Takami, M. Takahashi, T. Imai, A. Yamamoto, W. Kumita, K. Masumura, S. Matsuda, N. Kinae, T. Matsuda, T. Nohomi, M. Honma:Child-adult differences in evaluation of in vivo genotoxicity of acrylamide. 第37回日本環境変異原学会、沖縄、2008年12月4日～6日

8) J. Adachi, K. Kihara, T. Matsuda: Unbiased Quantification of oxidative modifications of cysteine thiols by double labeling approach using isotope amino acids and affinity tags (DLIAA). 第37回日本環境変異原学会、沖縄、2008年12月4日～6日

9) R. Nishi, Y. Totsuka, K. Wakabayashi, T. Matsuda: Analysis of DNA adducts in the lung of mice exposed to multi-wall carbon nanotube. 第37回日本環境変異原学会、沖縄、2008年12月4日～6日

10) H. Takemura, A. Matsui, M. Morita, H. Sakakibara, T. Matsuda, K. Shimoi: Inhibition of Benzo[a]pyrene-DNA adducts by chrysoeriol, a dietary methoxyflavonoid, in MCF-7 cells. 第37回日本環境変異原学会、沖縄、2008年12月4日～6日

11) H. Nagayoshi, M. Oka, Y. Yukawa, K. Hori, Y. Suwa, A. Yokoyama, M. Muto, T. Matsuda: Availability of DNA damage, N2-ethylidene-dG as a biomarker of acetakdehyde exposure. 第37回日本環境変異原学会、沖縄、2008年12月4日～6日

12) P.-H. Chou, H. Sugimura, K. Kawai, H. Kasai, T. Matsuda: Simultaneous analysis of various oxidative DNA adducts in human tissues using LC-MS/MS. 第37回日本環境変異原学会、沖縄、2008年12月4日～6日

13) K. Kawai, P.-H. Chou, T. Matsuda, S. Matsui, H. Kasai: 4-Oxo-2-hexenal-dA adduct formation in DNA in vitro and in mouse organs. 第67回日本癌学会学術総会、名古屋、2008年10月28日～30日

14) 松田知成:LC/MS/MSを用いたDNA損傷研究法、第35回BMSコンファレンス、福島県、2008年7月6日～9日

15) 松田知成、周　佩欣、永吉晴奈：ＨＰＬＣ－バイオアッセイ法による新規環境汚染物質の単離同定、第17回環境化学討論会、神戸、2008年6月11日～13日

16) 松田知成、周　佩欣、河井一郎、葛西宏：ＤＮＡアダクトーム解析による過酸化脂質由来ＤＮＡ塩基損傷の網羅的解析、第61回日本酸化ストレス学会学術集会、京都、2008年6月19日～20日

H. 知的所有権の取得状況

特になし

平成 20 年度 厚生労働科学研究費補助金(食品の安心・安全確保推進研究事業)
分担研究報告書

研究課題名:食品添加物等における遺伝毒性評価のための戦略構築に関する研究

分担研究課題名:動物個体を用いた遺伝毒性研究

分担研究者: 能美健彦 国立医薬品食品衛生研究所 変異遺伝部 部長

研究要旨

個体において遺伝毒性を検出する F344 系 *gpt* delta トランスジェニックラットに、発がん物質である 2,4-dianimotoluene (2,4-DAT)と非発がん物質である 2,6-dianimotoluene (2,6-DAT)を混餌で 13 週間投与し、発がんの標的臓器(肝臓)と非標的臓器(腎臓)で *gpt* 遺伝子突然変異体頻度を測定した。また末梢血を用いて小核を持つ赤血球の頻度を測定した。肝臓では、2,4-DAT 投与群(125 ppm, 250 ppm, 500 ppm)で非投与群よりも有意に高い変異体頻度が観察されたが、2,6-DAT では 500 ppm でも変異体頻度は増加しなかった。腎臓では、全ての投与群で変異体頻度が増加しなかった。小核の出現頻度は、いずれの投与群においても増加しなかった。以上の結果から F344 系 *gpt* delta トランスジェニックラットは発がんの標的臓器で変異を検出しうる有用な試験系であることが示唆された。

キーワード:遺伝毒性発がん物質、発がん標的臓器、*gpt* delta トランスジェニックラット

A. 研究目的

遺伝毒性を検出する試験系には in vitro 系(バクテリアを用いる復帰突然変異試験、哺乳類細胞を用いる染色体異常試験、哺乳類細胞を用いる遺伝子突然変異試験等)と in vivo 系(マウスを用いる小核試験、ラット肝臓を用いる不定期 DNA 合成試験等)が存在するが、発がんを検出する実験動物の標的臓器(発がんが起こる臓器)において遺伝毒性を検出する最も信頼できる手法は、現在のところ、変異検出用のレポーター遺伝子を導入したトランスジェニック動物遺伝毒性試験法である。

この試験では、トランスジェニックマウスあるいはラットを化学物質に曝露し、各種臓器(肝臓、腎臓、肺等)からレポーター遺伝子を in vitro パッケージング法によりλファージ粒子として回収して大腸菌に感染させ、動物個体で起きた変異を、大腸菌を用いて検出する。検出された変異体は、DNA シークエンス解析により分子解析することができる。

我々は、従来からのトランスジェニック遺伝毒性試験法では検出しにくかった欠失変異を効率良く検出することを目的に、新規なλファージλEG10 を開発し、これを C57BL6/J マウスの受精卵に導入することで点突然変異と欠失変異を検出できる *gpt*

delta トランスジェニックマウスを樹立した。またλEG10DNA を Sprague Dawley (SD) ラットに導入して SD *gpt* delta ラットを樹立した。

今回、発がん試験において汎用されている F344 ラットに SD *gpt* delta ラットをバッククロスし、F344 *gpt* delta ラットを樹立した。さらに F344 *gpt* delta ラットの有用性を検討するため、構造の類似した発がん物質 2,4-diaminotoluene(2,4-DAT) と非発がん物質 2,6-diaminotoluene(2,6-DAT) を投与し、その発がん標的臓器（肝臓）と非発がん臓器（腎臓）における変異頻度を比較した。

B．研究方法

1)　F344 *gpt* delta ラットの樹立

SD *gpt* delta ラットの雄を F344 ラットの雌と 15 世代戻し交配を行い、F344 *gpt* delta ラットを樹立した。

2)　2,4-DAT と 2,6-DAT の投与

7 週齢の雄 *gpt* delta ラットに 2,4-DAT(125 ppm, 250 ppm, 500 ppm)を、一群 5 匹、13 週間混餌投与した。500 ppm 投与群は、毒性影響のため 9 週以降は 400 ppm に用量を下げた。2,6-DAT は、500 ppm の用量で 13 週間混餌にて投与した。陽性対照としては、diethyl nitrosamine (DEN) を用い、20 mg/kg の用量で、13 週間、毎週一度、腹腔内投与した。陰性対照群には基礎食を 13 週間与えた。

3)　末梢血小核試験

解剖時に、尾静脈より約 60 μL 血液採取し、MicroFlow^{PLUS} キットの抗凝固剤と混和後、超低温メタノールで固定し、フローサイトメーター用サンプルとして、-80℃で保存した。キットの方法に従いサンプルを洗浄後、RNase、抗 CD71 抗体及び抗 CD61 抗体と、氷上及び室温（遮光）で、それぞれ 30 分間反応させた。測定まで氷冷下で保存し、測定時に propidium iodide (PI) と反応させた。測定は、Becton Dickinson FACSCalibur フローサイトメーターを用いて、FITC 標識抗 CD71 抗体、PE 標識抗 CD61 抗体及び PI で標識した細胞を、アルゴンレーザー光 (488 nm) で励起し、それぞれ FL1 (530/30 nm)、FL2 (585/42 nm) 及び FL3 (630/22 nm) で蛍光を検出し、CellQuest 3.3 software (Becton Dickinson) を用いて解析した。評価は 1 サンプルにつき CD71 陽性幼若赤血球 20000 個をカウントし、その中で小核を有する網状赤血球の割合 (%MN-RET) を算出した。

4)　*gpt* 変異体頻度(MF)の測定

肝臓および腎臓細胞から RecoverEase DNA isolation kit (Stratagene)を用いてゲノム DNA を採取した。その後、Transpack (Stratagene)を用いて in vitro パッケージング反応を行い、ゲノム DNA からλEG10 をファージ粒子として回収した。ファージは大腸菌 YG6020 に感染させ、6-チオグアニンとクロラムフェニコールを含む培地上に播種し耐性となったコロニー(*gpt* 変異体候補コロニー)を検出した。検出したコロニーは、再度、6-チオグアニンとクロラムフェニコールを含む培地上にストリークして、真の *gpt* 変異体を検出した。回収したファージの一部は適宜希釈した後 YG6020 に感染させ、クロラムフェニコールのみを含む培地上に播種し、耐性コロニー数を計測して回収したレポーター遺伝子の総数を求めた。*gpt* MF は、真の *gpt* 変異体数を回収したレポーター遺伝子数で除して算出した。

5)　統計的手法

すべての測定値について平均値と標準偏差を求め、統計的な有意差は Student's

t-test により評価した。

(倫理面への配慮)

本研究は、実験動物を用いたものであり、ヒトに関する倫理上の問題はない。また、全ての実験は、遺伝子組換え実験、動物実験に関する所内の規定に準拠して行った。

C. 研究結果

1) 2,4-DAT および 2,6-DAT による毒性

2,4-DAT 投与群では、全ての投与群で体重の減少が観察され、特に 500 ppm 投与群では顕著であった(2,4-DAT 500 ppm, 235.2 ±11.8g versus control, 340.7±11.5g $P<0.01$)。また、肝臓および腎臓の体重全体に対する比重量も投与量に依存して増加した(肝臓 2,4-DAT 500 ppm, 6.54±0.35% versus control 2.92±0.14% $P<0.01$; 腎臓 2,4-DAT 500ppm, 0.66±0.01% versus control 0.54±0.01% $P<0.01$)。一方、2,6-DAT 投与群では体重の減少、肝臓および腎臓の体重全体に対する比重量の増加は観察されなかった。陽性対照群では、体重の減少、肝臓および腎臓の体重全体に対する比重量の有意な増加が観察された。

2) 小核試験

末梢血を用いて小核試験を行ったが、2,4-DAT, 2,6-DAT ともに、用量にかかわらず% MN-RET の有意な増加は観察されなかった(control, 0.09±0.048%; 2,4-DAT 125ppm, 0.087±0.041%; 2,4-DAT 250 ppm, 0.104±0.041%; 2,4-DAT 500 ppm, 0.125 ±0.067%; 2,6-DAT 500 ppm, 0.078± 0.031%)。また遺伝子突然変異の陽性対照として用いた DEN 投与群でも、% MN-RET の有意な増加は観察されなかった(DEN 0.138± 0.046%)。

3) *gpt* 遺伝子突然変異試験

肝臓の *gpt* MF は、2,4-DAT 投与群においては非投与群に比較して有意に増加し、125ppm 投与群において非投与群の約 2 倍、250ppm および 500ppm 投与群で約 7 倍高い MF を示した(第 1 表)。一方、2,6-DAT は、500ppm 投与群において、非投与群に比べ低い値を示した。陽性対照は非投与群に比べ 250 倍以上高い値を示した。

第 1 表　肝臓の *gpt* MF

	gpt MF (10^{-6})
非投与群	6.0±2.4
DEN 投与群	1625.8±517.9***
2,4-DAT 125 ppm	13.7±5.7*
250 ppm	42.9±23.9**
500 ppm	44.2±19.6**
2,6-DAT 500 ppm	3.0±1.5*

gpt MF は 5 匹の値の平均値±標準偏差を示す
各群の MF の非投与群に対する統計的有意差を t テストにより検定した。*は P < 0.05、**は P < 0.01、***は P < 0.001 を表す。

一方、腎臓においては、2,4-DAT、2,6-DAT ともに、非投与群よりも有意に高い *gpt* MF を示さなかった(第 2 表)。陽性対照である DEN は、非投与群に比べ 30 倍以上高い値を示した。

第 2 表　腎臓の *gpt* MF

	gpt MF (10^{-6})
非投与群	4.6±5.0
DEN 投与群	147.8±111.1*
2,4-DAT 125 ppm	4.3±2.9
250 ppm	4.4±2.7
500 ppm	5.5±2.6
2,6-DAT 500 ppm	4.7±5.7

gpt MF は 5 匹の平均値±標準偏差を示す
各群の MF の非投与群に対する統計的有意差を t テ

ストにより検定した。*は P < 0.05 を表す。

D. 考 察

発がん物質は、DNA と反応して突然変異や染色体異常を起こす遺伝毒性発がん物質と、遺伝毒性以外のメカニズム(細胞増殖、ホルモン作用など) で発がん作用を示す非遺伝毒性物質発がん物質に分類され、遺伝毒性物質発がん物質には閾値がないとされている。このため遺伝毒性物質発がん物質には一日許容摂取量 (ADI) が設定されず、食品添加物、残留農薬、動物用医薬品としては原則使用が禁止される。だがどのような試験の結果を用いて遺伝毒性物質とするかは明確ではない。一般にバクテリアを用いる Ames 試験の結果をもって遺伝毒性とすることが多いが、本来は、発がん試験を行った実験動物の発がん標的臓器において遺伝毒性を検出すべきである。この点に関し、今回樹立した F344 *gpt* delta ラットは、発がん試験に汎用される F344 ラットを遺伝的背景にしており、発がんと遺伝毒性の関連を調べる上で有用な試験法と考えられる。

F344 *gpt* delta ラットに構造の類似した発がん物質(2,4-DAT)と非発がん物質(2,6-DAT)を投与し、標的臓器 (肝臓) と非標的臓器(腎臓) での遺伝毒性を検索した。その結果、2,4-DAT のみに肝臓で変異頻度の上昇を認めた(第 1 表)。腎臓では 2,4-DAT、2,6-DAT ともに遺伝毒性を示さなかった(第 2 表)。以上の結果は、F344 *gpt* delta ラットが、遺伝毒性を発がんの標的臓器において検出しうる可能性を示している。

2,4-DAT と 2,6-DAT については、トランスジェニックマウス (Big Blue マウス) を用いて肝臓での遺伝毒性を検討した報告があり、2,4-DAT では陽性、2,6-DAT は陰性と報告されている(M.L. Cunningham et al., Environ. Health Perspect., 104, Suppl. 3, 683-686, 1996) 。しかしマウスでの遺伝毒性は、1,000 ppm の用量で 90 日間混餌投与し MF の増加は約 2 倍とされており、ラットに比べて感受性が低い。これはマウスでは解毒代謝系 (グルタチオン転移酵素活性) がラットに比べて高いためと考えられる。

2,4-DAT と 2,6-DAT は代謝活性化系(S9) の存在下で、ともに Ames 試験で陽性となる(E. Zeiger et al., Environ. Mol. Mutagen., 11, 1-157, 1988)。このことは両者の代謝物が DNA に損傷作用を持つ可能性を示している。だが in vivo (ラット、マウス) においては、2,4-DAT が選択的に遺伝毒性、発がん性を示す。この原因としては、(1) in vivo では 2,6-DAT の解毒代謝が 2,4-DAT に比べて効率良く進むため(P.M. Wilson et al., Arch. Toxicol., 70, 591-598, 1996) (2) 2,4-DAT のみが肝臓において細胞増殖活性を示すため等が考えられる。

末梢血を用いる小核試験では、2,4-DAT、2,6-DAT ともに陰性の結果となった。2,4-DAT については、マウス末梢血を用いる小核試験でも陰性と報告されている(T. Morita et al., Mutat. Res., 389, 3-122, 1997)。幼弱ラットの肝臓を用いる小核試験で 2,4-DAT は陽性と報告されていることから(H. Suzuki et al., Mutagenesis, 24, 9-16, 2009)、肝臓で生じた活性代謝物が骨髄に到達しないために、末梢血は小核を生じないものと考えられる。遺伝子突然変異の陽性対照として用いた DEN も末梢血での小核試験は陰性であったが、マウス骨髄を用いる小核試験でも DEN は陰性と報告されている(D. Wild, Mutat. Res., 56, 319-327, 1978)。2,4-DAT と同様に、肝臓で生じた活性代謝物が骨髄に到達しないためと考えられる。この結果は、in vivo の遺伝毒性を検索する場合に、発がんの標的臓器で遺伝毒性を調べることの重要性を示している。

E．結　論

F344 *gpt* delta ラットに 2,4-DAT（発がん物質）と 2,6-DAT（非発がん物質）を投与し、肝臓（標的臓器）で陽性、腎臓（非標的臓器）で陰性の結果を得た。この結果は、F344 *gpt* delta ラットの有用性を示唆している。

F．健康危機情報

特になし

G．研究発表

1．論文発表

1) A. Shibata, D. Maeda, H. Ogino, M. Tsutsumi, T. Nohmi, H. Nakagama, T. Sugimura, H. Teraoka and M. Masutani, Role of Parp-1 in suppressing spontaneous deletion mutation in the liver and brain of mice atadolescence and advanced age, Mutat. Res., in press.

2) A.H. Hashimoto, K. Amanuma, K. Masumura, T. Nohmi and Y.Aoki, in vivo mutagenesis caused by diesel exhaust in the testis of *gpt* delta transgenic mice, Genes and Environ., in press.

3) T. Umemura, M. Tasaki, A. Kijima, T. Okamura, T. Inoue, Y. Ishii, Y. Suzuki, N. Masui, T. Nohmi and A. Nishikawa, Possible participation of oxidative stress in causation of cell proliferation and in vivo mutagenicity in kidneys of gpt delta rats treated with potassium bromate, Toxicol., 257, 46-52 (2009)

4) K. Masumura and T. Nohmi, Spontaneous mutagenesis in rodents: spontaneous gene mutations identified by neutral reporter genes in *gpt* delta transgenic mice and rats, J. Health Sci., 55, 40-49 (2009)

5) K. Yamauchi, S. Kakinuma, S. Sudo, S.Kito, Y. Oota, T. Nohmi, K. Masumura, M. Nishimura and Y. Shimada, Differential effects of low- and high-dose X-rays on N-ethyl-N-nitrosourea-induced mutagenesis in thymocytes of B6C3F1 *gpt*-delta mice, Mutat. Res., 640, 27-37 (2008)

6) T. Nohmi, Possible mechanisms of practical thresholds for genotoxicity, Genes and Environ., 30, 108-113 (2008)

7) T. Nohmi, N. Toyoda-Hokaiwado, M. Yamada, K. Masumura, M. Honma and S. Fukushima, International symposium on genotoxic and carcinogenic thresholds, Genes and Environ., 30, 101-107 (2008)

8) A. Nishikawa, T. Umemura, Y. Ishii, M. Tasaki, T. Okamura, T. Inoue, K. Masumura and T. Nohmi, in vivo approaches to study mechanism of action of genotoxic carcinogens, Genes and Environ., 30, 120-124 (2008)

2．学会発表

1) T. Nohmi, Transgenic in vivo genotoxicity assays: basic features and the possible applications, 14th Alexander Hollaender Course on Genetic Toxicology: Genomic and Proteomic Approaches & Special Workshop on Arsenic Exposure Assessment in Kolkata, India, December 10-12, 2008.

2) T. Nohmi, Combined genotoxic

effects of a methylating agent and radiation on human cells, New Nuclear Research Symposium, Biological Responses to Low Dose Radiation in Kitakyushu, Japan, November 2008.

3) T. Nohmi, Recent topics of mutation research with *gpt* delta mice and rats, 39th Annual Meeting of Environmental Mutagen Society in Puerto Rico, October 2008.

4) M. Ikeda, K. Masumura, Y. Sakamoto, B. Wang, M. Nenoi, K. Sakuma, I. Hayata, A. Yamamoto, M. Honma and T. Nohmi, *gpt* delta transgenic mice for in vivo genotoxicity assays: application for analysis of combined effects of radiation and chemicals, the 7th Japan-France Workshop on Radiation Biology, Chiba, October, 2008.

5) Y. Totsuka, T. Ichinose, K. Hiyoshi, T. Kato, S. Masuda, T. Nohmi, T. Sugimura and K. Wakabayashi, Genotoxicity of nanoparticles in *in vivo* mutation assay systems, 38th European Environmental Mutagen Society, Covtat, Croatia, September 2008.

6) T. Nohmi, Possible Mechanisms of Practical Genotoxic Thresholds, International Symposium on Genotoxic and Carcinogenic Thresholds, Tokyo, July, 2008.

7) T. Nohmi, *gpt* delta transgenic mouse and rat for analysis of in vivo mutagenesis, The 37th Annual Meeting of the Japanese Environmental Mutagen Society (37th JEMS) (2008, 12)

8) N. Koyama, A. Kimura, M. Yasui, S. Takami, M. Takahashi, T. Imai, A. Yamamoto, W. Kumita, K. Masumura, S. Masuda, N. Kinae, T. Matsuda, T. Nohmi and M. Honma, Child-adult differences in evaluation of in vivo genotoxicity of acrylamide, The 37th Annual Meeting of the Japanese Environmental Mutagen Society (37th JEMS) (2008, 12)

9) Y. Ishii, T. Okamura, M. Tasaki, T. Inoue, T. Nohmi, T. Umemura and A. Nishimura, *in vivo* mutagenicity of madder color and its constituents using *gpt* delta rats, The 37th Annual Meeting of the Japanese Environmental Mutagen Society (37th JEMS) (2008, 12)

10) Y. Tosuka, T. Kato, S. Masuda, T. Nohmi, T. Sugimura and K. Wakabayashi, Genotoxicity of nanoparticles in *in vivo* assay systems, The 37th Annual Meeting of the Japanese Environmental Mutagen Society (37th JEMS) (2008, 12)

11) N. Toyoda-Hokaiwado, T. Inoue, K. Masumura, H. Hayashi, Y. Kawamura, Y. Kurata, T. Umemura, A. Nishikawa and T. Nohmi, Difference in mutagenicity of structural isomers 2,4-DAT and 2,6-DAT in the target organ for carcinogenicity of F344 *gpt* delta transgenic rat, The 37th Annual Meeting of the Japanese Environmental Mutagen Society (37th JEMS) (2008, 12)

12) N. Toyoda-Hokaiwado, K. Masumura, H. Hayashi, Y. Kawamura, Y. Kurata, H. Sanada, T. Inoue, T. Umemura, A. Nishikawa, T. Nohmi, In vivo

mutagenicity of structural isomers 2,4-DAT and 2,6-DAT in F344 *gpt* delta transgenic rat, The 67th Annual Meeting of the Japanese Cancer Association (2008. 10)

13) K. Masumura, Y. Sakamoto, M. Ikeda, T. Tsukamoto, H. Ikehata, Y. Kuroiwa, T Umemura, A. Nishikawa, M. Tatematsu, T. Ono, T. Nohmi, p53 suppresses deletions in the epidermis of mice unirradiated or irradiated with low doses of UVB, The 67th Annual Meeting of the Japanese Cancer Association (2008. 10)

14) N. Toyoda-Hokaiwado, T. Inoue, K. Masumura, H. Hayashi, Y. Kawamura, Y. Kurata, T. Umemura, A. Nishikawa, T. Nohmi, Applying F344 *gpt* delta transgenic rat for in vivo genotoxicity study of structural isomer 2,4-diaminotoluene and 2,6-diaminotoluene, The 25th Annual Meeting of the Japanese Society of Toxicologic Pathology (2009. 1)

H. 知的所有権の取得状況

特になし

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
T. Nohmi, N. Toyoda-Hokaiwado, M. Yamada, K. Masumura, M. Honma, S. Fukushima	International symposium on genotoxic and carcinogenic thresholds	Genes Environ.	30	101-107	2008
F. Yatagai, Y. Umebayashi, M. Honma, K. Sugasawa, Y. Takayama and F. Hanaoka	Mutagenic radioadaptation in a human lymphoblastoid cell line	Mutat. Res.	638	48-55	2008
M. Yasui, E. Suenaga, N. Koyama, C. Masutani, F. Hanaoka, P. Gruz, S. Shibutani, T.Nohmi, M. Hayashi and M. Honma	Miscoding properties of 2'-deoxyinosine, a nitric oxide-derived DNA Adduct, during translesion synthesis catalyzed by human DNA polymerases	J. Mol. Biol.	377	1015-1023	2008
F. Yatagai, M. Suzuki, N. Ishioka, H. Ohmori and M. Honma	Repair of I-SceI Induced DSB at a specific site of chromosome in human cells: influence of low-dose, low-dose-rate gamma-rays	Radiat. Environ. Biophys.	47	439-444	2008
本間正充	遺伝毒性物質に閾値はあるのか？	ファルマシア	45	143–148	2009
M. Nagao, S. Ishikawa, H. Nakagama and M. Watanabe	Extrapolation of the animal carcinogenesis threshold to humans	Genes Environ.	30	160-165	2008
T. Sakai, S. Tokishita, K. Mochizuki, A. Motomiya, H. Yamagata and T. Ohta	Mutagenesis of uracil-DNA glycosylase deficient mutants of the extremely thermophilic eubacterium *Thermus thermophilus*	DNA Repair	7	663-669	2008
H. Nagayoshi, A. Matsumoto, R. Nishi, T. Kawamoto, M. Ichiba and T. Matsuda	Increased formation of gastric *N2*-ethylidene-2'-deoxyguanosine DNA adducts in aldehyde dehydrogenase-2 knockout mice treated with ethanol	Mutat. Res.	673	74-77	2009

Y. Okamoto, P.H. Chou, S.W. Kim, N. Suzuki, Y.R. Laxmi, K. Okamoto, X. Liu, T. Matsuda and S. Shibutani	Oxidative DNA damage in XPC-knockout and its wild mice treated with equine estrogen	Chem. Res. Toxicol.	21	1120-1124	2008
K. Misaki, M. Suzuki, M. Nakamura, H. Handa, M. Iida, T. Kato, S. Matsui and T. Matsuda	Aryl Hydrocarbon Receptor and Estrogen Receptor Ligand Activity of Organic Extracts from Road Dust and Diesel Exhaust Particulates	Arch. Environ. Contam. Toxicol.	55	199-209	2008
A. Shibata, D. Maeda, H. Ogino, M. Tsutsumi, T. Nohmi, H. Nakagama, T. Sugimura, H. Teraoka and M. Masutani	Role of Parp-1 in suppressing spontaneous deletion mutation in the liver and brain of mice atadolescence and advanced age	Mutat. Res.	in press		
A.H. Hashimoto, K. Amanuma, K. Masumura, T. Nohmi and Y.Aoki	in vivo mutagenesis caused by diesel exhaust in the testis of *gpt* delta transgenic mice	Genes Environ.	31	1-8	2009
T. Umemura, M. Tasaki, A. Kijima, T. Okamura, T. Inoue, Y. Ishii, Y. Suzuki, N. Masui, T. Nohmi and A. Nishikawa	Possible participation of oxidative stress in causation of cell proliferation and in vivo mutagenicity in kidneys of *gpt* delta rats treated with potassium bromate	Toxicol.	257	46-52	2009
K. Masumura and T. Nohmi	Spontaneous mutagenesis in rodents: spontaneous gene mutations identified by neutral reporter genes in *gpt* delta transgenic mice and rats	J. Health Sci.	55	40-49	2009
K. Yamauchi, S. Kakinuma, S. Sudo, S.Kito, Y. Oota, T. Nohmi, K. Masumura, M. Nishimura and Y. Shimada	Differential effects of low- and high-dose X-rays on N-ethyl-N-nitrosourea-induced mutagenesis in thymocytes of B6C3F1 *gpt*-delta mice	Mutat. Res.	640	27-37	2008
T. Nohmi	Possible mechanisms of practical thresholds for genotoxicity	Genes Environ.	30	108-113	2008
A. Nishikawa, T. Umemura, Y. Ishii, M. Tasaki, T. Okamura, T. Inoue, K. Masumura and T. Nohmi	in vivo approaches to study mechanism of action of genotoxic carcinogens	Genes Environ.	30	120-124	2008

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*

Contents

Volume 30 Number 4, November 2008, 101–170

[Continue]

Genes and Environment, Vol. 30, No. 4 pp. 101–107 (2008)

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]

[1]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[2]Japan Bioassay Research Center, Japan Industrial Safety and Health Association, Kanagawa, Japan

(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.

[3]Correspondence to: Takehiko Nohmi, Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-9872, Fax: +81-3-3700-2348, E-mail: nohmi@nihs.go.jp

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.