Table 3. cont.

Type of mutation	Nucleotide	Sequence Change	Number			
			Control		DE	
			12 weeks	24 weeks	12 weeks	24 weeks
Deletion	8–12	gAAAAAt → gAAAAt	1		2	1
−1 base	126–128	cGGGt → cGGt			1	
	133–134	gTTa → gTa			1	
	155–156	aTTc → aTc	1			
	179–181	aTTTc → aTTc				2
	230	gCa → ga				1
	237	gCg → gg	1			1
	244	cGa → ca			1	
	249	gCt → gt	1			
	277	tAc → tc			1	
	387–389	tCCCg → tCCg			1	
	416–418	tGGGa → tGGa		1	7†	5†
	420	aTa → aa				1
	426	gCg → gg			1	
	431	gTa → ga		1		
	442–443	gCCa → gCa				1
	451–452	cGGt → cGt				1
	454	tCg → tg			1	
>2	26–34	tGGGACATGTTg → tg		1		
	170–171	aCCg → ag	1			
	238–249	cGATGGCGAAGGCt → ct	1			1
Insertion	75	ct → cAt	1			
	107	ag → aTg	1			
	214–216	taaag → tAaaag			1	
Total			33	16	54	46

* and †: Mutations found in 2 and 3 different mice, respectively.

nents in DE, such as 1,6-DNP and related compounds, may also contribute to enhance spontaneous mutations *via* the generation of ROS in the lung and also in the testis in response to DE inhalation.

Potent mutagens such as B[*a*]P and 1,6-DNP in DE are suspected to cause tumors in the lung, but their effect on the germline remains to be investigated. Previously, B[*a*]P was shown to induce a dominant-lethal mutation in the germ cells of male mice (35). We show that inhalation of DE, a major air pollutant in urban air, induces mutations in the testis, suggesting that mutagenic PAH and other mutagenic compounds in DE cause germline mutations. Previously, a germline mutation has been reported to occur in herring gulls living in an urban area (36). Recently, heritable DNA mutations in micro-satellite DNA were identified in mice that inhaled polluted ambient air in an industrial area (37,38); exposure to polluted ambient air for 10 weeks, followed by 6 weeks in the laboratory, was required for a significant increase in the sperm mutant frequency in these mice (38). This observation (38) corresponds to the delayed induction of point mutations in the testis in our study. Mutagenic compounds in ambient air may contribute to the induction of germline mutations. However, further studies are required to confirm that DE and other air pollutants cause mutations in germline cells, which are good markers for assessing the health risk of air pollution.

Acknowledgements: We thank Drs. Hiroaki Shiraishi and Michi Matsumoto (National Institute for Environmental Studies) for their support and advice. This research was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Sciences (#14207100).

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ISSN 1880-7046 Coden:GEENGI

Sciences for genome safety

Vol.30 No.4 2008

Genes and Environment

Formerly Environmental Mutagen Research

Special Issue

International Symposium on Genotoxic and Carcinogenic Thresholds

Edited by T. Nohmi and M. Nagao

JEMS The official journal of the Japanese Environmental Mutagen Society

www.j-ems.org

The Official Journal of
the Japanese Environmental Mutagen Society

Genes and Environment

Formerly *Environmental Mutagen Research*

The Japanese Environmental Mutagen Society (JEMS) Office
c/o Oral Health Association of Japan
Komagome TS Building, 4F,
1-43-9 Komagome, Toshima-ku, Tokyo 170-0003, JAPAN
TEL: +81-3-3947-8891, FAX: +81-3-3947-8341
E-mail: gakkai13@kokuhoken.or.jp
URL: http://www.j-ems.org

Editorial Desk
c/o Komiyama Printing Co., Ltd.
78 Tenjin-cho, Shinjuku-ku,
Tokyo 162-0808, JAPAN
TEL: +81-3-3260-5211, FAX: +81-3-3268-3023
E-mail: j-ems@tokyo.kopas.co.jp

Subscription Information
Published by The Japanese Environmental Mutagen Society
ISSN 1880-7046 (Print), Frequency: four times per year
ISSN 1880-7062 (Online), Available at J-STAGE (http://www.jstage.jst.go.jp/browse/jemsge)
Printed by Komiyama Printing Co. Ltd.
78 Tenjin-cho, Shinjuku-ku, Tokyo 162-0808, JAPAN

The Official Journal of the Japanese Environmental Mutagen Society

Genes and Environment

Formerly *Environmental Mutagen Research*

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Meeting report

International Symposium on Genotoxic and Carcinogenic Thresholds

Takehiko Nohmi[1,3], Naomi Toyoda-Hokaiwado[1], Masami Yamada[1], Kenichi Masumura[1], Masamitsu Honma[1] and Shoji Fukushima[2]

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(Received October 17, 2008; Accepted November 10, 2008)

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 (*ΔadaΔogt*), 8-oxo-guanine DNA glycosylase (*ΔmutM*) or endonuclease III and VIII (*ΔnthΔnei*). These strains exhibit hypersensitivity to mutagenicity of alkylating agents (*ΔadaΔogt*), oxidizing agents that damage purine bases (*ΔmutM*) or pyrimidines bases (*ΔnthΔnei*) 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 Radiaological 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.

Genes and Environment, Vol. 30, No. 4 pp. 108–113 (2008)

Review

Possible Mechanisms of Practical Thresholds for Genotoxicity[1]

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(Received September 24, 2008; Revised October 10, 2008; Accepted October 15, 2008)

An axiom in regulatory sciences is that there are no thresholds for genotoxicity of chemicals. It leads to another default assumption that genotoxic carcinogens impose cancer risk on humans without thresholds, i.e., a linear non-threshold model. Therefore, no acceptable daily intake (ADI) is set for food additives, pesticides and veterinary drugs when they have genotoxic and carcinogenic activities. However, humans possess a number of defense mechanisms such as metabolic inactivation, DNA repair, error-free translesion DNA synthesis and so on. These mechanisms may constitute practical thresholds for genotoxicity. Error-free translesion DNA synthesis is a process where DNA polymerases bypass lesions in DNA by insertion of correct bases opposite the lesion and continue replication of whole chromosomes. These mechanisms might have been evolved because organisms from bacteria to humans are exposed to endogenous as well as exogenous genotoxic compounds. In fact, levels of spontaneous mutagenesis are strongly influenced by ability of DNA repair and translesion DNA synthesis of the host cells. Here, I show evidence that DNA repair and translesion DNA synthesis play roles in practical genotoxic thresholds in *Salmonella typhimurium* used for bacterial mutation assays, and discuss future directions of the research on genotoxic thresholds *in vivo*.

Key words: genotoxic thresholds, DNA repair, translesion DNA synthesis, ADI

Introduction

Human chromosome is exposed to a variety of endogenous and exogenous agents (1,2). The most prominent endogenous genotoxic agents are reactive oxygen species (ROS), which are generated as by-products of oxygen metabolism (3,4). These reactive molecules include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. ROS is also generated in cells by exposure to radiation and chemical carcinogens. Because ROS damages nearby cellular components such as DNA, proteins and lipids in membrane, cells must have evolved multiple defense mechanisms to combat the oxidative stress. Enzymes such as catalase or superoxide dismutase detoxify ROS, and low-molecular-weight scavengers such as glutathione alleviate the toxicity of ROS. Nevertheless, some ROS molecules escape from the defense systems and inevitably damage the biomolecules. DNA repair mechanisms, e.g., 8-hydroxyguanine (8-OH-G) DNA glycosylase encoded by *OGG1* in humans and *mutM* in *Escherichia coli*, remove the damage and convert the modified bases to unmodified ones (5,6).

Another class of endogenous genotoxic agents is alkylating agents such as *S*-adenosylmethionine (SAM) (7). SAM is an S_N2-(bimolecular) alkylating agent and induces 7-methylguanine and 3-methyladenine in DNA non-enzymatically (8). Although 7-methylguanine is formed more abundantly than 3-methyladenine, it is an innocuous modified base. 3-Methyladenine in DNA blocks DNA replication and is cytotoxic. It is estimated that about 600 3-methyladenine residues are formed by SAM in the DNA of a mammalian cell per day (9). Other S_N2-alkylating agents, e.g., naturally occurring methyl halides, induce N^1-methyladenine and N^3-methylcytosine in particular in single-stranded DNA (10). Endogenous S_N1-(monomolecular) alkylating agents such as nitrosamines may induce O^6-methylguanine and O^4-methylthymine, which are mutagenic and toxic lesions in DNA. As in the case of ROS, cells possess a number of defense mechanisms against alkylation damages in DNA (10). O^6-methylguanine DNA methyltransferase (MGMT) directly removes methyl groups from O^6-methylguanine and O^4-methylthymine, and 3-methyladenine DNA glycosylase excises 3-methyladenine from DNA, followed by gap-filling by DNA polymerases (DNA Pols). AlkB in *E. coli* and the counterparts in humans, i.e., ABH2 and ABH3, oxidize methyl groups modified in N^1-adenine and N^3-cytosine, and remove the methyl groups, thereby reverting them into intact adenine and cytosine bases, respectively.

[1]Presented at the International Symposium on *Genotoxic and Carcinogenic Thresholds*, Tokyo, July 22/23, 2008

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In addition to endogenous genotoxic agents, human DNA is damaged by exogenous chemical and physical genotoxic insults (11). These include ultraviolet light (UV), radiation, cigarette smoke, polluted air, mutagenic heterocyclic amines, asbestos and so on. The detrimental factors induce bulky DNA adducts, single- or double-strand breaks in DNA. Again, humans possess repair mechanisms against the lesions, such as nucleotide excision repair and homologous or non-homologous recombination, which are responsible for the repair of bulky DNA adducts and strand breaks in DNA, respectively (12). Even when adducts in DNA are not removed, error-free translesion DNA synthesis (TLS) bypasses the damage, thereby reducing the chance of induction of mutations and chromosome aberrations (13) (see below for more detail).

Here, I discuss the possibility that the abovementioned defense mechanisms, i.e., DNA repair and translesion DNA synthesis, may contribute to establish "practical thresholds" for genotoxicity. The term "practical thresholds" is defined as the doses below which no mutations are detectable (14,15). We developed sets of repair-deficient derivatives of *Salmonella typhimurium* TA1535, which is widely used in Ames genotoxicity assay, and used them for demonstration of the practical thresholds for genotoxicity (16–18). I also briefly discuss the *in vivo* (mouse) research on genotoxic thresholds.

MGMT is a Constituent of Oractical Thresholds for Alkylation-induced Genotoxicity

First, we constructed an MGMT-deficient derivative of strain TA1535, namely YG7108, and compared the dose-responses against alkylating agents (16,17) (Fig. 1). MGMT is encoded by two genes in *Salmonella*, namely ada_{ST} and ogt_{ST} (16,19). Both gene products remove mutagenic lesion, i.e., O^6-methy, ethyl, propyl and butyl guanine in DNA, which are induced by a variety of alkylating agents. The alkylating agents used in the study are *N*-methyl-*N'*-nitro-*N*-nitorosoguanidine (MNNG), *N*-ethyl-*N'*-nitro-*N*-nitorosoguanidine (ENNG), *N*-propyl-*N'*-nitro-*N*-nitorosoguanidine (PNNG), *N*-butyl-*N'*-nitro-*N*-nitorosoguanidine (BNNG) and methyl methanesulfonate (MMS). MNNG, ENNG, PNNG and BNNG are different in the length of alkyl chains and MMS induces 3-methyladenine, a cytotoxic lesion, in addition to O^6-methylguanine. The *ada*- and *ogt*-deficient strain YG7108 exhibited superior sensitivity to the genotoxicity of all the alkylating agents used compared to the repair proficient strain TA1535. In particular, the mutagenicity of ENNG and MMS is clear in YG7108 while the mutagenicity is almost completely suppressed in the repair proficient strain TA1535. In the low dose range of MNNG, the mutagenicity was only observed with YG7108 but not TA1535. These results strongly suggest that MGMT is a constituent of practical thresholds for alkylating agents in *Salmonella* strains.

8-OH-G DNA Glycosylase is a Constituent of Practical Thresholds for Oxidation-induced Genotoxicity

Next, we compared the dose responses between $mutM_{ST}$-deficient and proficient derivatives of *S. typhimurium* TA1535 and TA1975, i.e., YG3001 and YG3002, respectively, against oxidative mutagens (Fig.

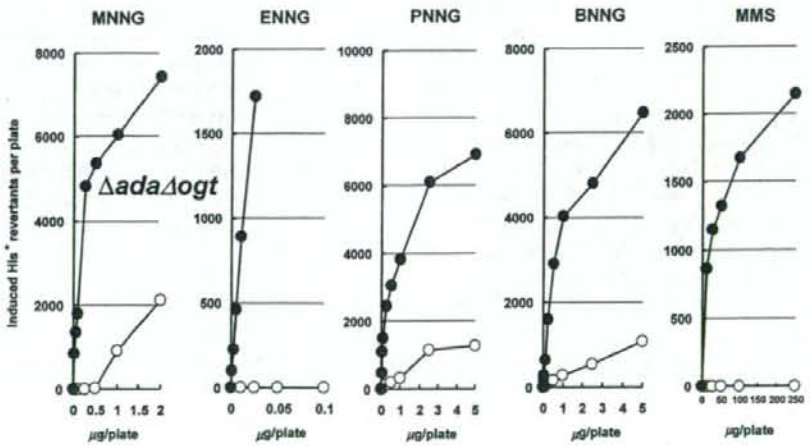

Fig. 1. Dose response curves of *Salmonella typhimurium* YG7008 (*ΔadaΔogt*) and its parent strain TA1535 against MNNG, ENNG, PNNG, BNNG and MMS. Closed circles, YG7008; open circles, TA1535. The assay was conducted as described by Maron and Ames (44), with preexposure of the cells to the alkylating agents for 20 min at 37°C before plating without removal of the alkylating agents. Data are from references (13,14).

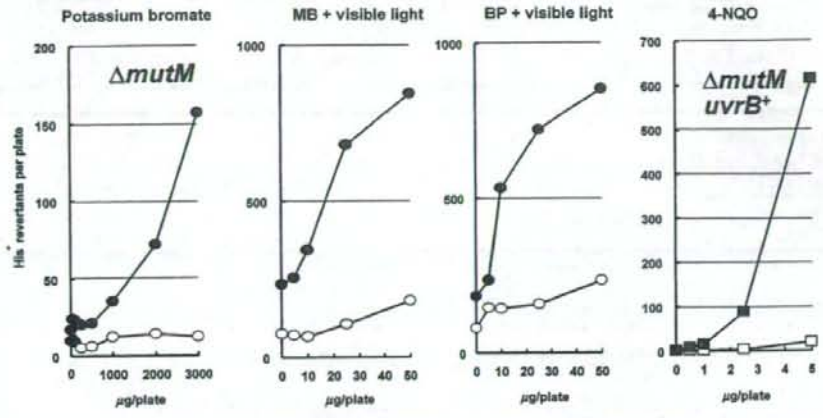

Fig. 2. Dose response curves of *Salmonella typhimurium* YG3001 (*ΔmutM*$_{ST}$, *ΔuvrB*), and its parent strain TA1535 (*ΔuvrB*) against potassium bromate, MB + visible light and BP + visible light. Closed circles, YG3001; open circles, TA1535. The most right panel shows dose response curves of *Salmonella typhimurium* YG3003 (*ΔmutM*$_{ST}$, *uvrB*$^+$) and its parent strain TA1975 (*uvrB*$^+$) against 4-NQO. Closed squares, YG3003; open squares, TA1975. The assay was carried out as written in the legend of Fig. 1. When the mutagenicity of MB and BP was assayed in the presence of visible light, plates were exposed to fluorescent light 15 W lamps at a distance of 30 cm during incubation at 37°C for two to three days. Data are from (18,26).

2). The *mutM*$_{ST}$ gene encodes 8-OH-G DNA glycosylase in *S. typhimurium* (18). Both YG3001 and YG3002 are deficient in *mutM*$_{ST}$ but YG3001 is also deficient in functions of nucleotide excision repair (*ΔuvrB*). The oxidative mutagens used are potassium bromate, methylene blue (MB) plus visible light, benzo[a]pyrene (BP) plus visible light and 4-nitroquinoline *N*-oxide (4-NQO). Potassium bromate is a rat renal carcinogen and induces 8-OH-G in DNA (20). MB is a photosensitizer and induces 8-OH-G in DNA in the presence of visible light (MB plus visible light) (21). BP is a well known genotoxic carcinogen upon metabolic activation, but the mutagenicity was assayed without metabolic activation in this study (22). Instead, BP was activated by exposure to visible light (BP plus visible light). 4-NQO is a genotoxic carcinogen too. Although 4-NQO induces bulky DNA adducts and oxidative lesions (23), the bulky adducts are removed by nucleotide excision repair in the backgrounds of TA1975 and YG3002 and thus the mutagenicity in the backgrounds depends on the oxidative lesion, namely 8-OH-G in DNA. The *mutM*$_{ST}$-deficient stains exhibited much higher sensitivity compared to the proficient strains (18). The mutagenicity of potassium bromate and 4-NQO was clearly observed in the *mutM*$_{ST}$-deficient strains, i.e., YG3001 and YG3002, respectively, while the mutagenicity was almost completely suppressed in the proficient strains, i.e., TA1535 and TA1975. Strain YG3001 also exhibited much higher sensitivity against MB plus visible light and BP plus visible light. These results suggest that 8-OH-G DNA glycosylase contributes to establish practical thresholds against oxidative mutagens.

8-OH-G DNA glycosylase is present not only in bacteria but also in humans (24). The glycosylase in humans is encoded by *OGG1*. Interestingly, there is a genetic polymorphism in the human *OGG1* gene (25). We conducted a functional complementation assay where three polymorphic forms of human *OGG1*, i.e., hOGG1-Ser326, hOGG1-Cys326 and hOGG1-Gln46, are expressed in *Salmonella* strain YG3001 deficient in the bacterial *mutM*$_{ST}$ gene and the mutagenicity of MB plus visible light was assayed with the strains (26). Although human OGG1 proteins suppressed the photomutagenicity of MB, the extent of suppression was different among three polymorphic forms where hOGG1-Gln46 exhibited the weakest suppression (hOGG1-Ser326 and hOGG1-Cys326 have Arg at amino acid 46 and hOGG1-Gln46 has Ser at amino acid 326). The results suggest that each polymorphic form of OGG1 may have different ability to suppress mutations induced by the oxidative DNA damage and also that the genetic polymorphism may affect the practical thresholds for oxidative mutagenesis.

TLS may be a Constituent of Practical Thresholds for Genotoxicity

Recent progress in research on DNA Pols revealed that humans possess more than 14 DNA Pols per cell and about half of them participate in DNA repair and TLS (27). TLS is a process where DNA Pols continues DNA synthesis across lesions (11). If correct bases are inserted opposite the lesions, TLS will reduce the chance of induction of mutations and contribute to DNA damage tolerance. However, if incorrect bases are in-

serted opposite the lesions or skip the lesion, it will induce point mutations such as base substitutions or frameshifts. If no TLS occurs, DNA replication may stall and DNA strands may be broken, which leads to chromosome aberrations. Therefore, TLS is a critical molecular event whether DNA damage is converted to mutations including chromosome aberrations or not. Even in *S. typhimurium*, whose genome size is about 1/1,000 of the size of human genome, there are six DNA Pols (13,28). Five of the Pols are encoded by the genes in the chromosome and the remaining one is encoded by the gene on the cryptic plasmid (29). Moreover, there is an additional plasmid pKM101 in *S. typhimurium* TA98 and TA100, where the *mucAB* genes encoding DNA Pol R1 are present (30). The presence of plasmid pKM101 carrying the *mucAB* genes strongly affects the sensitivity of *S. typhimurium* strains to a variety of chemical. In particular, the mutagenicity of furylfuramide (AF-2) and aflatoxin B_1 can be clearly detected with strain TA100 harboring plasmid pKM101 while no mutagenicity is observed with strain TA1535, the same as TA100 but has no plasmid pKM101 (31). AF-2 is a food additive that has been banned in Japan because of the carcinogenicity in the mice, and aflatoxin B_1 is a fungal toxin that can induce liver tumors in humans. It is supposed that DNA Pol R1 bypasses DNA adducts induced by AF2 and aflatoxin B_1 in an error-prone manner while other six DNA Pols in *S. typhimurium* can not. In contrast, human DNA Pol η is responsible for protection of genomic DNA from mutagenic effects of UV (32,33). This enzyme carries out error-free TLS across pyrimidine dimers in DNA and reduces the chance of mutations induced by UV. Lack of DNA Pol η leads to induction of Xeroderma pigmentosum variant, which is a genetic disease whose patients are highly sensitive to sunlight-induced skin cancer. Interestingly, bacterial DNA PolRI and human DNA Pol η belong to the same family of DNA Pol, i.e., Y-family. Therefore, TLS mediated by Y-family DNA Pols may enhance or reduce the frequencies of mutations, thereby influencing the practical thresholds for genotoxicity.

Both DNA Repair and Error-prone TLS Affect Levels of Spontaneous Mutagenesis

As written in Introduction, chromosome DNA is continuously exposed to not only exogenous genotoxic agents but also to endogenous ones. These endogenous lesions are causes for so-called spontaneous mutations (1). Interestingly, both DNA repair and TLS play important roles in regulations of spontaneous mutagenesis. When ada_{ST} and ogt_{ST} encoding MGMT are deleted in *S. typhimurium* TA1535, the number of spontaneous His^+ revertants per plate increases two- to three-fold (16). Similar extent of an increase in the number of spontaneous revertants per plate was observed in $\Delta mutM_{ST}$ strain, i.e., YG3001 (18). Introduction of plasmid pKM101 enhances the number of spontaneous revertants per plate of strain TA1535 more than five times (31). Both deletions of the repair genes and introduction of plasmid pKM101 exhibit additive effects on the spontaneous mutagenesis. These results suggest that the levels of spontaneous mutagenesis, which may play important roles in determination of threshold levels for genotoxicity, is strongly affected by the ability to repair DNA damage and to bypass lesions by DNA Pols in host cells.

Discussion

In theory, even a single molecule of mutagens could interact with DNA and induce genetic alterations, which might lead to cancer (15). Therefore, it is supposed that there are no thresholds for the risk of genotoxic and carcinogenic compounds and also that even a small amount of such compounds can impose carcinogenic loads on humans. Because of the assumption, no accepted daily intake (ADI) is set for food additives, pesticides and veterinary drugs when they have genotoxic and carcinogenic activities. The assumption is counterintuitive, however, because humans possess a number of defense mechanisms against endogenous and exogenous genotoxic insults. The mechanisms include antioxidants, detoxication metabolisms, DNA repair and error-free TLS (Fig. 3). These mechanisms may suppress genotoxicity and reduce it below the detection limits. In fact, both MGMT and 8-OH-G DNA glycosylase strongly affect the sensitivity of *S. typhimurium* strains for genotoxicity assays, thereby suggesting the possibility that they may be constituents of practical thresholds for genotoxicity. In some cases, however, that linear non-

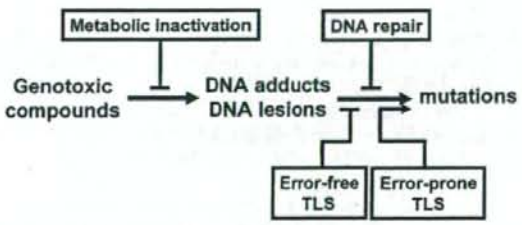

Fig. 3. Possible mechanisms underlying practical thresholds for genotoxicity. Detoxication mechanisms inactive genotoxic compounds. When active genotoxic compounds induce DNA adducts, DNA repair mechanisms remove them, thereby reverting the modified bases into intact ones. Error-free translesion DNA synthesis (TLS) inserts correct bases opposite the lesions and reduces the chance of induction of mutations. Therefore, these mechanisms, i.e., metabolic inactivation, DNA repair and error-free TLS, may be constituents of practical thresholds for genotoxicity. In contrast, error-prone TLS enhances mutations by insertion of incorrect bases opposite the lesions or skipping the lesions. These molecular events lead to base substitutions and frameshift mutations, respectively.

threshold dose response can be observed for genotoxicity even in the presence of wild-type DNA repair (34). Although error-free TLS can reduce the levels of mutations, error-prone TLS has an opposite effects and enhances the sensitivity to genotoxic compounds. Genetic approaches with cells deficient in DNA repair capacity and/or TLS are powerful tools to analyze possible mechanisms underlying practical thresholds for genotoxicity. Since risk assessment of chemical carcinogens is usually conducted with experimental animals, i.e., rats and mice, it is necessary to expand the genetic approaches to *in vivo*. In this respect, *gpt* delta rats and mice may be useful backgrounds to investigate constituents of the practical thresholds (35,36). These transgenic rodents harbor reporter genes for mutations, which enable to identify genotoxicity in target organs of chemical carcinogens (37). So far, *gpt* delta mice have been crossed with a number of knockout mice such as *p53*, *Ogg1*, *Parp-1*, *Atm*, *IL-10* and *Nrf-2* (38–43). It is important to examine which factors, e.g., detoxication, DNA repair or TLS, most strongly affect the levels of practical thresholds for genotoxicity and carcinogenicity *in vivo*. These studies are currently in progress in our laboratory.

Acknowledgements: Part of this work is financially supported by grant-in-aid by the Ministry of Health, Labour and Welfare, Japan.

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