ment, and a reliable detection method for the existence of α_{2u}-globulin is therefore necessary.

Using both immunochemical staining for paraffin-embedded sections and the immuno-electron microscopy technique, we demonstrated that our prepared antibody reacted specifically to α_{2u}-globulin in renal hyaline droplets in the male rats administered d-limonene, a well-known α_{2u}-globulin nephropathy inducer. The dose-dependent positive immuno-reaction of the antibody in both the tissue sections and the homogenates from d-limonene-treated rat kidneys indicated that the antibody could be applicable for semi-quantitative analysis. In addition, computational image analysis revealed that classical visual microscopic grading was also useful for semi-quantitative analysis of α_{2u}-globulin accumulation.

Although immunohistochemical α_{2u}-globulin analysis of the glycolmethacrylate-embedded sections had already been reported by Burnett *et al.* (1989), our method was advantageous from the standpoint of applicability to the paraffin-embedded sections. The paraffin-embedded specimens were usually prepared and stored for the general toxicity studies. In fact, all the sections used in experiment 2 in this study originated from study specimens which were prepared in the Japanese Existing Chemicals Survey Program conducted previously and stored for a long time. It indicated that our method is applicable to specimens derived directly from ordinary toxicology studies retrospectively. Hashimoto and Takaya (1992) previously investigated the application of α_{2u}-globulin immunostaining to paraffin sections by modifying the protocol of Burnett *et al.* (1989). The protocol includes pronase E treatment owing to enhancement of the antigen reactivity and removal of the non-specific reaction. Our method also includes the pronase E treatment, but

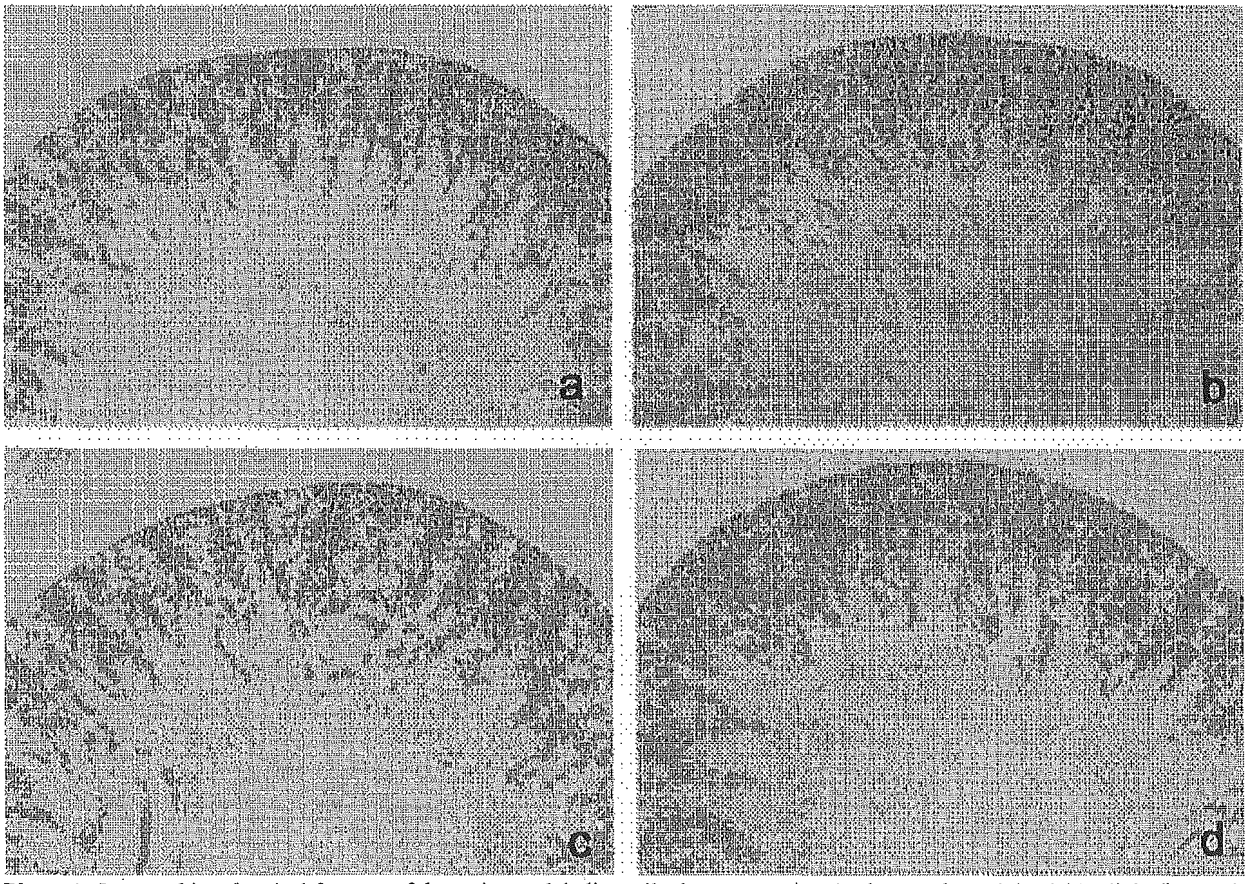

Photo 4. Immunohistochemical features of the anti-α_{2u}-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.

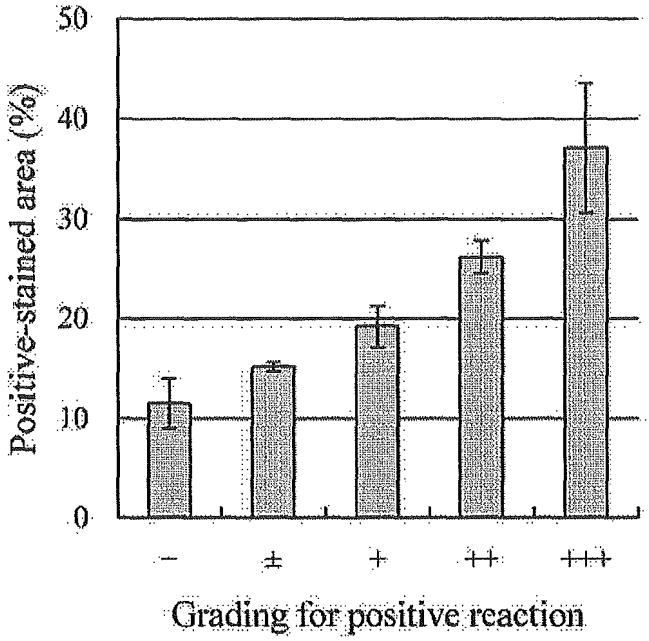

Fig. 2. Correlation between semi-quantitative and quantitative analyses for immuno-stained sections.
Results are expressed as mean ± SD (n=3).

the treatment is performed only in order to enhance the antigen activity and not to remove the non-specific reaction. This may suggest that our prepared antibody has a high specificity for α_{2u}-globulin. Caldwell *et al.* (1999) had conducted a similar quantitative immunohistochemical α_{2u}-globulin analysis, but it seems that the actual analyzed area was limited to narrower fields than in our study.

Urinary immunochemical analysis for detection of α_{2u}-globulin accumulation in male rat kidneys has been developed by Saito *et al.* (1996). Although the convenient urinary analysis is sufficient for detecting CIGA, the detectability is weaker than with kidney soluble protein analysis. The aim of the present analysis is not only to detect CIGA, but also to exclude the α_{2u}-globulin-induced nephrotoxic effects from risk assessment of chemicals. For 10 chemicals suspected of being CIGA, the occurrence of hyaline droplets in the kidneys with treatment was the lowest endpoint. In the process of evaluating chemical toxicity, if the most sensitive nephrotoxicity is concluded to be a neglected effect for human health, the NOAEL could be set based on other kinds of toxicological effects.

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Bull. Natl. Inst. Health Sci., **123**, 46-52 (2005) **Notes**

OECD化学物質対策の動向（第7報）
第15回OECD高生産量化学物質初期評価会議（2002年ボストン）

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Progress on OECD Chemicals Programme (7) — SIAM 15 in Boston, 2002

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The 15th Screening Information Data Set (SIDS) Initial Assessment Meeting (SIAM 15) was held in Boston, USA. The initial assessment documents of twelve substances at SIAM 15 (CAS numbers: 79-39-0, 88-60-8, 92-70-6, 102-76-1, 110-83-8, 135-19-3, 7647-01-0, 8007-18-9, 10043-52-4, 11070-44-3, 25321-09-9, 68186-90-3) were submitted by the Japanese Government with or without the International Council of Chemical Associations (ICCA) and all of them on the human health effect parts were agreed at the meeting. In this report, the human health effect parts in their 12 substance documents are introduced.

Key Words: OECD, HPV program, SIDS Initial Assessment Meeting

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はじめに

経済協力開発機構（Organisation for Economic Co-operation and Development: OECD）加盟各国における高生産量化学物質（High Production Volume Chemical: HPV）について，1992年に始まったOECD高生産量化学物質点検プログラム（HPV Program）により安全性の評価が行われている[1]．日本政府は初回より評価文書を提出しており，第14回までの初期評議会議（Screening Information Data Set (SIDS) Initial Assessment Meeting: SIAM）において日本政府が担当し結論及び勧告が合意された化学物質の評価文書のヒトの健康影響部分については既に紹介してきた[2~6]．国際化学工業協会協議会（International Council of Chemical Associations: ICCA）による評価文書の原案作成に伴い日本においても2001年から，日本政府に加え日本化学工業協会加盟企業も評価文書の原案を作成し，政府レビューの時OECDに提出している．

評価文書は，物性，環境毒性及びヒトの健康影響に関する記述から構成されているが，著者らがヒトの健康影響部分の担当であるため，本稿ではSIAM15でヒトの健康影響部分について合意に至った化学物質名及び日本担当物質の評価文書の記述の概要を紹介する．なお，OECDガイドラインに則した単回及び反復投与試験についてはガイドライン番号を示した．

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ヒトの健康影響部分についてSIAM15で合意された化学物質名と日本担当物質の初期評価内容

2002年10月にボストン（米国）で開催されたSIAM15において，再審議として6物質，新規審議として26物質，カテゴリーとして2グループ（3物質及び4物質），計39化学物質の初期評価文書が検討され，表1に示す物質の初期評価結果及び勧告が合意された．SIAMにおける合意はFW（The chemical is a candidate for further work.）またはLP（The chemical is currently of low priority for further work.）として示されている．FWは「今後も追加の調査研究作業が必要である」，LPは「現状の使用状況においては追加作業の必要はない」ことを示す．日本政府が担当した化学物質の初期評価報告書のヒトへの健康影響についての記述の概要を以下に示す．

Methacrylamide（79-39-0）（原案作成：ICCA日本企業）

本化学物質は紙や布地の仕上げ剤やコート剤の原料として主に使用される．

放射性標識体を用いた実験において，ウサギへの静脈内投与では24時間以内に86％が，ウサギ（雄）及びラット（雄）への15～30分間の皮膚暴露では24時間後にそれぞれ23-52％及び3.7-5.7％が，尿中に排泄される．

ラットの単回経口投与毒性試験（OECD TG 401）でのLD_{50}は1,653-1,938 mg/kgであった．毒性症状として振戦，流涎，よろめき歩行，被毛の汚染等が認められて

Table.1　Chemical substances discussed at SIAM15 and their outcomes

CAS No.	Name of substance	Sponsor country	Outcome
74-87-3	Chloromethane	US/ICCA	LP
79-34-5	1,1,2,2-Tetrachloroethane	FR/ICCA	LP
79-39-0	Methacrylamide	JP/ICCA	LP
88-60-8	6-tert-Butyl-m-cresol	JP/ICCA	LP
89-78-1 1490-04-6 2216-51-5 15356-60-2	Category: Menthols	DE/ICCA	ENV: − HH: LP
92-70-6	2-Hydroxy-2-naphthoic acid	DE & JP	ENV: − HH: FW
94-36-0	Benzoyl peroxide	KO	FW
98-92-0	Nicotinamide	CH/ICCA	LP
100-00-5	1-Chloro-4-nitrobenzene	DE/ICCA	LP
100-37-8	2-Diethylaminoethanol	DE/ICCA	LP
102-76-1	Triacetin	JP/ICCA	LP
106-63-8	Isobutyl acrylate	US/ICCA	LP
107-06-2	1,2-Dichloroethane	DE/ICCA	LP
110-83-8	Cyclohexene	JP	ENV: FW HH: LP
115-86-6	Triphenyl phosphate	DE/ICCA	ENV: FW HH: LP
120-83-2	2,4-Dichlorophenol	FR/ICCA	LP
121-91-5	Isophthalic acid	US/ICCA	LP
135-19-3	2-Naphthol	DE & JP	ENV: FW HH: LP
141-32-2	Butyl acrylate	US/ICCA	LP
143-22-6 1559-34-8 23783-42-8	Category: High Boiling Ethylene Glycol Ethers	US/ICCA	LP
144-55-8	Sodium hydrogencarbonate	BE/ICCA	LP
497-19-8	Sodium carbonate	BE/ICCA	LP
528-44-9	1,2,4-Benzenetricarboxylic acid	US/ICCA	LP
552-30-7	1,2,4-Benzenetricarboxylic anhydride	US/ICCA	LP
1330-20-7	Xylene	HU	−
2432-99-7	11-Aminoundecanoic acid	FR/ICCA	LP
7647-01-0	Hydrochloric acid	JP/ICCA	LP
7791-25-5	Sulfuryl chloride	DE/ICCA	LP
8007-18-9	C.I. Pigment Yellow 53	JP/ICCA	LP
10043-52-4	Calcium chloride	JP/ICCA	LP
11070-44-3	Tetrahydromethyl-1,3-isobenzofuranedione	JP/ICCA	LP
25321-09-9	Diisopropylbenzene	JP	ENV: FW HH: LP
68186-90-3	C.I. Pigment Brown 24	JP/ICCA	LP
90387-57-8	Formaldehyde, reaction products with sulfonated 1,1'-oxybis[methylbenzene], sodium salts	DE/ICCA	FW

Note. Abbreviations show BE: Belgium, CH: Switzerland, DE: Germany, FR: France, JP: Japan, HU: Hungary, KO: Korea and US: the United States of America in the sponsor country column, and ENV: environment parts, HH: human health parts and −: not finalized in the outcome column.

いる.

ウサギの皮膚に対する刺激性は認められなかったが，眼に対しては中程度の刺激性が認められている.

28日間反復経口投与毒性試験（OECD TG 407）では，ラットの雌雄に0，30，100及び300 mg/kg/dayを強制経口投与した．100 mg/kg/day以上の雄，30 mg/kg/day以上の雌で自発運動の低下が認められた．300 mg/kg/dayの雌雄の体重増加量が減少し，臨床的及び機能的変化（筋緊張の低下，歩行失調）や病理組織学的変化（坐骨神経の変性，小脳脚の軸索膨化）がみられた．雌では100 mg/kg/dayでも体重増加量が減少した．さらに，300 mg/kg/dayの群ではヘマトクリット，ヘモグロビン，平均赤血球色素量（MCH），尿素窒素，クレアチニン，α1-グロブリン，α2-グロブリン，アルカリホスファターゼ活性の減少，アルブミン及びトリグリセリドの増加が認められ，100 mg/kg/dayの群ではヘモグロ

ビン及びMCHの減少が認められた．本試験でのNOAELは30 mg/kg/day（雄），30 mg/kg/day未満（雌）と考えられた．

雄のラットとマウスに0，200，400，800及び1,200 ppm（ラット：0，4.6，9.1，19.5及び31.6 mg/kg/day，マウス：0，24.3，49.6，120及び220.6 mg/kg/day）を12ヶ月間飲水投与した反復投与試験で，ラットでは19.5 mg/kg/day以上の群でロータロッドの成績低下，膀胱の拡張，座骨神経有髄線維の収縮と消失，腓腹筋の萎縮がみられ，31.6 mg/kg/day群で握力低下や歩行異常などの神経症状，血清総コレステロールとリン脂質の増加がみられた．マウスでは120 mg/kg/day以上の群でロータロッドの成績低下，握力低下や歩行異常などの神経症状，腓腹筋萎縮，膀胱拡張，体重増加量の減少がみられ，49.6 mg/kg/day以上の群で後肢麻痺，座骨神経有髄線維の収縮と消失がみられた．本試験でのNOAELはラットで9.1 mg/kg/day，マウスで24.3 mg/kg/dayと判断された．

経口投与簡易生殖毒性試験（OECD TG 421）では，ラットの雌雄に0，12.5，50及び200 mg/kg/dayを強制経口投与した．200 mg/kg/day群で交尾率の減少，分娩遅延，哺育異常，児の低体重，生児数減少がみられた．本試験での生殖毒性のNOAELは50 mg/kg/dayと判断された．

二世代生殖毒性試験では，マウスに0，24，80及び240 ppm（F0：0，4.5，15.4及び49 mg/kg/day，F1：0，6.8，23.8及び71.3 mg/kg/day）が二世代にわたり飲水投与されたが，F0とF1の生殖能に影響はみられなかった．本試験でのNOAELはそれぞれ最高投与量となり，49 mg/kg/day（F0）及び71.3 mg/kg/day（F1）と判断された．これらの結果から生殖毒性のNOAELは49 mg/kg/dayと判断された．

マウスの妊娠6-17日に0，60，120及び180 mg/kg/dayを強制経口投与して発生毒性試験を行った．180 mg/kg/day群で着床後胚死亡の増加，120 mg/kg/day以上の群で胎児体重の低下がみられた．出生児に被験物質投与に起因した外表異常はみられなかった．本試験での発生毒性のNOAELは60 mg/kg/dayと判断された．前述の二世代生殖毒性試験では，全ての被験物質投与群において後肢握力が3週齢の雌雄F1で低下した．しかしながら，この影響は6.8及び23.8 mg/kg/day群ではF1が成長するに従い有意差はなくなった．これらの結果から発生毒性のNOAELは6.8 mg/kg/day未満と判断された．

細菌を用いた復帰突然変異試験ではS9 mix存在及び非存在下のいずれでも陰性であった．優性致死試験でも陰性であった．

6-tert-Butyl-m-cresol（88-60-8）（原案作成：ICCA日本企業）

本化学物質は日本では主にポリマーやゴムに添加する酸化防止剤の中間体である．

ラットの単回経口投与毒性試験（OECD TG 401）でのLD_{50}は320-800 mg/kg（雄），130-320 mg/kg（雌）であった．毒性症状として自発運動の低下，腹臥位あるいは側臥位姿勢，下腹部などの被毛の汚染が認められた．マウスを用いた試験では，経口LD_{50}は580 mg/kg（雄），740 mg/kg（雌）であった．毒性症状として，自発運動の低下，運動失調，四肢麻痺と過呼吸／呼吸困難が認められた．また，マウスの経皮LD_{50}は1,200 mg/kgであった．

ウサギの皮膚と眼に強い刺激性が認められた．

反復投与毒性・生殖発生毒性併合試験（OECD TG 422）では，ラットの雌雄に交配前2週間及び交配期間，さらに，雄では交配期間終了後2週間，雌では妊娠期間及び分娩後哺育3日まで，0，2.5，12.5及び60 mg/kg/dayを強制経口投与した．60 mg/kg/day群において体重増加抑制・摂餌量の減少（雌），小葉中心性肝細胞肥大（雌雄）がみられた．反復投与毒性のNOAELは12.5 mg/kg/dayと判断された．また，60 mg/kg/day群において母動物の体重増加抑制や黄体数，着床数のわずかな減少が認められ，生児数減少や児体重増加の抑制がみられたことから，生殖発生毒性のNOAELは12.5 mg/kg/dayと判断された．被験物質投与に起因した児の形態異常はみられなかった．

細菌を用いた復帰突然変異試験ではS9 mix存在及び非存在下で陰性であった．チャイニーズ・ハムスター培養細胞を用いた染色体異常試験では，連続処理及びS9 mix非存在下の短時間処理で陰性であったが，S9 mix存在下の短時間処理では染色体異常の誘発作用が認められたことから，染色体異常試験は陽性と判断された．しかしながら，*in vivo*でのマウスの小核試験は投与可能な最高用量において陰性であったことから，本化学物質は*in vivo*では遺伝毒性を発現しないと判断された．

2-Hydroxy-2-naphthoic acid（92-70-6）（日本政府及びドイツ政府作成）

本化学物質は染料や色素などの中間体である．

ラットの単回経口投与毒性試験（OECD TG 401）でのLD_{50}は823-1,040 mg/kgであった．毒性症状として自発運動の低下，呼吸亢進，閉眼，下痢が認められた．モルモットの皮膚に24時間塗布した試験では約2,000 mg/kgで死亡がみられた．

ウサギの皮膚に対しては弱い刺激性が認められ，モルモットの皮膚では壊死や皮下出血がみられた．ウサギの眼に対しては強い刺激性が認められた．また，皮膚感作

性が疑われた．

28日間反復経口投与毒性試験（OECD TG 407）では，ラットの雌雄に0，12，60及び300 mg/kg/dayを強制経口投与した．60及び300 mg/kg/day群の雌の副腎に壊死がみられた．雄では300 mg/kg/day群において血清中無機リンのレベルが低下し，血清及び尿中ビリルビンが上昇し，雌ではさらに肝重量の増加がみられた．NOAELは60 mg/kg/day（雄）及び12 mg/kg/day（雌）であった．また，ラットへの反復吸入投与毒性試験で300 mg/m^{3}を10日間暴露させたところ腎臓に壊死が認められた．

一世代生殖毒性試験（OECD TG 415）では，ラットの雄に交配前10週から剖検前日まで（98日間），雌に交配前2週間から哺育20日まで，0，12.5，50及び200 mg/kg/dayを強制経口投与した．200 mg/kg/day群で児体重低下，成長遅延，外表異常がみられた．生殖発生毒性のNOAELは50 mg/kg/day，雄の一般毒性のNOAELは12.5 mg/kg/day（50 mg/kg/dayでの前胃の病変），雌の一般毒性のNOAELは50 mg/kg/day（200 mg/kg/dayでの体重増加量の減少と前胃の病変）と判断された．

細菌を用いた復帰突然変異試験ではS9 mix存在及び非存在下で陰性であった．チャイニーズ・ハムスター培養細胞を用いた染色体異常試験では，S9 mix存在下の短時間処理で陰性であったが，連続処理及びS9 mix非存在下の短時間処理では染色体異常の誘発作用が認められたことから，染色体異常試験は陽性と判断された．*In vivo*における変異原性については今後の検討が必要（FW）とされた．

Triacetin（102-76-1）（原案作成：ICCA日本企業）

本化学物質はタバコフィルターの可塑剤，硝酸セルロース，セルロイド製品の溶剤，写真フィルム，化粧品の防カビ剤，食品添加物などとして使用される．また，間接食品添加物（接着コーティング用）としてFDAの承認を得ている．

*In vitro*で反転させたラットの腸管とともにインキュベートすると直ちにグリセロールと酢酸に加水分解された．犬に静脈内注射した場合，血管内で加水分解され，生じた酢酸の大部分が各組織で酸化分解された．

経口及び吸入による急性毒性は低い．ラットの単回経口投与毒性試験（OECD TG 401）では，最高用量の2,000 mg/kgでも死亡はみられず，LD_{50}は2,000 mg/kg以上と判断された．ウサギとモルモットの経皮LD_{50}も2,000 mg/kg以上であった．ラットの単回吸入投与毒性試験（OECD TG 403）のLC_{50}は1,721 mg/m^{3}以上であった．

ウサギの皮膚と目に対して刺激性は認められず，モルモットにおいて皮膚感作性は認められなかった．ヒトでも皮膚刺激性や皮膚感作性はみられなかったが，タバコ工場でのアレルギー性接触性湿疹に関する報告が1件ある．

反復投与毒性・生殖発生毒性併合試験（OECD TG 422）では，ラットの雄には交配前2週から44日間，雌には交配前2週から分娩後哺育3日まで，0，40，200及び1,000 mg/kg/dayを強制経口投与した．最高用量の1,000 mg/kg/dayでも毒性影響がみられず，反復投与毒性及び生殖発生毒性のNOAELはそれぞれ1,000 mg/kg/dayと判断された．

ラットへの反復吸入投与毒性試験において249 ppm（2,220 mg/m^3）を90日間暴露したところ毒性影響はみられず，NOAELは249 ppm（2,220 mg/m^3）と判断された．

細菌を用いた復帰突然変異試験ではS9 mix存在及び非存在下で陰性であった．チャイニーズ・ハムスター培養細胞を用いた染色体異常試験では，S9 mix存在下の最高用量で染色体異常がみられたが，本化学物質添加後の低pH（4.9）によると考えられた．これらの結果から本化学物質は遺伝毒性を発現しないと判断された．

Cyclohexene（110-83-8）（日本政府作成）

本化学物質はシクロヘキサノール，L-リジンの原料，特殊溶剤，シクロヘキセンオキサイド等各種有機合成原料として使用されている．

*In vitro*試験においてアリル位での酸化が示されているが，*in vivo*における代謝・動態に関する報告はない．

経口，経皮及び吸入による急性毒性は低い．ラットの単回経口投与毒性試験（OECD TG 401）による，LD_{50}は1,000-2,000 mg/kgであった．モルモットの経皮LD_{50}は16,220 mg/kg以上であった．単回吸入投与毒性試験において21,388 mg/m^{3}でもラットの死亡は認められなかった．

反復投与毒性・生殖発生毒性併合試験（OECD TG 422）では，ラットの雄には交配前2週から48日間，雌には交配前2週から分娩後哺育4日まで，0，50，150及び500 mg/kg/dayを強制経口投与した．150 mg/kg/day以上の雌雄で一過性の流涎が認められた．150 mg/kg/day以上の雄で血清中性脂肪の低値，500 mg/kg/dayの雄で総ビリルビンの高値，150 mg/kg/day以上の雌雄で総胆汁酸の高値が認められた．反復投与毒性のNOAELは50 mg/kg/dayと判断された．生殖発生に関する影響は最高用量の500 mg/kg/dayで認められず，生殖発生毒性のNOAELは500 mg/kg/dayと判断された．

細菌またはほ乳類細胞を用いた遺伝毒性試験ではS9 mix存在及び非存在下で陰性であった．

2-Naphthol（135-19-3）（日本政府及びドイツ政府作成）

本化学物質は合成ゴム工業における抗酸化剤の製造原料をはじめとして，医薬品，染料，香料などの原料として広く利用されている．

肝臓と腎臓におけるグルクロニド抱合及び硫酸抱合によって急速に除去される．

ラットの単回経口投与毒性試験（OECD TG 401）でのLD_{50}は1,320 mg/kgであった．毒性症状として自発運動の低下，呼吸促進，閉眼，鼻汁，下痢が認められた．ラットの単回吸入投与毒性試験（OECD TG 403）のLC_{50}は2,200 mg/m^{3}と判断された．毒性症状として自発運動の低下，運動障害，反射障害，鼻汁，角膜混濁，下痢が認められた．

ウサギの皮膚に対して刺激性は認められなかったが，眼に対しては強い刺激性が認められた．モルモットの皮膚で感作性が認められた．

ラットの28日間反復経口投与毒性試験（OECD TG 407）において，50 mg/kg/day以上の群の雌雄において副腎重量の増加がみられた．150 mg/kg/day群の雄で腎臓影響を示し，血清中クレアチニンの増加や電解質の変化がみられた．

イヌとラットの皮下及び吸入による反復投与毒性試験において肝臓と腎臓への影響がみられ，1.35及び10.1 mg/m^{3}群で血液凝固障害及び肝臓と腎臓の病理組織学的影響を伴う機能障害が報告されている．

一世代生殖毒性試験（OECD TG 415）では，ラットの雄に交配前10週から剖検前日まで（98日間），雌に交配前2週間から哺育20日まで，0，10，40及び160 mg/kg/dayを強制経口投与した．親世代の生殖能力への影響や児の形態異常は認められなかった．160 mg/kg/day群で哺育不良と児体重低下，生存数減少がみられたことから，生殖発生毒性のNOELは40 mg/kg/dayと判断された．雄の一般毒性のLOELは最低投与量の10 mg/kg/day（流涎），雌の一般毒性のNOELは10 mg/kg/day（40 mg/kg/dayでの流涎，鼻汁，自発運動低下，摂餌量減少）と判断された．

細菌を用いた数種の復帰突然変異試験ではS9 mix存在及び非存在下で陰性であった．

Hydrochloric acid（7647-01-0）（原案作成：ICCA日本企業）

本化学物質は化学工業における無機塩類の製造原料をはじめとして，肥料製造や醸造などの原料として広く利用されている．

雌ラットの単回経口投与毒性試験でのLD_{50}は238-277 mg/kgであり，単回吸入投与毒性試験のLC_{50}は，ラットで23.7-60.9 mg/L/5 min，5.7-7.0 mg/L/30 min，4.2-4.7 mg/L/60 min，マウスで20.9 mg/L/5 min，3.9 mg/L/30 min，1.7 mg/L/30 minであった．

ウサギの皮膚と目に対し濃度や接触時間に比例して「弱い」～「強い」刺激性が認められている．

ラット及びマウスの90日間反復吸入投与毒性試験において0，10，20及び50 ppm（0，15，30及び75 mg/m^3）を暴露した．鼻や口唇に対する局所刺激性は10 ppmで認められたが，両動物における全身毒性のNOAELは20 ppm（50 ppmでの体重増加量の減少など）と判断された．

生殖発生毒性について信頼性の高い試験報告はないが，本化学物質は水素イオンとクロルイオンから成り，これらは体液の構成要素であることから，低濃度の塩酸のガスや溶液は悪影響を及ぼさないと考えられる．実際，胃腺は塩酸を分泌し，また，pHが同様に変化する硫酸の経口投与でも実験動物において発生毒性は認められていない．これらの所見から，発生毒性のないことが示唆される．生殖毒性については，上述の90日間反復吸入投与毒性試験において最高用量の50 ppmまで生殖器への影響はみられなかった．

細菌を用いた復帰突然変異試験ではS9 mix存在及び非存在下で陰性であった．チャイニーズ・ハムスター培養細胞を用いた染色体異常試験では，低pHによると考えられる染色体異常がみられた．

雄ラットに10 ppmを128週間吸入させた試験では，鼻における腫瘍性の病変は認められなかった．吸入・経口・経皮投与毒性試験でも発がん性は示されていない．ヒトでの症例対照研究において，被験化学物質への暴露と腫瘍発生との因果関係は認められなかった．

C.I. Pigment Yellow 53（8007-18-9）（原案作成：ICCA日本企業）

本化学物質は，プラスチック，セラミックス，建材，コート剤の着色剤として利用されている．

ラットの単回経口投与毒性試験（OECD TG 401）では，最高用量の2,000 mg/kgでも死亡はみられず，LD_{50}は2,000 mg/kg以上と判断された．

ウサギの皮膚と目に対して弱い刺激性が認められている．

ラットの90日間反復混餌投与毒性試験（最高用量450 mg/kg/day）と反復投与毒性・生殖発生毒性併合試験（OECD TG 422，最高用量1,000 mg/kg/day）では，毒性影響は示されていない．OECD TG 422の試験に基づき，反復投与毒性及び生殖発生毒性のNOAELはそれぞれ1,000 mg/kg/dayと判断された．また，ラットの反復吸入投与毒性試験（60 mg/m^{3}に1日6時間，5日間）において毒性影響は認められていない．

細菌またはほ乳類細胞を用いた遺伝毒性試験ではS9 mix存在及び非存在下で陰性であった．

Calcium chloride（10043-52-4）（原案作成：ICCA日本企業）

本化学物質は氷結防止・道路安定・ダスト防除・コンクリート凝固などの用途や，食品添加物・薬品などとして使用される．

水中ではカルシウムイオンとクロルイオンに速やかに解離し，体内において別々に吸収・分布・排泄が行われる．

単回経口投与毒性は低く，マウスのLD_{50}は1,940-2,045 mg/kg，ラットでは3,798-4,179 mg/kg，ウサギでは500-1,000 mg/kgであった．単回経皮毒性も低く，ウサギの経皮LD_{50}は5,000 mg/kg以上と判断された．

ウサギの眼に対して強い刺激性が認められたが，皮膚に対しては弱い刺激性しか認められていない．しかしながら，塗布時間が長く高濃度溶液の場合には強い皮膚刺激性を示し，本化学物質またはその高濃度溶液への接触事故によりヒトの皮膚への損傷が認められている．

カルシウムイオンとクロルイオンは必須栄養素であり，各々1,000 mg/kg以上が1日の摂取量として推奨されている．OECD TG 414に準じた発生毒性試験において189 mg/kg/day（マウス），176 mg/kg/day（ラット），69 mg/kg/day（ウサギ）まで毒性影響は認められていない．

細菌またはほ乳類細胞を用いた遺伝毒性試験ではS9 mix存在及び非存在下で陰性であった．

Tetrahydromethyl-1,3-isobenzofuranedione（11070-44-3）（原案作成：ICCA日本企業）

本化学物質は主にエポキシ樹脂硬化剤として使用される．

代謝と動態についての動物試験は行われていないが，ヒトが吸入した場合は代謝によってジカルボン酸となり尿中に排泄されることが知られている．尿中濃度の半減時間は3-6時間であった．

ラットの単回経口投与毒性試験（OECD TG 401）では，最高用量の2,000 mg/kgでも死亡はみられず，LD_{50}は2,000 mg/kg以上であった．毒性症状として前胃の粘膜肥厚・扁平上皮過形成・肉芽性炎などの変化が認められている．

ウサギの皮膚には中程度の刺激性が認められ，眼に対しても刺激性が認められた．ヒトの疫学的調査により感作性が疑われている．

反復投与毒性・生殖発生毒性併合試験（OECD TG 422）では，ラットの雌雄に交配前2週間，その後さらに，雄では交配期間を含む35日間，雌では交配期間，妊娠期間及び分娩後哺育3日まで，0，30，100及び300 mg/kg/dayを強制経口投与した．300 mg/kg/day群において，雄に一過性の流涎，腎臓の相対重量の増加がみられ，雌雄に前胃の粘膜肥厚・扁平上皮過形成・肉芽性炎がみられた．反復投与毒性のNOAELは100 mg/kg/dayと判断された．また，生殖発生毒性に対する影響は認められず，生殖発生毒性のNOAELは300 mg/kg/day（最高用量）と判断された．

細菌またはほ乳類細胞を用いた遺伝毒性試験ではS9 mix存在及び非存在下で陰性であった．

Diisopropylbenzene（25321-09-9）（日本政府作成）

本化学物質はガソリンやディーゼル等の炭化水素燃料に混入される．また，Diisopropylbenzeneperoxideの合成に使われる．

単回投与毒性試験での異性体混合物の経口LD_{50}はラットで5,850 mg/kg，経皮LD_{50}はウサギで14,400 mg/kgであった．単回吸入投与毒性試験においてラットで4時間，マウスで2時間暴露した場合，5,300 mg/m^{3}以下の用量では死亡が認められなかった．

28日間反復経口投与毒性試験（OECD TG 407）では，ラットの雌雄に0，6，30，150及び750 mg/kg/dayを強制経口投与した．投与期間の後半に150 mg/kg/day以上の雌雄で散瞳がみられた．雄では150 mg/kg/day以上，雌では750 mg/kg/dayの群で肝の小葉中心性肝細胞肥大が認められた．これらの結果から反復投与毒性のNOAELは30 mg/kg/dayと判断された．

経口投与簡易生殖毒性試験（OECD TG 421）では，ラットの雌雄に，交配前2週間，その後さらに，雄では交配期間を含む36-38日間，雌では交配期間，妊娠期間及び分娩後哺育3日まで，0，6，30，150及び750 mg/kg/dayを強制経口投与した．生殖発生毒性に関する影響は認められず，生殖発生毒性のNOAELは750 mg/kg/day（最高用量）と判断された．

細菌またはほ乳類細胞を用いた遺伝毒性試験ではS9 mix存在及び非存在下で陰性であった．

C.I. Pigment Brown 24（68186-90-3）（原案作成：ICCA日本企業）

本化学物質は，プラスチック，セラミックス，建材，コート剤の着色剤として利用されている．毒性学的プロファイルは類似構造をもつC.I. Pigment Yellow 53（上述）と本質的に類似している．

ラットの単回経口投与毒性試験では，最高用量の10,000 mg/kgでも毒性影響がみられず，LD_{50}は10,000 mg/kg以上と判断された．

ウサギの皮膚に対して弱い刺激性が認められる．

ラットの90日間反復混餌投与毒性試験（最高用量500 mg/kg/day）において毒性影響はみられず，反復投与毒性のNOAELは500 mg/kg/dayと判断された．

細菌またはほ乳類細胞を用いた遺伝毒性試験ではS9

mix存在及び非存在下で陰性であった.

おわりに

本稿では，SIAM15で合意された化学物質名及び日本担当の12物質の初期評価の健康影響部分について紹介した．SIAMで合意された物質については，初期評価文書が出版されたのち，インターネットのOECD webサイト（http://cs3-hq.oecd.org/scripts/hpv/）より報告書の入手が可能である.

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Lack of potential of low dose *N*-nitrosodimethylamine to induce preneoplastic lesions, glutathione *S*-transferase placental form-positive foci, in rat liver

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Abstract

Induction of liver lesions in male F344 rats by the genotoxic and carcinogenic *N*-nitrosodimethylamine (NDMA) was studied at a wide range of dose levels, i.e. from 0.001 to 10 ppm, in drinking water for 16 weeks. Dose related and statistically significant increase of glutathione *S*-transferase placental form-positive foci, endpoint markers for hepatocarcinogenesis in rats, at 1 and 10 ppm dose groups was obtained, but no increment in foci could be detected with the lower doses (0.001, 0.01, and 0.1 ppm). This finding of a no-observed effect level supports our hypothesis that a threshold, at least in practical terms, exists in carcinogenesis proposed on the basis of extensive wide range dose-dependence studies of other genotoxic carcinogens.

Keywords: *N*-nitrosodimethylamine; Risk assessment; Carcinogenicity dose threshold

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1. Introduction

Chemical carcinogens are generally classified into two categories, genotoxic and non-genotoxic. Concerning cancer risk assessment, it is considered that genotoxic carcinogens exert carcinogenic potential regardless of the animal species, so that chemicals which are carcinogenic to rodents are carcinogenic to humans as well. Because genotoxic carcinogens are mutagenic and seem to act through interaction with DNA to produce irreversible genetic changes in target organ cells, it has been generally concluded that they have no dose threshold in their carcinogenic potential [1,2]. Therefore, there is widespread acceptance of a linear curve extending to zero at very low doses for chemicals found to be carcinogenic with in vivo carcinogenicity tests. However, while there are data supporting the non-threshold theory [3–5], and we recently provided evidence of thresholds for the hepatocarcinogenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and *N*-nitrosodiethylamine (NDEA) in rats [6,7]. Williams et al. [8,9] also earlier reported the existence of similar thresholds for NDEA and 2-acetylaminofluorene hepatocarcinogenicity.

N-nitrosodimethylamine (NDMA), an *N*-nitroso compound, is well established as a hepatocarcinogen in rodents. Humans are exposed to NDMA from occupational and environmental sources and through in vivo formation of ingested precursor amines and nitrosating agents [10]. In particular its endogenous formation from ingested precursors has been indicated to be a major source of exposure. Previously Peto et al. [5] reported non-threshold of NDMN hepatocarcinogenicity based on statistical analysis of results from a long-term carcinogenicity test at low doses in 4080 rats.

Recently in vivo medium-term bioassays for carcinogens have become accepted as alternatives to long-term carcinogenicity tests. Particularly, the liver medium-term bioassay has been developed as useful for detecting hepatocarcinogenicity of chemicals [11]. Recently we found a 21-day-old rat, a medium-term model to be very useful for assessment of low dose carcinogenicity of hepatocarcinogens such as MeIQx and NDEA because of high sensitivity [6]. In this medium-term bioassay, the animal treatment duration was 16 weeks and glutathione *S*-transferase placental form (GST-P)-positive foci, established preneoplastic lesions in the livers of rats [11,12], were taken as end-point markers of carcinogenicity.

In the present study, we examined low dose carcinogenicity of NDMA in the rat liver from viewpoint of 'weight of evidence' for clarification of human risk assessment of genotoxic carcinogens. For this purpose we employed the same experimental protocol with which MeIQx and NDEA were earlier examined for low dose carcinogenicity [6].

2. Materials and methods

2.1. Animals and chemicals

A total of 540 male 20-day-old F344 rats were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan) and housed in rooms maintained on a 12 h light/dark cycle, at constant temperature and humidity, and observed daily. Numbers of the rats employed in the present study were decided on the basis results of previous, low dose carcinogenicity studies [6,7]. NDMA (purity >99%) was purchased from Sakai Research Laboratory (Fukui, Japan).

2.2. Experimental procedures

The experiment was started when the animals were aged 21 days. They received NDMA at doses of 0 (group 1, a control, 90 rats), 0.001 (group 2, 89 rats), 0.01 (group 3, 89 rats), 0.1 (group 4, 90 rats), 1 (group 5, 91 rats), or 10 ppm (group 6, 91 rats), in drinking water for 16 weeks. The lowest level, 0.001 ppm, was established with reference to daily exposure of humans to this carcinogen [10]. The animals had free access to Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) throughout the experiment and were killed at the end of week 16 under ether anesthesia for examination of lesion development.

Ten percent phosphate-buffered formalin-fixed liver tissues (a total of 9 slices per animal, 3 each from the left lateral, medial, and right lateral lobes) were embedded in paraffin wax for immunohistochemical examination of GST-P-positive foci in the liver, as described previously [6]. Hepatocellular foci comprising of two and more cells were counted under a light microscope. They were categorized as

Table 1
Final average body, absolute and relative liver weights, and average total NDMA intakes

Groups	NDMA doses (ppm)	No. of rats	Final body weights (g)	Liver		Total NDMA intake (mg/rat)
				Absolute (g)	Relative (%)	
1	0	90	327±15[a]	9.6±0.9	3.0±0.2	0
2	0.001	89	325±17	9.6±0.7	2.9±0.2	0.00151
3	0.01	89	325±16	9.5±0.7	2.9±0.2	0.0145
4	0.1	90	327±18	9.5±0.8	2.9±0.1	0.1505
5	1	91	326±19	9.9±1.0	3.0±0.2	1.5117
6	10	91	315±19	9.1±0.9	2.9±0.2	15.0680

[a] Values are mean±SD.

having a total of 11 and more cells. Total areas of livers were measured using a color image processor (IPAP, Sumica Technos, Osaka, Japan) and the numbers of foci per cm^2 of liver tissue were calculated.

2.3. Statistical analysis

Statistical analysis of the data was performed using the StatView-J 5.0 program (Abacus Concepts, Inc., Berkeley, CA). Differences from control values were evaluated for significance with the Dunnett two-tailed post hoc test.

3. Results

3.1. General findings

All the rats survived in good condition until the scheduled sacrifice. No macroscopic lesions were apparent in any organ including the liver. No adverse effects on average body weight gain were observed in rats treated with NDMA at any of the doses (Table 1). Absolute liver weights were not increased in the groups given NDMA and relative liver weights did not differ among the groups. Average total NDMA intake in each group was dose-dependent.

3.2. Induction of GST-P-positive foci in the liver

In livers of rats treated with NDMA, total numbers of GST-P-positive foci per unit area in the groups receiving 0.001 to 0.1 ppm of the carcinogen did not differ from the control value (non-treatment group, Table 2 and Fig. 1), in contrast to the significant increase observed in rats treated with the 1 and 10 ppm doses. In fact, total values in groups treated with NDMA at a dose of 0.01 ppm showed a slight decrease as compared to the control value. Moreover, numbers of GST-P-positive foci comprising ≥11 cells in the groups given 0.001–0.1 ppm NDMA were not different from the control values, while these values in rats treated with 1 ppm NDMA, and particularly 10 ppm NDMA, were significantly increased.

Table 2
The development of GST-P-positive foci in the livers of rats treated with NDMA at various doses

Groups	NDMA doses (ppm)	No. of rats	Size distribution of GST-P-positive foci (no./cm^2)	
			Total	≥11 cells
1	0	90	0.375±0.545[a]	0.012±0.066
2	0.001	89	0.366±0.586	0.018±0.077
3	0.01	89	0.276±0.582	0.011±0.056
4	0.1	90	0.377±0.519	0.025±0.074
5	1	91	1.905±2.399*	0.117±0.200*
6	10	91	24.875±13.267*	11.063±6.986*

*$P<0.01$ (vs. group 1).
[a] Values are mean±SD.

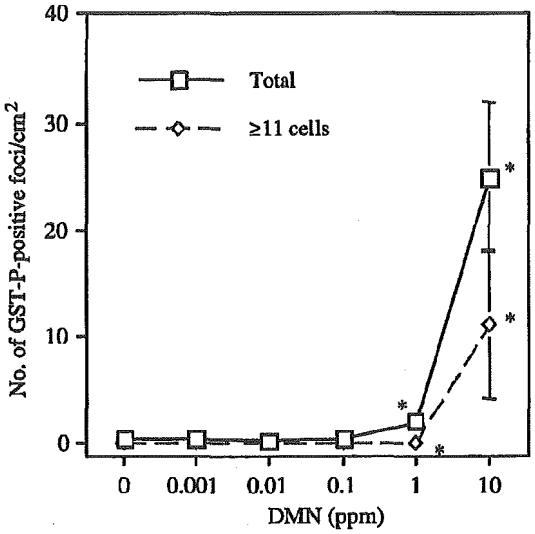

Fig. 1. Induction of GST-P-positive foci in the liver of rats treated with NDMA. *$P < 0.01$ (vs. group 1). Numbers of rats are shown in Table 1. Bars, SD.

4. Discussion

Previously Peto et al. [5] examined the carcinogenicity of NDMA or NDEA at low doses (the so-called ED01 study) and found no indication of any threshold for liver tumor induction of rats. They speculated that NDMA or NDEA at 0.1 ppm in drinking water cause about 2.5% of animals to develop liver tumors and therefore a dose of 0.01 ppm would yield a 0.25% incidence. However, the issue of true and practical thresholds for carcinogenicity has attracted increasing interest [13] and recently Waddell [14] reanalyzed data of the rat carcinogenicity study using NDEA at low doses. His speculation pointed to the existence of a threshold for NDEA carcinogenicity in the liver and esophagus. Recently Williams et al. [9] also suggested the existence of threshold for NDEA hepatocarcinogenicity in rats. In the present study, a dose related and statistically significant increase of GST-P-positive foci in the liver, established endpoint markers for hepatocarcinogenesis in rats [11,12], was obtained with the 1 and 10 ppm doses of NDMN, but the lower doses (0.001, 0.01, and 0.1 ppm) did not cause significant increment in the foci, in line with thresholds found for MeIQx and NDEA previously [6].

Recently we documented that MeIQx and NDEA do not induce GST-P-positive foci in rat liver at very low doses [6]. Moreover, formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), the most abundant species of adduct associated with oxidative stress, also demonstrated a no-observed effect level. We also reported that the curve for induction of aberrant crypt foci, preneoplastic markers in the colon of rats by 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine (PhIP) is not linear down to zero [15]. Similarly, no-response levels were evident for both PhIP–DNA adducts and 8-OHdG formation. The present study also clearly indicates that the curve for induction of GST-P-positive foci in the liver is not linear down to zero. Taking all the evidence together, we conclude that genotoxic carcinogens have a threshold, at least in practical terms, for their carcinogenicity.

The question of whether there is a threshold for chemical carcinogenesis, particularly with genotoxic agents is clearly controversial in risk assessment and the non-threshold theory continues to hold sway in the regulatory area for carcinogenic toxicology. However, the findings for a threshold in the genotoxicity of MeIQx [16,17] and the evidence of practical thresholds for genotoxic carcinogenicity from recent in vivo studies including the present result [6–9,15] indicates that this area requires more attention and careful consideration.

In conclusion, the present finding of no-observed effect level on induction of GST-P-positive foci supports our hypothesis that a threshold, at least in practical terms, exists with regard carcinogenesis due to genotoxic agents, from our extensive wide range dose-dependence studies of a variety of carcinogens.

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Review Article

High susceptibility of human c-Ha-ras proto-oncogene transgenic rats to carcinogenesis: A cancer-prone animal model

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Transgenic animals carrying human c-Ha-ras proto-oncogene, v-Ha-ras transgenic mice, pim-1 transgenic mice and several knockout mice deficient of tumor suppressor genes, such as p53, have been shown to exhibit increased carcinogen susceptibility. As a result, studies into practical application and medium-term screening of environmental carcinogens are under way. Given the advantages of rat models characterized by larger organ size, abundant information regarding preneoplasias and virus-free constitution, we have concentrated on the generation of transgenic rats bearing copies of the human c-Ha-ras proto-oncogene and shown the Hras128 strain to be extremely sensitive to the induction of mammary carcinomas, and to a lesser extent, lesions in the urinary bladder, esophagus and skin. In most, if not all, the mammary cancers mutations of the transgene but not the endogenous H-ras gene are present, appearing to occur early in the process of tumorigenesis, which involves proliferation of cells in TEB and intraductal hyperplasia before carcinomas arise. Preliminary findings suggest that this is independent of endogenous ovarian hormones, although inhibited by soy isoflavones and promoted by atrazine and nonylphenols. Although further studies of the mechanisms are clearly necessary, the model appears to have great potential for screening purposes, not only for modifiers active in the breast, but also other organs where tumors characterized by ras gene mutations develop. (*Cancer Sci* 2005; 96: 309–316)

Transgenic mice carrying the human c-Ha-ras proto-oncogene,[1–3] v-Ha-ras transgenic mice,[4,5] pim-1 transgenic mice[6,7] and several knockout strains of mice deficient in tumor suppressor genes such as p53 have been shown to exhibit increased carcinogen susceptibility. Therefore, there is a great deal of interest in their practical application for medium-term screening of environmental carcinogens, for example with rasH2 and Tg.AC mice.

For studies of chemical carcinogenesis, however, rats rather than mice are generally more frequently used for various reasons. For example, in addition to the benefits accruing with size, abundant information is available regarding biological characteristics of preneoplastic lesions that can be used as endpoint lesions appropriate as surrogate markers for cancer development.[8–11] Furthermore, tumors of the mammary glands and other organs can be induced without the complication of a possible viral etiology, which is not the case with mice. However, only limited types of transgenic rats have so far been developed for studying carcinogenesis. In the majority of established models, the transgene is under the control of an SV40 T antigen gene such as the probasin/SV40 T antigen gene for the prostate,[12,13] the albumin-SV40 T antigen gene for the liver,[8,14,15] and the phosphoenolpyruvate carboxykinase (PEPCK)-SV40 T antigen gene for pancreas islet cells.[16] This is clearly not optimal. In oncogene transgenic rats, the c-erbB-2 and TGFα-MMTV[17] and Neu proto-oncogene[18] have been applied for the study of mammary carcinogenesis. An example of a transgene related to thymus carcinogenesis is the pX gene encoding a major product of human T lymphocyte virus type I (HTLV-I). Others include the glutathione S-transferase (GST-P) gene[19] for the liver, and the Tsc2 gene[20,21] for the kidney but these are not directly relevant to enhancement of carcinogenesis, unlike the case with tumor oncogenes (Table 1).

We have concentrated attention on the generation of transgenic rats with the same human c-Ha-ras proto-oncogene used for establishment of transgenic mice.[3] As this transgene is under the control of its own promoter region, it is expressed in the whole body, allowing the study of carcinogenesis in different organs. Two rat lines have been found to exhibit very high susceptibility to chemically induced mammary carcinogenesis, with development of multiple carcinomas within an extremely short period.[23,30] Less remarkably, one has also been found to demonstrate increased sensitivity to skin, bladder and esophagus carcinogens.[24–26] Here we report our experience regarding susceptibility of our transgenic rats to chemically induced carcinogenesis, analysis of the mechanisms, and possible application as an animal model for short-term evaluation of carcinogenicity of chemical compounds.

Generation of H-ras transgenic rats

The DNA construct used for transgenic rats has been described in a previous study.[31] For the purpose of genarating the transgenic rata, a 6.8 kb *Bam*HI fragment of the human c-Ha-ras proto-oncogene with its own promoter region eluted from agarose gel was purified and then injected into the pronuclei of rat

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Abbreviations: TEB, terminal end buds; Hras128, human c-Ha-ras proto-oncogene transgenic rat; MNU, *N*-methyl-*N*-nitrosourea; DMBA, 7,12-dimethylbenz[a]anthracene; PhIP, 2-amino-1-methl-6-phenylimidazo[4,5-b]pyridine; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; PCNA, proliferating cell nuclear antigen; NLRR-3, neuronal leucine-rich repeat protein-3; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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Table 1. Transgenic rats generated for carcinogenesis studies

Carcinogenesis	Transgene	Promoter	Strain	Tumor site	Reference
Enhancement/ induction†	SV40	Albumin	SD	Liver‡	Hully *et al.* (1994)[8]
		Probasin	SD	Prostate	Asamoto *et al.* (2001)[12]
		PEPCK	SD	Pancreas islet	Haas *et al.* (1999)[22]
	Human c-Ha-ras¶	Human Hras	SD	Mammary gland‡	Asamoto *et al.* (2000)[23]
				Bladder‡	Ota *et al.* (2000)[24]
				Esophagus‡	Asamoto *et al.* (2002)[25]
				Skin‡	Park *et al.* (2004)[26]
	Neu	MMTV	SD	Mammary gland‡	*Watson et al.* (2002)[18]
	c-erbB2	MMTV	SD	Mammary gland‡	Davies *et al.* (1999)[17]
	TGFα	MMTV	SD	Mammary gland‡	Davies *et al.* (1999)[17]
	px(HTLV-1)	p53lck	F344	Thymus	Kikuchi K (2002)[27]
	HLA-B27	HLA-B27	F344	Colon	Hammer RE (1995)[28]
Inhibition†	Tsc2	Tsc2	Eker	Kidney	Kobayashi *et al.* (1997)[20]
	Rat H-, K-ras¶	Rat H-ras	SD	Mammary gland‡	Thompson *et al.* (2002)[29]
	GST-P	GST-P	Wistar	Liver‡	Nakae *et al.* (1998)[19]

†As compared to wild-type rats; ‡chemically induced tumor; §domionant negative; ¶protooncogene. SD, Sprague-Dawley; PEPCK, phosphoenolpyruvate carbokinase; MMTV, mouse mammary tumor virus; GST-P, glutathione S-transferase.

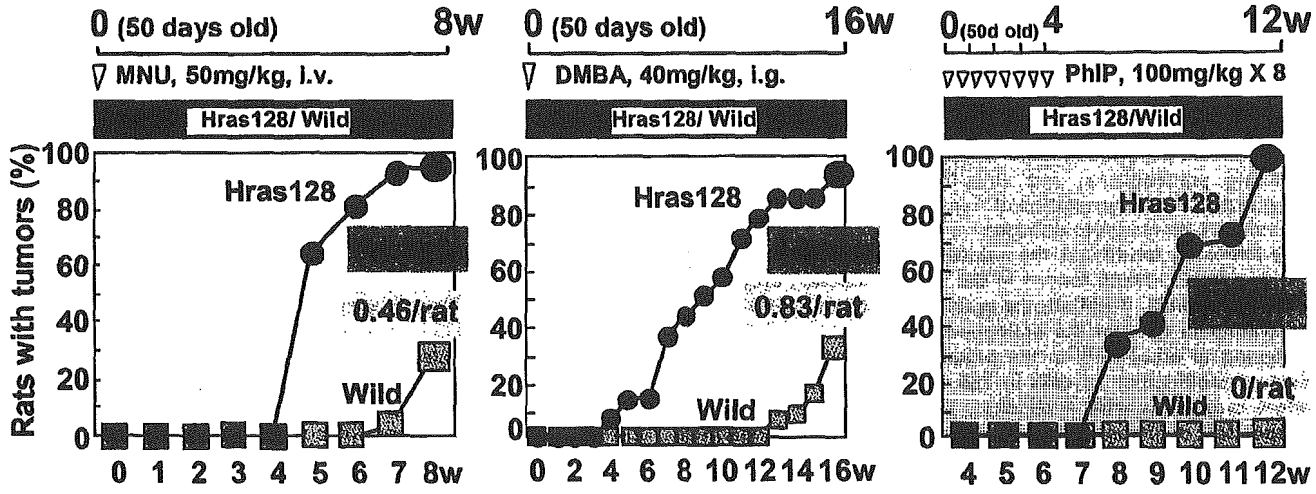

Fig. 1. Rapid development of mammary carcinomas with three different carcinogens, MNU, DMBA and PhIP. Hras128 and its wild-type counterparts were given a single dose of MNU (50 mg/kg, i.v.) or DMBA (40 mg/kg, i.g.) on day 50 after birth, then killed on week 8 or 12, respectively. PhIP (100 mg/kg) was given i.g. eight times over a 4 week-period followed by death on week 12. Multiple mammary carcinomas developed in almost all the transgenic rats, whereas the incidence and multiplicities were far lower in the wild-type rats.

embryos from Sprague-Dawley female rats. Two male progeny were shown to carry the transgene, one having three copies (Hras128) and the other one copy (Hras104).[23] Subsequent matings have been carried out between transgenic males and non-transgenic Sprague-Dawley female rats to maintain the heterozygote transgenic Hras128 strain. Expression of the transgene has been repeatedly detected in all organs by northern blot analysis. The strain is now being maintained and bred by Clea Japan (Tokyo, Japan).

Mammary carcinogenesis

To examine the susceptibility of the transgenic rat to mammary carcinogens, females at 50 days of age were treated with a single dose of N-methyl-N-nitrosourea (MNU) (50 mg/kg, i.v.), 7,12-dimethylbenz[a]anthracene (DMBA) (40 mg/kg, i.g.) or multiple doses (100 mg/kg, i.g., eight times in 4 week-period) of the food contaminant carcinogen, 2-amino-1-methl-6-phenylimidazo[4,5-b]pyridine (PhIP).[23,30,32] Almost all the transgenic rats rapidly developed multiple mammary carcinomas within 8–12 weeks (see Fig. 1). The histological appearance of the tumors was solid, tubular, papillary and, less frequently, undifferentiated and sarcomatous, as previously reported to be typical in rats.[33,34] As it has been established that the mode of DNA modification differs among the three compounds, O^6-methylguanine formation resulting with MNU,[29,35] depurinating adduct formation with DMBA[36] and guanine deletion in the 5′-GGGA-3′ sequence with PhIP,[37] our results indicate that Hras128 rats are highly susceptible to chemically induced mammary carcinogenesis, irrespective of the carcinogen applied.

Mutation of the transduced c-Ha-ras proto-oncogene and demonstration of activated ras in induced mammary tumors

PCR-SSCP and PCR-RFLP analysis and direct sequencing of the transgene indicated the large majority of carcinomas induced with MNU, DMBA and PhIP to contain cells with mutations,

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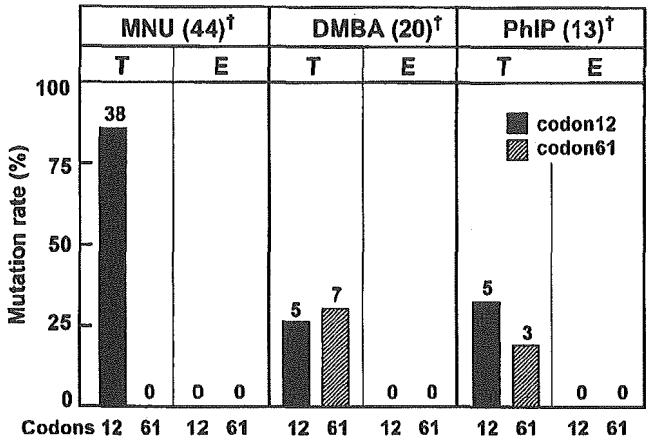

Fig. 2. Data for mutations that occurred in the transgene but not the endogenous Hras gene in all treatment groups. T, transgene; E, endogenous Hras gene; †numbers indicate the number of tumors examined.

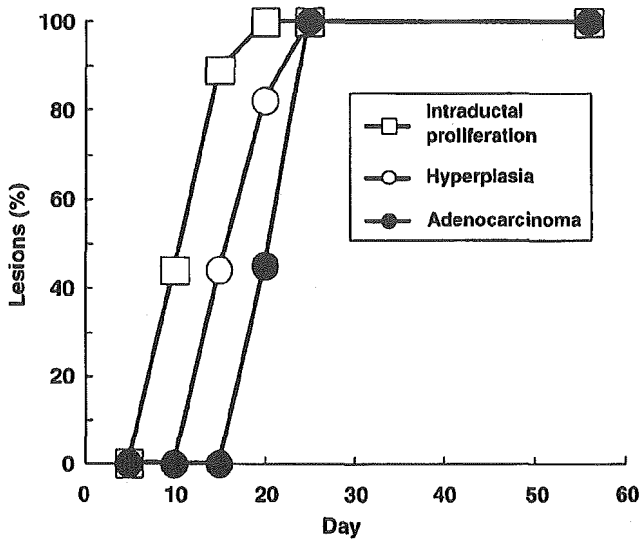

Fig. 3. Results of periodical observation of preneoplastic and carcinoma lesion development in abdominal-inguinal mammary glands after injection of N-methyl-N-nitrosourea (MNU) in Hras128.

many featuring GG → GA at codon 12[23,30] (Fig. 2). Furthermore, activated form ras protein could be detected in carcinomas induced by PhIP.[32] In contrast, no mutations whatsoever were found in the endogenous c-Ha-ras gene in the mammary tumors arising in the Hras128 strain.[23] Therefore, the results indicate that preferential mutation and activation of the transduced human c-Ha-ras proto-oncogene play dominant roles over the endogenous c-Ha-ras gene. It is of interest in this context that treatment with *d*-limonene, an inhibitor of ras protein isoprenylation, clearly inhibited tumor induction.[38]

Our results are in contrast to the observation that transgenic rats with copies of rat H- and K-ras gene showed less carcinoma development than their non-transgenic littermates following MNU exposure, with less transgene mutation than in the endogenous ras gene.[29] The findings indicate that particular mutations of the transgene are important for its functions as a modifier gene in mammary carcinogenesis.

Elevated cell proliferation and mutation of the transgene in TEB cells as early events

The observed transgene mutations as early events in carcinogenesis, periodic observation of mutations and whether proliferative focal lesions were performed in TEB were studied.[39] As TEB cells are precursors for mammary exocrine glands, they are the most likely targets of chemical carcinogens applied before sexual maturation, at 50 days old.[39] We focused on analysis of numbers, proliferative status and presence of any mutations in the transgene.

Number count and proliferative potential of TEB. Counts of TEB in the abdominal mammary glands of 49–91-day-old female Hras128 were compared with those in wild rats. The numbers were significantly greater in Hras128 rats until 81 days after birth. Confocal microscopy further revealed that the level of active protein kinase is clearly elevated in TEB cells. Thus, an increase in number with elevated proliferation activity would appear to play an important role in observed rapid tumor development.[40] As TEB is a precursor tissue for mammary glands, duct and acini, the induced tumors comprised epithelial, stromal and transitional cells. Indeed, a variety of histological patterns, from epithelial to stromal and epithelial-mesenchymal type, was found,[32] in line with observations in wild rats.[41,42]

Alteration of sensitivity to DMBA carcinogenesis during sexual maturation. With a time sequence observation of sensitivity to DMBA administration, made by shifting the application time from 7 to 25 weeks of age, the tumor yield was clearly decreased in line with the evolutionary decrease in the number of TEB.[40]

Early transgene mutations in TEB cells induced by MNU. Transgene mutations in codon 12 could be demonstrated in the laser captured normal-looking TEB from female Hras128 rats as early as 5 days after application of MNU, providing compelling evidence that the TEB is indeed a target of the carcinogen and that transgene mutation is an early event in carcinogenesis.[40]

Rapid development of tumors in abdominal-inguinal mammary glands. Sequential histological observation of the abdominal-inguinal mammary glands, taken as a representative of all six pairs of mammary glands, at different time points after a single injection of MNU showed rapid development of intraductal epithelial proliferation on day 5, atypical hyperplasia on day 15 and adenocarcinoma on day 20, as shown in Fig. 3. The data are clearly of great significance for application as a rapid carcinogen assay model for detection of environmental carcinogens. The postulated sequence of events is depicted in Fig. 4.

Spontaneous tumor development. To determine carcinoma development without exposure to carcinogens, we conducted a study to observe spontaneous lesions in virgin Hras128 rats. The tumor yield was 52.8% at week 40, slightly increased as compared to the value for parent Sprague-Dawley females. As preneoplastic lesions, incidences of intraductal epithelial and acinar cell hyperplasia, with increased PCNA labeling, were two-fold greater than in wild rats at week 10. Similar data were obtained for subsequent atypical hyperplasias. The mRNA and protein levels of cyclins D1 and D2 started to increase from week 17. The results confirmed proliferative features of TEB at early stages followed by duct epithelial and alveolar cell hyperplasia with elevated expression of the c-Ha-ras protooncogene are background lesions for carcinoma development.[40,43] Thus, the transduced human c-Ha-ras proto-oncogene in the TEB is a target of carcinogens and mutations may occur before obvious proliferative changes become evident. Studies of spontaneous carcinogenesis indicated that carcinomas directly arise from the duct and acinar cells, corresponding to human 'ductular' and 'lobular' carcinomas, respectively.

Cloning of a new gene involved in the ras-MAPK pathway

In a search for genes involved in ras gene activation and MAPK transduction, the rat neuronal leucine-rich repeat protein-3

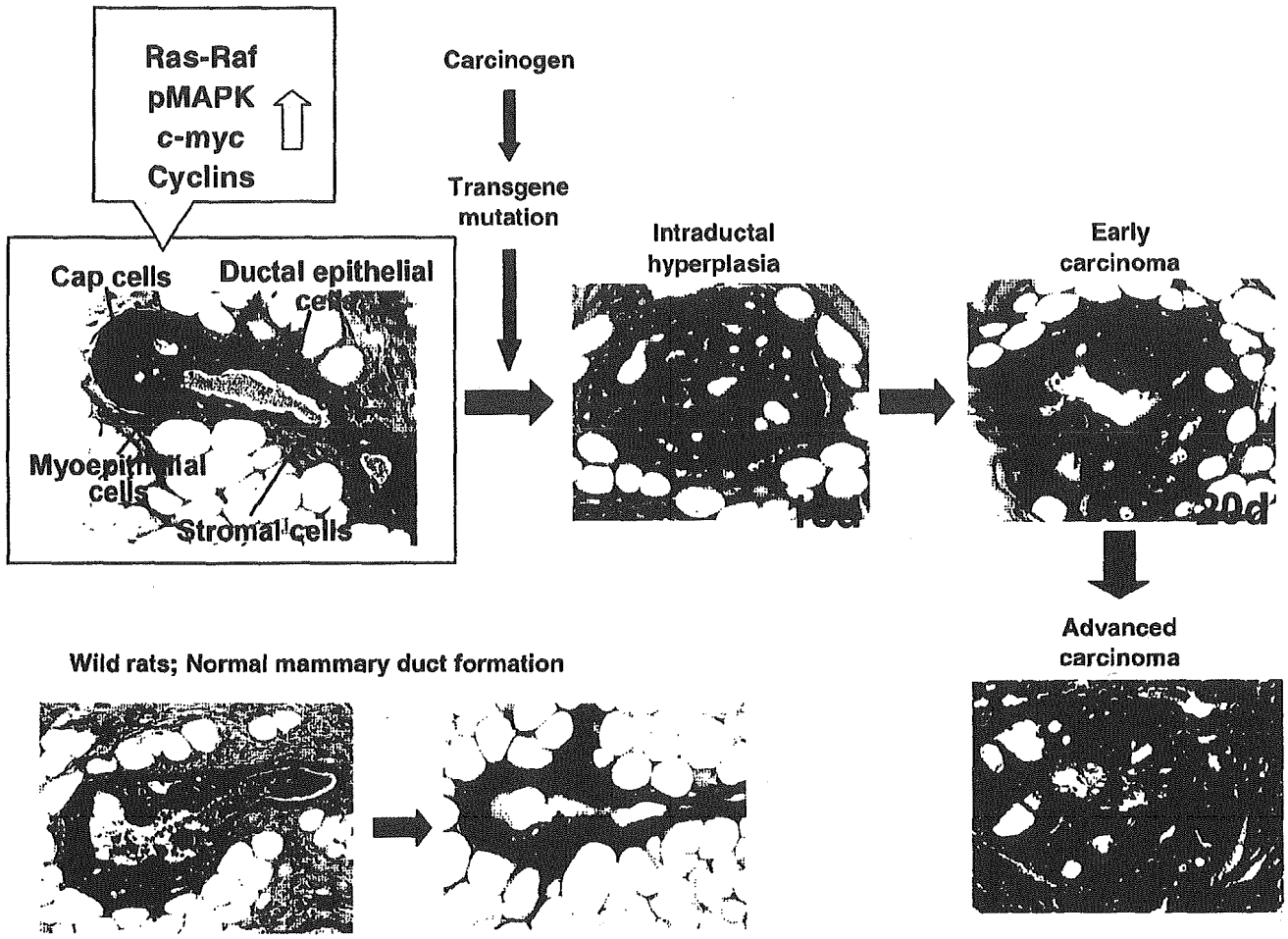

Fig. 4. Schematic presentation of carcinogenesis. Terminal endbuds, targets of carcinogens, have proliferative potential as a result of expression of the mutated transgene. Hyperplastic change occurs rapidly after application of carcinogens. The TEB shown at the lower left indicates normal mammary duct development in wild-type rats.

(NLRR-3) gene was cloned in a sarcoma spontaneously developing in the mammary region of an Hras128 rat. Stable expression of constitutively active forms of Ras (H-RasV12 or v-H-Ras) and treatment of epidermal growth factor (EGF) led to increase in NLRR-3 mRNA expression in normal fibroblasts. The carboxyl-terminal 30-aa region of NLRR-3 is responsible for the amplification of MAPK phosphorylation through association with endocytotic vesicles and NLRR-3 may amplify MAPK activity resulting in growth stimulation/Ras activation in malignant tumors.[44,45]

Modification of mammary carcinogenesis in the Hras128 rat

Effects of ovarian hormones. Effects of ovarian hormones on induction of mammary carcinogenesis were examined by performing ovariectomy before treatment with MNU. Although ovariectomy completely inhibited development of mammary carcinomas in wild counterparts, it did not affect either the incidence or the multiplicity of mammary carcinomas in the Hras128 rats,[38] indicating the high susceptibility to MNU carcinogenesis to be independent of ovarian hormones, including estrogens.

Environmental compounds: Possible application as a medium-term detection model for carcinogenesis modifying agents. After administration of MNU, suppressive effects of soy isoflavones could be clearly shown within 20 days in the Hras128 rat. Numbers of lesions, atypical hyperplasias and adenocarcinomas, in the isoflavone fed group in the post-initiation stage were clearly decreased as compared to the basal diet group (40 vs 100% incidence). The results indicate that this model may be useful for short-term screening for chemopreventive agents for mammary carcinogenesis.[43] To assess this possibility, effects of environmental compounds with estrogenic action, 4-n-octylphenols, atrazine and nonylphenols, were investigated. Female transgenic rats were given a single oral dose of DMBA and thereafter received diets containing one of these compounds. Although 4-*n*-octynophenols proved inactive,[46] atrazine at a dose of 50 p.p.m. and nonylphenol at 10 p.p.m. increased the incidence and multiplicity of adenocarcinomas ($P < 0.05$, by trend analysis).[47] These results suggest that endocrine disruptors may enhance mammary carcinogenesis, although the doses applied were extremely high as compared with feasible environmental human exposure levels, and that our transgenic rat can be used for medium-term assessment of the modification potential of environmental compounds.

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