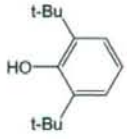
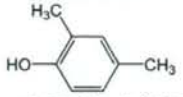
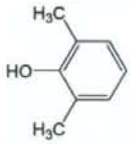
・2,6-tert-butylphenol (CAS No. 128-39-2)



・2,4-Xylenol (CAS No. 105-67-9)



・2,6-Xylenol (CAS No. 576-26-1)



物理化学的性状、環境毒性においては上記物質でのカテゴリー化については問題がないが、ヒト毒に関しては、t-Butyl を一つまたは二つを持つ場合、評価するための合理性の説明が必要等からカテゴリーの再考が指摘された。

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D. 研究成果による特許権等の知的財産権の出願・登録状況 (予定を含む)

1. 特許取得
(該当なし)
2. 実用新案登録
(該当なし)
3. その他
(該当なし)

厚生労働科学研究費補助金

化学物質リスク研究事業

化学物質リスク評価における(定量的)構造活性相関((Q)SAR)に関する研究

2／2

Ⅲ．研究成果の刊行物・別刷

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Performance of flow cytometric analysis for the micronucleus assay—a reconstruction model using serial dilutions of malaria-infected cells with normal mouse peripheral blood

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To confirm the performance and statistical power of a flow cytometric method for scoring micronucleated erythrocytes, reconstruction experiments were performed. For these investigations, peripheral blood erythrocytes from untreated mice, with a micronucleated erythrocyte frequency of ~0.1% were combined with known quantities of *Plasmodium berghei* (malaria) infected mouse erythrocytes. These cells had an infected erythrocyte frequency of ~0.7%, and mimic the DNA content of micronuclei (MN). For an initial experiment, samples with a range of MN/malaria (Mal) content were constructed and analysed in triplicate by flow cytometry until 2000, 20 000 and 200 000 total erythrocytes were acquired. In a second experiment, each specimen was analysed in triplicate until 2000, 20 000, 200 000 and 1 000 000 erythrocytes were acquired. As expected, the sensitivity of the assay to detect small changes in rare erythrocyte sub-population frequencies was directly related to the number of cells analysed. For example, when 2000 cells were scored, increases in MN/Mal frequencies of 3.9- or 2.7-fold were detected as statistically significant. When 200 000 cells were analysed, a 1.2-fold increase was detected. These data have implications for the experimental design and interpretation of micronucleus assays that are based on automated scoring procedures, since previously unattainable numbers of cells can now be readily scored.

Introduction

From a statistical point of view, in order to achieve a higher power of detection, sample size should be increased. For many experimental situations, it is not always feasible to increase the number of subjects studied. When the event under consideration is rare as to cause appreciable scoring error, then an alternative would be to enhance the precision of each measurement. For example, in the rodent erythrocyte micronucleus assay, the evaluation of 2000 immature erythrocytes per animal and 5 animals per dose group represents commonly cited minimum values. Owing to the rarity of micronucleated cells, even this minimal assay design results in tedious and time-consuming efforts. The use of flow cytometry (1–3) realizes the ability to evaluate high numbers of erythrocytes, something that is impossible to achieve by manual microscopy. By reducing scoring error in this manner, flow cytometry has the potential to increase statistical power.

In the present study, we evaluated the relationship between statistical power to detect a rare erythrocyte sub-population, i.e. micronucleated or malaria-infected erythrocytes (MN/Mal), and the total number of erythrocytes analysed. These experiments were accomplished using a reconstruction model whereby known quantities of malaria-infected erythrocytes were added to blood from an untreated mouse. Malaria is a known model for micronucleated erythrocytes, as they endow the target cells of interest with a micronucleus-like DNA content (4,5). The samples were analysed by flow cytometry to measure the MN/Mal frequency through the interrogation of 2000 (2k), 20 000 (20k), 200 000 (200k) and 1 000 000 (1m) erythrocytes. The results presented here show the capability of flow cytometric technology to reduce scoring error, and also the extent to which this affects the ability to detect small changes to baseline micronucleus frequencies.

Materials and methods

Staining of blood specimens

Methanol-fixed blood from untreated and malaria-infected mice used in this study were two 'biological standards' which accompany the Mouse MicroFlow®PLUS kits (Litron Laboratories, NY). MicroFlow PLUS kits were the source of these specimens.

Before analysis, malaria-infected specimens and untreated mouse specimens were washed out of fixative with ~12 ml Hank's Balanced Salt Solution. Procedures for the 3-colour labelling technique which appear in the MicroFlow®PLUS instruction manual (version 031230) were scaled up ~7-fold in order to provide at least 10 ml each of control and malaria blood in a cell density range that is recommended for this assay (between ~2000 and 6000 events/s). Anti-CD71-FITC, anti-CD61-PE and all other flow cytometry reagents were also supplied in the kits. After the labelling procedures were accomplished, the cell density of the malaria-infected sample was adjusted so that it was equal to that of the control blood sample. Initial cell densities were measured with a Coulter Counter, model ZM. After adjustment with additional propidium iodide staining solution, equal cell densities were confirmed by Coulter Counter measurements. Normalization of cell densities was an important experimental design consideration, as this allowed us to calculate the expected MN/Mal frequencies in the diluted samples once the frequencies of the original control (0.10 and 0.09% for Experiments 1 and 2, respectively) and malaria-infected (0.67 and 0.70% for Experiments 1 and 2, respectively) samples were determined with high precision (i.e. control and malaria-infected %MN/Mal frequencies are the mean value of triplicate analyses with 1m erythrocytes per analysis).

Dilution of malaria blood specimen

Malaria-infected blood (Sample H) was diluted with control blood (Sample A) in the following ratios (v/v): 1:1 (Sample G), 1:3 (Sample F), 1:7 (Sample E), 1:15 (Sample D), 1:31 (Sample C) and 1:63 (Sample B). These blood specimens were stored at 4°C until flow cytometric analysis, which occurred on the same day. Each sample was analysed three times to evaluate reproducibility.

Flow cytometric analysis

All samples were analysed according to the MicroFlow® PLUS 3-colour technique. One deviation to the kit-supplied data acquisition and analysis template was that the frequency of erythrocytes with malaria or micronuclei was determined without restriction to CD71-expression level. That is, the

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Mal and MN frequencies measured and reported here are based on total peripheral blood erythrocytes. A second deviation from standard practices is that the default stop mode of 20 000 reticulocytes was not utilized. Rather, each specimen was analysed until the following number of erythrocytes were acquired: 2k, 20k and 200k erythrocytes in the first experiment and 2k, 20k, 200k and 1m erythrocytes in the second experiment.

Statistical analysis

The average of triplicate MN/Mal measurements associated with the control blood sample were compared with those associated with each of the other seven specimens by the Fisher's exact method. A P-value of 0.05 divided by 7 (number of sample groups) was considered evidence of a statistically significant difference. Expected versus observed MN/Mal frequencies were graphed for each measurement performed in the second experiment. Microsoft Excel (Microsoft Corp., Seattle, Washington) was used to determine a best-fit line. The associated equations and r^2 values were determined.

Results

Data from Experiments 1 and 2 are summarized in Table I and include the expected and observed MN/Mal frequencies. The MN/Mal frequencies shown are the average of triplicate analyses. As shown in Table I, for measurements based on 2k erythrocytes, samples with expected MN/Mal frequencies of 0.39 and 0.24% were found to be significantly different from control samples, in Experiments 1 and 2, respectively. These values correspond to fold increases of 3.9 and 2.7 for the first and second experiment, respectively. As more erythrocytes were analysed per sample, the detection limit was improved. For instance, measurements based on the evaluation of 200k erythrocytes per analysis show statistical significance for expected MN/Mal samples of 0.12 and 0.11%. These values correspond to an increase of ~1.2-fold. In fact for the second experiment, when a stop mode of 1m erythrocytes was investigated, statistical significance was observed between the control blood sample (0.09% MN/Mal) and the specimen with the lowest frequency of malaria (0.10% MN/Mal; $P = 0.00005$).

As an aid for visualizing the performance characteristics associated with the various number of cells analysed, scattergrams showing %MN/Mal measurement are presented (Fig. 1). Best-fit lines and equations are included with these graphs, and illustrate the degree to which the experimentally derived data agree with the linear relationship that is known to exist among MN/Mal frequencies for these specimens.

Discussion

To evaluate the performance and statistical power of a flow cytometric approach to score micronucleated erythrocytes, we performed a reconstruction model experiment by the serial dilution of malaria-infected mouse blood with normal mouse

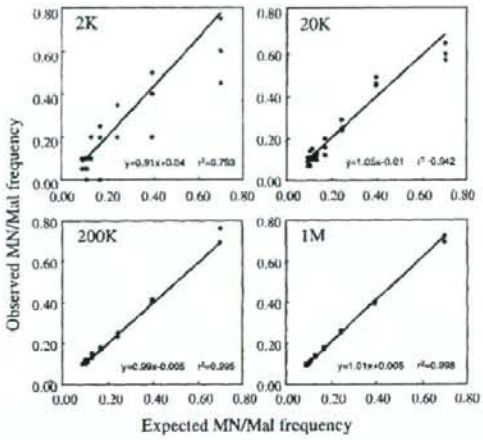

Fig. 1. Scattergram of expected versus observed MN/Mal frequencies. Each of three replicate measurements is plotted for these specimens. Best-fit linear lines are graphed, with associated equations. r^2 values document the degree of reproducibility observed.

Table I. MN/Mal frequencies (%) and *P*-values for comparisons with sample A

Sample	Expected (%)	Number of cells analysed/sample							
		2k		20k		200k		1m	
		(%)	*P*-value	(%)	*P*-value	(%)	*P*-value	(%)	*P*-value
Experiment 1									
A	0.10	0.07		0.09		0.10			
B	0.11	0.08	*0.50000*	0.11	*0.20764*	0.11	*0.13403*		
C	0.12	0.05	*0.77349*	0.10	*0.46272*	0.12	*0.00002*		
D	0.14	0.08	*0.50000*	0.13	*0.03463*	0.14	*0.00000*		
E	0.18	0.22	*0.02452*	0.25	*0.00000*	0.18	*0.00000*		
F	0.25	0.18	*0.05924*	0.30	*0.00000*	0.25	*0.00000*		
G	0.39	0.52	*0.00000*	0.36	*0.00000*	0.43	*0.00000*		
H	0.67	0.57	*0.00000*	0.73	*0.00000*	0.67	*0.00000*		
Experiment 2									
A	0.09	0.08		0.09		0.10		0.09	
B	0.10	0.08	*0.62305*	0.11	*0.20374*	0.11	*0.04822*	0.10	*0.00005*
C	0.11	0.05	*0.85547*	0.12	*0.06479*	0.12	*0.00001*	0.11	*0.00000*
D	0.13	0.13	*0.29053*	0.12	*0.08887*	0.14	*0.00000*	0.13	*0.00000*
E	0.17	0.15	*0.21198*	0.16	*0.00938*	0.17	*0.00000*	0.17	*0.00000*
F	0.24	0.30	*0.00531*	0.26	*0.00000*	0.24	*0.00000*	0.25	*0.00000*
G	0.40	0.37	*0.00026*	0.47	*0.00000*	0.41	*0.00000*	0.40	*0.00000*
H	0.70	0.60	*0.00000*	0.61	*0.00000*	0.71	*0.00000*	0.70	*0.00000*

Shading indicates those samples that are significantly different from respective control samples.

blood. As expected, the present results illustrate that the power of rare event detection is directly related to the number of cells analysed per specimen. By analysing 3m (triplicate of 1m) cells per group, 0.10% is significantly different ($P = 0.00005$) when compared with 0.09%. Even so, it must be appreciated that the biological significance of minute changes must be considered in addition to statistical significance.

Previously, we have shown that individual differences were negligible in the mouse micronucleus assay when 1000 cells per animal and 5 or 6 mice per dose group were analysed (6–8) and the statistical unit for the evaluation can be assigned to a cell but not to an animal. According to the present results and also results by Asano *et al.* (9), the variability of the data was high when 2k cells were analysed. Under these circumstances, the difference among animals is not apparent, as they are likely to be smaller than the scoring error. While, in the case of the present malaria dilution experiments, when 200k or 1m cells per sample were analysed, the scoring error decreased and converged to a value. This, however, is not true in the case of the actual micronucleus assays using model chemicals (9). When 200k or 1m immature erythrocytes were analysed, differences between individual animals became apparent and there was data variability within each dose group. Therefore, even if the experimental size in the animal experiments is increased, we cannot expect the same increment of detecting power. This finding suggests that optimizing the statistical procedure also includes evaluating individual differences.

Based on the present results, we confirm the accuracy and high performance of the micronucleus assay system using flow cytometry and we propose that the number of reticulocytes analysed for the micronucleus assay using flow cytometry be a minimum of 20k. We suggest that the analysis of 20k reticulocytes is approximately equivalent to the manual microscopic analysis according to test guideline OECD 474 (9,10). We anticipate that the experimental size of the MN assay will be recommended and set by expert committees based on the evaluated data. In addition to statistical sensitivity, biological variability between animals and as a consequence of treatment should also be considered. There appears to be diminishing value to analyses based on 200k or even 1m per animal. These may be useful in certain special circumstances, for instance when looking for evidence of threshold or practical threshold effects (9).

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Practical threshold for micronucleated reticulocyte induction observed for low doses of mitomycin C, Ara-C and colchicine

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Micronucleus induction was studied for the DNA target clastogens mitomycin C (MMC) and 1-β-D-arabinofuranosylcytosine (Ara-C), and also the non-DNA target aneugen colchicine (COL) in order to evaluate the dose–response relationship at very low dose levels. The acridine orange (AO) supravital staining method was used for microscopy and the anti-CD71-FITC based method was used for flow cytometric analysis. In the AO method, 2000 reticulocytes were analysed as commonly advised, but in the flow cytometric method, 2000, 20 000, 200 000 and 1 000 000 reticulocytes were analysed for each sample to increase the detecting power (i.e. sensitivity) of the assay. The present data show that increasing the number of cells scored increases the statistical power of the assay when the cell was considered as a statistical unit. Even so, statistically significant differences from respective vehicle controls were not observed at the lowest dose level for MMC and Ara-C, or the lower four dose levels for COL, even after one million cells were analysed. When the animal was considered as a statistical unit, only the top dose group for each chemical showed significant increase of micronucleated reticulocytes frequency. As non-linear dose–response curves were obtained for each of the three chemicals studied, these observations provide evidence for the existence of a practical threshold for the DNA target clastogens as well as the non-DNA target aneugen studied.

Introduction

As chemical safety evaluations are performed, the existence of a threshold is an important issue when considering genotoxic carcinogens. There are two important threshold concepts, i.e. 'absolute' threshold and 'practical or biological' threshold in genotoxicity. The 'absolute' threshold is defined as a concentration below which a cell would not 'notice' the presence of the chemical, and the 'practical or biological' one is considered as a concentration below which any effect is biologically unimportant (1–3). Some chemicals clearly exhibit a threshold, and non-DNA target mechanisms of action (e.g. spindle apparatus disturbance, topoisomerase II inhibition, DNA synthesis inhibition, overloading homeostatic defence, and physiological perturbation) provide rationale for the non-linear responses that are observed (4). For instance, the spindle poison, colchicine (COL), damages spindle fibres, but the effect on chromosome movement should be detected only at the concentration that damages enough microtubules to impair the anchorage of the chromosome. This mechanism is thought to explain the threshold that is observed for this particular non-DNA target chemical (5). Up to the present, a widely held view is that genotoxic carcinogens do not have a threshold, and thus it has been difficult to determine the acceptable daily intake (ADI) safety exposure level. For this reason, such chemicals have been banned for use in daily life, most notably in food and food-related chemicals. Recently, however, discussion on the strategy for evaluating genotoxicity for risk assessment has been initiated (6–8). Moreover, in many cases and especially in the European Union, the principle of reducing exposure to unavoidable toxic compounds to levels that are as-low-as-reasonably-achievable (ALARA) has been advocated (9–11).

Adding to this complex discussion are reports by Fukushima and his group (12–14) who have demonstrated the existence of practical thresholds for the genotoxic hepatocarcinogens 2-amino-3, 8-dimethylimidazo *[4,5-f]* quinoxaline (MeIQx) and *N*-nitrosodiethylamine, and even hormesis for phenobarbital. These investigators studied carcinogenicity, glutathione *S*-transferase placental form (GSTP) positive focus, gene mutation, DNA oxidative damage and DNA adduct formation at very low dose levels. They showed GSTP positive focus induction at the dose level at which carcinogenicity could not be detected; gene mutation could be observed at the dose level at which GSTP positive foci could not be detected, and so on. Therefore, they concluded that at least practically, a threshold for carcinogenicity existed. It has been claimed that one of the shortcomings of their proposal is the lack of discussion of the sensitivity of the assays they performed, because, from a statistical view point, the power of the assay (i.e. sensitivity) largely depends on the number of cells analysed.

The micronucleus assay has been widely used for evaluating chemical genotoxicity *in vivo*. One of the characteristics of the rodent peripheral blood micronucleus assay is its simple endpoint, i.e. a small DNA containing cell inclusion in the cytoplasm of enucleated erythrocytes. Because of this simplicity, automation of analysis has been achieved by image analysis (15–17) and flow cytometry (18–23). We have developed a high performance manual method using acridine orange (AO) supravital staining (24,25). We have also developed a flow cytometric method utilizing an erythrocyte surface antigen for CD71 to identify young erythrocytes and the use of malaria infected erythrocytes as an instrument calibration standard for accurate measurement (21–23,26). In the present study, we aimed to show the dose–effect relationship of micronucleus inducers with different modes of action at extremely low dose levels. We applied two methods, i.e. the manual AO supravital staining method and flow cytometry, on three model

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chemicals: mitomycin C (MMC), which is a cross-linking agent and typical micronucleus inducer (27) frequently used as a positive control in the micronucleus assay; 1-β-D-arabinofuranosylcytosine (Ara-C), which is a long-patch repair inhibitor and known inducer of small micronuclei (28); and COL, a spindle poison that induces large micronuclei (29). Another aim was to evaluate the degree to which the statistical power of the assay depends on the number of target cells interrogated.

Further impetus to study low dose effects came from reports by Grawé *et al.* (30) and Abramsson-Zetterberg (31), who measured genotoxicant-induced micronuclei using an alternate, dual laser flow cytometric technique. We discuss the interpretation of very weak micronucleus induction, if any, at low dose levels and also the existence of a threshold effect.

Materials and methods

Chemical substances

Three micronucleus inducers with different modes of action, MMC (CAS number: 50-07-7), Ara-C (CAS number: 147-94-4), and COL (CAS number: 64-86-8) were obtained from Kyowa Hakko (Tokyo, Japan), Merck (PA, USA) and Sigma (Mo, USA), respectively. MMC was dissolved with sterile distilled water and COL and Ara-C were dissolved with physiological saline. Solvents were used as negative controls.

AO pre-coated slides for supravital staining were obtained from Toyobo Co., Osaka. Fixative, anticoagulant and other all materials necessary for diluting, fixing and shipping specimens for flow cytometric analysis were from Mouse MicroFlow® Basic Kits (transported from Litron Laboratories, NY).

Animals

Healthy seven-week-old male CD-1 mice (ICR, Charles River Japan Inc., Hino, Japan) with a mean body weight of 34.6 g were used after a week's acclimation. The mice were housed at 21 ± 1°C and 55 ± 10% relative humidity and exposed to 12-h light-dark cycle. Five mice per group were assigned randomly and were given commercial food pellets (CE-2, CLEA Japan Inc., Tokyo Japan) and tap water *ad libitum* throughout the acclimation and the experimental period.

Micronucleus assay

The highest dose level of each chemical was determined experimentally or by referring to the published data (27,32–34). The objective was to choose a high dose level that induced micronuclei slightly, but statistically significantly. We selected 0.3 mg/kg for MMC, 6 mg/kg for Ara-C, and 0.8 mg/kg for COL as the high dose levels. The six dose levels for MMC and five dose levels for Ara-C and COL were spaced by the square root of 10. A solvent control was assigned to each experiment as reference. Each chemical was delivered intraperitoneally (10 ml/kg) once and blood was collected 48 h after treatment based on data from a previous paper (25). Treatment, sample preparation and AO scoring were performed at Nitto Denko, Osaka, Japan and fixed blood samples were sent by air on dry ice to Litron Laboratories, NY for flow cytometric analysis.

All AO supravital staining slides were coded and analysed without knowledge of treatment information. All tubes containing fixed blood cells for flow cytometric analysis were also coded. All codes were broken only after analysis was completed.

AO supravital staining micronucleus assay

The AO supravital staining micronucleus assay was performed according to the method of Hayashi *et al.* (24,25). Aliquots of 5 μl of peripheral blood, obtained from the tail blood vessel of each mouse, was put on an AO coated glass slide, and immediately covered with a glass coverslip. Two thousand reticulocytes were analysed by fluorescence microscopy (Model:AHBT3-RFC, Olympus, Tokyo Japan) with blue-excitation filter set, and the number of micronucleated reticulocytes (MNRET) was scored.

Flow cytometric analysis

At the same time of AO supravital sampling, another 100 μl blood was collected via orbital sinus into the Mouse MicroFlow® basic kit-supplied anticoagulant solution using a cleaned glass capillary. Each sample was fixed in duplicate with ultracold (−80°C) methanol, agitated vigorously and immediately placed at −80°C until shipment on dry ice from Nitto Denko to Litron Laboratories for flow cytometric analysis.

On the day of analysis, samples were washed out of fixative with ~12 ml Hank's Balanced Salt Solution (HBSS). A high-density/CD71-associated fluorescence thresholding technique was used (35,36). Briefly, with this method, 80 μl of each washed cell pellet was added to polypropylene tubes

Table I. Frequencies of micronucleated reticulocytes (%) assay by manual and flow analysis

Chemical	Dose (mg/kg body wt)	Number of cells analysed				
		Manual	Flow cytometry			
		2 K	2 K	20 K	200 K	1 M
MMC	0	0.22 ± 0.06	0.23 ± 0.16	0.25 ± 0.09	0.23 ± 0.06	0.23 ± 0.05
	0.00095	0.33 ± 0.06	0.17 ± 0.04	0.21 ± 0.05	0.24 ± 0.06	0.24 ± 0.05[a]
	0.00300	0.24 ± 0.08	0.25 ± 0.11	0.27 ± 0.05	0.25 ± 0.05	0.25 ± 0.05[b]
	0.00948	0.24 ± 0.17	0.22 ± 0.09	0.28 ± 0.04	0.27 ± 0.03[b]	0.27 ± 0.04[b]
	0.03000	0.20 ± 0.06	0.22 ± 0.08	0.24 ± 0.08	0.19 ± 0.08	0.23 ± 0.08
	0.09480	0.35 ± 0.14	0.27 ± 0.10	0.25 ± 0.08	0.28 ± 0.07[b]	0.27 ± 0.06[b]
	0.30000	0.64 ± 0.15[d,b]	0.36 ± 0.10	0.40 ± 0.11[c,b]	0.41 ± 0.06[d,b]	0.39 ± 0.05[d,b]
Ara-C	0.000	0.32 ± 0.10	0.21 ± 0.05	0.21 ± 0.05	0.23 ± 0.04	0.22 ± 0.02
	0.060	0.26 ± 0.15	0.19 ± 0.07	0.24 ± 0.07	0.24 ± 0.07	0.24 ± 0.07
	0.190	0.28 ± 0.18	0.30 ± 0.06	0.23 ± 0.06	0.25 ± 0.04[c]	0.25 ± 0.03[c]
	0.600	0.32 ± 0.03	0.21 ± 0.13	0.25 ± 0.05	0.27 ± 0.05[b]	0.27 ± 0.05[b]
	1.890	0.42 ± 0.21	0.24 ± 0.11	0.29 ± 0.07[b]	0.29 ± 0.05[b]	0.28 ± 0.04[b]
	6.000	0.81 ± 0.38[d,b]	0.34 ± 0.10[d]	0.41 ± 0.11[c,b]	0.39 ± 0.09[c,b]	0.39 ± 0.09[c,b]
COL	0.00000	0.32 ± 0.08	0.22 ± 0.10	0.25 ± 0.05	0.27 ± 0.05	0.28 ± 0.06
	0.00800	0.18 ± 0.11	0.21 ± 0.09	0.22 ± 0.06	0.22 ± 0.04	0.22 ± 0.03
	0.02520	0.31 ± 0.13	0.16 ± 0.09	0.20 ± 0.04	0.22 ± 0.04	0.21 ± 0.04
	0.08000	0.13 ± 0.08	0.22 ± 0.11	0.23 ± 0.06	0.21 ± 0.05	0.21 ± 0.03
	0.25200	0.34 ± 0.19	0.21 ± 0.08	0.28 ± 0.05	0.26 ± 0.04	0.27 ± 0.05
	0.80000	0.56 ± 0.19 [b,d]	0.41 ± 0.14	0.41 ± 0.13[b]	0.44 ± 0.13[b]	0.44 ± 0.13[b]

Data are shown as mean ± SD (%) of five mice.
[a]Only 881 389 instead of 1 M cells were analysed in one of five mice.
[b]Fisher's exact test (all groups) significant difference at 1%.
[c]Fisher's exact test (all groups) significant difference at 5%; no mark, not significant.
[d]Student's *t*-test (20 and 200 K, and 1 M groups) significant difference at 1%.
[e]Student's *t*-test (20 and 200 K, and 1 M groups) significant difference at 5%.

containing 80 µl RNase/antibodies (1.0 ml HBSS, 10 µl anti-CD71-FITC, 5 µl anti-CD61-PE and RNase A at 5 mg/ml). Antibodies, and all other flow cytometry reagents, including fixed malaria-infected rodent blood (malaria biostandard), were from Mouse MicroFlowPLUS® Kits (available from Litron Laboratories, Rochester, NY, and BD Biosciences-Pharmingen).

Following successive 30-min incubations at 4°C and ~37°C, the cells were placed at 4°C until analysis (same day). For analysis, each sample was resuspended in approximately 1.5 ml propidium iodide (PI) staining solution. Of the stained blood sample 100 µl was transferred to a separate tube containing 400 µl PI. This diluted sample was used to determine the percentages of reticulocytes and micronucleated normochromatic erythrocyte (MN-NCE) of each blood sample by the analysis of 1 000 000 (1 M) total erythrocytes.

The corresponding undiluted sample was then analysed using the CD71-thresholding technique whereby CD71-negative erythrocytes (the majority of the cells) were omitted from acquisition (35,36). The frequency of micronuclei was then measured for each sample using each of the following stop modes: 2000 (2 K), 18 000 (18 K), 180 000 (180 K) and 800 000 (800 K) reticulocytes. By adding the successive values, percentage of MNRET frequencies could be calculated based on the analysis of 2 K, 20 000 (20 K), 200 000 (200 K) and 1 M reticulocytes.

Statistical analysis

P-values for each comparison with respective controls were calculated by Fisher's exact method. For the flow cytometry data based on 20 K or more cells analysed, a Student's *t*-test was used after normality of the data was confirmed. When determining statistical significance, a Bonferroni correction was used to adjust for the multiple (i.e. 5) comparisons made.

Results

The group means of five mice/group are summarized in Table I for the AO supravital staining method based on the observation of 2 K reticulocytes and for the flow cytometric method based on the observation of 2, 20 and 200 K, and 1 M reticulocytes. The *P*-values for all three chemicals were <0.01 at the highest dose group when the Fisher's exact test was applied. However, when considering individual differences, this was not the case for all COL high dose datasets when evaluated using the Student's *t*-test. Dose-response relationship curves of MMC, Ara-C and COL are shown in Figure 1. Dose-response curves of each chemical were similar between AO supravital analysis and flow cytometric analysis, although there was a tendency for the absolute values of induced MNRET to be higher by the AO supravital method than by the flow cytometric method, especially at higher dose levels.

It is evident that the variation among mice in each dose group decreased depending on the number of cells analysed. Even so, individual differences among animals in each group were observed even when 1 M reticulocytes were scored per sample. Likewise, the smoothness of the dose–response curves tended to increase as the number of cells analysed increased. As a representative example, Figure 2 shows individual scattergrams of MMC at 2, 20 and 200 K, and 1 M reticulocytes analysed. There are not, however, essential differences among results based on the number of cells analysed.

Discussion

According to the present data, MNRET frequencies obtained using the AO supravital staining method tended to be higher than those by flow cytometric analysis. This phenomenon may be explained by modest differences in the age cohort of reticulocytes analysed by each method, i.e. AO supravital staining, where the analysis is restricted to Types I, II and III reticulocytes, and the flow cytometric method, where the analysis was restricted to reticulocytes with the CD71-positive phenotype. In both analysis procedures, the method of defining reticulocytes was kept consistent for all samples.

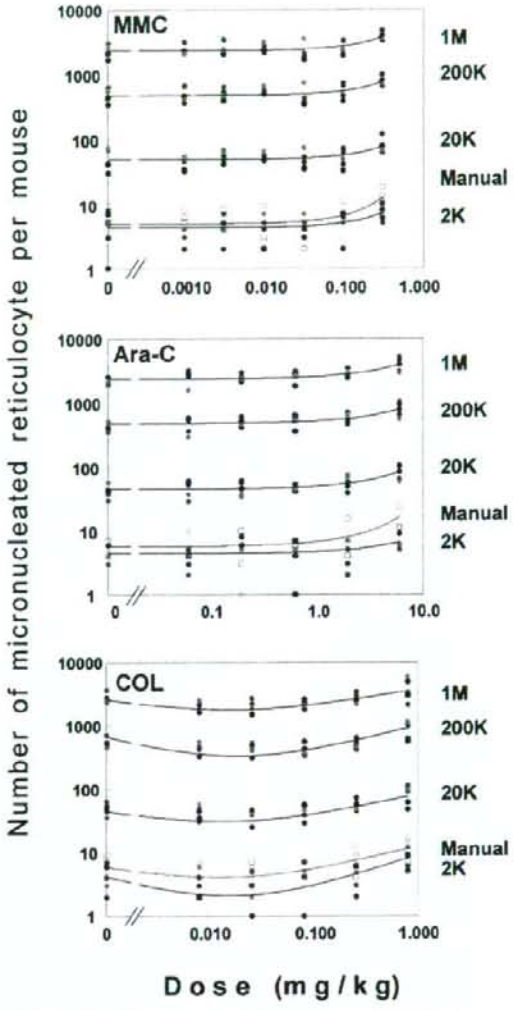

Fig. 1. Dose–response curves for MMC, Ara-C and COL. Each symbol shows individual number of micronucleated reticulocytes. In each dose group, a colour shows identical mouse for each experimental size for flow cytometry (closed symbol) and manual analysis (open symbols).

For flow cytometry, our data show that 20 and 200 K were sufficient to obtain reliable data for the evaluation of micronucleus induction. While flow cytometric data associated with analysis of 2 K reticulocytes were slightly more variable than corresponding microscopy-based measurements, automated acquisition of 20 K reticulocytes yielded essentially equivalent power of detection compared to the AO method. When 200 K and 1 M reticulocytes were analysed per specimen, assay sensitivity was observed to improve significantly, as evidenced by the lower doses at which statistical significance was noted when evaluated by Fisher's exact test. This was true when cells were the statistical unit evaluated, but not when individual mice were considered to be the unit. This issue is discussed

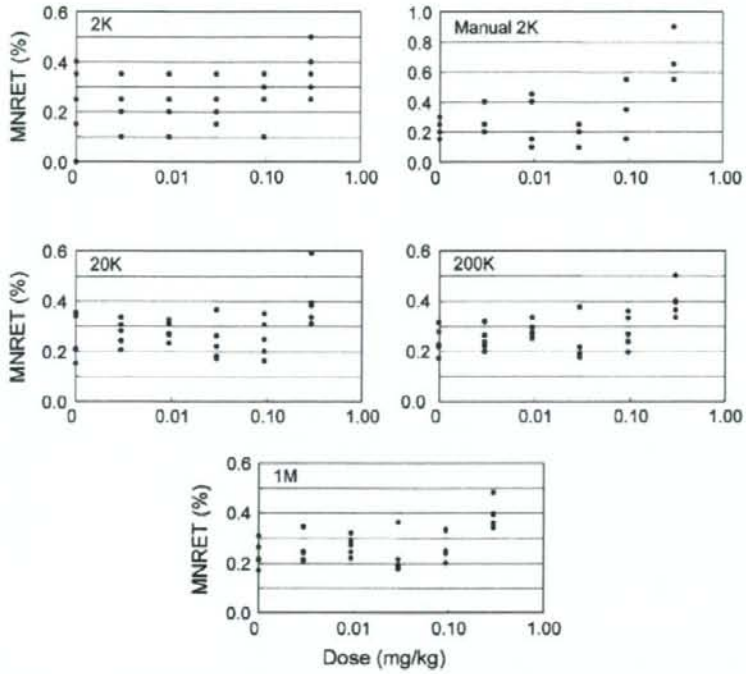

Fig. 2. Frequencies of micronucleated reticulocytes (%) for MMC based on 2, 20 and 200 K, and 1 M for flow cytometry and 2 K for manual microscopy. Each symbol shows the frequency of one mouse with exceptions because of overlapping (five animals per group).

in a companion paper wherein data from a reconstruction experiment are presented (37). In that study it was observed that when 200 K or 1 M cells per sample were analysed, the scoring error decreased and converged to a respective value. However, in the present study, the differences among the individual animals became apparent and there was more data variability within each dose group.

Grawé *et al.* (30) reported low dose effects on MMC, diepoxybutane, cyclophosphamide and COL using flow cytometry (stained with Hoechst 33342 for DNA and thiazole orange for RNA). Generally, one animal per dose level was used and 200 K polychromatic erythrocytes were analysed. Our present data agree with the COL data showing a non-linear dose–response, but in contrast to our present results, they showed linear dose–response relationships for extremely low dose levels of MMC, 0.007 mg/kg; diepoxybutane, 0.44 mg/kg; and cyclophosphamide, 0.3 mg/kg. Abramsson-Zetterberg *et al.* (31) also showed linear dose–response curves for acrylamide down to very low dose levels (1 mg/kg body wt) and observed no threshold. Although we did not evaluate acrylamide as a model chemical in this study, we did not observe linear dose–response curves, even for MMC and Ara-C, which are DNA-reactive clastogens. We could not find any rationale to explain such differences at the present time, and believe it is necessary to continue studying chemicals that interact directly with DNA to better understand their effect at extremely low dose levels.

To confirm biological and statistical relevance of the present study data, we performed a reconstruction model experiment using serial dilutions of malaria-infected blood with non-treated mouse blood (37). The samples were analysed by flow cytometry based on 2, 20 and 200 K (Experiment 1) and up to 1 M (Experiment 2) cells. These data show extremely high performance of the flow cytometric assay in terms of accuracy, especially when 200 K or more reticulocytes are evaluated per specimen. This result shows that the statistical power of the assay depended on the number of analysed cells. This dilution experiment supports our conclusion that thresholds were present for micronucleus induction in reticulocytes for the three model chemicals analysed at very low dose levels.

Table II. Individual data in the Ara-C study by flow cytometry analysed 1 M cells

Mouse ID	Dose (mg/kg body wt)					
	0.00	0.06	0.19	0.60	1.89	6.00
1	0.24	0.26	0.25	0.28	0.34	0.39
2	0.21	0.32	0.30	0.31	0.24	0.31
3	0.19	0.16	0.23	0.31	0.27	0.50
4	0.25	0.28	0.21	0.18	0.24	0.46
5	0.22	0.16	0.24	0.25	0.29	0.29
Mean	0.22	0.24	0.25	0.27	0.28	0.39
SD	0.02	0.07	0.03	0.05	0.04	0.09

It might be difficult to prove the existence of thresholds in toxicology or biology in general using statistical methods (38), and it is not easy to discuss the threshold concept from the biological viewpoint. However, when we only consider mean values, for example in the case of Ara-C, the MNRET frequencies appeared to increase linearly, but when the individual values (Table II) were evaluated, the differences among

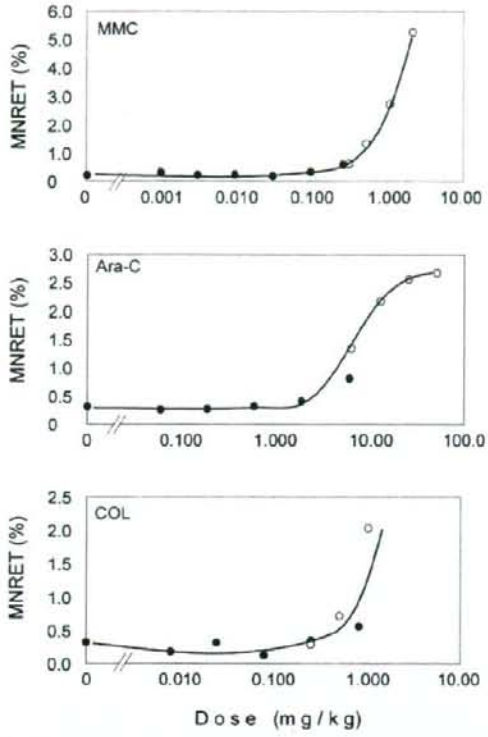

Fig. 3. Dose–response curves of MMC, Ara-C and COL in wider range of dose levels superimposed to the present data with the results of our published data. MMC: Hara *et al.*, 1992 (Table I) Study 1, 48 h; Ara-C: Iwakura *et al.*, 1992 (Table I) Laboratory B, 48 h; COL: Kondo *et al.*, 1992 (Table II) Laboratory 4, 48 h.

animals became clearer. Two individuals at the lowest dose group (mouse ID 3 and 5) demonstrated the lowest MNRET frequency (0.16%) even when including the vehicle control group. The control data variation in the Ara-C experiment was less than that in the MMC or COL experiments. When the Student's *t*-test was applied to the data obtained by flow cytometry based on the analysis of ≥20 K cells, only the highest dose groups were significantly different from the concurrent control for MMC and Ara-C. Therefore, we should consider the individual animal differences to determine the micronucleus induction ability of the chemical being studied. It is likely that at very low doses of genotoxicant, as were studied here, individual differences in DNA repair activity, metabolism related cytochrome P450 activity, or anti-oxidant concentration etc. play a larger role in dictating the micronucleus incidence of each individual of an exposure group.

In addition, Figure 3 shows the dose–response curves of these three model chemicals in a wider range of dose levels superimposed on the results of published data (27,32,34) using the same strain of mouse and same manner of experiments by AO supravital staining. Closed circles represent the data from the present study and open circles are published data for higher dose levels. The dose–response curves became clearer by adding the data from higher dose levels and the practical threshold or the threshold were shown. Moreover, COL even shows the tendency of inhibition in induction of micronuclei at extremely low dose levels (U- or J-shape response). Hormesis usually implies increased repair capability or some other protective, adaptive response in the field of radiobiology (1). The COL data presented herein is suggestive of a hormesis-like effect, and further work aimed at elucidating the mechanisms and significance of this observation is warranted.

Considering the data detailed above, an important conclusion is the existence of a biological or practical threshold in the genotoxicity assay on DNA target chemicals as well as non-target chemicals. Although we used only three model chemicals in the present study, we could draw the following conclusions: (i) the flow cytometric micronucleus assay method is a powerful tool when ≥20 K cells were analysed; (ii) the AO supravital staining micronucleus assay method and the flow cytometric method gave qualitatively similar results; (iii) when the cell is considered the statistical unit and more cells are analysed, both power and assay sensitivity at lower dose levels is significantly enhanced as evidenced by the significant differences observed when compared to vehicle control; and (iv) non-linear dose–response curves were obtained for the model chemicals studied here when evaluating the individual animal as a unit, suggesting the existence of a practical threshold for the DNA target micronucleus inducers (MMC and Ara-C) as well as the non-DNA target chemical (COL).

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Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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Abstract

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (*TK*) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and *TK* assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at ≥0.5 mM. Molecular analysis of the *TK* mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the in vitro and in vivo genotoxicity of AA and GA.

Keywords: Acrylamide; Gycidamide; Genotoxicity; *TK* mutation; Metabolic activation

1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in in vitro and in vivo [8]. In vivo examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some in vitro mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations in vitro and in vivo [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblatoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the in vitro micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the TK mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

2. Materials and methods

2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 10^5 to 10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions (5.0×10^5 cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PE0). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 μg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37 °C in 5% CO_2 in a humidified incubator. The TK assay produces two distinct phenotypic classes of *TK* mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the *TK* gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the *TK* gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing *TK* mutants in the TFT plates at 14th day after plating. We then re-fed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [21].

2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human *TK* gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that contains frameshift mutations. Another primer set for amplifying parts of the β-globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

3. Results

3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (*TK* assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first *TK* assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced *TK* mutation frequencies that were about 20 times and MN fre-

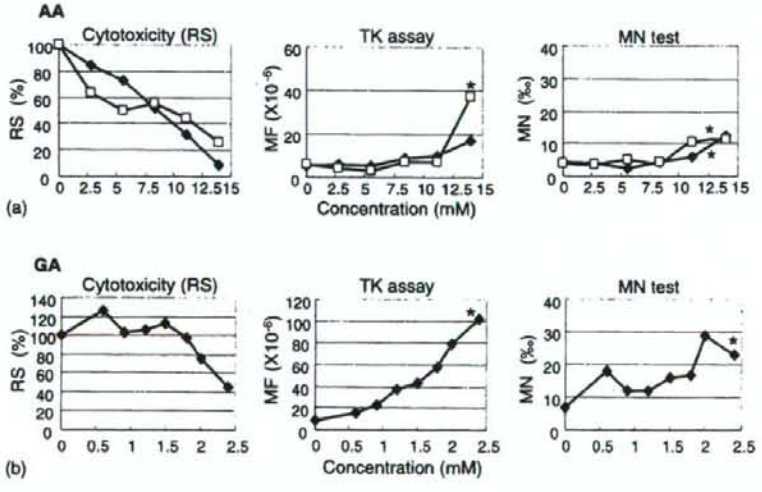

Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

Table 1
Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ($\times 10^{-6}$)	% SG	No.	None LOH	Hemi-LOH	Homo-LOH
Vehicle [16]	100	2.19	56	56			
NG mutants				19	14(74)	3(16)	2(11)
SG mutants				37	0(0)	9(24)	28(76)
AA (14 mM, 4 h)	40	18.9	54	48			
NG mutants				22	11(50)	11(50)	0(0)
SG mutants				26	0(0)	13(50)	13(50)
GA (2.2 mM, 4 h)	12	55.5	36	44			
NG mutants				28	26(93)	2(7)	0(0)
SG mutants				16	0(0)	6(38)	10(62)

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

3.2. Molecular analysis of TK mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK* mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ($n = 37$), GA-induced ($n = 17$) and spontaneous ($n = 29$) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

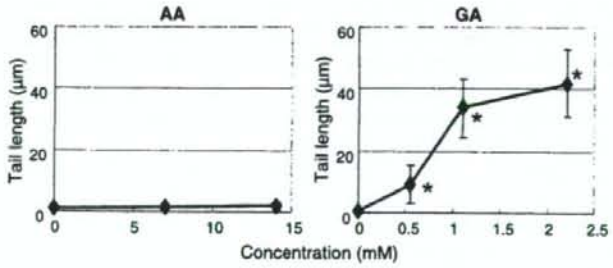

Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P < 0.05$).