ことを考えると、ヒトへの健康影響を考えるうえで、今後さらに毒性データの蓄積が必要である。特に食生活は、ヒト発がんの大きな要因の一つであることから、4-OHEのヒト発がんへの関わりに注目したい。

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河井一明、Savela Kirsti、葛西宏：脂質過酸化物 4-oxo-2-hexenal による DNA 損傷とアポトーシス誘導
日本環境変異原学会第35回大会（堺）平成１８年１１月

G. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

特になし

別添 4

厚生労働科学研究費補助金（国際医学協力事業）
分担研究報告書

大腸発がん感受性および抵抗性要因の解明

分担研究者　中釜　斉　国立がんセンター研究所生化学部長

研究要旨
加熱した魚・肉食品中に含まれる変異原性・がん原性物質であるヘテロサイクリックアミン類（HCAs）の中で最も含量が多い 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine(PhIP)により誘発されるラット大腸がんモデルを用いた解析により、RNA-induced silencing complex (RISC)の構成因子の一つで新規の翻訳抑制因子の可能性がある SND1 蛋白質が、大腸発がんの極めて早期の病変である non-dysplastic ACF において発現亢進していることが分かった。SND1 の過剰発現を介する新たなエピジェネティック機構による WNT シグナル経路の活性化が、大腸がんの初期発生過程において重要な役割を果たしている可能性がある。さらに、PhIP への曝露により大腸上皮に発現誘導されるストレス応答性の microRNA の一つである microRNA-X が大腸上皮細胞の増殖抑制に関与することや、その発現誘導の系統差が PhIP による大腸発がんの感受性にも寄与している可能性が示唆された。SND1 や microRNA-X の発現誘導の分子機構や発現誘導の個体差の機構を明らかにすることにより、ヒト集団における大腸がんの高リスク群の掌握にも資することが期待される。

A. 研究目的

加熱魚肉食品中に含まれる変異原性物質ヘテロサイクリックアミン類(HCAs)の中で最も含量が多い 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine(PhIP)はヒト大腸がんの原因物質である可能性が示唆されている。本研究では、PhIP 誘発ラット大腸発がんモデルを用いて大腸発がんの感受性に寄与する種々の遺伝的要因を解明し、遺伝学的情報に基づいたヒト大腸がん予防策の構築に資する基礎的資料を提供することを目的とする。

B. 研究方法

① 化学発がん物質誘発ラット大腸がんの早期微小病変における翻訳制御因子 SND1 の発現：ヒト大腸がんにおいて過剰発現が既に報告されていた SND1 蛋白質の発現について、PhIP 大腸発がん過程の種々の初期病変を用いて抗 SND1 抗体による免疫組織染色を行った。
② Snd1 過剰発現細胞の樹立と増殖能の検討：ラット小腸上皮由来の IEC-6 細胞を用いて、マウス Snd1 を安定に過剰発現している細胞株を樹立し、その増殖能を調べた。
③ Snd1 の過剰発現による細胞接着関連分子の発現変化の検討:細胞間接着に関与する Apc 蛋白質の発現量を western blotting 法により検討した。Snd1 の一過性の発現誘導による影響についても併せて検討した。
④ Snd1 発現誘導の分子機構に関する検討：PhIP 誘発大腸発がんにおける Snd1 過剰発現の分子機構について新たな知見を得るため、RKO 大腸がん細胞株を PhIP-OAc に曝露させ、Snd1 蛋白質の発現誘導様式について検討した p53 蛋白質の発現についても併せて調べた。
⑤ PhIP 投与によるストレス応答性 microRNA 種の発現誘導：F344 ラットに PhIP 含有飼料を投与した際に、ラット大腸上皮に発現誘導される microRNA-X の発現誘導を real-time RT-PCR 法により調べた。
⑥ PhIP 誘導性のストレス応答性 microRNA-X による細胞増殖抑制効果の検討：2 種類の大腸がん細胞株（HCT116 及び RKO 細胞）にストエス応答性 microRNA-X を導入し、in vitro 及び in vivo での増殖及び造腫瘍性に対する抑制効果を検討した。
⑦ ストレス応答性 microRNA の発現誘導のラット系統差：PhIP による大腸発がん性の異なるラット系統間におけるストレス応答性 microRNA の発がん誘導の違いを検討した。

C. 研究結果

① 異常腺窩(ACF)における Snd1 の発現亢進：Snd1 蛋白質は PhIP 及び AOM 誘発ラット大腸がんにおいて過剰発現していた（データは示さず)。大腸発がん過程における Snd1 過剰発現の時期を調べるため、初期微小病変である ACF における発現を免疫組織染色法により検討した。AOM 及び PhIP 誘発の dysplastic ACF

において高率に過剰発現していることが分かった（図 1）。β-catenin の細胞質内蓄積が極く軽微な病変においても、Snd1 の過剰発現が認められた（図 1 右下）。興味深いことに、non-dysplastic ACF においても約半数の病変で既に Snd1 の過剰発現が認められた(表 1)。

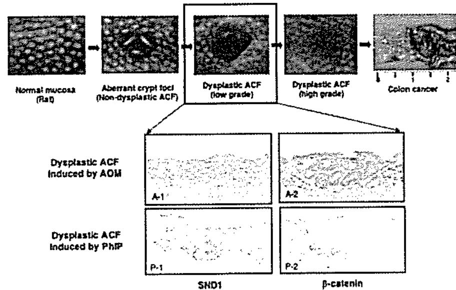

図 1 Dysplastic ACF における Snd1 の過剰発現

Types of lesion	No. of lesions analyzed	Cytoplasmic β-catenin accumulation		SND1 accumulation
Dysplastic ACF	6	positive	4	4
		(-)	2	2
Non-dysplastic ACF	12	positive	2	2
		(-)	10	5

表 1 PhIP 誘発大腸微小病変における Snd1 の発現

② Snd1 過剰発現細胞における増殖能亢進：ラット IEC-6 細胞において Snd1 を恒常的に過剰発現している 5 種類のクローンを樹立した。いずれの細胞クローンも細胞増殖能が亢進していた。特に、細胞-細胞間の接着形成後も細胞は増殖を続けることから、contact inhibition の消失が示唆された（図 2）。

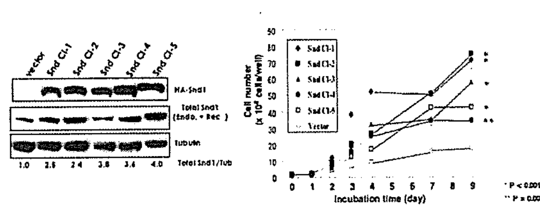

図 2　Snd1 過剰発現 IEC-6 細胞の増殖曲線

③ Snd1 の過剰発現による Apc 蛋白質の発現低下：Snd1 過剰発現 IEC-6 細胞クローンのいずれにおいても、Apc 蛋白質の発現低下が認められた（図 3A）。Snd1 蛋白質を一過性に過剰発現させた場合にも同様に Apc 蛋白質の発現低下が認められた（図 3B）。この時、Apc 遺伝子の mRNA 発現量には有意な変化を認めないことから（図 3C）、Apc 蛋白質の発現低下は post- transcriptional な分子機構による発現制御を受けていることが示唆された(図 3D)。

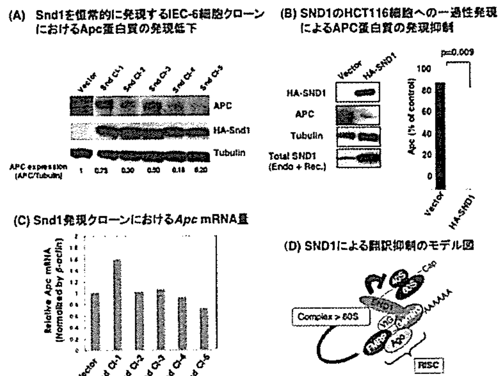

図 3　Snd1 過剰発現による Apc 蛋白質の発現抑制

④ PhIP-OAc 処理した RKO 大腸がん細胞株での SND1 発現誘導：PhIP の活性化体である PhIP-OAc（5, 50mM）で RKO 大腸がん細胞株を処理すると、処理後 24 時間後において SND1 蛋白質の顕著な発現誘導が認められた(図 4)。p53 蛋白質の発現誘導も同様に観察された。

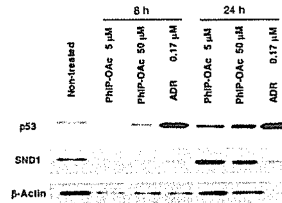

図 4 RKO 細胞株の PhIP-OAc 処理による SND1 の発現誘導

⑤ PhIP 投与によるストレス応答性 microRNA 種の発現誘導：PhIP400ppm 含有飼料を F344 ラットに投与し、投与開始後 1 週及び 2 週目に大腸上皮における microRNA の発現誘導を調べた。PhIP 投与開始後 1 週目において、ストレス応答性 microRNA の一つとして我々が同定した microRNA-X の発現が PhIP 非投与群に比較して 3~4 倍に上昇することが分かった（図 5）。この傾向は PhIP 投与開始後 2 週目でも認められた。

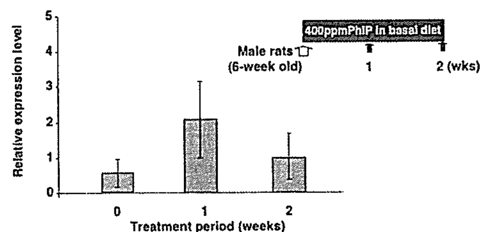

図 5 PhIP によるストレス応答性 microRNA-X の発現誘導

⑥ PhIP 誘導性のストレス応答性 microRNA-X による細胞増殖抑制効果の検討：PhIP への曝露により大腸上皮に発現誘導される micro-RNA-X を 2 種類のヒト大腸がん細胞株(HCT116, RKO)に導入すると、細胞増殖が顕著に抑制されることが分かった（図 6)。これらの大腸がん細胞株をヌードマウスに移植して得られた腫瘍に対しても同様に増殖抑制効果を示すことが分かった（データは示さず)。これらの結果から、PhIP に曝露されたラット個体では、ストレス応答性 microRNA の発現誘導を介して、大腸上皮細胞の強い増殖抑制が働くことが示唆された。この現象が PhIP 誘発大腸発がんに対するバリアー機構として作用している可能性がある。実際、PhIP による大腸発がんにおいて抵抗性を示す ACI ラット系統では、PhIP による microRNA-X の発現誘導が F344 系統に比較して有意かつ顕著に高いことが分かった（図 7)。

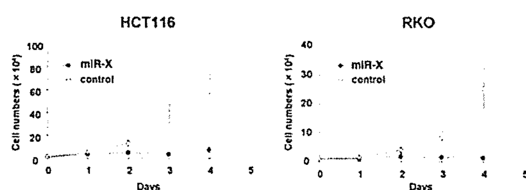

図 6 ストレス応答性 microRNA-X による大腸がん細胞株の増殖抑制作用

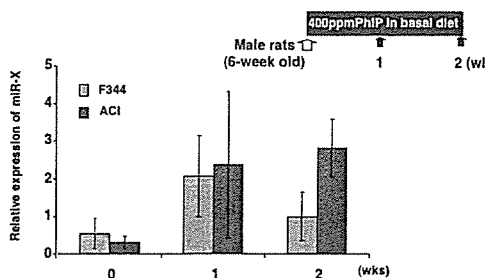

図 7 PhIP による microRNA-X 発現誘導のラット系統差

D. 考察

RNA-induced silencing complex (RISC)の構成因子の一つで、新規の翻訳抑制因子の可能性がある SND1 蛋白質が、大腸発がんの極めて早期の病変である non-dysplastic ACF において発現亢進していることから、SND1 の過剰発現を介した新たなエピジェネティック機構による WNT シグナル経路の活性化が、大腸発がんの初期発生過程において重要な役割を果たしている可能性がある。また、PhIP への曝露により大腸上皮に発現誘導されるストレス応答性 microRNA の発現誘導の差異が、PhIP による大腸発がんの感受性に寄与している可能性がある。

E. 結論

本研究成果により、*Apc* 遺伝子や *β-catenin* 遺伝子変異の有無に依存せず、翻訳抑制因子 SND1 の発現異常により WNT シグナルの活性化が起こり得るという全く新規の分子機構が、ラットだけでは無くヒト大腸がんの初期発生に重要な役割を果たしている可能性がある。SND1 発現誘導の分子機構及び発現誘導の程度の個体差の機構を明らかにすることにより、大腸発がんの高リスク群の掌握にも資することが期待される。

F. 健康危惧情報

（特になし）

G. 研究発表

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H. 知的財産権の出願・登録状況

1． ~~特許取得~~・特許出願
「マイクロ RNA を有効成分として含有する腫瘍増殖抑制剤、および癌治療用医薬組成物」（中釜斉、田澤大、土屋直人）（特願 2007-50908）

2． 実用新案登録（特になし）

3． その他（特になし）

別添 4

厚生労働科学研究費補助金（国際医学協力研究事業）
分担研究報告書

酸化的DNA傷害修復遺伝子MYHの新規多型と胃がん症例対照研究における評価

分担研究者　相村春彦　浜松医科大学第一病理

研究要旨　DNA修復遺伝子MYHの上流に、新たな遺伝子多型を見いだし大腸癌の症例対照で環境因子を考慮したロジスティック解析をおこなうとリスク上昇との関連を見いだした。また、肺癌の早期病変とされる腺腫様過形成における染色体数の異常をパラフィンブロックで解析することを可能にし、早期からの染色体異常を同定した。また、これらの病変と喫煙歴との関連はなかった。

A．研究目的

環境要因を考慮した、がん感受性と遺伝子多型、さらに、早期病変におけるゲノム変化との関連を解析する。

B．研究方法

喫煙歴などの情報のある、マッチさせた症例対照DNAを用いて、遺伝子多型との関連を調整ロジスティック解析をおこなってリスク形で算出する。また、病理保存標本におけるFISH法の感度を上昇させる方法を開発し、その方法で、5　mmmまでの小病変や早期癌病変の染色体変化を検出する。

（倫理面への配慮）

症例対照研究においては、疫学チームとともに当該施設の倫理委員会の審査承認を得ており、また、病理標本を使用する際の個人情報の保護についても留意している。

C．研究結果

DNA 修復酵素MYHに新たな多型を発見し、その多型のvariantで、散発性大腸癌のリスクの有意な上昇をみた。また、肺癌の前癌病変とされる小病変の一部で染色体数の増加がすでにおこっていること、喫煙などとの関連がみられないことをしめした。

D．考察

MYHは非密生型のポリポーシスの原因遺伝子としてもその多型（変異）がみとめられているが、本邦をふくむアジア諸国での同様の変化がいまのところ検出されておらず、かわりにいくつかの多型がみいだされている。

そのなかで、上流部の新規多型が大腸癌のリスクであることをしめし、その機序や、普遍性が関心をもたれる。肺癌など頻度の高い腫瘍での前癌病変の染色体変化と環境要因、遺伝要因との相関が興味深い。

E．結論

本邦にあらたにみられた修復遺伝子多型が、大腸癌のリスクになる可能性をしめし、病理ブロックを用いた微少病変の染色体異常の検索に道を開いた。

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H．知的財産権の出願・登録状況
（予定を含む。）

パラフィン切片保存シート
特願　2006-59424、(2006.3.6、PS06-1915)

別添 4

厚生労働科学研究費補助金（国際医学協力研究事業）
分担研究報告書

沖縄産薬草のNO合成酵素に及ぼす影響

分担研究者　安仁屋洋子　　琉球大学大学院医学研究科教授

研究要旨
沖縄産薬草ベニバナボロギクの肝薬物代謝酵素への作用を検討し、シトクロムP450の1A2と2E1の活性及び発現を選択的に抑制することを明らかにした。

A．研究目的
昨年、ベニバナボロギク（BB）熱水抽出物は劇症肝炎モデルラットでTNF-α、iNOS、COX-2を抑制し、肝保護作用を有することを明らかにしたが、その際薬物代謝酵素活性を抑制することが示唆されていた。今回、BBの薬物代謝酵素のシトクロムP-450（CYP）への作用を明らかにする目的でin vitroおよびin vivoで検討した。

B．研究方法
ベニバナボロギクは熱水抽出液の凍結乾燥品を用いた。無処置または各種P450誘導剤を投与したラットの肝ミクロソームにinvitroでBB抽出液を作用させ、P450活性を測定した。また、無処置および3-methylcholanthreneを投与したラットにBB 100mg/kgまたはイソクロゲン酸40mg/kgを2日間腹腔内投与し、経時的に肝ミクロソームのP-450活性、蛋白ならびにmRNAの発現量を測定した。
（倫理面への配慮）
動物実験は琉球大学動物実験委員会の許可を得て行った。

C．研究結果
BB熱水抽出液をラット肝ミクロソームに作用させると、特にCYP2E1および1A2活性の阻害が見られた。BB100mg/kgをラットに投与した場合、CYP 1A1、3A1/2、2B1 および2C活性に変化は見られなかったが、CYP2E1および1A2活性は有意に低下し、蛋白の発現量も低下していた。BBの20mg/kg投与でもCYP2E1および1A2の活性抑制が見られたが、BBの抗酸化成分として単離されたイソクロゲン酸投与ではこれらのCYP活性の低下は見られなかった。これより、BBは選択的にCYP2E1と1A2を抑制し、この抑制作用にはイソクロゲン酸はほとんど寄与してないことが示唆された。

D．考察
沖縄産食用・薬用素材であり強い抗酸化作用を有するベニバナボロギク熱水抽出液が薬物代謝酵素の特にCYP1A2、2E1を抑制することが確認された。CYP1A2、2E1は医薬品のみならず多くの発がん物質の代謝的活性化に関与している。化学発がんは発がん物質の代謝過程で生じた活性酸素、フリーラジカルがDNAの変異を起こすことが大きな要因であり、これらを消去したり、産生を抑制する物質はがん化学予防となりうる。これまでに共同研究でBBの抗酸化作用、ラットの大腸がん予防効果が確認されており、今回得られたBBのCYP1A2、2E1抑制作用は抗酸化作用に加え、BBがん原物質の代謝的活性化を抑制することによりがん化学予防に寄与する可能性を示唆している。

E．結論
ベニバナボロギクの熱水抽出液は薬物代謝酵素のCYP1A2 ならびに2E1を選択的に抑制することが確認された。このことからベニバナボロギクが抗酸化作用に加え、この代謝酵素抑制作用により、がん化学予防に寄与する可能性が示唆された。

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H．知的財産権の出願・登録状況
（予定を含む。）
1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

別添 5

研究成果の刊行に関する一覧表レイアウト（参考）

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別添 5

Genes and Environment, Vol. 28, No. 3 pp. 108-119 (2006)

Regular article

Mutagenicity and Levels of 2-Phenylbenzotriazole (PBTA)-type Mutagens in Sewage Effluent, River Water, Sediment and Drinking Water Collected from the Yodo River System, Japan

Takeshi Ohe[1,6], Tomoko Mizuno[1], Tamie Morisawa[1], Sayo Kiritani[1], Shinobu Suzuki[1], Hitomi Takehana[1], Satoko Kasetani[1], Tetsushi Watanabe[2], Haruo Nukaya[3], Tatsushi Shiozawa[4], Yoshiyasu Terao[4] and Keiji Wakabayashi[5]

[1]Department of Food and Nutrition, Kyoto Women's University, Kyoto, Japan
[2]Department of Public Health, Kyoto Pharmaceutical University, Kyoto, Japan
[3]School of Pharmaceutical Science, University of Shizuoka, Shizuoka, Japan
[4]Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan
[5]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo, Japan

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In order to assess the potential hazards to human health and aquatic ecosystem, we examined the mutagenic activity of sewage effluents, river waters, sediments and drinking water collected from the Yodo River system, Japan. We also compared the levels of mutagenic activity with the levels of 2-phenylbenzotriazole (PBTA)-type mutagens formed from corresponding dinitrophenylazo dyes *via* reduction and subsequent chlorination. We assessed mutagenicity in the *O*-acetyltransferase-overexpressing frameshift strain YG1024 of Salmonella with S9 mix. Sixty-six samples among 133 adsorbates (50%) obtained by the blue rayon hanging method collected from 1996 to 2005 were classified as extreme mutagenicity with more than 100,000 revertants per g blue rayon equivalent (BRE). The average mutagenicity of both sewage effluents and river waters at sites located below sewage plants was 382,400 revertants per g BRE ($n=86$), which was 4.4 times as higher than the downstream river waters (87,900 revertants per g BRE, $n=47$). PBTA-1 was detected in 33 samples among 76 (43%), and PBTA-2 was detected in 66 samples among 76 (87%), however, the concentration of these compounds fluctuated widely among the samples. Average concentrations of PBTA-1 and PBTA-2 were also much higher in sewage effluents and river waters at sites located below sewage plants ($n=50$, PBTA-1, 24.1 ng/g BRE; PBTA-2, 88.4 ng/g BRE) than they were in downstream river water samples ($n=26$, PBTA-1; 1.3 ng/g BRE, PBTA-2; 19.7 ng/g BRE). PBTA-1 and PBTA-2 accounted for 6% and 26% on average, respectively, of the total mutagenicity in all samples analyzed. Based on the concentrations of the PBTA-type mutagens and the effluent volume discharged from three sewage plants, we estimated that ~5 kg/year of PBTA-type mutagens including PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-6, PBTA-7 and PBTA-8, were discharged from three sewage plants into rivers. Further studies showed that these PBTA-type mutagens in river water might not easily accumulate in the river sediment and these PBTA-type mutagens were not detected in drinking water. In the final study, we monitored quantitatively the mutagenic potency of water samples collected at twelve sites, including six sites mentioned above, from the Yodo River system using Sep-Pak C18 cartridge columns and Blue-Chitin columns. The average mutagenic activities recovered by these columns were 10,000 and 5,800 revertants/L. These findings demonstrate that the Yodo River system has been continually and heavily polluted with not only polycyclic planar mutagens, but also by a wide range of chemical mutagens released from sewage plants located along the tributaries for many years.

Key words: *Salmonella typhimurium* YG1024, 2-phenylbenzotriazole (PBTA)-type mutagen, sewage effluent, river water, the Yodo River system

Introduction

A wide range of genotoxic/mutagenic compounds are released from industrial, agricultural and domestic sources into surface waters such as rivers, lakes and the sea. Mutagenicity/genotoxicity tests of complex mixtures such as surface waters using variety of bioassays demonstrate that these environmental mixtures contain many unidentified and unregulated toxicants that may

[6]Correspondence to: Takeshi Ohe, Kyoto Women's University, 35 Kitahiyoshi-cho, Imakumano, Higashiyama-ku, Kyoto 605-8501, Japan. Tel: +81-75-531-7124, Fax: +81-75-531-7170, E-mail: ooe@kyoto-wu.ac.jp

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be carcinogenic and pose a health risk of unknown magnitude (1–4). Analyses of surface waters are necessary to identify areas potentially contaminated by genotoxic compounds from the different sources (5–12). Although many attempts to identify the chemicals responsible for the mutagenicity/genotoxicity of river waters have been reported, only a limited number of new mutagens have been identified. Increased efforts are needed to identify and characterize the likely vast array of unidentified mutagens in surface waters using bioassay-directed chemical analysis. Such work would help to clarify the post-emission transport and fate of these identified toxicants for risks of adverse effects to humans and indigenous biota.

In 1997 and 1998, we identified the chemical structures of two novel potent mutagens with a 2-phenylbenzotriazole structure that accounted for 21% and 17%, respectively, of the total mutagenicity of the adsorbates collected at sites below a sewage plant in the Yodo River system, Japan, by blue cotton hanging method developed by Sakamoto and Hayatsu (12). These two mutagens were confirmed to be 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)-amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), and 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and they were further synthesized from the corresponding dinitrophenylazo-type dyes, which were used for industrial purposes, by reduction with sodium hydrosulfite followed by chlorination with sodium hypochlorite mainly used in sewage plants for disinfection purposes (13–15). We also demonstrated that a major source of PBTA-1 and PBTA-2 in the Yodo River system was effluents from sewage plants and that discharged mutagens were diluted and/or decomposed while moving down the river (16). It was further suggested that other PBTA-type compounds besides PBTA-1 and PBTA-2 might also contribute to the mutagenicity of the river water samples, because various kinds of dinitrophenylazo-type dyes were used as industrial materials. Between 2000 and 2002, six PBTA-type compounds, i.e. 2-[2-(acetylamino)-4-[(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3), 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4), 2-[4-[bis(2-acetoxyethyl)-amino]-2-(acetylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-5), 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6), 2-[2-(acetylamino)-4-(diethylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-7) and 2-[2-(acetylamino)-4-(diallylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-8) were confirmed and were suggested to be formed from the corresponding dinitrophenylazo dyes *via* reduction with sodium hydrosulfite and subsequent chlorination with sodium hypochlorite (17–20). In fact, these PBTA-type compounds with the exclusion PBTA-5 were detected in concentrates collected at sites downstream from municipal sewage plants or textile-dying factories along several rivers flowing in geographically different areas in Japan (16–23). The Yodo River system is an especially important one in Japan, serving as the main source of drinking water for more than 17 million people living in the Osaka area (24).

In the first part of this paper we demonstrate the mutagenicity and levels of PBTA-1 and PBTA-2 in sewage effluents and river waters collected from the Yodo River system between 1996 and 2005. Subsequently, we estimated the concentrations of eight kinds of PBTA-type mutagens discharged from sewage plants into the Yodo River system. To make a comprehensive survey of the occurrence of PBTA-type mutagens in surface waters, we also surveyed the level of mutagenicity and PBTA-type mutagens in river sediments and drinking water.

In this report, we used blue rayon to collect organic pollutants in water samples such as sewage effluents, river water and drinking water. Blue rayon is a solid matrix with covalently linked copper phthalocyanine trisulfonate that can selectively adsorb polycyclic planar molecules with three or more fused rings and enables easy monitoring of water pollution with these chemicals by hanging the rayon in a plastic net in the flowing river for one day or packing blue rayon into glass column. Furthermore, Blue-Chitin columns allow highly efficient quantitative concentration of polycyclics in river water (25–28). On the other hand, adsorption on Amberlite XAD resins and Silica C18 is the most commonly applied method for concentrating organic substances from different kinds of water samples in aquatic environment. It is known that they can generally adsorb a broad class of organic substances, including polycyclic aromatic hydrocarbons, arylamines, nitro-compounds, quinolines, anthraquinones and so on (29). In the latter part of this paper we report here the comparative analysis of mutagenicity monitoring of the Yodo River system, using two different solid matrixes, one with Silica C18 (Sep-Pak C18 cartridges) and the other with covalently linked phthalocyanine trisulfonate (Blue-Chitin columns).

Materials and Methods

Materials: Eight PBTA-type mutagens (PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-5, PBTA-6, PBTA-7 and PBTA-8) shown in Fig. 1 were synthesized according to methods reported previously (13–15, 17–20). Blue rayon and Blue-Chitin column were obtained from

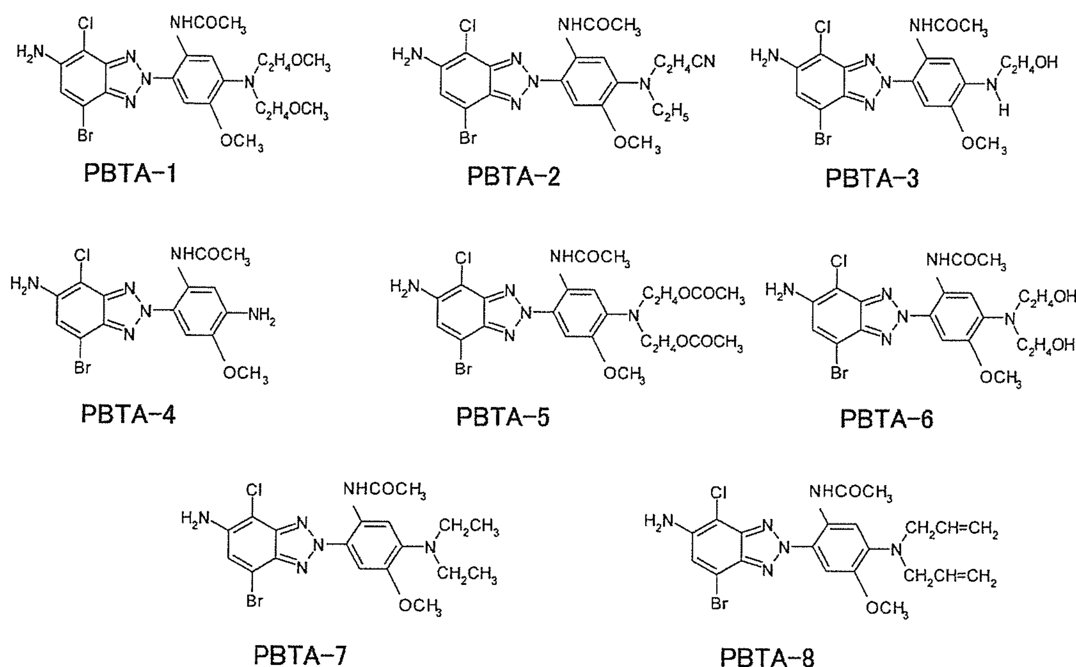

Fig. 1. Structure of 2-phenylbenzotriazole (PBTA)-type mutagens.

Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan), and Sep-Pak C18 cartridges were obtained from Waters Co. Ltd. (Milford, Ireland). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile, 2-aminoanthracene and 3-amino-1-methyl-5*H*-pyrido[4, 3-*b*]indole (Trp-P-2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were of analytical-reagent grade.

Sample collection and extraction procedures: Sample collection and extraction procedure for sewage effluents, river waters, sediments and drinking waters are shown in Table 1. As shown in Fig. 2, the Yodo River system is mainly composed of the Yodo River (Y) and tributaries of the Uji River (U), Katsura River (KA), Nishitakase River (N), and the Kizu River (KI). The annual mean flow rate of the Yodo River system was ca. 240 m^3/s in 1995 (30). Sampling collection sites are also shown in Fig. 2. All extracts of samples prepared by the following method were stored at −20°C until assayed for mutagenicity and characterized for the levels of PBTA-type mutagens.

Sewage effluents and river waters—Blue rayon hanging method (Exp. 1): Water samples were collected at six sites: two outlets of the sewage plants (Site U-1 and KA-1), two river waters at sites located below sewage plants (Site N-1 and Site N-2), and their two downstream river waters (Site KA-2; 5 km downstream from SP 2 and Y-2; 10 km downstream from Site KA-2). At each point, 5 g of blue rayon in a meshed plastic bag was hung for 24 h between 1996 and 2005 according to the method by Sakamoto and Hayatsu (12). After hanging, blue rayon was washed with distilled water several times. The materials adsorbed to blue rayon were extracted by shaking in 200 mL of methanol/ammonia water (50:1, v/v) or methanol for 20 min three times. The eluates were combined and were evaporated to dryness under reduced pressure, and the residues were dissolved in 2 mL of 75% methanol to prepare a sample to be subjected to the mutagenicity assay and for quantification of PBTA-1 and PBTA-2.

Sewage effluents and river waters—Blue rayon column method (Exp. 2): The blue rayon column method was used for the estimation of PBTA-type mutagen levels discharged from sewage plants into river water, according to the method described previously. Ten liter of water samples was collected at two outlets (Sites U-1 and KA-1) of two sewage plants SP 2 and SP 3, treating effluents from the textile dyeing plants, and at Site N-2 receiving effluents from two sewage plants, SP 1 and SP 2. Each water sample was passed through a glass column (25 mm × 300 mm) packed with 2 g of blue rayon at the flow rate of 30 mL/min at room temperature. After passing the water sample through the column, the blue rayon was washed with distilled water several times and excess water was absorbed onto paper towels. Adsorbed materials were then extracted by shaking the blue rayon in 100, 100, and 50 mL respectively, of methanol three times for 20 min each, because it was suggested that bis(2-acetoxyethyl)amino group of PBTA-5 was hydrolyzed to the bis(2-hydroxyethyl)amino group in alkaline solution (19). The extracts were

Table 1. Sample collection and extraction method for water environmental samples in the Yodo River system

Experiment No.	Sample	Sampling period	Sampling site*	Collection and extraction method
Exp. 1	Sewage effluent and river water	March 1996–October 2005	U-1, KA-1, KA-2, N-1, N-2 and Y-2	Blue rayon hanging method
Exp. 2	Sewage effluent and river water	November and December 1999, and December 2001	U-1, KA-1 and N-2	Blue rayon column method
Exp. 3	Sediment	March and September 2002	U-1 and Y-2	Ultrasonication
Exp. 4	Drinking water	April and July 2002	Y-6	Blue rayon column method
Exp. 5	Sewage effluent and river water	June, July and October 2004, and May, July, October and December 2005	U-1, U-2, KA-1, KA-2, N-1, N-2, KI-1, Y-1, Y-2, Y-3, Y-4 and Y-5	Sep-Pak C18 cartridge column and Blue-Chitin column method

*Sampling sites are shown in Fig. 2.

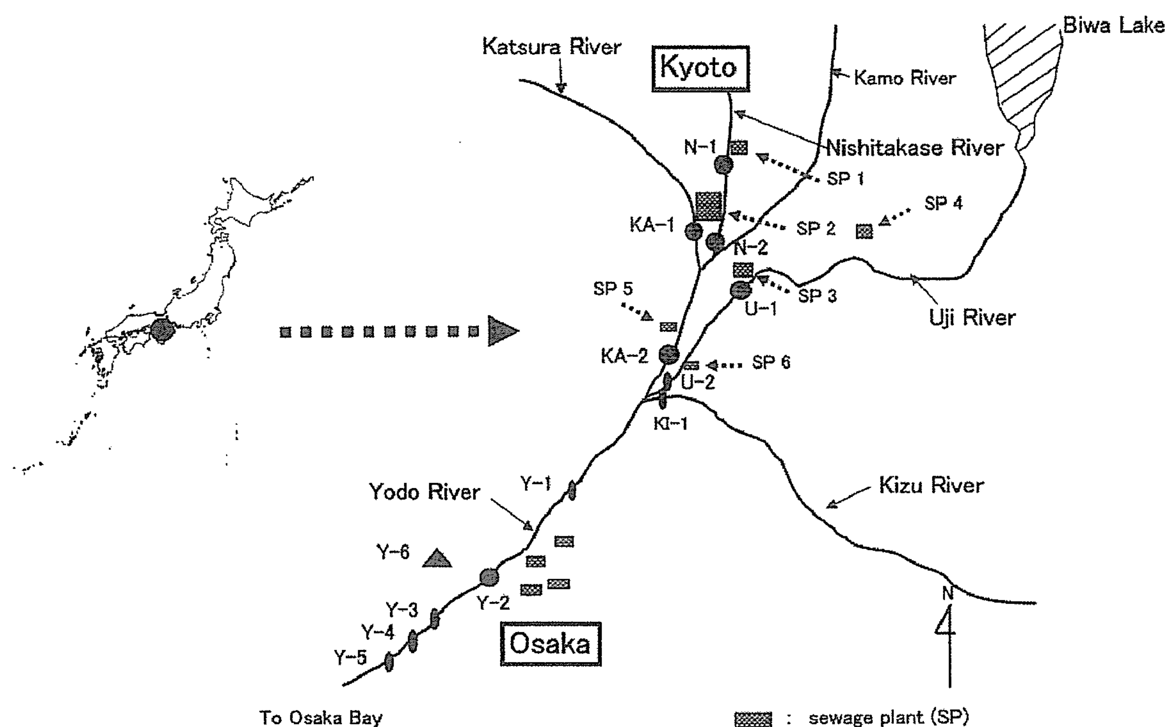

Fig. 2. Sampling locations in the Yodo River system. SP 1–6 shows the place of sewage plants, which their discharges flow into the Nishitakase, Katsura and Uji Rivers. Treatment capacities are: SP 1, 114; SP 2, 1,047; SP 3, 155; SP 4, 140; SP 5, 199 and SP 6,132 $\times 10^3$ m^3 per day.

combined and evaporated to dryness, and the residue was dissolved in 2 mL of 75% methanol. A portion of the extract equivalent to 7 L was used for the purification and determination of eight kinds of PBTA-type mutagens and another portion equivalent to 3 L was evaporated to dryness and the residue was dissolved in dimethylsulfoxide for Salmonella mutagenicity assay.

River sediments (Exp. 3): River sediments were collected at Sites U-1 and Y-2 in the Yodo River system on May 31 and September 6, 2002. The sediments were spread on paper towel and allowed to stand to dryness for 3 days at room temperature in the dark. The dried sediments were screened through a 60-mesh sieve (250 μm) to remove large gravel and trash. The sieved sediment (30 g, dry basis) was extracted with each 300 ml of methanol or a mixture of methanol/benzene (1:1) by triplicate ultrasonication. The combined extracts were reduced to dryness under reduced pressure. Condensed sample solutions were used for the determination of PBTA-type mutagens and the Salmonella mutagenicity assay.

Drinking water (Exp. 4): Each 30 L of drinking water was collected in three pre-cleaned polyethylene bottles at Ibaraki City (Site Y-6 in Fig. 2), whose source for drinking water is located close to Site Y-2 of the Yodo River system. Sampling was performed on April

and July 2002, respectively, and each drinking water sample was passed through a glass column (25 mm × 300 mm) packed with 2 g of blue rayon at the flow rate of 30 mL/min at room temperature after de-chlorination. Each blue rayon adsorbate was treated as mentioned above for the Salmonella mutagenicity assay and the determination of PBTA-type mutagens.

Mutagenicity monitoring using Sep-Pak C18 cartridge column and Blue-Chitin column (Exp. 5): Water samples were collected at twelve sites shown in Fig. 2 on 3 June, 8 July and 16 October 2004, and 26 May, 21 July, 14 October and 9 December 2005. Each water sample (3 L) was passed through Sep-Pak C18 cartridge column and Blue-Chitin column at the flow rate of 10 mL/min at room temperature. Both columns were then washed with 20 mL distilled water and eluted with 2 mL of dimethyl sulfoxide. Both dimethyl sulfoxide eluates obtained here were used for mutagenicity assays with *S. typhimurium* YG1024 in the presence of S9 mix.

Salmonella mutagenicity assay—Ames test: The mutagenic activity of the extracts was measured in using *Salmonella typhimurium* YG1024, an *O*-acetyltransferase-overexpressing derivative of the frameshift strain TA98 (31), with metabolic activation according to the method previously reported (32,33). *Salmonella typhimurium* YG1024 was kindly provided by Dr. Takehiko Nohmi from the National Institute of Health Sciences, Tokyo, Japan. The S9 mix contained 25 μL of S9 (25 mg of protein/mL), prepared from livers of male Sprague-Dawley rats intraperitoneally administered phenobarbital and 5,6-benzoflavone, at a total volume of 500 μL. Dose-response curves of samples were obtained between 10^{-4}–0.2 g blue rayon equivalent (BRE) in the blue rayon hanging method, 2–20 mL water in blue rayon column method, 0.0625–1 g sediment, 0.0625–2 L drinking water, and 20–150 mL water in the Sep-Pak C18 cartridge and Blue-Chitin column method, respectively, per plate. Mutagenic activities of test samples were calculated from the linear portions of the dose-response curves obtained with three or four nontoxic doses, and duplicate plates in two independent experiments, and results are the mean of two independent experiments. The positive controls were 2-aminoanthracene (0.1 μg/plate) and Trp-P-2 (0.01 μg/plate) in YG1024 with S9 mix. The mutagenic potencies were expressed as revertants per one g of BRE or one L of water. A positive result was refined as a reproducible and dose-related response that at least induced a two-fold increase in revertants over the control. Mutagenic activities of authentic PBTA-type mutagens, calculated from linear portions of the dose-response curves in this study were as follows: PBTA-1, 883; PBTA-2, 786; PBTA-3, 1,279; PBTA-4, 2,396; PBTA-5, 723; PBTA-6, 487; PBTA-7, 1,430 and PBTA-8, 2,213 revertants per ng.

Purification and quantification of PBTA-type mutagens by HPLC: PBTA-type mutagens were purified by HPLC on reverse-phase columns, then quantified by HPLC with UV and an electrochemical detector according to the method described previously (16, 20–22). The residue dissolved in 75% methanol was fractionated by HPLC using a Nanospace SI-1 chromatograph (Shiseido, Tokyo, Japan) on a semi-preparative TSK-GEL ODS-10A column (10 μm particle size, 7.8 × 300 mm; Tosoh Corp., Tokyo, Japan) in purification step 1. The mobile phase of 80% methanol was pumped in isocratically at a flow rate of 1.6 mL/min at ambient temperature, and eluates containing corresponding authentic PBTA-type mutagens were collected. The eluates were then reduced to 400 μL. The condensed eluate was further purified with a reverse-phase CAPCELL PAK C18 (UG120, 5 μm, 4.6 × 150 mm, Shiseido) in purification step 2. A mobile phase of 25–42% acetonitrile in 25-mM H_3PO_4/Na_2HPO_4 (pH 6.5) was pumped in at a flow rate of 0.8 mL/min at 35°C. The absorption of the eluate at 260 nm was monitored and the fractions corresponding to eight PBTA-type mutagens were separately collected and reduced to 400 μL. The condensed fraction was finally analyzed on a reverse-phase YMC-Pack ODS A column (5 μm, 4.6 × 250 mm, YMC Co. Ltd. Kyoto, Japan) with an UV detector (260 nm, Shimadzu Co. Ltd. Kyoto, Japan) and an electrochemical detector (900 mV, Irica 985, Kyoto, Japan) in a determination step. The mobile phase of 20–45% acetonitrile in 25-mM H_3PO_4/Na_2HPO_4 (pH 2.0) was pumped in at a flow rate of 0.8 mL/min at 35°C. The eluates were also monitored for the structural confirmation with the Shiseido nanospace SI-1 photodiode array detector. In cases where it was not possible to perform definitive structural confirmation, eluates were analyzed further on a YMC-Pak ODS A column with a mobile phase of 25–50% acetonitrile in 20-mM acetic acid/sodium acetate (pH 4.7) at a flow rate of 0.8 mL/min at 35°C.

Results and Discussion

Salmonella mutagenicity of blue rayon extracts from sewage effluents and river waters collected between 1996 and 2005 (Exp. 1): Thirty-nine effluent samples were collected at outlets of two sewage plants (Site U-1 and KA-1 in Fig. 2); 47 river water samples were collected at sites located below sewage plants (Site N-1 and N-2 in Fig. 2); and 47 river water samples were collected at their downstream sites (Site KA-2 and Y-2 in Fig. 2) in the Yodo River system, Japan, between 1996 and 2005, using the blue rayon hanging method, which gives a one-day exposure. Table 2 summarize the mutagenic potency values analyzed by the *Salmonella* mutagenicity assay in strain YG1024 with S9 mix

Table 2. Mutagenicity of sewage effluents and river waters collected from the Yodo River system

Sampling date	Salmonella YG1024 with S9 mix (revertants/g BRE)					
	Uji River	Katsura River		Nishitakase River		Yodo River
	U-1	KA-1	KA-2	N-1	N-2	Y-2
10 March 1996	—	9,100	16,400	76,300	27,400	17,700
15 June 1996	—	108,800	303,200	165,600	340,100	—
21 July 1996	—	366,600	53,500	455,700	339,500	10,900
18 September 1996	—	185,200	47,600	859,400	606,000	83,600
13 May 1997	—	—	184,000	501,500	670,200	—
13 June 1997	—	—	12,900	565,500	1,095,000	66,000
25 July 1997	—	—	24,800	16,200	303,800	5,500
7 October 1997	2,898,400	—	8,700	3,500	93,200	4,900
20 October 1999	418,100	280,400	203,600	—	450,600	23,300
9 November 1999	129,800	943,500	—	116,400	171,800	18,200
19 July 2000	702,400	549,400	8,900	23,300	170,500	2,600
8 September 2000	49,600	212,300	6,800	1,400	44,000	2,500
28 March 2001	546,500	885,900	75,100	810,400	413,000	556,200
29 March 2001	552,800	2,073,700	92,800	528,300	929,700	1,075,200
30 May 2001	593,300	618,900	135,700	720,100	2,194,500	86,400
29 May 2002	586,800	30,700	95,000	3,600	369,200	12,800
30 May 2002	698,800	33,400	98,100	3,600	760,000	21,400
31 May 2002	817,100	24,900	45,700	3,000	877,600	11,000
6 September 2002	460,100	143,900	30,300	9,100	253,100	7,600
12 September 2002	614,400	132,500	61,100	3,200	258,400	13,200
15 January 2004	63,500	103,800	37,700	—	65,800	42,000
16 July 2004	186,000	29,600	36,200	—	174,800	6,300
21 December 2004	176,700	205,700	236,200	4,900	73,000	57,000
21 July 2005	266,500	178,600	84,100	nd*	128,800	13,600
13 October 2005	136,200	77,000	42,500	28,000	87,400	50,400
Total number	18	21	24	22	25	23
Average	726,700	394,300	82,500	233,300	435,900	95,100
Median	586,800	185,200	53,500	28,000	303,800	17,700
SD	757,400	522,800	79,900	306,400	474,000	241,800
Maximum	2,898,400	2,073,700	303,200	859,400	2,194,500	1,075,200
Percentage with "extreme mutagenicity"†	89%	71%	21%	41%	76%	9%

Samples were collected by blue rayon hanging method between 1996 and 2005.
—: Data are not available.
*not detected (< 400 revertants/g BRE).
†Percentage of samples with "extreme mutagenicity" among total numbers.
"extreme mutagenicity": >100,000 revertants/g BRE according to mutagenic potency classification by Ohe *et al.* (4).

calculated from the linear regression equation in dose-response effect of blue rayon extracts. All but one sample was mutagenic, and the mutagenic activities fluctuated widely among samples. An average mutagenicity of the total 133 samples was 278,300 rev/g BRE with a maximum of 2,898,400 rev/g BRE and a median value of 98,100 rev/g BRE. Average mutagenicities of sewage effluent samples at Site U-1 and KA-1 were 726,700 and 394,300 rev/g BRE; those of river water samples at Site N-1 and N-2 located below sewage plants were 233,300 and 435,900 rev/g BRE; and those of downstream river waters at Site KA-2 and Y-2 were 82,500 and 95,100 rev/g BRE, respectively. The results showed that levels of mutagenic potency of sewage effluents (Site U-1 and KA-1) and river water samples at sites located below sewage plants (N-1 and N-2) were remarkably higher than those for river water samples collected from their downstream sites (Site KA-2 and Y-2). We (4) have classified the mutagenic potencies of samples obtained from the combination of the blue rayon hanging method as a collecting method and YG1024 strain as a bioassay system as low, moderate, high and extreme mutagenic activity. Based on this scheme, 50% of all samples had extreme mutagenicity, with more than 100,000 rev/g BRE. Samples from sewage plants and river waters at sites located below sewage plants were classified as extreme mutagenicity in high frequency as shown in Table 2. Exceedingly high mutagenicities, at 1,000,000 or more revertants per gram BRE, were found in samples collected at Site U-1 (Uji River) in October 1997, Site KA-1 (Katsura River) in March 2001, Site N-2 (Nishitakase River) in June

Table 3. Levels of PBTA-1 and PBTA-2 and their contribution ratio to the mutagenicity of water samples in *S. typhimurium* YG1024 with S9 mix

Sampling date	Levels of PBTA-1/ng per g blue rayon equivalent					
	Uji River	Katsura River		Nishitakase River		Yodo River
	U-1	KA-1	KA-2	N-1	N-2	Y-2
15 June 1996	—	7.9 (6.4)	1.0 (0.3)	104.5 (55.7)	24.4 (6.0)	nd (0)
18 September 1996	—	3.2 (1.5)	2.0 (3.7)	78.5 (8.1)	29.4 (4.3)	3.0 (3.2)
25 July 1997	—	—	2.4 (8.5)	0.5 (2.7)	1.0 (0.3)	0.2 (3.2)
7 October 1997	4.3 (0.1)	—	0.7 (7.1)	0.3 (7.6)	1.0 (0.9)	0.4 (7.2)
20 October 1999	nd (0)	nd (0)	nd (0)	—	nd (0)	—
19 July 2000	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
28 March 2001	7.6 (1.2)	53.7 (5.4)	7.4 (8.7)	76 (8.3)	211 (42.6)	3.5 (0.6)
29 March 2001	7.5 (1.2)	63.0 (2.7)	11.3 (10.8)	268 (44.8)	235 (22.3)	2.8 (0.2)
30 May 2002	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
31 May 2002	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
16 July 2004	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
21 December 2004	nd (0)	9.5 (4.1)	nd (0)	nd (0)	nd (0)	nd (0)
21 July 2005	nd (0)	10.5 (5.2)	nd (0)	nd (0)	nd (0)	nd (0)
13 October 2005	nd (0)	8.2 (9.6)	nd (0)	nd (0)	nd (0)	nd (0)
Number	11	12	14	13	14	12
Average	1.76 (0.2)	13.8 (2.9)	1.77 (2.8)	40.6 (9.8)	35.8 (5.5)	0.83 (1.1)
Median	0	8.1	0	0	0	0
SD	3.14	21.3	3.39	78.1	80	1.39
Range	nd–7.6 (0–1.2)	nd–63.0 (0–9.6)	nd–11.3 (0–10.8)	nd–268 (0–44.8)	nd–235 (0–42.6)	nd–4.8 (0–7.2)

Sampling date	Levels of PBTA-2/ng per g blue rayon equivalent					
	Uji River	Katsura River		Nishitakase River		Yodo River
	U-1	KA-1	KA-2	N-1	N-2	Y-2
15 June 1996	—	25.2 (18.2)	18.6 (4.8)	101.8 (48.3)	114.4 (26.4)	nd (0)
18 September 1996	—	15.1 (6.4)	4.0 (6.6)	84.0 (7.7)	50.1 (6.5)	10.1 (9.5)
25 July 1997	—	—	15.7 (49.8)	2.1 (10.2)	20.1 (5.2)	nd (0)
7 October 1997	138.8 (3.8)	—	2.2 (19.9)	1.1 (24.7)	4.0 (3.4)	nd (0)
20 October 1999	154.0 (29.0)	109.0 (30.6)	79.0 (30.5)	—	200.0 (34.9)	—
19 July 2000	54.1 (6.1)	54.8 (7.8)	5.5 (48.6)	2.3 (7.8)	6.6 (3.0)	nd (0)
28 March 2001	31.9 (4.6)	279.2 (24.8)	33.8 (35.4)	83.9 (8.1)	109.6 (20.9)	19.6 (2.8)
29 March 2001	75.0 (10.7)	883.1 (33.5)	27.5 (23.3)	67.9 (10.1)	136.0 (25.9)	26.8 (2.0)
30 May 2002	61.8 (7.0)	11.0 (25.9)	9.4 (7.5)	2.4 (52.4)	158.0 (14.2)	21.5 (79.0)
31 May 2002	97.0 (9.3)	16.2 (51.1)	5.0 (8.6)	1.6 (41.9)	54.6 (4.9)	7.9 (56.4)
16 July 2004	nd (0)	22.2 (59.0)	36.5 (79.3)	nd (0)	184.3 (82.9)	nd (0)
21 December 2004	49.2 (21.9)	242.1 (92.5)	106.0 (35.3)	nd (0)	51.6 (55.6)	46.0 (63.4)
21 July 2005	173.5 (51.2)	100.7 (44.3)	31.4 (29.3)	nd (0)	98.9 (60.4)	nd (0)
13 October 2005	140.3 (81.0)	102 (104)	12.9 (23.9)	nd (0)	48.4 (43.5)	12.9 (20.1)
Number	11	12	14	13	14	13
Average	88.7 (20.4)	155.1 (41.5)	27.7 (28.7)	26.7 (17.6)	88.3 (27.7)	11.1 (17.9)
Median	75	77.8	17.2	2.1	76.8	7.9
SD	56.1	245.9	30.2	40.6	64.5	14.1
Range	nd–173.5 (0–81.0)	11.0–883.1 (7.8–104)	2.2–106.0 (4.8–79.3)	nd–101.8 (0–52.4)	4.0–200.0 (3.0–82.9)	nd–46.0 (0–63.4)

—: Data are not available.
Figures in parenthesis show the contribution ratio (%) of PBTA-1 or PBTA-2 to total mutagenicity.

1997 and May 2001, and Site Y-2 (Yodo River) in March 2001. The fact that extreme mutagenicity was also detected at the downstream site with a frequency of 9% was a serious problem, because there are several sources for drinking water supply upstream and downstream from Site Y-2.

Maruoka *et al.* (34,35) demonstrated that XAD resin extracts collected in 1982 and 1983 at sites downstream from sewage plants along the Katura River and the Nishitakase River, exhibited consistently strong mutagenic activity to strain TA1538 and TA98 in the presence of S9. Since then, some researchers reported that these tributaries of the Yodo River system were polluted especially with potent frameshift-type indirect-