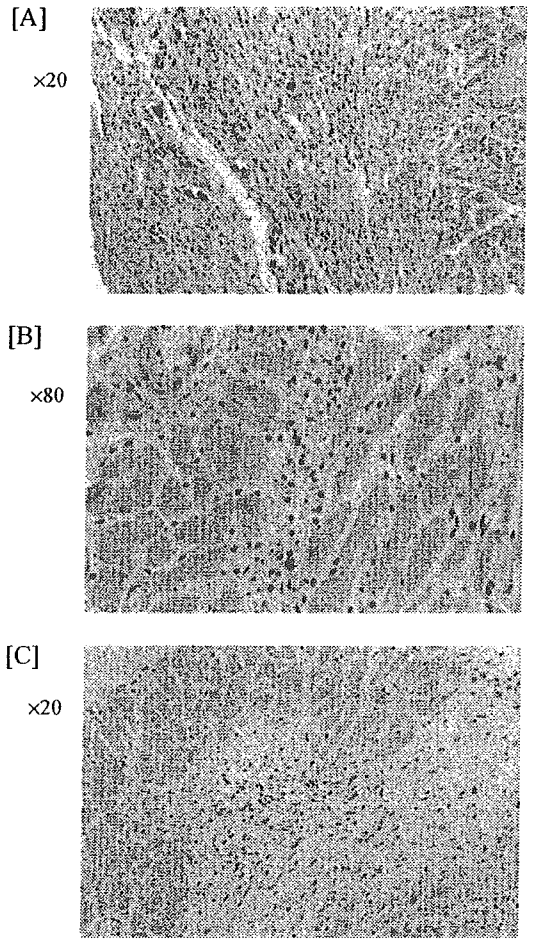

Figure 3. Histological analysis of dead DBA/2 mice administered with CAWS. [A, B, C] CAWS (4mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days in the 1st and 5th week. Thereafter, the hearts of dead mice were stained with hematoxylin-eosin.

Cytokine Production by Spleen Cells of CAWS-Administered Mice on Stimulation with CAWS

As splenocyte counts in DBA/2 mice were high, we examined the production of cytokines in response to CAWS. Spleen cells of mice administered CAWS to induce coronary arteritis were prepared at a concentration of 1×10^7 cells/ml, and cultured for 48 hours in a 5% CO_2 incubator at 37° for observing cytokine production. Following the culture, IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-α, and TNF-α levels in the culture supernatant were measured by ELISA. The strains with severe coronary arteritis, C3H/HeN, DBA/2 and C57BL/6 mice, showed IL-1β and IL-6 production by spleen cells in the CAWS -administered group (Fig. 5). In contrast, IL-10 was significantly produced

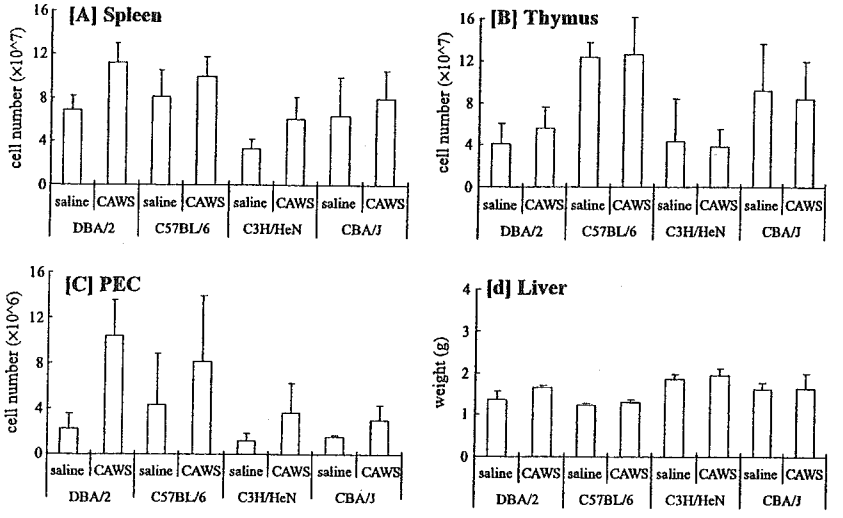

Figure 4. Cell number in peripheral blood and organ weight from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, the internal organs were collected from each mouse. Total cell number was counted with a hemocytometer and organ weight was measured with a analytical balance. The results show the mean ± standard deviation (S.D.). *; $P<0.05$ compared with the control using Student's *t-test*. [A]: Spleen, [B]: Thymus, [C]: PEC, [D]: Liver.

by spleen cells of the CBA/J mouse, which is the strain resistant to the coronary arteritis induced by CAWS.

In order to observe CAWS-specific reactions, the spleen cells of mice administered CAWS were stimulated with CAWS (0, 2.5, 5 or 10 μg/ml) and cultured for 48 hours in a 5% CO_2 incubator at 37°C. IFN-γ, IL-6 and IL-10 levels correlated well with the degree of coronary arteritis induced by CAWS (Fig. 6). IFN-γ and IL-6 production in DBA/2 and C57BL/6 mice tended to increase during the CAWS-specific response in the CAWS groups as compared with the saline groups, but no response was observed in C3H/HeN and CBA/J mice. IL-10 production was particularly enhanced in CBA/J mice treated with CAWS and slightly increased in the saline group, but no significant increase in production was observed even with the administration of CAWS in DBA/2 and C57BL/6 mice, and only a slight increase in C3H/HeN mice. Levels of other cytokines were not correlated with the coronary arteritis caused by CAWS. The amount of IL-4 production was small in all strains, but increased somewhat in CBA/J and C3H/HeN mice. IL-12 was hardly detected in any of the strains.

Measurement of Anti-CAWS Antibody Titer

Serum was obtained from mice administered CAWS in accordance with the coronary arteritis induction protocol, and anti-CAWS antibody in serum was detected with anti-mouse IgG + IgM Ab. Anti-CAWS antibodies were detected in all of the

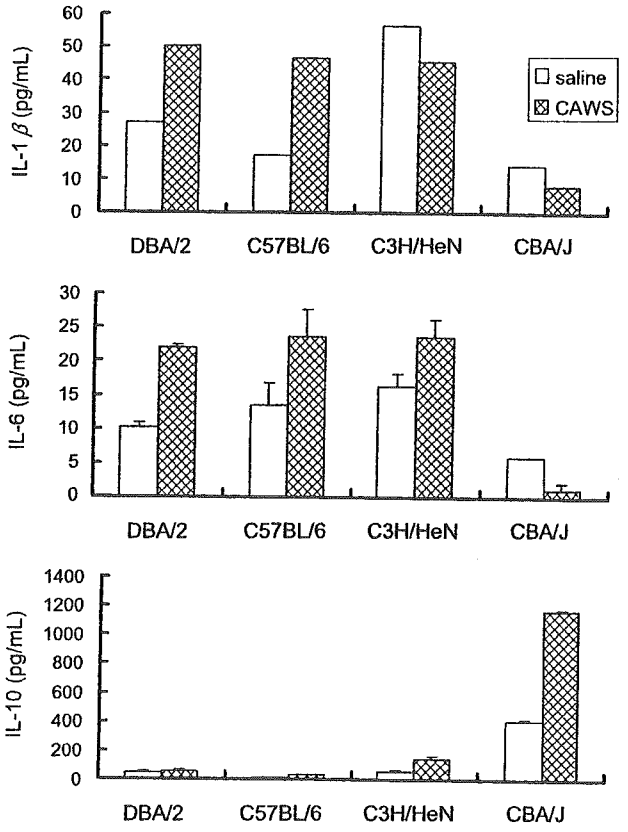

Figure 5. Cytokine production in culture supernatants of splenocytes in vivo from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, splenocytes were collected from each mouse. The splenocytes were cultured for 48 hour at a density of 1×10^7 cells/ml. The culture supernatants were collected and the level of each cytokine was measured by ELISA. The data shows one of four (C3H/HeN and CBA/J), three (DBA/2) or two (C57BL/6) experiments performed with similar results. The results show the mean ± standard deviation (S.D.).

mice, and the titers were extremely high (Fig. 7). Although the IgM titer varied depending on the mouse strain, there was no relationship between the IgM and the incidence of coronary arteritis (Fig. 8). Little IgE was detected in any of the strains. Conversely, IgG1 was detected at high levels in all the strains. CBA/J mice exhibited the lowest IgG2a titers.

On the basis of the above results, the antibodies produced in the form of anti-CAWS antibodies consisted mainly of IgG, followed by IgM. With respect to IgG2a production, although the titers were low in CBA/J and C57BL/6 mice, as the levels in CBA/J mice were roughly half those in C57BL/6 mice, and when considering the results of cytokine production in the spleen, it is possible that CBA/J mice exhibit Th2 bias.

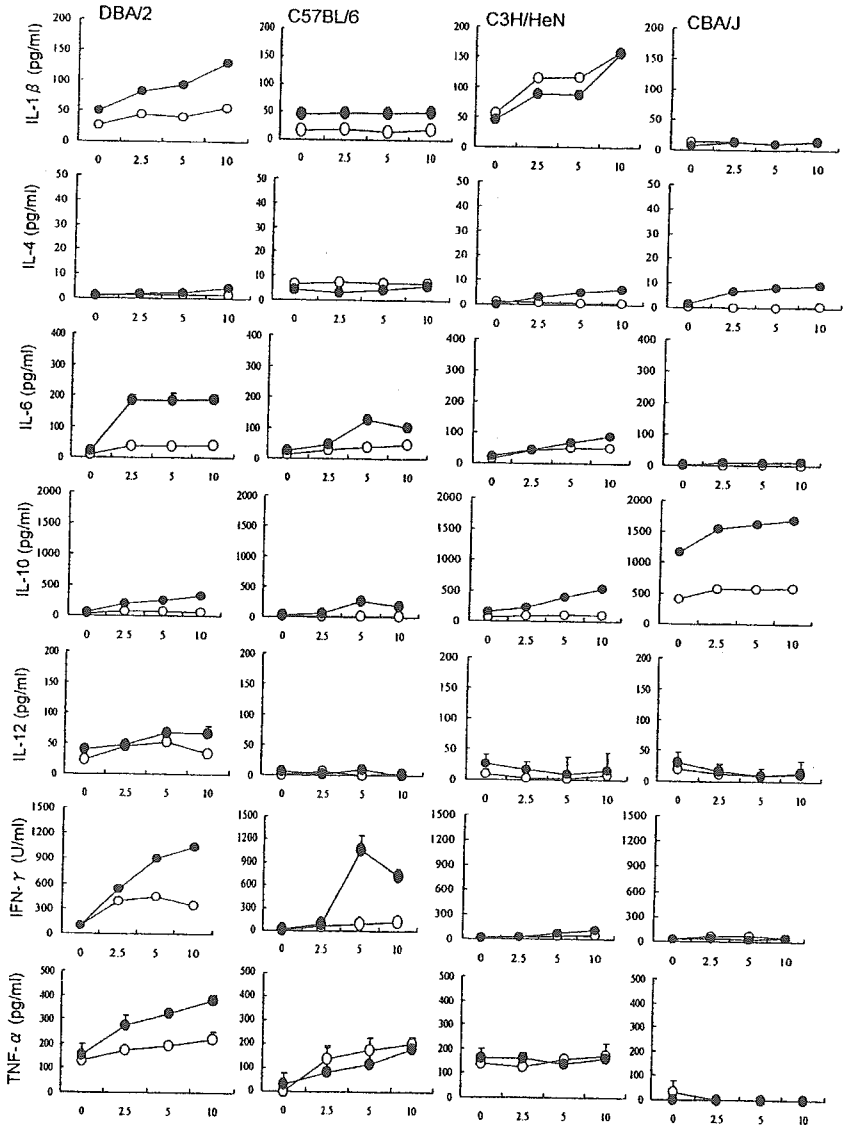

Figure 6. Cytokine production in culture supernatants of splenocytes stimulated with CAWS in vivo from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, splenocytes were collected from each mouse. The splenocytes were cultured with CAWS (0, 2.5, 5 or 10 μg/ml) for 48 hour at a density of 1×10^7 cells/ml. The culture supernatants were collected and the level of each cytokine was measured by ELISA. The data shows one of four (C3H/HeN and CBA/J), three (DBA/2) or two (C57BL/6) experiments performed with similar results. The results show the mean ± standard deviation (S.D.). *; $P < 0.05$ compared with the control using Student's *t-test*.

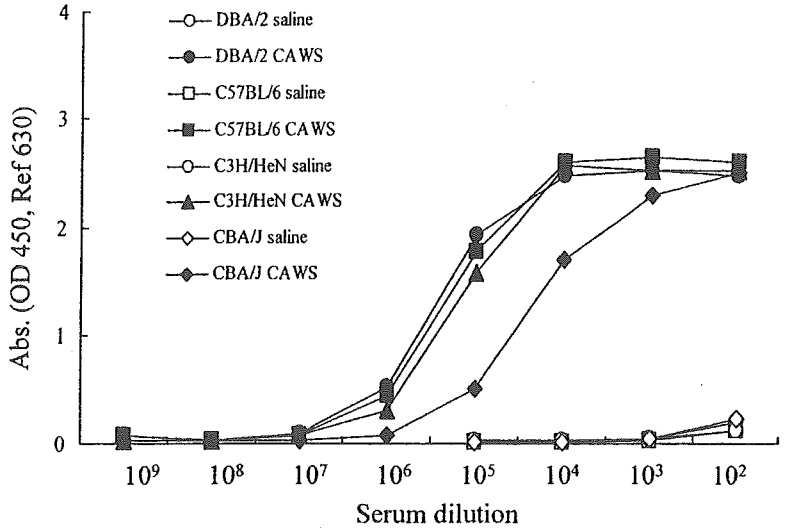

Figure 7. Anti-CAWS antibody in sera from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, sera were collected from each mouse. Anti-CAWS antibody was measured by ELISA. Color development was stopped after 10 min.

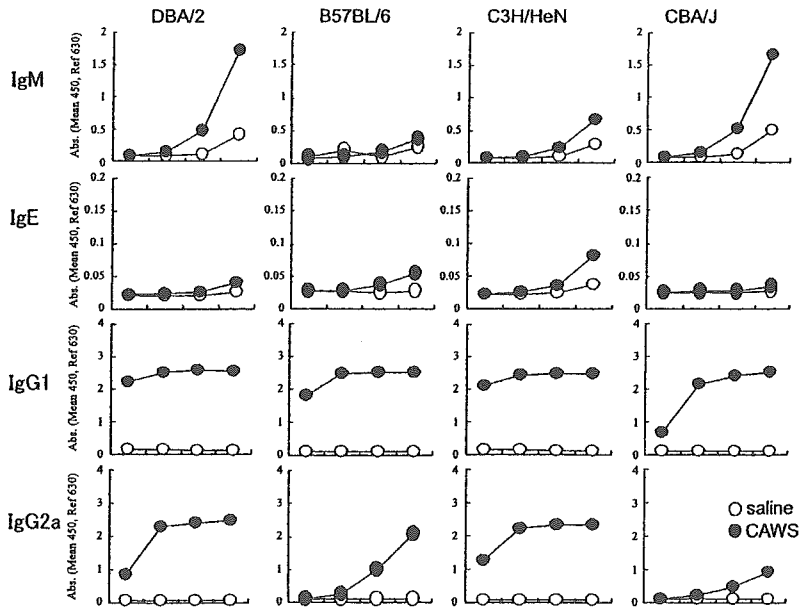

Figure 8. Immunoglobulin subclasses of anti-CAWS antibody in sera from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, sera were collected from each mouse. Anti-CAWS immunoglobulin subclasses were measured by ELISA. Color development was stopped after 10 minute.

DISCUSSION

Kawasaki disease is a febrile inflammatory disease that presents with systemic arteritis, and can be fatal particularly in the case of an exacerbation of coronaritis. When CAWS is administered to mice in accordance with the protocol of Murata et al.,[7,8] a Kawasaki-disease-like angiitis is induced at the origin of the coronary arteries. In the present study, strain differences were found to exist with respect to the incidence of the disease induced by CAWS with DBA/2, C3H/HeN and C57BL/6 mice exhibiting sensitivity and CBA/J mice exhibiting resistance. Moreover, histological observation of the sites of coronaritis in DBA/2 mice revealed hypertrophy of the tunica intima and cellular invasion, and the disease occurred with extremely high levels of severity and frequency. DBA/2 mice developed a much more severe coronary arteritis than the other strains. In addition, DBA/2 mice exhibited high mortality during the course of the disease's induction, and based on findings obtained from histological examination of the hearts, the cause of death was suspected to be myocardial infarction attributable to coronary occlusion. Because the difference in sensitivity to CAWS among the mouse strains examined in this study correlates strongly with the diversity of prognoses for Kawasaki disease patients,[19] this model is considered to be effective for elucidating the cause of coronary arteritis associated with Kawasaki disease, analyzing the characteristic condition, and developing more effective treatment methods.

Among patients with Kawasaki disease in the acute stage, cytokines, including IL-1, IL-2, IL-2 receptor, IL-6 and TNF-α, are detected in the serum.[20–23] When the production of cytokines from spleen cells was measured in mice stimulated with CAWS in vitro, a similar trend was demonstrated by the three strains that were sensitive to coronary arteritis induction, namely, DBA/2, C3H/HeN and C57BL/6, and in the case of DBA/2 mice in particular, a prominent CAWS-specific response involving inflammatory cytokines such as IL-6, IFN-γ and TNF-α, was observed, indicating the occurrence of an inflammatory immune response. The levels of inflammatory cytokines produced were high in DBA/2 mice in particular. On the other hand, an increased production of IL-10, which exhibits an immunosuppressive action, was observed in CBA/J mice that exhibited resistance to the occurrence of coronary arteritis. It is interesting to note that these cytokine production patterns resemble those observed in Kawasaki disease patients.

However, as there is one report indicating increased production of IL-4 and IL-10 in patients with Kawasaki disease,[24] dynamics that do not necessarily coincide with the findings of this study are observed. The discrepancies may be related to the stage of the disease (acute, subacute or recovery stage), thus indicating the need for further study of the relationship between cytokine production and the condition of Kawasaki disease.

Analyses have been conducted on the background genes of various diseases, and in the case of Kawasaki disease as well, there is a possibility of some form of involvement by genetic factors.[25,26] During the course of research on genes associated with coronaritis in rodents, numerous analyses have been conducted on the relationship between arteriosclerosis and hyperlipemia, and the IL-10 gene has been reported to have a close relationship with the lesions of coronary arteritis.[27,28] This finding also supports the findings obtained in the present study. In addition, although CBA is a strain derived from DBA, it is quite interesting that there are large differences in the

incidences of coronary arteritis and myocarditis induced by CAWS between the two strains. A survey of the genes involved in mouse coronaritis also suggested the existence of multiple inductive genes and repressor genes. This is a subject that requires detailed analysis.

On the basis of the above results, the activation of lymphocytes, vascular endothelial cells and so forth was prominently induced by means of hypercytokinemia in DBA/2 mice, and the resulting coronaritis promoted the occurrence of chronic myocardial ischemia, which, as a result, was thought to ultimately lead to death caused by fibrosis, infarction and cardiac insufficiency. It is hoped that this model will contribute not only to elucidation of the stage of Kawasaki disease and the associated coronary arteritis, but also to the improvement and development of treatment methods.

ACKNOWLEDGMENTS

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Environ. Mutagen Res., 27: 7-12 (2005) Original Article

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

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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* WP2*uvrA*/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 $\mu W/cm^2$ and that Trp^+ reverse mutations in *Escherichia coli* WP2*uvrA*/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 WP2*uvrA*/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 $\mu W/cm^2$(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 $1-3\times10^9$ 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 WP2*uvrA*/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

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

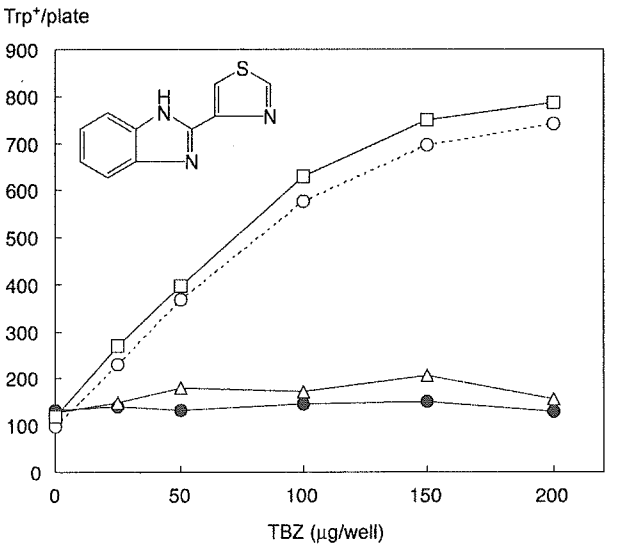

Fig. 1 Effect of S9 mix and co-factors on photo-mutagenicity of TBZ. UVA was irradiated for 10 min. ○, control (buffer); □, S9 fraction (50 μL/well); ●, S9 mix (0.5 mL/well); △, co-factors solution (0.5 mL/well)

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-

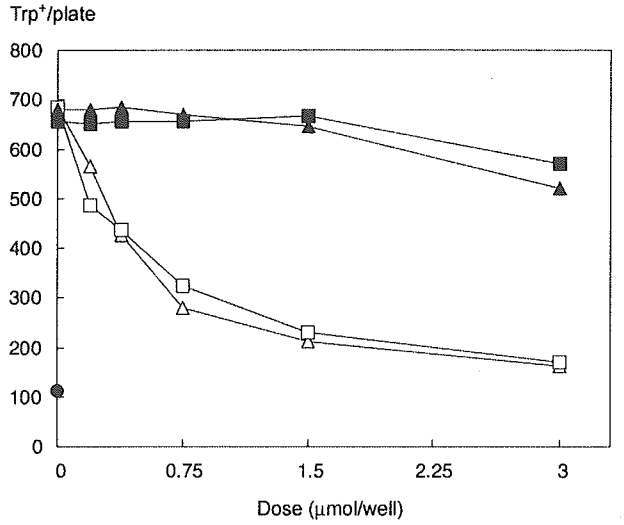

Fig. 2 Inhibitory effect of NADH and NADPH on photo-mutagenicity of TBZ. NAD^+ (▲), $NADP^+$ (■), NADH (△), or NADPH (□) was added to the mixture of bacteria and TBZ (0.75 μmol/well) in 0.5 mL phosphate buffer and then the mixture was irradiated for 10 min. ●, control without TBZ

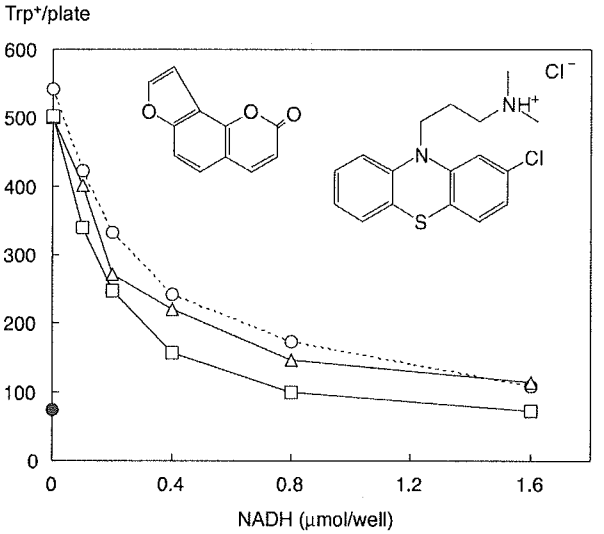

Fig. 3 Inhibition of photo-mutagenicity of TBZ, chlorpromazine, and angelicin by NADH. UVA was irradiated for 10 min. ○, TBZ (100 μg/well); △, angelicin (1 μg/well); □, chlorpromazine (10 μg/well); ●, control

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.

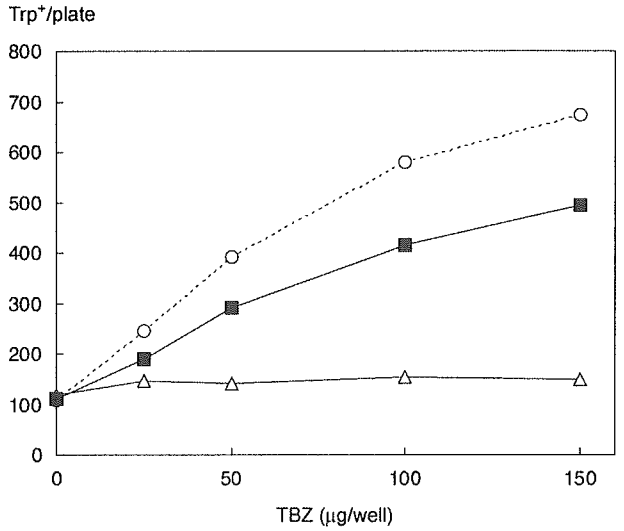

Fig. 4 Photo-mutagenicity of TBZ in the presence of S9 mix. UVA was irradiated for 10 min. ○, control (buffer); ■, NAD^+/$NADP^+$-based S9 mix, △, NADH/NADPH-based S9 mix

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 photo-genotoxic activity only when irradiated in the presence of S9 mix have been reported, the effects of S9 mix on photo-mutagens 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 photo-mutagenicity. 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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A novel genotoxic aspect of thiabendazole as a photomutagen in bacteria and cultured human cells

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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.

Keywords: Photogenotoxicity; Thiabendazole; UVA; DNA damage; Micronucleus; Gene mutation

1. Introduction

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

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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*, Δ*lac*] (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

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

TBZ (μg/ml)	UVA (min)	Cell growth	β-Galactosidase activity	
		OD600	OD420	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.

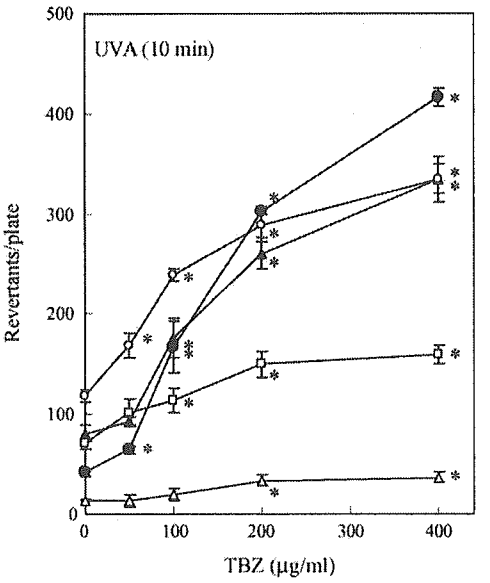

Fig. 1. Induction of reverse mutations in *E. coli* and *S. typhimurium* by TBZ with UVA-irradiation. Cells were treated with TBZ + UVA for 10 min and washed before plating. □, TA100; △, TA98; ▲, TA97; ○, TA104; ●, WP2s/pKM101 (*$p < 0.05$).

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

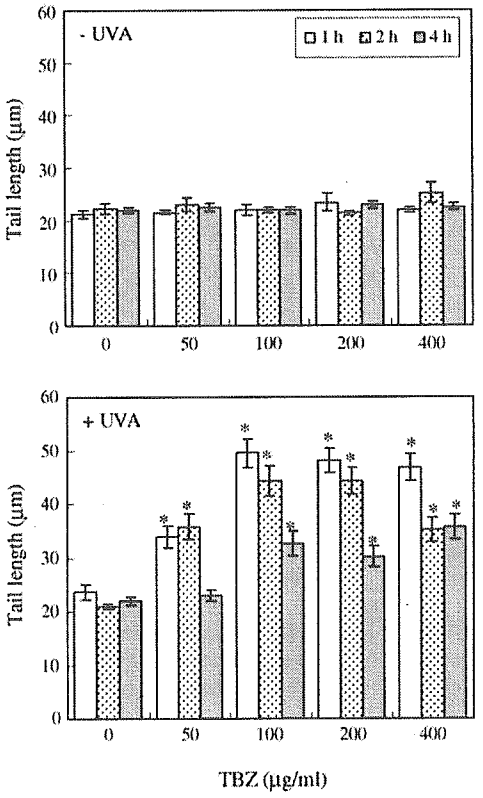

Fig. 2. Induction of DNA damage in WTK1 cells by pulse treatment with TBZ + UVA for 10 min in the comet assay. Assay was conducted 1 h (open bar), 2 h (dotted bar), and 4 h (closed bar) after the irradiation. (*Upper*) un-irradiated control cells, (*lower*) UVA-irradiated cells ($^{*}p < 0.05$).

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-carriying 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.