female)	22/22	22/22	19/20	19/18
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/88.9
Surface righting reflex response time (s)[a]				
Male	2.1 ± 1.7	2.0 ± 1.5	2.8 ± 2.5	2.2 ± 2.3
Female	2.3 ± 0.9	2.4 ± 1.7	2.1 ± 0.9	3.7 ± 3.7 (16)[b]
Negative geotaxis reflex completion rate (%)				
Male/female	100/100 (21)[b]	95.5/100	100/100	81.3 (16)[b]/88.2 (17)[b]
Negative geotaxis reflex response time (s)[a]				
Male	17.3 ± 8.6	14.7 ± 6.8 (21)[b]	15.2 ± 6.4	14.1 ± 6.7 (13)[b]
Female	12.4 ± 5.3 (21)[b]	12.0 ± 5.2	16.7 ± 6.4	14.6 ± 6.6 (15)[b]
Mid-air righting reflex completion rate (%)				
Male/female	100/100 (21)[b]	100/100	94.4 (18)[b]/90.0	100 (13)[b]/76.9 (13)[b,*]

Surface righting reflex on postnatal day 5 (three trials), negative geotaxis reflex on postnatal day 8 (one trial) and mid-air righting reflex on postnatal day 18 (three trials) were examined. Completion rate (%) = (no. of animals showing all positive responses of the trials/no. of animals examined) × 100.
[a] Values are given as the mean ± S.D.
[b] Data were obtained from the numbers of pups in parentheses.
[*] Significantly different from the control, $P < 0.05$.
[**] Significantly different from the control, $P < 0.01$.

also observed in F0 males at 1500 ppm and higher, and in F0 females at 1500 ppm.

Fig. 3 shows the number of the primordial follicles in the ovary of F1 females. The number of primordial follicles (mean ± S.D.) was significantly decreased at 1500 (197.9 ± 76.9) and 15,000 ppm (203.4 ± 79.5), but not at 150 ppm (294.2 ± 66.3), compared to controls (316.3 ± 119.5). The range of the background control data in the laboratory performed current study was 189.5–353.4 (mean = 295.6) in 4 studies using 10 females per study in 2005–2006.

Table 4
Sexual development in F1 males and females

HBCD (ppm)	0 (control)	150	1500	15,000
F1 rats				
Male preputial separation				
No. of males examined	24	24	24	24
Age (days)[a]	42.8 ± 1.7	41.7 ± 1.8	42.8 ± 2.2	43.7 ± 1.5
Body weight (g)[a]	225.6 ± 17.1	219.6 ± 20.0	235.0 ± 20.8	226.5 ± 16.2
Female vaginal opening				
No. of females examined	24	24	24	24
Age (days)[a]	30.9 ± 2.0	30.3 ± 2.6	30.1 ± 1.8	30.8 ± 2.2
Body weight (g)[a]	106.0 ± 13.8	102.9 ± 13.8	106.0 ± 10.6	100.7 ± 13.0

[a] Values are given as the mean ± S.D.