Inhibitory effects of NADH/NADPH in S9 mix on photo-mutagenicity of thiabendazole following UVA-irradiation in $E.$ $coli$

Mie Watanabe-Akanuma[1][*] and Toshihiro Ohta[2]

[1] Kureha Chemical Industry Co., Ltd. Biomedical Research Laboratories
3-26-2, Hyakunin-cho, Shinjuku, Tokyo 169-8503, Japan
[2] School of Life Science, Tokyo University of Pharmacy and Life Science
1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

Summary

Thiabendazole (TBZ), a post-harvest fungicide commonly used on imported citrus fruits, exhibited photo-mutagenicity following UVA-irradiation (320-400 nm) in Trp[+] reverse mutation assay using $Escherichia$ $coli$ WP2uvrA/pKM101 strain. The photo-mutagenicity was not observed in the presence of S9 mix, a rat liver homogenate microsome fraction with co-factors for metabolic activation. We found that NADH and NADPH used as co-factor in the S9 mix efficiently suppressed the photo-mutagenicity of TBZ. This evidence strongly suggested that non-mutagenicity in the presence of S9 mix was not due to the metabolic detoxification of TBZ or the scavenging of UVA-activated TBZ by macromolecules in the S9 mix. Rather quenching effect of NADH and NADPH (λmax=338 nm) may be more responsible for suppression of UVA-activation of TBZ, because oxidized forms of NAD^{+} and NADP^{+} did not show inhibitory effects. Mutagenicity of the UVA-irradiated photo-mutagens such as angelicin and chlorpromazine was also suppressed by the addition of NADH or NADPH. Our present results suggest the possible underestimation in risk evaluation for photo-mutagenic compounds when they are assayed in the presence of S9 mix.

Keywords: photo-mutagenicity, TBZ, chlorpromazine, UVA, S9 mix

Introduction

Benzimidazole fungicides such as thiabendazole (TBZ) and benomyl are widely used because of their non-toxicity to higher plants. TBZ is approved in Japan as a post-harvest fungicide for imported citrus fruits during transport and storage. Although TBZ is reported to be cytotoxic to the spindle apparatus and mitosis in mammalian cells (Styles and Garner, 1974; Mudry de Pargament et al., 1987; Parry and Sors, 1993), it is not mutagenic in bacterial reverse mutation tests with or without metabolic activation (Cancer Assessment Document, EPA, 2000). We recently reported that TBZ shows potent mutagenicity following UVA-irradiation (320-400 nm) for 10 min at 250 μW/cm^{2} and that Trp[+] reverse mutations in $Escherichia$

$coli$ WP2uvrA/pKM101 strain is more sensitive than His[+] reverse mutations in $Salmonella$ $typhimurium$ strains TA100 and TA98 (Watanabe-Akanuma et al., 2003). The predominant mutations induced by UVA-activated TBZ were G:C → A:T transitions and A:T → T:A transversions. Since pre-irradiated TBZ solution just before adding bacterial cells did not show any mutagenicity, it seems likely that the photo-mutagenic TBZ products are unstable and/or rapidly react with other molecules before being incorporated into the cells (Watanabe-Akanuma et al., 2003). The photo-mutagenicity of TBZ in the $E.$ $coli$ strain was also observed with a fluorescent lamp, probably due to a low dose of UVA form the lamp (unpublished observation). For further investigation of the photo-mutagenic activation of TBZ by UVA-irradiation, we have conducted a screening assay to find effective inhibitors. As far as tested, several scavengers for reactive oxygen species such as ethanol, dimethyl sulfoxide, mannitol, histidine, ascorbic acid, epigallocatechin did not show apparent inhibitory effects (unpublished observation). On the other hand, the

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photo-mutagenic activation of TBZ was not observed in the presence of exogenous metabolic activation system (S9 mix) in our preliminary study. We, therefore, tested the possible suppressing effect of macromolecules like DNA, proteins, and enzymes as well as S9 fraction (rat liver microsome fraction) in this study. We report here that photo-mutagenicity of TBZ was completely abolished by the addition of NADH and NADPH which were commonly used as co-factors to prepare the S9 mix, but not by the S9 fraction or other co-factor components, suggesting a mechanism other than metabolic detoxification of TBZ.

Materials and Methods

Bacterial strain, media, and chemicals

E. coli strain WP2uvrA/pKM101 (trpE65, uvrA155, malB15, lon-11, sulA1) was used for Trp$^+$ reverse mutation assay. Bacteria were cultured in Oxoid nutrient broth No. 2 at 37℃. Minimal glucose agar plates, and top agar for the Trp$^+$ reversion assay were described previously (Watanabe-Akanuma et al., 2003). S9 mix consisted of 10% rat liver homogenate S9 fraction, 4 mM NADH, 4 mM NADPH, 5 mM glucose-6-phosphate (G-6-P), 33 mM KCl, 8 mM MgCl$_2$, 100 mM sodium phosphate buffer (pH 7.4), according to the Guideline for Screening Toxicity Testing in Chemicals, Japan (1997).

Thiabendazole [TBZ, 2-(thiazol-4-yl)benzimidazole, CAS Registry number 148-79-8, (chemical structure in Fig. 1)], chlorpromazine hydrochloride [CAS No. 69-09-0, (chemical structure in Fig. 3 right)], dimethyl sulfoxide (DMSO), L-cysteine, pyridoxal hydrochloride, bovine serum albumin (BSA) and salmon sperm DNA were purchased from Wako Pure Chemical Industries, Japan. β-NADH and β-NADPH (reduced forms), β-NAD$^+$ and β-NADH$^+$(oxidized forms), G-6-P, S9 fraction, and co-factors mix solution were purchased from Oriental Yeast Co., Japan. Angelicin [CAS No. 523-50-2, (chemical structure in Fig. 3 left)], glutathione (reduced forms), and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma-Aldrich Co. MO, USA. Catalase from beef liver was purchased from Roche Diagnostics Co., IN, USA. UV absorption spectra from 280–400 nm of NADH, NADPH, and TBZ were measured using a U-2000A spectrophotometer (Hitachi Ltd., Japan).

UVA-irradiation

A black-light fluorescent lamp (National Black Light Blue, FL15BL-B, 15W, Matsushita Electric Industrial Co., Japan) that emitted wavelengths of 300–400 nm was used as the UVA source. To filter out UVB wavelengths below 320 nm, which are weakly mutagenic to the tester strain, a 5-mm thick soft glass plate was used. UVA was irradiated from a distance of 22 cm at 250 μW/cm^2(UVX Radiometer, Model UVX-36, Ultra-Violet Products, Upland, CA, USA) for 10 min on a 24-well multiplate with lid.

Mutagenicity assay

Bacteria were grown overnight in nutrient broth to a density of 1–3×10^9 cells/mL. A 0.1 mL aliquot of overnight culture was added to each well of a 24-well multiplate containing 0.5 mL of either S9 mix or 100 mM sodium phosphate buffer (pH 7.4). TBZ solution (2.5–20 μL) dissolved in DMSO at a concentration of 10 mg/mL was added at doses of 25–200 μg, and mixed well by pipetting. There was no precipitation of TBZ. The mixture was irradiated by UVA for 10 min at room temperature. Photomutagens, angelicin (Venturini et al., 1980; 1981) and chlorpromazine (Ciulla et al., 1986; Oppenländer, 1988; Gocke, 1996) dissolved in DMSO and sterile water respectively, were also used. In other experiments, one of the following compounds was added to the well containing phosphate buffer, TBZ, and bacteria: 1–100 μg of DNA, BSA, catalase, and SOD (10 μL of 0.1–10 mg/mL solutions), 1–50 μL of S9 fraction, 0.1–3 μmol NADH, NADPH, NAD$^+$, and NADP$^+$(10 μL of 10–300 μmol/mL solutions). After the mixtures were irradiated by UVA for 10 min, they were transferred to 2 mL of molten top agar in a test tube kept at 46℃, and immediately poured onto a minimal glucose agar plate. Plates were incubated for 2 days at 37℃ and the number of Trp$^+$ revertant colonies was counted. Experiments were conducted with duplicate plates for each dose and triplicate plates for the control. Data presented in the figures are the averages of duplicate or triplicate plates.

Results

Suppressing effect of macromolecules such as proteins and DNA in the irradiation mixture on photo-mutagenicity of TBZ was first investigated. At a dose of 150 μg (0.75 μmol) TBZ, UVA-irradiation for 10 min caused about 7-fold increase in the number of Trp$^+$ revertants of WP2uvrA/pKM101 over the corresponding control plates, while neither TBZ nor UVA alone was mutagenic (Table 1). Addition of sermon sperm DNA, BSA, reactive oxygen eliminating enzymes (SOD and catalase) up to a dose of 100 μg did not cause any suppressing effects. On the other hand, photo-mutagenicity of TBZ was largely reduced in the presence of S9 mix, but not S9 fraction alone (Table 1), suggesting either that the UVA-activated TBZ easily reacted with compounds in the S9 mix before entering the cells, or that the metabolites of TBZ were no longer photo-mutagenic. Further investigations, however, revealed that the photo-mutagenicity of TBZ was completely inhibited by the addition of 0.5 mL of co-factors solution alone as shown in Fig. 1. The results implied that the lack of photo-mutagenicity of TBZ in the presence of S9 mix was not due to simple metabolic detoxification of TBZ. Among the ingredients (G-6-P, NADH, NADPH, KCl, and MgCl$_2$) of co-factors, NADH and NADPH were found to be responsible for the suppressing effects. As

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Table 1 Effect of macromolecules on the photo-mutagenicity of TBZ with UVA-irradiation

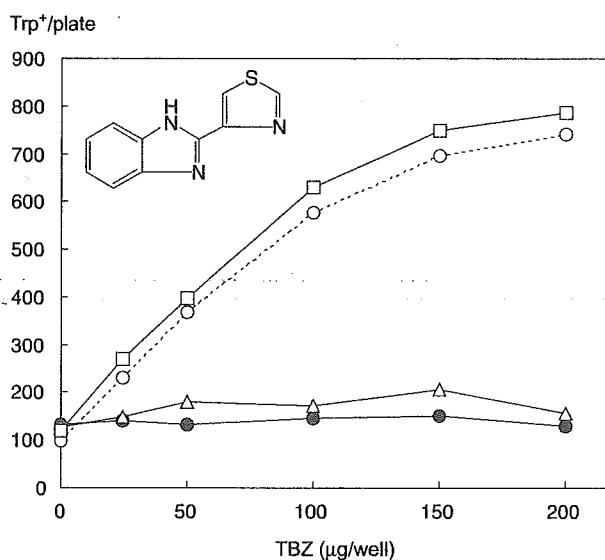| Compound (Dose)	TBZ (µg)	UVA (min)	Trp⁺/plate (mean)	Induced Trp⁺/plate	Relative mutagenicity (%)
—	0	0	102	—	—
—	150	0	100	0	—
—	0	10	103	1	—
(control)	150	10	684	582	100
DNA (0.1 mg)	150	10	694	592	101.7
BSA (0.1 mg)	150	10	685	583	100.2
Catalase (0.1 mg)	150	10	712	610	104.8
SOD (0.1 mg)	150	10	677	575	98.8
S9 (0.05 mL)	150	10	728	626	107.6
S9 mix (0.5 mL)	150	10	149	47	8.1

Fig. 1 Effect of S9 mix and co-factors on photo-mutagenicity of TBZ. UVA was irradiated for 10 min. ○, control (buffer); □, S9 fraction (50 µL/well); ●, S9 mix (0.5 mL/well); △, co-factors solution (0.5 mL/well)

Fig. 2 shows, equimolar NADH or NADPH suppressed the photo-mutagenicity of TBZ at a dose of 0.75 µmol, but oxidized forms of NAD^+ or $NADP^+$ were not. At a dose of 3 µmol $NAD^+(NADP^+)$, slight decrease in the number of Trp^+ revertants induced by TBZ with UVA was observed.

Since the reducing agents such as cysteine and glutathione did not inhibit the photo-mutagenicity of TBZ (data not shown), it was considered that the reducing potential of the compounds was not related to the inhibitory effects. It is known that NADH and NADPH have an absorption peak of 338 nm (ε=6,200), while TBZ has a peak of 302 nm (ε=24,530). Around 320–330 nm, which is considered to be the wavelength responsible for the UVA-activation of TBZ, NADH and NADPH but not NAD^+ and $NADP^+$ showed high absorbance (data not shown). Thus, it is suggested that NADH and NADPH might be quenching UVA irradiation, and suppressed photo-mutagenicity

of TBZ. The possibility that NADH and NADPH might interfere with UVA absorption by TBZ might be supported by an observation that an addition of pyridoxal (λmax=318 nm, ε=8,200) at 0.75–3 µmol showed similar inhibitory effect on photo-mutagenicity of TBZ, as did NADH (data not shown).

We carried out further experiments with other photo-mutagenic agents, angelicin (UV absorption peak around 300 nm) and chlorpromazine (UV absorption peak around 255 and 305 nm). As shown in Fig. 3, the photo-mutagenicity of these compounds as well as TBZ was also suppressed in the presence of 0.1–1.6 µmol NADH in a dose-dependent manner. Similar inhibition of photo-mutagenicity was observed by adding NADPH, but not by NAD^+ or $NADP^+$(data not shown). Thus, the use of NADH and NADPH as components of S9 mix is likely to inhibit UVA-activated photo-genotoxicity. In standard in vitro genotoxi-
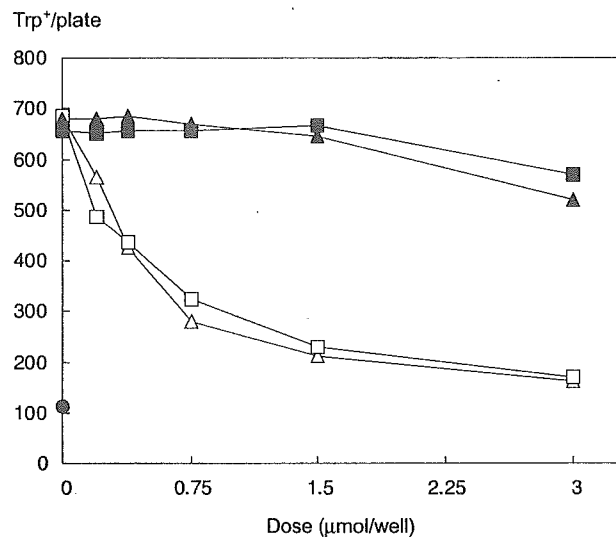
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Trp+/plate

Fig. 2 Inhibitory effect of NADH and NADPH on photo-mutagenicity of TBZ. NAD+ (▲), NADP+ (■), NADH (△), or NADPH (□) was added to the mixture of bacteria and TBZ (0.75 μmol/well) in 0.5 mL phosphate buffer and then the mixture was irradiated for 10 min. ●, control without TBZ
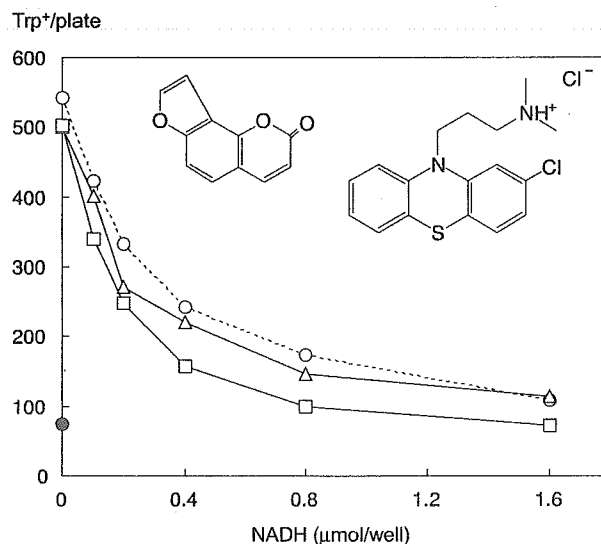
Trp+/plate

Fig. 3 Inhibition of photo-mutagenicity of TBZ, chlorpromazine, and angelicin by NADH. UVA was irradiated for 10 min. ○, TBZ (100 μg/well); △, angelicin (1 μg/well); □, chlorpromazine (10 μg/well); ●, control

city tests, 0.5 mL of S9 mix is incorporated into the assay system. This amount of S9 mix contains 2 μmol each of NADH and NADPH and that is enough for complete inhibition of TBZ photo-mutagenicity. On the other hand, S9 mix supplemented with NAD+ and NADP+ instead of NADH and NADPH was generally used in in vitro genotoxicity tests in USA (Ames et al., 1975; Maron and Ames, 1983). Therefore, we compared the effect of S9 mix containing NAD+ and NADP+ with the effects of S9 mix containing NADPH and NADH on photo-mutagenicity of

TBZ. UVA-induced photo-mutagenicity of TBZ was observed in the presence of NAD+/NADP+-based S9 mix, but not in NADH/NADPH-based S9 mix (Fig. 4). The slight decrease in photo-mutagenicity in the presence of NAD+/NADP+-based S9 mix compared with the phosphate buffer control may have been caused in part by the intracellular reduction of NAD+(NADP+) to NADH (NADPH) during the irradiation of 10 min or by metabolically detoxification of TBZ.
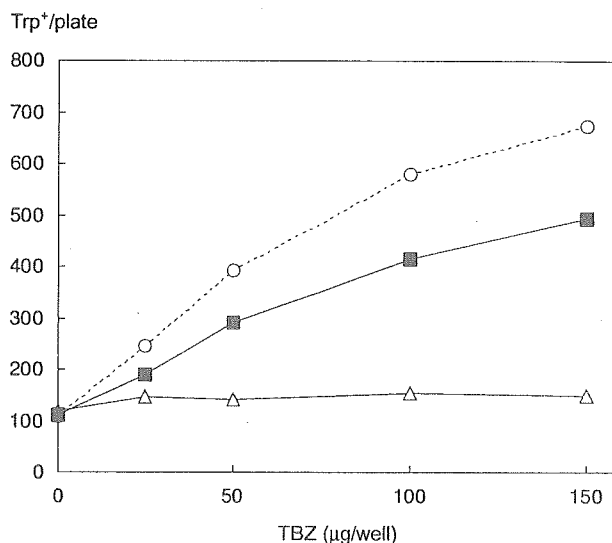
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Trp⁺/plate

Fig. 4 Photo-mutagenicity of TBZ in the presence of S9 mix. UVA was irradiated for 10 min. $\bigcirc$, control (buffer); ■, NAD⁺/NADP⁺-based S9 mix, $\triangle$, NADH/NADPH-based S9 mix

Discussion

Several biological systems for detecting photo-mutagenic compounds have been described (Gocke et al., 2000; Marrot et al., 2001). Averbeck et al.(1993) reported that 1,6-dioxapyrene was strongly photo-mutagenic to *S. typhimurium* TA100 in the absence of S9 mix but less so in its presence. They assumed that 1,6-dioxapyrene was metabolically deactivated by S9 mix. It has been reported that all of the photo-mutagenic compounds were detected in the absence of S9 mix and their mutagenicity was generally disappeared or decreased in its presence (De Flora et al., 1989; Gocke et al., 2000), probably due to metabolic detoxification or, in another case, scavenging reactive oxygen radicals generated by UVA-irradiation by proteins in the S9 mix. Since no compounds that show clear photogenotoxic activity only when irradiated in the presence of S9 mix have been reported, the effects of S9 mix on photomutagens have not been fully examined. Under these circumstances, the Scientific Committee for Cosmetology (SSC) guideline on photo-genotoxicity testing in vitro has not included the use of an exogenous metabolic activation system (S9 mix), because there was no appropriate positive control compound (Loprieno, 1991).

TBZ also showed photo-mutagenicity following UVA-irradiation in the absence of S9 mix but not in the presence of S9 mix. In our present study, NADH and NADPH were shown to inhibit photo-mutagenicity of angelicin and chlorpromazine as well as TBZ. On the other hand, oxidized forms of NAD⁺ and NADP⁺ did not inhibit photomutagenicity. Neither catalase nor SOD, reactive oxygen eliminating enzymes, suppressed the photo-mutagenicity of TBZ. We, therefore, suppose that NADH and NADPH interfere with UVA absorption by TBZ and suppress photo-mutagenicity. For the interpretation of decreased photo-mutagenicity in the presence of S9 mix, we should consider the third possibility of quenching effects by NADH (NADPH) in addition to the metabolic detoxification of photo-mutagens and scavenging of oxygen radicals. In genotoxicity assays in vitro, either NADH/NADPH-based S9 mix or NAD⁺/NADP⁺-based S9 mix are commonly used. When photo-mutagens are being investigated in the presence of an exogenous metabolic activation system, the effect of UVA-absorbing compounds such as NADH and NADPH must be considered, and it seems that the use of NAD⁺/NADP⁺-based S9 mix would be better for photo-mutagenicity assays.

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Toxicology Letters

A novel genotoxic aspect of thiabendazole as a photomutagen in bacteria and cultured human cells

Mie Watanabe-Akanuma [a,*], Toshihiro Ohta [b], Yu F. Sasaki [c]

[a] Biomedical Research Laboratories, Kureha Chemical Industry Co., 3-26-2 Hyakunin-cho, Shinjuku, Tokyo 169-8503, Japan
[b] School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[c] Faculty of Chemical and Biological Engineering, Hachinohe National College of Technology,
Tamonoki Uwanotai 16-1, Hachinohe, Aomori 039-1192, Japan

Abstract

Thiabendazole (TBZ) is a post-harvest fungicide commonly used on imported citrus fruits. We recently found that TBZ showed photomutagenicity with UVA-irradiation in the Ames test using plate incorporation method. In the present study, potential of DNA-damaging activity, mutagenicity, and clastogenicity were investigated by short pulse treatment for 10 min with TBZ (50–400 μg/ml) and UVA-irradiation (320–400 nm, 250 μW/cm^2) in bacterial and human cells. UVA-irradiated TBZ caused DNA damage in *Escherichia coli* and human lymphoblastoid WTK1 cells assayed, respectively, by the *umu*-test and the single cell gel electrophoresis (comet) assay. In a modified Ames test using *Salmonella typhimurium* and *E. coli*, strong induction of −1 frameshift mutations as well as base-substitution mutations were detected. TBZ at 50–100 μg/ml with UVA-irradiation significantly induced micronuclei in WTK1 cells in the in vitro cytochalasin-B micronucleus assay. Pulse treatment for 10 min with TBZ alone did not show any genotoxicity. Although TBZ is a spindle poison that induces aneuploidy, we hypothesize that the photogenotoxicity of TBZ in the present study was produced by a different mechanism, probably by DNA adduct formation. We concluded that UVA-activated TBZ is genotoxic in bacterial and human cells in vitro.
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Keywords: Photogenotoxicity; Thiabendazole; UVA; DNA damage; Micronucleus; Gene mutation

1. Introduction

Benzimidazole derivative fungicides such as thiabendazole (TBZ), benomyl, and carbendazim (MBC)

* Corresponding author. Tel.: +81 3 3362 7420;
fax: +81 3 3362 8522.
E-mail address: akanuma@kureha.co.jp
(M. Watanabe-Akanuma).

are widely used in agriculture. TBZ is also used as a post-harvest fungicide for imported citrus fruits during transport and storage. The compounds inhibit fungal microtubular function and thereby cause non-disjunction of chromosomes at cell division. Many studies report the induction of aneuploidy by these fungicides (Mudry de Pargament et al., 1987; McCarroll et al., 2002). They also induce micronuclei

in mouse bone marrow cells, probably through an aneugenic mechanism (Barale et al., 1993; Sarrif et al., 1994; Marrazzini et al., 1994). Regarding the in vitro cytochalasin-B micronucleus/kinetochore assay on TBZ, contradictory results were reported, negative in human lymphocytes (Van Hummelen et al., 1995) and positive in Chinese hamster Luc2 cells (Lynch and Parry, 1993). Since aneugens affect DNA indirectly, negative results for TBZ in the *Salmonella* reverse mutation test (Moriya et al., 1983; Georgieva et al., 1990) seem consistent.

We recently reported that TBZ shows potent mutagenicity following UVA irradiation (320–400 nm) for 10 min at 250 μW/cm^2 in *Escherichia coli* strains and *Salmonella typhimurium* strains in the standard plate incorporation assay (Watanabe-Akanuma et al., 2003). Unlike the aneugenic effects, the photomutagenic effects were not general for the benzimidazole derivative fungicides (no photomutagenicity was detected with benomyl, MBC, benzimidazole, thiazole, and phenylbenzimidazole; unpublished observation). Therefore, a different mechanism, perhaps direct interaction with DNA, must be involved in the photomutagenicity of UVA-irradiated TBZ. In the present study, we used short pulse treatments with TBZ and UVA to investigate the photogenotoxic properties of TBZ in bacteria and cultured human cells.

2. Materials and methods

2.1. Chemicals, media, and cells

Thiabendazole (2-(4-thiazolyl)-1H-benzimidazole), dimethyl sulfoxide (DMSO), cytochalasin-B, streptomycin, and ampicillin were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). TBZ was dissolved in DMSO at a concentration of 10 mg/ml or 20 mg/ml just before the experiments. The final concentration of DMSO in cell suspension during UVA irradiation was 4.5% or less for bacterial cells and 2% or less for WTK1 cells. TBZ and DMSO were removed immediately after UVA-irradiation by centrifugation of cells.

E. coli strain ZA201/pSK1002 [*trpE, uvrA, Δlac*] (Kato et al., 1994) was obtained from Dr. K. Matsumoto

(Institute of Environmental Toxicology, Japan) and used for the *umu*-test in which expression of the DNA-damage inducible gene (*umuC*) was monitored by a *umuC–lacZ* fusion gene on the plasmid pSK1002 (Oda et al., 1985). Cells were grown in nutrient medium (Oxoid, No. 2) supplemented with 25 μg/ml of ampicillin at 37 °C with shaking. *S. typhimurium* strains TA100 [*hisG46, uvrB, rfa*/pKM101], TA98 [*hisD3052, uvrB, rfa*/pKM101], TA97 [*hisD6610, uvrB, rfa*/pKM101], and TA104 [*hisG428, uvrB, rfa*/pKM101] (Maron and Ames, 1983; Levin et al., 1984) and *E. coli* strain WP2s/pKM101 [*trpE65, uvrA*] (Kato et al., 1994) were used for the reverse mutation assays. These strains were obtained from Dr. T. Matsushima (Japan Bioassay Research Center). Bacteria were cultured in Oxoid nutrient medium at 37 °C with shaking. Minimal glucose agar plate consisted of Vogel–Bonner E medium supplemented with 2% glucose and 1.5% agar (Maron and Ames, 1983). Top agar (0.6% agar and 0.5%) contained 0.05 mM D-biotin and 0.05 mM L-histidine for the *S. typhimurium* strains or 0.05 mM L-tryptophan for the *E. coli* strain.

Human lymphoblastoid cell line WTK1 (Levy et al., 1968; Xia et al., 1995) was used for assessment of DNA damage and micronucleus formation. Cells were provided by Dr. Honma (National Institute of Health Science, Japan) and maintained in logarithmic growth in RPMI-1640 medium (Nissui Pharmaceuticals Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Utah, USA), 300 μg/ml L-glutamine, 200 μg/ml sodium pyruvate, and 200 μg/ml streptomycin at 37 °C under a 5% CO$_2$ atmosphere. The doubling time was approximately 15 h.

2.2. UVA-irradiation

A black-light lamp (FL15BL-B, 15 W, National, Japan) that emitted wavelengths of 300–400 nm was used as the light source. To filter out wavelengths below 320 nm (UVB), we inserted a 5-mm thick soft glass plate between the culture dishes and the lamp. UVA irradiation was delivered at 250 μW/cm^2 (UVX Radiometer, Model UVX-36, Ultra-Violet Products, Upland, CA, USA). Immediately after irradiation, the cells were centrifuged and resuspended in fresh culture medium.

2.3. E. coli umu-test

We used three different treatment protocols. For the standard irradiation protocol, 0.45 ml bacterial suspension in phosphate buffer ((1–2) × 10^9 cells/ml) was dispensed into 24-well multiplates. TBZ solution (50 μl) was added to a final concentration of 100–900 μg/ml and then the cells were irradiated with UVA for 5–15 min at room temperature. The cells were then collected by centrifugation and resuspended in 2.5 ml nutrient broth. For the post-irradiation protocol, 0.45 ml bacterial suspension was mixed with TBZ solution (50 μl) and incubated for 15 min at 37 °C. The cells were centrifuged, resuspended in 0.5 ml phosphate buffer, irradiated with UVA for 10 min, and suspended in 2.5 ml of nutrient broth. For the pre-irradiation protocol, 50 μl TBZ solution was added to 4.5 ml phosphate buffer and irradiated with UVA for 10 min. The bacterial suspension (0.5 ml) was added and incubated for 15 min at 37 °C. Cells were centrifuged and resuspended in 2.5 ml nutrient broth. Duplicate cultures were used for each dose point.

TBZ-treated cells were cultured for 60 min at 37 °C with shaking. β-Galactosidase activities were assayed with ONPG as a substrate by the method described previously (Ohta et al., 1984). Enzyme activity (units) was calculated by the formula of Miller (1992). More than a doubling of enzyme units was judged as a positive response (Oda et al., 1985), indicating induction of the DNA-damage inducible gene *umuC*.

2.4. Reverse mutation test

A 0.5-ml aliquot of overnight culture ((1–2) × 10^9 cells/ml) was dispensed to each well of a 24-well multiplate. TBZ was added at 50–400 μg/ml and the mixture was UVA irradiated for 10 min. Cells were collected by centrifugation and suspended in 0.5 ml 100 mM sodium phosphate buffer (pH 7.4). A 0.15-ml aliquot of the irradiated cells was transferred to 2 ml of molten top agar in a test tube kept at 46 °C and immediately poured onto a minimal glucose agar plate. Plates were incubated for 2 days at 37 °C and the number of His[+] or Trp[+] revertant colonies was counted. Experiments were conducted in triplicate plates. The Dunnett's test was used to compare the counts of each dose with the control (Gatehouse et al., 1994).

2.5. Alkaline comet assay

For TBZ and UVA treatment, WTK1 cells were centrifuged and suspended in saline (approximately 1 × 10^7 cells/ml). TBZ was added to 2 ml aliquot of the cell suspension and layered in 5 cm dishes. The cells were irradiated with UVA for 10 min at room temperature. The cells were then washed and cultured in fresh medium. The cells were harvested 1 h, 2 h, or 4 h after the irradiation, embedded in 1% GP42 agarose (Nakalai Tesque, Kyoto, Japan) dissolved in saline, and layered on a glass slide as described previously (Sasaki et al., 1997). Slides were placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Tris–HCl, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept for more than 60 min in the dark at 0 °C, then in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 20 min in the dark at 0 °C. Electrophoresis was conducted at 0 °C in the dark for 20 min at 25 V (0.96 V/cm) and approximately 250 mA. The slides were then neutralized and stained with ethidium bromide. The length of the whole comet was measured for 50 nuclei for each dose with the aid of a fluorescence microscope (200× magnification) with a green filter. We routinely use "tail length" to measure DNA damages (Sasaki et al., 1997, 2000; Tice et al., 2000), because it is a simple and reliable parameter that can be measured without an image analyzer. The difference between the mean of the treated and control plates was evaluated with the Dunnett's test after one-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

2.6. In vitro micronucleus assay

The micronucleus test was performed as described elsewhere (Fenech and Morley, 1985; Kersten et al., 2002; Stopper and Lutz, 2002). WTK1 cells were irradiated with UVA for 10 min in the presence or absence of TBZ as was done in the comet assay. The cells were then washed to remove TBZ and cultured in fresh medium containing cytochalasin-B (3 μg/ml) for 20 h. At the end of incubation period, cells were fixed with ice-cold methanol/acetic acid (1:1) and their nuclei and micronuclei were stained with acridine orange (Hayashi et al., 1990). Duplicate slides were viewed with the aid of a microscope and the number of binucleated cells with micronuclei was counted in 1000

binucleated cells. The criteria for scoring micronuclei were those of Fenech (2000). The assay was repeated once and Kastenbaum and Bowman (1970) tables were used for statistical evaluation.

3. Results and discussion

Table 1 shows the results of the *umu*-test with *E. coli* ZA201/pSK1002. Neither TBZ alone nor UVA pre-irradiated TBZ induced DNA damage. DNA damage was induced when the cells were irradiated with UVA in the presence of TBZ. The results of experiments conducted with the post-irradiation protocol suggest that the amount of TBZ incorporated into the cells during a 15-min incubation were not sufficient for subsequent UVA-irradiation to induce DNA damage. Negative results in the pre-irradiation protocols suggest that photo-activated TBZ was considerably unstable, or photo-activated TBZ was not able to penetrate cells. Induction of reverse mutations by pulse treatment with TBZ and UVA-irradiation was clearly observed in strains TA104, TA97, and
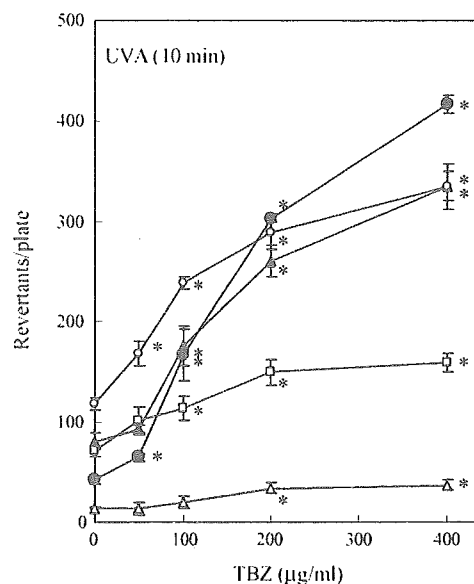

Fig. 1. Induction of reverse mutations in *E. coli* and *S. typhimurium* by TBZ with UVA-irradiation. Cells were treated with TBZ + UVA for 10 min and washed before plating. □, TA100; △, TA98; ▲, TA97; ○, TA104; ●, WP2s/pKM101 ($^*p < 0.05$).

WP2s/pKM101, while a weak response was observed in TA100 and TA98 (Fig. 1). Both strains TA104 and WP2s/pKM101, whose mutational targets for base-substitution are the TAA ochre codon (Levin et al., 1984; Ohta et al., 2002), were more sensitive than TA100, whose target for base-substitution is the *hisG46* missense mutation (CCC) (Levin and Ames, 1986), suggesting that UVA-irradiated TBZ caused DNA damage at A:T base pairs as well as G:C base pairs. In addition to the base-substitution mutations, −1 frameshift mutations (TA97) were more efficiently induced than −2 or +1 frameshift mutations (TA98). Although the photomutagenic mechanism has not been clarified, we suspect that the production of radicals followed by DNA bulky adduct formation may be involved. Another mechanism is the possible formation of DNA–DNA and/or DNA-protein cross-linking by UVA-irradiated TBZ.

We further investigated the photogenotoxicity of TBZ using human lymphoblastoid cell line WTK1. The single cell gel electrophoresis assay (comet assay) is a sensitive and rapid method for detecting DNA damage such as single-stranded DNA breaks and alkali-labile sites such as apurinic and apyrimidinic (AP) sites in

Table 1
Photogenotoxicity of TBZ in the *E. coli umu*-test

TBZ (μg/ml)	UVA (min)	Cell growth OD600	β-Galactosidase activity OD420	β-Galactosidase activity Units
0	0	0.323	0.183	60
0	10	0.344	0.193	63
300	0	0.497	0.147	55
300	5	0.563	0.551	276[a]
300	10	0.524	0.947	551[a]
300	15	0.532	0.787	447[a]
100	10	0.298	0.442	285[a]
200	10	0.290	0.546	370[a]
300	10	0.322	0.697	434[a]
600	10	0.306	0.687	451[a]
900	10	0.265	0.573	425[a]
Post-irradiation of TBZ-treated cells				
0	10	0.341	0.191	65
200	10	0.313	0.184	81
300	10	0.319	0.185	79
600	10	0.301	0.205	98
900	10	0.318	0.207	86
Cells treated with pre-irradiated TBZ				
300	10	0.393	0.143	64
600	10	0.379	0.151	68

Values were the average of duplicate culture.
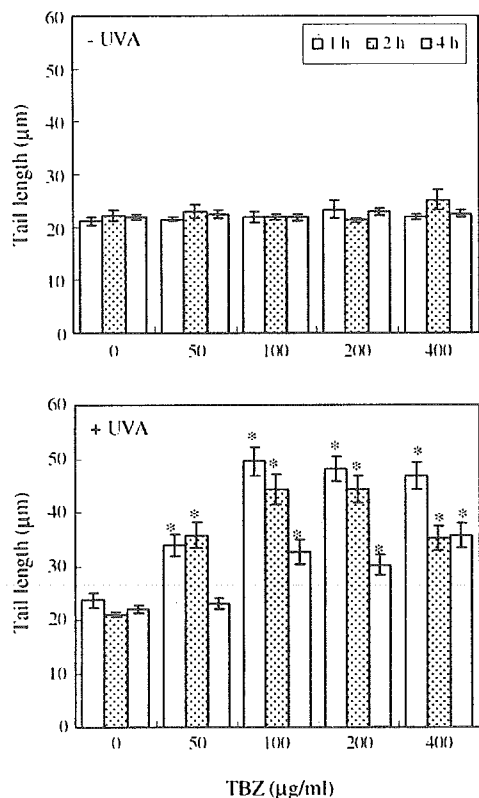[a] More than 2-fold increase.

Fig. 2. Induction of DNA damage in WTK1 cells by pulse treatment with TBZ + UVA for 10 min in the comet assay. Assay was conducted 1 h (open bar), 2 h (dotted bar), and 4 h (closed bar) after the irradiation. (*Upper*) un-irradiated control cells, (*lower*) UVA-irradiated cells ([*]$p < 0.05$).

DNA (Singh et al., 1988). In the comet assay, cells were pulse treated with TBZ (50 μg/ml, 100 μg/ml, 200 μg/ml, or 400 μg/ml) and UVA irradiation for 10 min, and sampled for preparation 1, 2, and 4 h after irradiation. Without UVA-irradiation, TBZ did not cause DNA damage at any dose or any sampling time. A significant increase in the mean tail length was observed when cells were irradiated in the presence of TBZ as shown in Fig. 2. DNA damage induced by 50–400 μg/ml TBZ was almost the maximum levels 1 h after the irradiation and apparent decrease was observed at 4 h. At the lowest dose of 50 μg/ml TBZ, the mean tail length at 4 h was control level, suggesting that the DNA damage provoked by TBZ + UVA treatment and detected by the comet method was efficiently repaired. Since the type of DNA damage detected in the

comet assay is subject to DNA repair and therefore does not necessarily result in fixed genetic alterations such as mutations and chromosome aberrations, we also measured micronucleus formation as the end-point of structural chromosome aberrations. In the in vitro micronucleus assay, the same treatment condition as employed in the comet assay was used. Since the doubling time of WTK1 cells in our experiment was about 15 h, cells were sampled for preparation after 20 h incubation in the presence of cytochalasin-B. As shown in Fig. 3, the % BNC was slightly decreased at a 200 μg/ml TBZ without UVA, while that was markedly decreased at doses of 50 μg/ml or more with UVA irradiation. Since only 3.0–3.2% BNC was observed at a 200 μg/ml TBZ with UVA irradiation, micronuclei induction was only evaluated at lower doses of 50 and 100 μg/ml TBZ. A weak but significant increase in the frequency of binucleated cells with micronuclei (MNBNC) was observed at 50 and 100 μg/ml. The result suggests that in human cells, 50 and 100 μg/ml TBZ + UVA induced both significant DNA damage (Fig. 2) and micronucleus formation (Fig. 3).

Micronucleus induction by TBZ itself in mouse bone marrow cells is associated with its aneugenic properties (Marrazzini et al., 1994). In the in vitro tests to detect aneuploidy induction, cells were usually treated for more than one cell cycle. On the other hand, the micronucleus induction demonstrated in our present study occurred in cells treated with TBZ and UVA for 10 min. Together with the fact that UVA-irradiated TBZ caused DNA damage in bacterial and human cells and mutations in bacteria, we hypothesize that direct interaction with DNA, such as adduct formation is involved in the photogenotoxicity of TBZ like other photomutagens, angelicin (Venturini et al., 1981) and chlorpromazine (Gocke, 1996), that have known to form DNA adduct. In a previous study with *E. coli* strains WP3101–WP3106 and their corresponding pKM101-carrying strains WP3101P–WP3106P (Ohta et al., 1999), we reported that G:C→T:A transversions induced by TBZ with UVA irradiation was very weak compared with the other predominant A:T→T:A transversions and A:T→G:C transitions (Watanabe-Akanuma et al., 2003). Since induction of G:C→T:A transversions, which are typical of mutations caused by oxidative DNA damage (Cheng et al., 1992), was low, 8-hydroxyguanine would not have been the responsible mechanism.
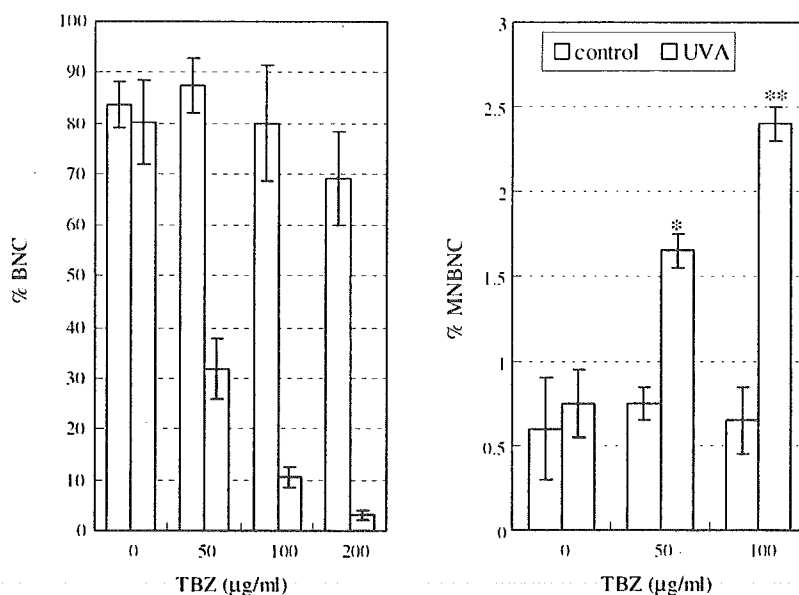
Fig. 3. Induction of micronuclei in WTK1 cells by pulse treatment with TBZ+UVA for 10 min in the cytochalasin-B/micronucleus test. Cell preparations were made 20 h after irradiation and micronuclei were counted in populations of 2000 binucleate cells. Open bar shows control cells without UVA-irradiation and shaded bar shows UVA-treated cells in both of the figures for % BNC (binuclear cells) and % MNBNC (micronucleated binuclear cells; $p<0.05$, $p<0.01$).

On the other hand, TBZ photomutagenicity in *E. coli* was observed following fluorescent illumination (15 W, 1860 lx, 10 min), probably due to small amounts of UVA and UVB emitted from the lamp (unpublished observation). UVA is the predominant source of radiant energy in sunlight. On the other hand, residue values of TBZ in citrus fruits were recommended not to exceed 10 ppm (FAO/WHO, 1973). We report here the induction of DNA damage and micronuclei by TBZ with UVA-irradiation in a human cell line. The photogenotoxic property of TBZ may be an important factor to consider when evaluating the risk to workers who spray TBZ on growing plants and on comported citrus fruits rather than residual intake from foods.

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げっ歯類を用いる小核試験の基礎研究ならびに
その行政面への応用

林　真*

国立医薬品食品衛生研究所変異遺伝部　〒158-8501　東京都世田谷区上用賀 1-18-1

The rodent micronucleus test
—The basic research and application to regulatory use—

Makoto Hayashi

Division of Genetics and Mutagenesis, NIHS, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Summary

Genotoxicity is an important consideration in the safety evaluation of chemicals. It is well known that there are in vitro and in vivo assay systems with different endpoints for evaluating chemical genotoxicity. Bacterial gene mutation test and chromosomal aberrations test using mammalian cultured cells are representative examples. It is apparent that there are limitations of in vitro assay systems for chemical safety evaluation and risk assessment for human health, and in vivo assay systems are becoming more important from the viewpoint of weight of evidence. There are several in vivo assay systems that have been developed and which are based on various endpoints. Among these, the rodent micronucleus test using hematopoietic cells has been most widely and frequently used to detect induction of chromosomal aberration. It is evident that there are chemicals that gave a positive result in the in vitro chromosome aberration test but were negative in the rodent micronucleus test. In such case, as a rule, the in vivo negativity is considered dominant to the in vitro positivity.

It is important and necessary to reduce use of test animals without any loss of evaluation accuracy. In the micronucleus test, development of the method using peripheral blood instead of bone marrow cells succeeded in reducing the total number of animals required for chromosomal aberration evaluation in vivo. Sampling of very small amounts of blood can be done without killing animals, which is one of the most important advantages of the method; it also permits combining with other assays for different endpoints that require different optimal sampling times. Based on this development, in vivo multiple endpoint assay systems will be realized and will lead to further reduction of animal use for the evaluation of chemical genotoxicity. In this manuscript, I describe the history of development and applications of the peripheral blood micronucleus assay.

Keywords: genotoxicity assay, rodent micronucleus assay, peripheral blood, acridine orange supravital staining

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Table 1　Representative genotoxicity assays

	Gene mutation	Chromosomal aberration
In vitro	Ames assay	Metaphase analysis using cultured cells
	MLA	MLA
In vivo	Transgenic animal model	Bone marrow metaphase analysis
		Erythrocyte micronucleus assay

MLA: mouse lymphoma assay using L5178Y cells

緒　　言

　化学物質の安全性を評価するのに，変異原性は重要な意味を持つ．また，がん原性は安全性を考える上で最も大きな関心事の一つであり，実験動物を用いて，その生涯にわたって処理し，多大な経費と労力を用いての試験が現在も行われている．このがん原性を短期間で予測する手法として細菌を用いる復帰突然変異試験を始めとする多くの変異原性試験が考案されてきた．当初はがん原性の予測のためにこれらの試験結果が用いられたが，現在ではがん原性のメカニズムの解明のための方法として重要視されるようになってきた．すなわち，がん原性のメカニズムに変異原性の関与が認められたときには，閾値が存在しない，との考えが定着しており，この考えを基に化学物質の評価がなされる．特に，発がんの部位において変異原性が認められるか否かは重要で，in vivo試験系が重要な役割を果たす．

　変異原性試験は，その指標と試験に用いる材料で分類される．変異原性の主な指標は遺伝子突然変異と染色体異常である．また，材料面からは大別してin vitro系とin vivo系がある．1976年の10月に当時の国立衛生試験所薬品病理部に入所し，すぐにげっ歯類を用いる小核試験に取り組むことになった．この試験は染色体異常誘発性を指標とした動物個体を用いるin vivo試験である．試験を始めた当時は，げっ歯類の骨髄細胞を用い，分裂中期像を直接顕微鏡観察して評価していた．最初に，小核の生成機構に関して検討し，骨髄細胞で見られる切断型の構造異常の90%程度が小核の生成に寄与するが，交換型異常では35%程度しか寄与しない場合もあることを明らかにした（Hayashi et al., 1984）．その後，手法の開発も行い，アクリジンオレンジ蛍光染色法を導入して観察の精度を高め（Hayashi et al., 1983），今ではOECD等のガイドラインでも推奨されている（OECD, 1997）．また，超生体染色法を小核試験に適用し，正確で，パフォーマンスの高い手法の確立に成功した（Hayashi et al., 1990）．

　この間，日本環境変異原学会，特にMMS研究会（分科会）で多くの共同研究を行い，試験結果に影響する要因（性差，系統差，投与回数，投与経路等）について質の高いデータを世界に向けて発信してきた（CSGMT, 1986;

1988; 1990; 1995a; Hayashi et al., 1989）．また，新しい手法のバリデーションに関する共同研究の成果も含め（CSGMT, 1992; Morita et al., 1997; Wakata et al., 1998; Hamada et al., 2001），ICHやOECDのガイドライン策定に大きな影響と貢献を果たしてきた．現在は，造血組織以外での染色体異常誘発性評価（Ohyama et al., 2002; Nishikawa et al., 2001; 2002; Suzuki et al., 2004），観察の自動化（Asano et al., 1998; Dertinger et al., 2005）等に注目が集まっているとともに，結果の評価（Hauschke et al., 1997; Adler et al., 1998; Hayashi et al., 1985; 1989; 1994），解釈（Kirkland et al., 2000; 2003; Müller et al., 2003）についても多くの議論がなされている．ここでは末梢血を用いる小核試験を中心に，その開発と応用，行政への反映についてまとめる．

1．小核試験

　化学物質の遺伝毒性を評価する試験法は試験の指標と用いる実験生物に基づき，Table 1のように分類できる．国内外の多くの試験ガイドラインでは，細菌を用いる復帰突然変異試験，ほ乳類培養細胞を用いる染色体異常試験またはマウスリンフォーマTK試験（MLA），およびげっ歯類を用いる小核試験（以下小核試験）が標準的な試験バッテリーと考えられている．小核試験は染色体異常誘発性を指標とし，動物個体を用いるin vivo試験系として最も汎用されている代表的な試験系である（Hayashi et al., 1994; 2000; Heddle et al., 1991）．汎用されている理由としては，染色体異常誘発性を検出するin vivo試験系としては感度が高く，パフォーマンスの高い試験系であることと，遺伝子突然変異をin vivoで検出するすぐれた試験系が確立されていない点が考えられる．

　げっ歯類の骨髄における小核生成機構をFig. 1に示す．赤血球の生成過程において，最終の細胞分裂時に染色体異常が誘発されると，その一部が小核を形成し，脱核の過程で細胞質内に取り残され，本来無核の赤血球中に小核が出現する．小核形成の効率は，染色体異常の型により異なり，単純な切断ではそれらの約90%が小核を形成するが，交換型の異常はその約35%程度しか小核を形成しないものもある（Hayashi et al., 1984）．脱核後間もない幼若な赤血球を顕微鏡下に観察し，小核を有する幼若赤血球の出現頻度から，最終細胞分裂時における染色体異常誘発性を推定する．これまでは，骨髄中の
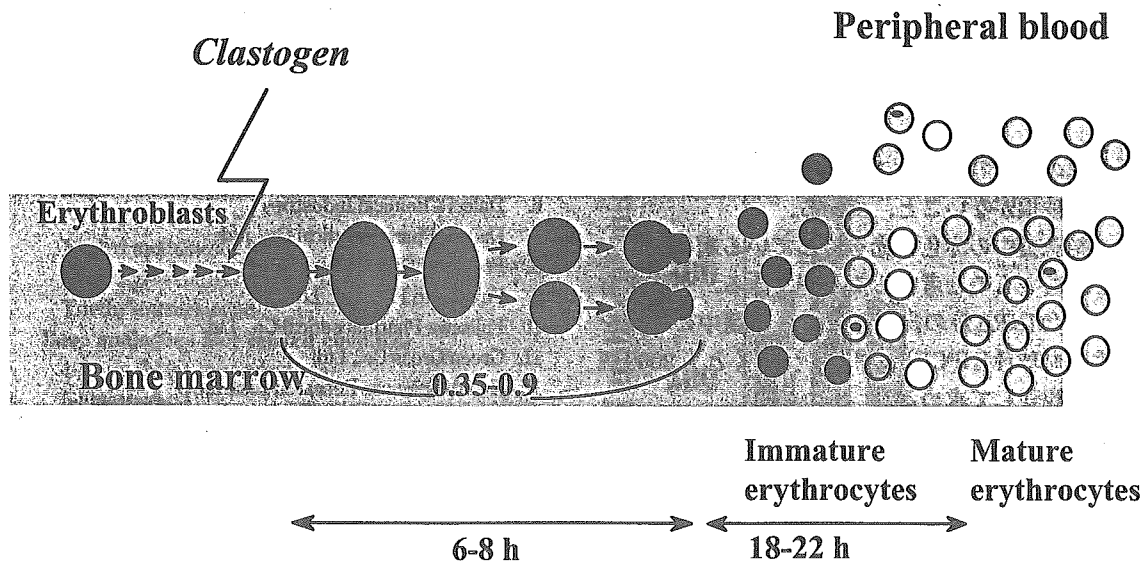
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Fig. 1 Micronucleus formation in erythrocyte

幼若赤血球である多染性赤血球を観察対照としてきた．小核試験全般に関しては成書（林，1991）を参照されたい．

2．末梢血を用いる小核試験

Fig. 1に示したように，幼若な赤血球は骨髄中にしばらくとどまるとともに，確率論的に血流に出ていく．末梢血を用いる小核試験に関しては，MacGregorら（1980）によって紹介され，その後も彼らのグループによって数多くの報告がなされている．しかし，末梢血を用いることによる多くの利点が強調された割には，あまり普及しなかったようである．末梢血を用いる小核試験は，被験物質を長期間投与し，成熟した赤血球を観察して化学物質の慢性的影響を評価しようとするものと，末梢血中の幼若赤血球を観察し，骨髄中の多染性赤血球を用いる小核試験と同等の結果を得ようとするものに大別できる．

一般の変異原性試験は，短期試験法とも呼ばれるように，化学物質の急性効果を評価するための手法である．しかし，ヒトが変異原に暴露されるのは低濃度で長期間のことが多く，遺伝子の突然変異や染色体異常の誘発についても，化学物質の慢性的な影響を評価できる系が必要となる．骨髄中の幼若な赤血球を観察対象とする通常の小核試験が，化学物質の急性効果を評価するものであるのに対し，末梢の成熟赤血球を観察する方法は，慢性的な影響を評価できる試験系として期待できる．

ヒトやラットでは，末梢血中に出た小核を持つ赤血球は異常赤血球として脾臓で効率良く壊されてしまうが，幸いなことにマウスではこの機能が不完全であるため，小核を有する赤血球も壊されることなく，正常な赤血球と同様の寿命（約30日）を全うする．従って，少なくとも赤血球の寿命の期間中小核を有する赤血球を蓄積することができ，被験物質の長期間投与の影響を評価できる．

さらに，末梢血は非常に均質な細胞集団であり，観察の機械化に最も適した材料である．末梢血を用いる小核試験と，観察の機械化が結びついてこの分野での大きな進歩となることが期待される（Hayashi et al., 1992a; 1992b; Asano et al., 1998; Dertinger et al., 2005）．

末梢血中の幼若赤血球である網赤血球を観察して，骨髄中の幼若赤血球を観察するのと同等の結果を得ることができれば，動物をその都度殺さなくとも経時的に標本作製することが可能となり，標本時期決定のための予備試験も必要なくなる．少なくともマウスについては，一般の単回投与試験の動物より採血することにより，実験動物を共有することも可能である．

1）標本作成法

アクリジン・オレンジ（A.O.）は未固定の細胞にも取り込まれ，核酸と結合して蛍光を発する色素である．2重鎖のDNAには塩基対間に入り込み，530 nmにピークを持つ黄緑色の蛍光を発し，単鎖のRNAとも結合し，590 nmにピークを持つ赤色の蛍光を発する．この性質を利用し，一定量のAOをあらかじめスライドグラスに塗布しておき，そこへ微量の末梢血をのせ，カバーグラスで覆うと，AOが血清中に溶け出し，それが細胞中に取り込まれ，自動的に蛍光染色が施される（超生体染色）．手法の詳細については文献（Hayashi et al., 1990; 林，1991; CSGMT, 1995b）を参照されたい．

2）観察方法

標本作成後すぐにも蛍光顕微鏡により観察可能であるが，細胞の動きがおさまるのを数時間待って観察する．観察は波長490 nm付近の励起光，観察用フィルタとして515－530 nm以上の波長の光を透過するものを備えた蛍光顕微鏡で行う．以下の条件を満たす良好な観察箇所

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を選定する.

(1) 白血球の核が黄緑色の蛍光を発していること

(2) 網赤血球の網状構造が赤色の蛍光を発していること

(3) 赤血球の重なりがないこと

(4) 細胞の破損がないこと, である.

赤血球の細胞質中の赤色蛍光を発している網状構造が大きいほど幼若なものと考えることができる. 小核を有する網赤血球(MNRET)の出現頻度は, 骨髄中の小核を持つ多染性赤血球(MNPCE)と同様, 少なくとも2000個の網赤血球を観察して求める.

3) 実験動物の削減

さて, 末梢血を用いる小核試験を行うことにより, 骨髄を用いるこれまでに行われてきた標準的な方法と比較して, どれくらい実験動物の削減につながるかについて考えてみる. 1群5匹の動物を用い, 陰性, 陽性対照群と3用量群で試験を行うとする. 単回投与の場合は, 2回の標本作製が必要とされており, 骨髄を用いる方法では, その都度動物を安楽死させる必要があるので, 陽性対照群は1回の標本作製と考えても合計で45匹の動物が必要となる. ただし, 複数回の投与の場合には, 1回の標本作製で良いとされているので, その場合は25匹となる. 一方, 末梢血小核試験では2回の標本作製を行うとしても, 同一の動物を用いることができるので, 投与回数にかかわらず25匹で試験が完結する. しかも, 陰性対照は投与直前の末梢血のデータで代表することが可能なので, 陰性対照群を別個に設ける必要がなくなる. 従って20匹の動物で良いことになる. また, 最近小核試験における陽性対照群をその都度設ける必要はないとの議論が国際的になされており, ICHのメンテナンスの議題と考えられている. もしそのようなことが認められるなら, 15匹の動物で試験が完結することとなり, 骨髄の45匹と比較すると1/3の動物で試験が可能になり, 大きな動物資源の節約になる.

3. バリデーション

行政的な安全性の評価に新しい試験手法を用いる場合には, その試験法の信憑性に関する十分な保証が必要である. 末梢血を用いる小核試験法に関しても, 安全性評価の一手法として用いるには, その性質を十分理解する必要があり, 多くの確認試験を行うことになる. 日本環境変異原学会MMS研究会でこの方法のバリデーション共同研究が企画され, 実施された. 44機関が参加し(Table 2), 原則として1つの化学物質を2機関で比較検討した. また, 評価に用いるモデル化学物質は, 小核の誘発が知られており, かつその作用機構が異なるものを選択した(Table 3). 実際の共同研究を始める前に, 技術移転のための講習会を開催し, 試験方法の伝授を行っ

Table 2 Participants in collaborative study

1. Biological Research Center for the Protection of Environment
2. Biomedical Laboratories, Inc.
3. Biosafety Research Center, Foods, Drugs and Pesticides
4. Central Institute for Experimental Animals
5. Chemicals Inspection & Testing Institute
6. Daicel Chemical Industries
7. Daiichi Pharmaceutical Co., Ltd.
8. Food and Drug Safety Center
9. Fuji Photo Film Co., Ltd.
10. Fujisawa Pharmaceutical Co., Ltd.
11. Green Cross Co., Ltd.
12. Health Sciences Research Institute
13. Institute of Environmental Toxicology
14. Itoham Central Research Institute
15. Japan Tobacco, Inc.
16. Kaken Pharmaceutical Co., Ltd.
17. Kanagawa Prefectural Public Health Laboratories
18. Kissei Pharmaceutical Co., Ltd.
19. Kumiai Chemical Industry Co., Ltd.
20. National Institute of Hygienic Sciences
21. Nihon Bioresearch Center, Inc.
22. Nihon Noyaku Co., Ltd.
23. Nippon Glaxo, Ltd.
24. Nippon Shinyaku Co., Ltd.
25. Nitto Denko Corporation
26. Ono Pharmaceutical Co., Ltd.
27. Otsuka Pharmaceutical Factory, Inc.
28. Pfizer Pharmaceutical, Inc.
29. Sandoz Pharma, Ltd.
30. Sankyo Co., Ltd.
31. Shionogi & Co., Ltd
32. Shiseido Toxicological Analytical Research Center
33. Sumitomo Chemical Co., Ltd.
34. Suntory Co., Ltd.
35. Taiho Pharmaceutical Co., Ltd.
36. Taisho Pharmaceutical Co., Ltd.
37. Takeda Chemical Industries, Ltd.
38. Tanabe Seiyaku Co., Ltd.
39. Teijin, Ltd.
40. Toyama Institute of Health
41. Toyobo Co., Ltd.
42. University of Shizuoka
43. Yamanouchi Pharmaceutical Co., Ltd.
44. Yoshitomi Pharmaceutical Industries, Ltd.

*Organizer

た. 結果は, ほとんどの参加者にとって初めての手法であるにもかかわらず, ばらつきの少ないデータが得られた. 化学物質の投与直前に採血した対照データのまとめをFig. 2に示す. ヒストグラムが実際の観察値であり, 丸印が平均値を実測値に合わせた2項分布から予測された値を示している. このように実測値と理論値が一致していることは, 偶然のばらつき以外にデータのふれをもたらす要因がなかったことを意味する. すなわち, 経験のないものが本試験を実施しても, 十分満足のいく結果を得ることが可能であることを示している. モデル化学

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Table 3 List of chemicals tested

Chemical tested
Alkylating agents
Cyclophosphamide monohydrate
Dimethylnitrosamine
Ethyl methanesulfonate
N-Ethyl-*N*-nitrosourea
Methyl methanesulfonate
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine
Triethylenemelamine
Base analogues and related chemical
1-*β*-D-arabinofuranosylcytosine
5-Fluorouracil
6-Mercaptopurine
Methotrexate
Aromatic amines
2-Acetylaminofluorene
Phenacetin
Polycyclic aromatic hydrocarbons
Benzo[*a*]pyrene
7,12-Dimethylbenz[*a*]anthracene
Crosslinking agent
Mitomycin C
Inorganic chemicals
Potassium bromate
Potassium chromate (VI)
Spindle poisons
Colchicine
Vincristine sulfate
Miscellaneous chemicals
Benzene
Procarbazine hydrochloride
Urethane

Fig. 2 Number of MNRETs/1000 RETs

物質に関しては，ほとんどのものが陽性結果を示し，さらに，単回の腹腔内投与48時間後にMNRET出現頻度が最大になることを示した．骨髄細胞を用いて小核の出現を観察するときには，化学物質によって経時的な変化が異なることが知られているが，末梢血の場合には48時間後に観察すればほとんどの化学物質の染色体異常誘発性を検出することが可能であることが判明した．

4．特徴と展望

　末梢血を用いる小核試験の特徴はいろいろ考えられるが，実験動物を殺すことなく微量の採血により染色体異常誘発性を評価できることが最も重要な点であると考える．これは，動物愛護の観点からもすぐれた特徴であるが，それ以上に化学物質の経時的な変化を同一動物からとることが可能となり，メカニズムの解析にも大きな意味を持つ．

　本法の特徴をよく示す一例として，マウスの各系統で，自然小核出現頻度が加齢に伴って変化するか否かを毎月同一個体から採血して評価する研究をMMS研究会として行った（Sato et al., 1995）結果を示す．系統により多少
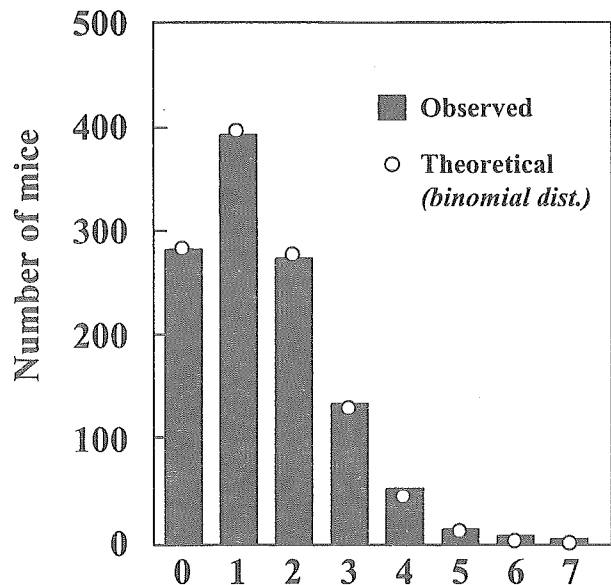
差はあるが1年以上にわたりほとんど変化のないことが判明した（Fig. 3）．従って，試験の材料とするときに動物の週齢に対してはロバストであり，その他の要件により週齢を選ぶことが可能である．

　各種試験法はそれぞれに最適な標本作製時期があり，同一個体から各種のデータをとることに無理があったが，微量の採血のみであれば多染性赤血球の指標に大きな影響を与えることはないので，他の試験と組み合わせることが可能になった．現在トランスジェニックマウスを用いる in vivo での遺伝子突然変異を検出する系が，安全性評価に用いられるようになっている．その系と末梢血を用いる小核試験を組み合わせると，突然変異と染色体異常を同時にin vivo で調べることができ，この分野に大きな展開をもたらすことが期待できる（Hayashi et al., 1994; Kohara et al., 2002a; 2002b; Suzuki et al., 1993; 1994; 1995）．

　網赤血球を用いる方法は，脾臓で小核赤血球が破壊されない点を考えてマウスを対象とすることに問題はない．しかし，小核試験の結果を総合的に評価するには，一般毒性のデータや薬物動態学的なデータが重要であり，これらのデータはラットを用いたものの方が圧倒的に多い．そこで，ラットを用いる小核試験が重要な役割を果たすことになるが，ラットでは脾臓で小核赤血球は破壊されてしまい，末梢血中では観察が困難であるとされている．しかし，MNRETが末梢血流に出てから破壊されるまでにごく幼若な網赤血球を観察することにより，小核が観察できることがMMS研究会での大規模なバリデーション研究により証明され，ラットもマウス同様試験動物として用いることが可能であることが判明し
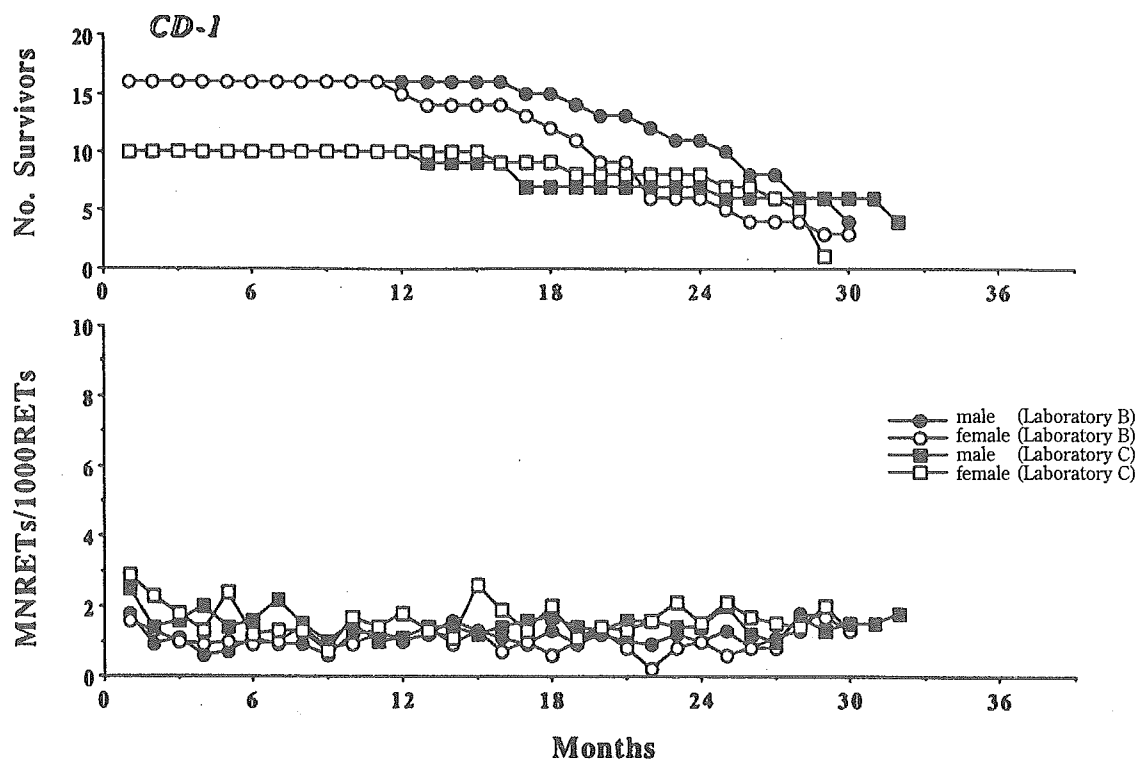
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Fig. 3 Aging effect on the spontaneous frequencies of MNRET in CD-1 mice

た（Wakata et al., 1998）．さらに，末梢血小核試験を一般
の毒性試験に組み込むことが可能か否かを調べるための
共同研究を MMS 研究会で行った（Hamada et al., 2001）．
その結果，紡錘体形成阻害剤での反応が明確でなかった
が，その他のモデル化学物質では小核の誘発性が確認さ
れ，今後の発展が期待できる結果となった．

　ここに一つの試験法の開発の歴史を示した．日本環境
変異原学会 MMS 研究会の非常に強力な共同研究の成果
に支えられ，多くの新しいことが判明した．それらの結
果は多くの論文にまとめられ，Mutation Research,
Mutagenesis, Environmental and Molecular Mutagenesis,
MMS Communications 等の雑誌を通じて広く海外にも
発信された．現在のガイドラインでは，小核試験に末梢
血を用いることも認めており，行政面への貢献を述べて
本稿の終わりとしたい．「医薬品の遺伝毒性試験に関す
るガイドライン：動物個体を用いて染色体損傷を検出す
る試験としては，通常げっ歯類の骨髄細胞を用いる染色
体異常試験と造血系細胞を用いる小核試験が用いられ
る．染色体異常を観察する試験では，様々な染色体の変
化が検出できる．また，染色体異常に起因して生成する
小核を骨髄または末梢血を用いて検出する小核試験は，
染色体異常誘発物質を検出する簡便な試験法として，前
者と同等に受け入れられる．さらに小核試験では異数性
誘発物質を検出できる可能性がある（医薬審　第1604号
平成11年11月1日）．」

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