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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 881389 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.

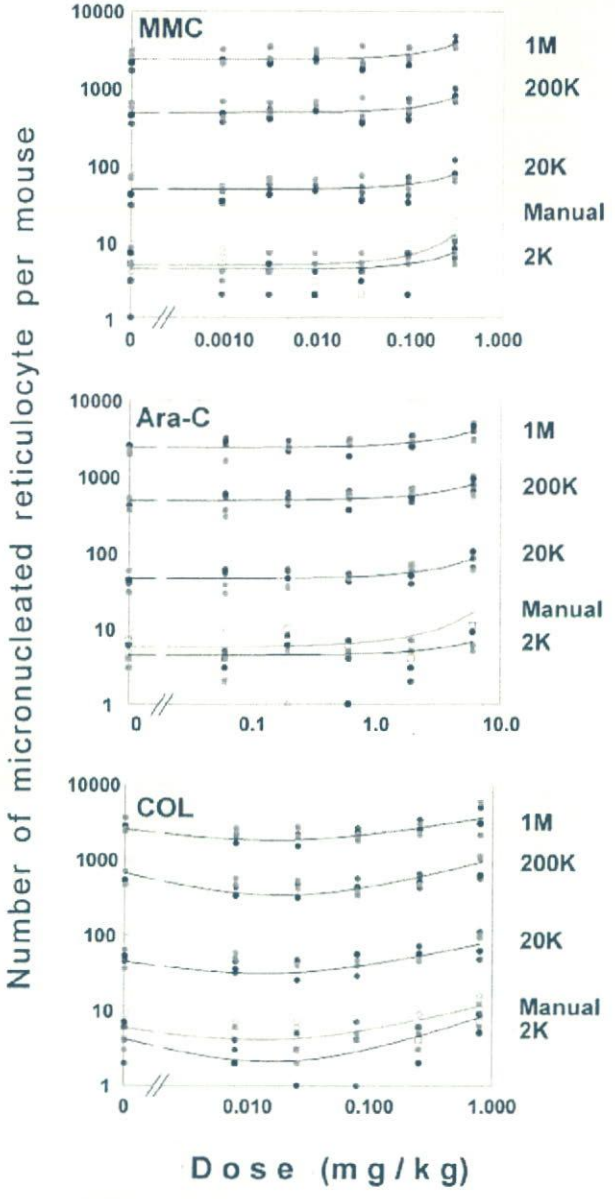

Fig. 1. Dose–response curves for MMC, Ara-C and COL. Each symbol shows individual number of micronucleated reticulocytes. In each dose group, a colour shows identical mouse for each experimental size for flow cytometry (closed symbol) and manual analysis (open symbols).

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

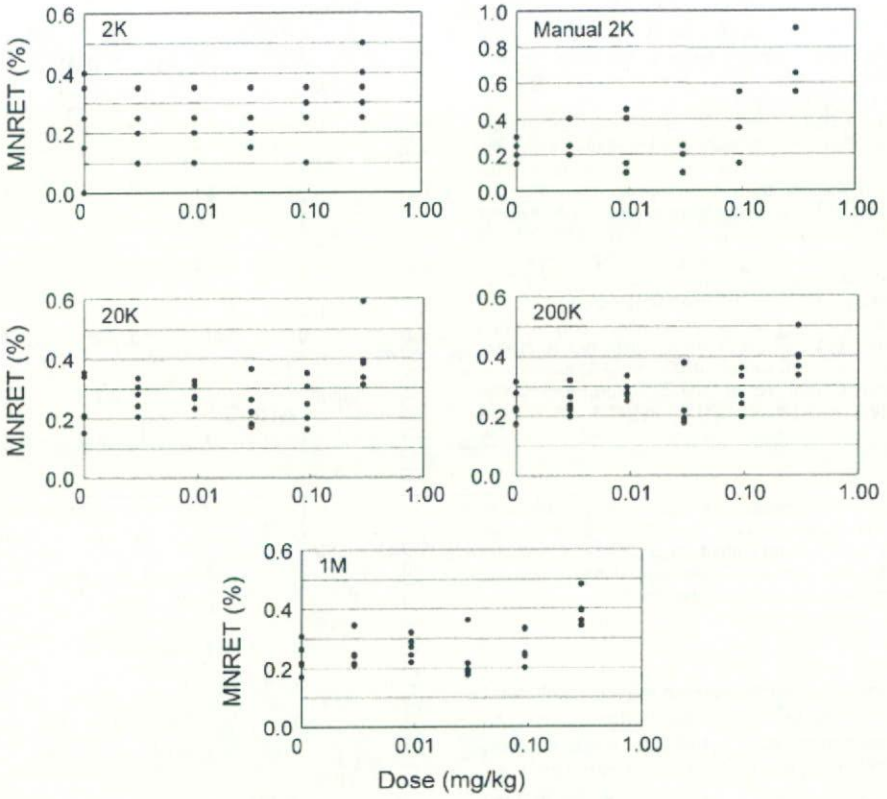

Fig. 2. Frequencies of micronucleated reticulocytes (%) for MMC based on 2, 20 and 200 K, and 1 M for flow cytometry and 2 K for manual microscopy. Each symbol shows the frequency of one mouse with exceptions because of overlapping (five animals per group).

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

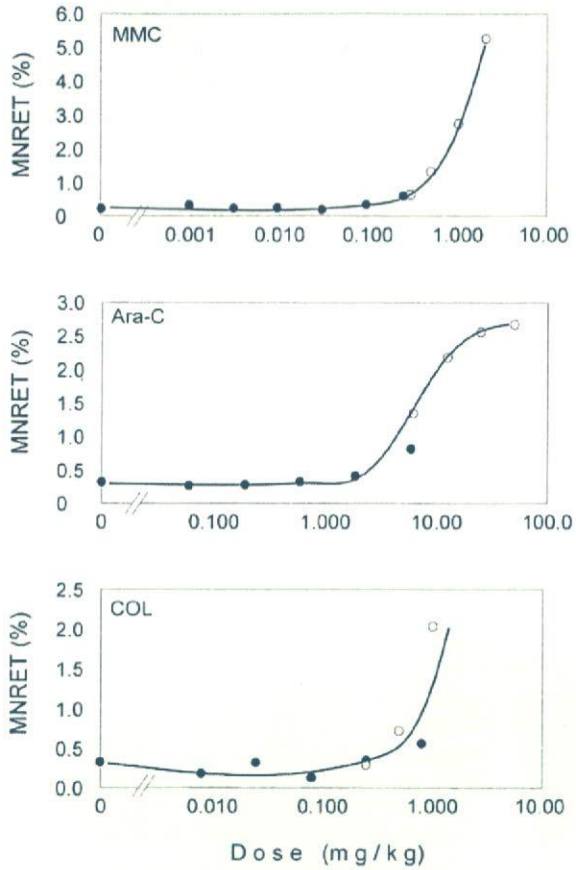

Fig. 3. Dose–response curves of MMC, Ara-C and COL in wider range of dose levels superimposed to the present data with the results of our published data. MMC: Hara *et al.*, 1992 (Table I) Study 1, 48 h; Ara-C: Iwakura *et al.*, 1992 (Table I) Laboratory B, 48 h; COL: Kondo *et al.*, 1992 (Table II) 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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Flow Cytometric Analysis of Micronuclei in Peripheral Blood Reticulocytes: I. Intra- and Interlaboratory Comparison with Microscopic Scoring

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Accumulating evidence suggests that reticulocytes (RETs) in the peripheral blood of rats may represent a suitable cell population for use in the micronucleus assay, despite the ability of the rat spleen to selectively remove micronucleated erythrocytes from the peripheral circulation. To evaluate the analytical performance of a previously described flow cytometric method (Torous *et al.*, 2003, *Toxicol. Sci.* 74, 309–314) that may allow this assay to be conducted using peripheral blood *in lieu* of bone marrow sampling, we compared the sensitivity and performance characteristics of the flow cytometric technique with two established microscopy-based scoring methods. Peripheral blood samples from single Sprague-Dawley rats treated for 6 days with either vehicle or cyclophosphamide were prepared in replicate for scoring by the three methods at different laboratories. These blood-based measurements were compared to those derived from bone marrow specimens from the same animals, stained with acridine orange, and scored by microscopy. Through the analysis of replicate specimens, inter- and intralaboratory variability were evaluated for each method. Scoring reproducibility over time was also evaluated. These data support the premise that rat RETs harvested from peripheral blood are a suitable cell population to assess genotoxicant-induced micronucleus formation. The inter-laboratory comparison provides evidence of the general robustness of the micronucleus endpoint using different analytical approaches. Furthermore, data presented herein demonstrate a clear advantage of flow cytometry–based scoring over microscopy—significantly lower inter- and intralaboratory variation and higher statistical sensitivity.

Key Words: flow cytometric analysis; reticulocytes; micronucleus test; CD71.

The *in vivo* rodent erythrocyte micronucleus (MN) test is widely used in research and regulatory safety assessment to evaluate the potential of chemical and physical agents to cause chromosomal damage. Historically, MN studies based on rat peripheral blood have been avoided as it has been assumed that the efficiency by which the rat spleen filters out erythrocytes with intracellular inclusions would reduce assay sensitivity (Hayashi *et al.*, 2000; Wakata *et al.*, 1998). However, accumulated data suggest that peripheral blood from intact rats can be used effectively to detect chemical-induced genotoxicity (Abramsson-Zetterberg *et al.*, 1999; Asanami *et al.*, 1995; Hamada *et al.*, 2001; Hayashi *et al.*, 1992; Hynes *et al.*, 2002; Romagna and Staniforth, 1989; Torous *et al.*, 2000, 2003; Wakata *et al.*, 1998). Thus, it appears that MN studies using peripheral blood sampling in the rat have the potential to substitute for labor-intensive, bone marrow–based tests. In addition, the ability to use low-volume blood samples will facilitate integration of the assay into routine toxicology and/or pharmacokinetic studies and may make it unnecessary to conduct separate assays for the evaluation of chromosomal damage (Asanami *et al.*, 1995; Hamada *et al.*, 2001; MacGregor *et al.*, 1995; Wakata *et al.*, 1998).

Before rat blood–based MN assays gain wider acceptance, especially in the context of regulatory testing requirements, additional information that allows direct comparisons between bone marrow and blood data is needed. Furthermore, the performance characteristics of the most widely utilized scoring techniques require further study. The experiments described herein were designed to address these issues of analytical performance by directly comparing values in blood and bone marrow obtained at different laboratories with three widely used methodologies, comparing values derived from two microscopy-based methods with a flow cytometry–based method that incorporates a calibration standard.

For each of the three scoring techniques, at least three proficient laboratories received replicate, coded samples for reticulocyte

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TABLE 1
Participating Laboratories

Laboratory	Code	Specimens analyzed	Scoring method	Instrumentation (magnification)
U.S. FDA-NCTR, Rockville, MD and Jefferson, AR	L1	BM, PB	MeOH-AO	Zeiss Axioskop 50 (×630), Zeiss PlanApomat ×63 oil objective
			FCM	BD-FACSort, BD-FACScan
Litron Laboratories, Rochester, NY	L2	PB	FCM	BD-FACSCalibur
Health Canada, Ottawa, Ontario, Canada	L3	PB	FCM	BD-FACSCalibur
National Institute of Health Sciences, Tokyo, Japan	L4	Coordinated SV-AO laboratories		
Nitto Denko Corporation, Osaka, Japan	L5	PB	SV-AO	Olympus AHBT3-RFC (×600)
An-Pyo Center, Shizuoka, Japan	L6	PB	SV-AO	Olympus BX50-RFL (×800)
Astellas Pharma Inc., Tokyo, Japan	L7	PB	SV-AO	Olympus BH-RFL (×600)
N/A to this study	L8			
Contract testing laboratory 1*	L9	BM, PB	MeOH-AO	Leitz Laborlux 12 (×1000)
Contract testing laboratory 2*	L10	BM	MeOH-AO	Olympus BH2 (×1000)
Contract testing laboratory 3*	L11	BM, PB	MeOH-AO	Zeiss STD 14 (×1000)

Note. Abbreviations: FDA-NCTR = U.S. Food and Drug Administration, National Center of Toxicological Research; BM = bone marrow; PB = peripheral blood; MeOH-AO = acridine orange staining of methanol-fixed smears; FCM = flow cytometry; SV-AO = supravital staining using acridine orange–coated slides; N/A = not applicable. *The three contract testing laboratories are BioReliance, Covance, and SRI International, but their identities as L9, L10, or L11 is confidential.

(RET) and MN-RET scoring. Proficiency was assumed based on the high level of training that has occurred at these laboratories (L1, L2, L3, L5, L6, and L7) and/or the frequency with which they contribute *in vivo* rodent MN data for regulatory submission purposes (L9, L10, and L11). See Table 1 for more detailed information regarding collaborating laboratories.

Data presented herein describe the performance characteristics of the three scoring methods evaluated, address the sensitivity of the rat peripheral blood compartment for detecting genotoxicant-induced micronuclei, and support recommendations concerning the minimum number of rat blood RET that should be evaluated for micronuclei.

MATERIALS AND METHODS

Chemicals and other reagents. Cyclophosphamide (CP) (CAS No. 6055-19-2) was purchased from Sigma, St Louis, MO. Acridine orange (AO)–coated slides used for supravital staining, prepared according to the method of Hayashi *et al.* (1990), were provided by the National Institute of Health Sciences, Japan. Flow cytometry reagents, including fixed malaria-infected rat blood (malaria biostandard) were from Rat MicroFlow^{PLUS} Kits contributed by Litron Laboratories (available from Litron Laboratories, Rochester, NY and BD Biosciences PharMingen, San Diego, CA).

Animals and treatment regimens. Animal studies were conducted in compliance with guidelines of the National Research Council (1996) "Guide for the Care and Use of Laboratory Animals" and were approved by the appropriate Institutional Animal Care and Use Committees. Two female Sprague-Dawley rats, 4- to 5-weeks old, were purchased from Taconic, Germantown, NY. Animals were housed singly and were assigned randomly to treatment groups. The animals were acclimated for approximately 2 weeks before the experiment was initiated. Food and water were available *ad libitum* throughout the acclimation and experimentation periods. One rat was treated via oral gavage with distilled water, and the other rat was treated by the same route with 10 mg CP/kg/day for 6 consecutive days.

Blood/bone marrow sample collection and storage. Each day, before vehicle or CP treatment, low-volume blood samples (approximately 100 µl) were collected from the tail vein using a 26.5-gauge needle and syringe after a brief warming period under a heat lamp. These samples were fixed for flow cytometric analysis of RET and MN-RET frequencies according to procedures described in the Rat MicroFlow^{PLUS} manual (v020213). Fixed samples were stored at – 85°C until analysis. Approximately 24 h after the last administration of vehicle or CP, blood samples were collected into tubes containing heparin solution (500 USP units heparin per milliliter of phosphate buffered saline) as follows: into a small tube containing 75 µl heparin solution, blood was collected until a final volume of approximately 750 µl was obtained; into a second tube containing 5 ml heparin solution, approximately 1 ml blood was collected. To tubes with the 750 µl blood suspension, an equal volume of heat-inactivated fetal bovine serum (FBS) was added. These FBS-diluted suspensions were used to prepare replicate AO-supravital (SV) slides (8 µl per slide) according to the method of Hayashi *et al.* (1990, 1992). These slides were frozen, shipped to collaborating SV-AO laboratories on dry ice, and stored frozen until analysis. FBS-diluted blood suspensions were also used to prepare slides for conventional acridine orange staining of methanol-fixed smears (MeOH-AO) staining (5 µl per slide). These blood smears were prepared by drawing the cell suspensions behind a second slide with smoothed edges (a "spreader slide"). These smears were allowed to air dry and were then fixed with absolute methanol for 10 min. The slides were stored in a slide box until they were shipped to collaborating MeOH-AO laboratories for MN scoring according to their standard operating procedures. Replicate bone marrow slides were prepared as smears, air dried, methanol fixed, and shipped similarly. These bone marrow cells were harvested from two femurs per rat, whereby both ends of each femur were cut and its contents flushed with 1 ml FBS. The cells were centrifuged at approximately at 1100 rpm for 5 min and then resuspended with approximately 600 µl FBS. As with the peripheral blood, 5 µl of cell suspension was applied to each slide. The 6 ml heparinized peripheral blood suspensions were fixed with ultracold methanol according to procedures described in the Rat MicroFlow^{PLUS} manual (v020213) in order to preserve cells for flow cytometric analysis. These cell suspensions were stored at – 85°C until analysis or shipment on dry ice to collaborating flow cytometry laboratories.

The samples obtained were divided into three identical pools, and replicate samples of each pool were provided to participating laboratories with three separate codes. Thus, laboratories received triplicate samples of each condition, but were not aware that they were from an identical pool. Thus, the analyses

conducted allow assessment of both intralaboratory variability of replicate analysis of identical samples and interlaboratory variability of the same analysis. Each laboratory also conducted analysis of each of these pools on three separate occasions, allowing assessment of variability of analysis over time.

Standard acridine orange slide scoring (MeOH-AO). Blood and bone marrow smears were scored using the MeOH-AO scoring technique at the Food and Drug Administration-National Center for Toxicological Research laboratory (L1) and three contract testing laboratories (L9, L10, and L11). Methanol fixation leads to a diffuse distribution of RNA, and erythrocytes are classified as normochromatic or as RETs based on the presence or absence of RNA-associated fluorescence. This technique is not well suited for visually classifying subpopulations of RETs. RET frequencies were determined by inspecting 500 or 1000 total erythrocytes per bone marrow or blood sample, respectively. MN-RET incidence was determined by inspecting 2000 RETs per sample. At L1, micronuclei were defined by the criteria of Schmid (1976) with the added requirements that they exhibit the characteristic yellow to yellow-green fluorescence characteristic of AO staining and that they exhibit the smooth boundary expected from a membrane-bound body. Laboratories L9, L10, and L11 were instructed to follow the standard operating procedures they use for regulatory submissions to support new drug or food additive development. Thus, the acquisition of data by these facilities allows for comparisons with three highly experienced contract laboratories under conditions associated with regulatory testing.

Supravital acridine orange slide scoring (SV-AO). Laboratories L5, L6, and L7 scored peripheral blood samples using the SV-AO scoring technique. This staining procedure aggregates RNA, leading to punctate staining patterns. These staining characteristics allow RET to be classified into four age cohorts: Type I (youngest) through Type IV (oldest) RETs as described by Hayashi *et al.* (1990, 1992). The frequency of MN-RETs was determined by analyzing 2000 Type I and Type II RETs (L5 and L7) or 2000 Type I RETs (L6). An index of cytotoxicity was obtained by inspecting at least 400 RETs and calculating the percentage of Type I and Type II RET among total RETs (L5 and L7) or the percentage of Type I RETs among Type I and Type II RETs (L6). AO-coated slides were purchased from TOYOBO (Osaka, Japan). Supravitally stained triplicate slides were frozen and sent to the Japanese reference laboratory (Nitto Denko) with dry ice. Each set of slides was also sent to two other laboratories for replicate scoring by fluorescence microscopy.

Flow cytometry–based scoring. Methanol-fixed blood samples were washed and labeled for flow cytometric analysis by L1, L2, and L3 according to procedures described in the Rat MicroFlow^{PLUS} Kit (v020213). Samples were analyzed with 488-nm capable instruments (FACSCalibur, FACSort, and FACScan, all from Becton Dickinson, San Jose, CA). Anti-CD71-FITC and propidium iodide fluorescence signals were detected in the FL1 and FL3 channels, respectively (stock filter sets). Calibration of the flow cytometers for the MN scoring application, across laboratories and between experiments within each laboratory, was accomplished by staining *Plasmodium berghei*–infected rat blood (malaria biostandards) in parallel with test samples on each day of analysis (Dertinger *et al.*, 2000; Tometsko *et al.*, 1993; Torous *et al.*, 2001). By adjusting voltages applied to the photomultiplier tube, it was possible to standardize the FL3 fluorescence channel into which erythrocytes with single (MN like) parasites fell. In this manner, analysis regions were consistent across laboratories and between experiments. Flow cytometry–based MN-RET measurements reported herein are based on an immature fraction of peripheral blood RETs (approximately the youngest 30–50% of propidium iodide–positive erythrocytes, based on CD71 expression level; Torous *et al.*, 2001, 2003). This is thought to be analogous to scoring the youngest (Types I and II) RETs using the SV-AO method, which may be beneficial in view of reports which have suggested that the influence of rat spleen filtration function can be minimized by scoring the younger RETs (Abramsson-Zetterberg *et al.*, 1999; Hayashi *et al.*, 1992; Hynes *et al.*, 2002; Torous *et al.*, 2000, 2003). Data were acquired with CellQuest software (v3.3, BD-Immunocytometry Systems, San Jose, CA), with the stop mode set so that 20,000 high CD71-expressing RETs were analyzed per blood sample. The number of mature (CD71 negative) erythrocytes was determined concurrently, providing an index of cytotoxicity (%RETs).

Calculations. All calculations were performed with Excel (Office X for Mac or Microsoft Office Excel 2002 for XP Windows Professional, Microsoft Corp., Seattle, WA). The incidences of MN-RETs are expressed as frequency percent. The percentage of RETs among total erythrocytes was measured by the flow cytometric and MeOH-AO laboratories and served as an index of bone marrow cytotoxicity. The three SV-AO laboratories used percentage of RETs in different stages of maturity as an index of toxicity; therefore, these indices are not directly comparable to those obtained by the flow cytometric and MeOH-AO microscopy laboratories. Percent coefficient of variance values (%CV, i.e., standard deviation (SD) as percent of the mean) were used to describe intralaboratory variability associated with multiple readings of replicate samples and also interlaboratory variation of vehicle control and CP-induced MN-RET measurements that were pooled according to scoring method.

RESULTS AND DISCUSSION

Confirmation of Steady State

RET and MN-RET measurements obtained from the daily low-volume blood specimens were analyzed to confirm that the

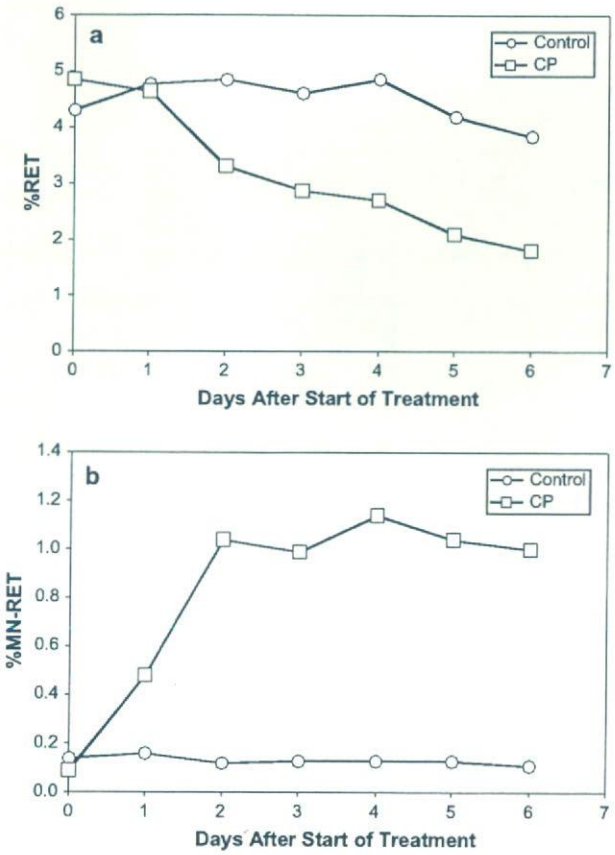

FIG. 1. The frequency of peripheral blood RETs (%RET, panel a) and peripheral blood micronucleated RETs (%MN-RET, panel b) as a function of time in the individual rats used to generate reference samples for analytical comparison. These data were acquired by flow cytometric analysis (laboratory L2) and demonstrate the attainment of a steady-state MN-RET frequency, facilitating subsequent comparisons between bone marrow and peripheral blood compartments.

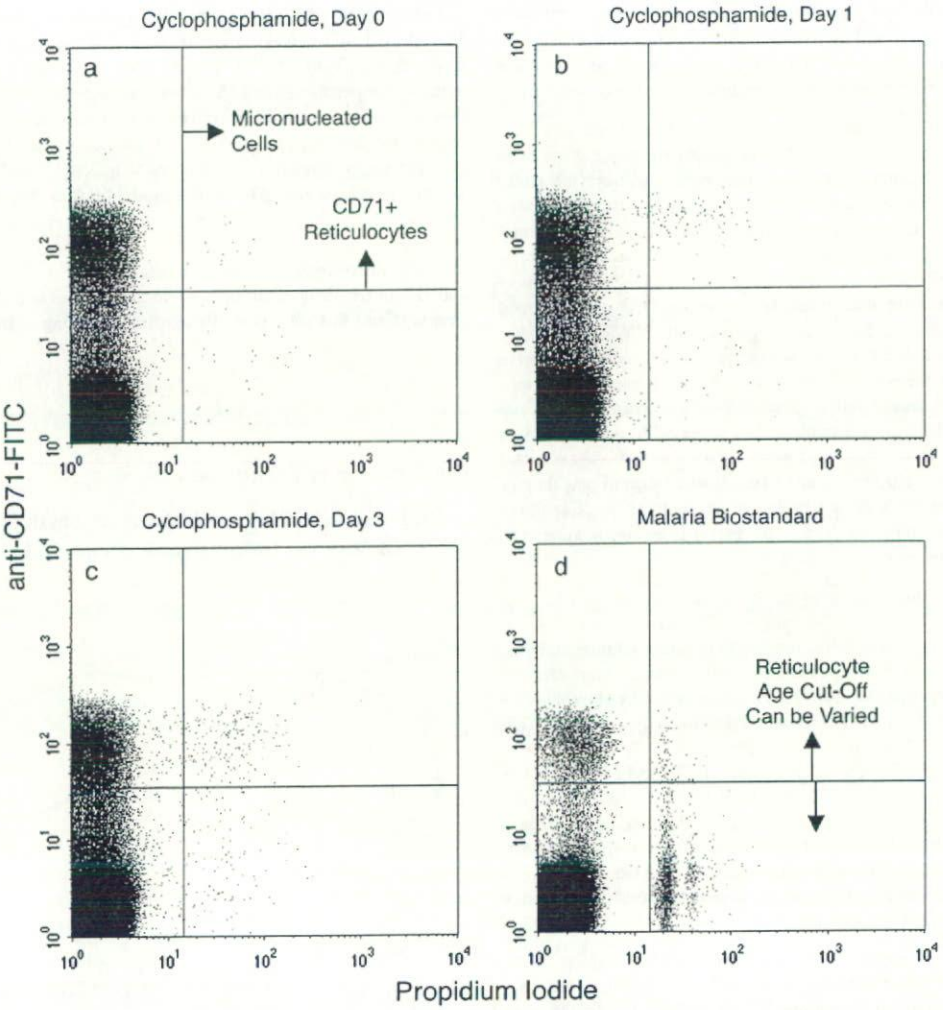

FIG. 2. Panels (a–c): Bivariate graphs illustrate the staining characteristics of rat blood specimens over the course of several days of CP treatment. Note the appearance of micronuclei at Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution among RETs after a steady state has been reached on Day 3 of treatment (Panel c). Panel (d) illustrates the use of the malaria standard, with distinct fluorescence intensities corresponding to inclusion of one, two, or three parasites. This allows the instrument settings to be standardized to the DNA content of the parasite, which is controlled biologically to a quantity similar to that in an average MN.

MN-RET frequency of the vehicle-treated animal was stable over the duration of the experiment and that CP treatment caused the MN-RET frequency to increase to a steady-state level of approximately 10-fold the control frequency (Fig. 1). Since the frequency of MN-RETs was at steady state in both cases, the values in bone marrow and peripheral blood should be directly comparable—that is, expected to be equal in the absence of selective removal of MN-RETs from blood or methodological differences in measurement. Thus, the samples collected in this manner allow the direct comparisons between measurements in the bone marrow and blood compartments that follow. The use of large samples from a single treated and a single control rat allows differences in methodology and scoring laboratory to be assessed independently of sample variation.

The dose of CP (10 mg/kg/day) had a moderate effect on erythropoiesis, as indicated by the decline in RET frequency (terminal day specimen showed a greater than 50% decrease from pretreatment value; see Fig. 1, panel a). This level of bone marrow cytotoxicity is well within the range of target toxicity recommended by current regulatory guidances (i.e., $\leq$ 80%, see Organisation for Economic Cooperation and Development, 1997, Guideline 474; U.S. Food and Drug Administration, 2000.

To illustrate the nature and source of the flow cytometry–based data described above, bivariate fluorescence intensity plots are provided (Fig. 2). Note the appearance of micronuclei on Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution

TABLE 2
Reticulocyte Data (cytotoxicity determinations)

Laboratory	Method	Compartment	Treatment	Cytotoxicity index[a]	Average %RET[b] ± SEM	%CV	%Change
L1	MeOH-AO	BM	Vehicle	%RET	81.0 ± 0.70	1.5	
			CP		67.8 ± 2.36	6.0	− 16
L9	MeOH-AO	BM	Vehicle	%RET	65.4 ± 1.03	2.7	
			CP		51.9 ± 1.83	6.1	− 21
L10	MeOH-AO	BM	Vehicle	%RET	58.2 ± 1.65	4.9	
			CP		60.0 ± 0.66	1.9	+ 3
L11	MeOH-AO	BM	Vehicle	%RET	63.1 ± 1.67	4.6	
			CP		57.1 ± 1.14	3.5	− 10
Pooled[b] L1, 9, 10, 11			***Vehicle***		***66.9 ± 2.63***	***13.6***	
			CP		***59.2 ± 1.87***	***10.9***	***− 12***
L1	MeOH-AO	PB	Vehicle	%RET	7.7 ± 0.19	4.2	
			CP		5.7 ± 0.27	8.2	− 26
L9	MeOH-AO	PB	Vehicle	%RET	6.2 ± 0.27	7.4	
			CP		5.6 ± 0.90	27.8	− 9
L11	MeOH-AO	PB	Vehicle	%RET	6.6 ± 0.52	13.5	
			CP		4.9 ± 0.33	11.7	− 26
Pooled L1, 9, 11			***Vehicle***		***6.9 ± 0.29***	***12.5***	
			CP		***5.4 ± 0.32***	***17.6***	***− 21.7***
L5	SV-AO	PB	Vehicle	%Type I + II/I–IV	55.2 ± 1.95	6.1	
			CP		42.2 ± 0.12	0.5	− 24
L6	SV-AO	PB	Vehicle	%Type I/I + II	42.4 ± 2.8	11.5	
			CP		29.1 ± 2.1	12.4	− 31
L7	SV-AO	PB	Vehicle	%Type I + II/I–IV	52.3 ± 2.1	6.8	
			CP		34.8 ± 3.1	15.6	− 34
Pooled[c] L5, 7			***Vehicle***		***53.7 ± 1.4***	***6.5***	
			CP		***38.5 ± 2.2***	***13.8***	***− 28***
L1	FCM	PB	Vehicle	$\%RET^{High\ CD71+}$	3.40 ± 0.02	1.18	
			CP		1.53 ± 0.01	0.75	− 55
L2	FCM	PB	Vehicle	$\%RET^{High\ CD71+}$	3.32 ± 0.02	1.26	
			CP		1.44 ± 0.01	1.44	− 57
L3	FCM	PB	Vehicle	$\%RET^{High\ CD71+}$	3.32 ± 0.05	2.42	
			CP		1.40 ± 0.08	9.88	− 58
Pooled L1, 2, 3			***Vehicle***		***3.34 ± 0.02***	***1.93***	
			CP		***1.46 ± 0.03***	***6.33***	***− 56***

Note. Abbreviations: RET = reticulocyte; MeOH-AO = acridine orange staining of methanol-fixed smears; SV-AO = supravital staining using acridine orange–coated slides; FCM = flow cytometry; BM = bone marrow; PB = peripheral blood; CP = cyclophosphamide; SEM = standard error of the mean.

[a]Each laboratory evaluated cytotoxicity based on immature erythrocyte parameters. This was accomplished in several different manners: %RET = percentage of RETs relative to total erythrocytes; %Type I + II/I–IV = percentage of Type I and Type II RETs relative to total RETs; %Type I/I + II = percentage of Type I RETs relative to Type I and Type II RETs; and $\%RET^{High\ CD71}$ = percentage of RETs that express high levels of CD71 relative to total erythrocytes.

[b]Values are the mean of three separately coded, but identical, samples. By "Pooled" it is meant that like-method data from two, three, or four laboratories were combined for these calculations.

[c]Only data from the two SV-AO laboratories that measured toxicity similarly (%Type I + II/I–IV) were combined for these calculations.

among RETs after a steady state has been reached on Day 3 of treatment (Panel c). Panel (d) illustrates the use of the malaria biostandard, with distinct fluorescence intensities corresponding to inclusion of one, two, or three parasites. This allows the instrument settings to be standardized to the DNA content of the parasite, which is controlled biologically to a quantity similar to that in an average MN. For research purposes, the regions may be adjusted to allow measurements in different age populations of RETs and/or micronuclei with different DNA contents. For analytical purposes, the standard can be used to achieve comparable instrument performance across time within a laboratory or across different instruments in different laboratories.

Intra- and Interlaboratory Variability

Replicate bone marrow and/or peripheral blood specimens obtained after 6 consecutive days of treatment were provided to each collaborating laboratory. As noted above, the frequencies of MN-RETs were at steady state and therefore not changing as a function of time. Each laboratory received three separately coded samples from each of the high and low MN-RET frequency pools but were not aware that the three separately coded samples were identical. Tabular values are the means of the values of the three separately coded samples.

TABLE 3
Intra- and Interlaboratory Micronucleated Reticulocyte Data

Laboratory	Method	Compartment	Treatment	%MN-RET		
				Average[a] ± SEM	%CV	Fold difference
L1	MeOH-AO	BM	Vehicle	0.15 ± 0.03	33.3	
			CP	3.35 ± 0.10	5.4	22.3
L9	MeOH-AO	BM	Vehicle	0.05 ± 0.05	173.2	
			CP	1.63 ± 0.27	28.4	32.6
L10	MeOH-AO	BM	Vehicle	0.03 ± 0.02	86.6	
			CP	2.33 ± 0.23	17.3	77.7
L11	MeOH-AO	BM	Vehicle	0.18 ± 0.03	31.5	
			CP	2.44 ± 0.20	13.9	13.6
Pooled[a] L1, 9, 10, 11			***Vehicle***	***0.10 ± 0.02***	***80.5***	
			CP	***2.44 ± 0.21***	***29.1***	***24.4***
L1	MeOH-AO	PB	Vehicle	0.05 ± 0.03	100.0	
			CP	1.77 ± 0.17	16.6	35.4
L9	MeOH-AO	PB	Vehicle	0.05 ± 0.00	0.0	
			CP	0.50 ± 0.03	10.0	10.0
L11	MeOH-AO	PB	Vehicle	0.18 ± 0.04	41.7	
			CP	1.42 ± 0.10	12.3	7.9
Pooled L1, 9, 11			***Vehicle***	***0.09 ± 0.03***	***85.6***	
			CP	***1.23 ± 0.20***	***48.2***	***13.7***
L5	SV-AO	PB	Vehicle	0.13 ± 0.03	43.3	
			CP	1.83 ± 0.15	13.7	14.1
L6	SV-AO	PB	Vehicle	0.12 ± 0.07	99.0	
			CP	1.77 ± 0.32	31.2	14.8
L7	SV-AO	PB	Vehicle	0.22 ± 0.14	113.8	
			CP	1.47 ± 0.27	31.7	6.7
Pooled L5, 6, 7			***Vehicle***	***0.16 ± 0.05***	***94.3***	
			CP	***1.69 ± 0.14***	***24.7***	***10.6***
L1	FCM	PB	Vehicle	0.12 ± 0.02	24.8	
			CP	0.99 ± 0.04	6.5	8.3
L2	FCM	PB	Vehicle	0.11 ± 0.02	31.5	
			CP	1.04 ± 0.04	6.7	9.5
L3	FCM	PB	Vehicle	0.11 ± 0.02	32.9	
			CP	1.11 ± 0.04	6.8	10.1
Pooled L1, 2, 3			***Vehicle***	***0.11 ± 0.01***	***26.5***	
			CP	***1.05 ± 0.03***	***7.6***	***9.5***

Note. Abbreviations: MN-RET = micronucleated reticulocyte; MeOH-AO = acridine orange staining of methanol-fixed smears; SV-AO = supravital staining using acridine orange–coated slides; FCM = flow cytometry; BM = bone marrow; PB = peripheral blood; CP = cyclophosphamide; SEM = standard error of the mean; %CV = percent coefficient of variance.

[a]Values are the mean of three separately coded, but identical, samples. By "Pooled" it is meant that like-method data from three or four laboratories were combined for these calculations.

Most laboratories detected a reduction in %RET for the CP-treated rat (see Table 2). However, this was somewhat variable across microscopy-based laboratories, especially when the MeOH-AO technique was used to evaluate bone marrow specimens. In two of the three laboratories that scored both bone marrow and peripheral blood, peripheral blood measurements demonstrated greater CP-associated reduction of %RETs than in bone marrow. Intra- and interlaboratory %CV values for the replicate RET analyses are presented in Table 2. Flow cytometric measurements were more consistent within and across laboratories than microscopic scoring. For instance, vehicle control specimens' %CV for pooled laboratory MeOH-AO/bone marrow data was 13.6%, while the corresponding blood-based analyses for flow cytometric, SV-AO, and MeOH-AO techniques were 1.93, 6.5, and 12.5%, respectively.

The interlaboratory %CV values for MN-RET determinations and the intralaboratory %CV values for the triplicate blinded analyses conducted within each laboratory are provided in Table 3. The flow cytometric analyses demonstrate superior intra- and interlaboratory consistency relative to both microscopy-based methods. %CV values for MN-RET measurements performed on vehicle control blood specimens pooled across like-method laboratories were 26.5, 94.3, and 85.6% for the flow cytometric, SV-AO, and MeOH-AO methods, respectively, and 80.5% for MeOH-AO scored bone marrow. Analogous %CV values for CP blood samples were 7.6, 24.7,

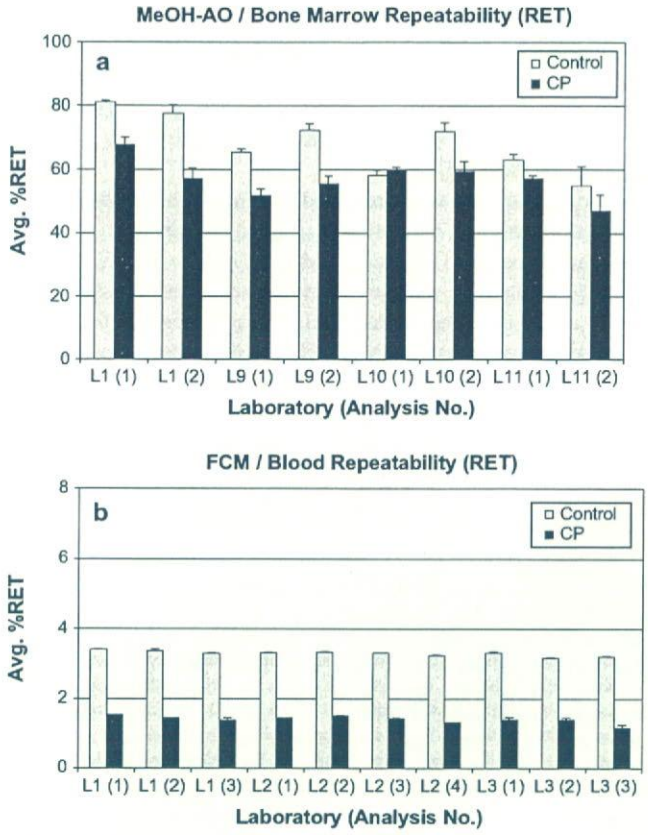

FIG. 3. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow RETs (%RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two separate occasions at each laboratory. Panel (b): The average frequency of peripheral blood RET as measured by the flow cytometric (FCM) technique are graphed (with SEM bars). These data were collected on three or four separate occasions.

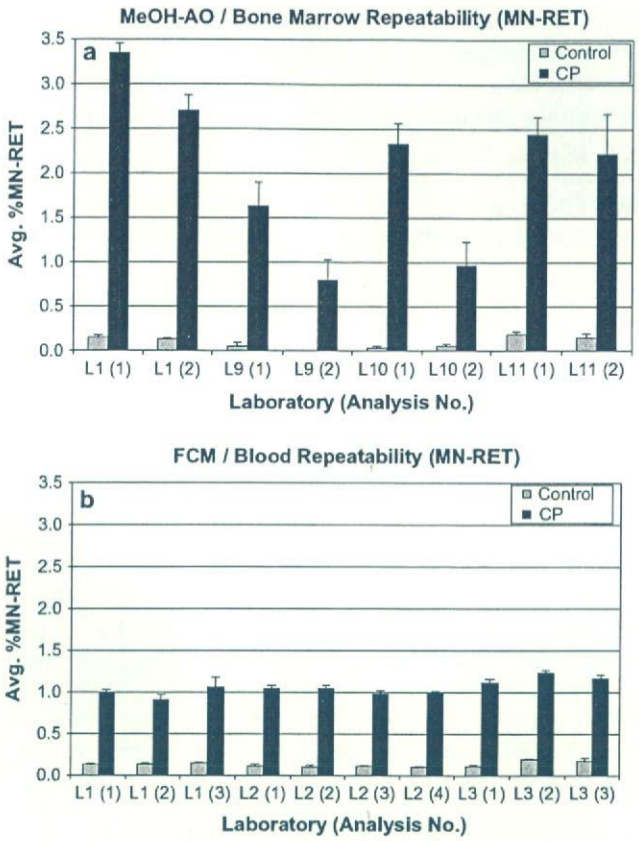

FIG. 4. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow micronucleated RETs (%MN-RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two separate occasions at each laboratory. Panel (b): The average frequency of peripheral blood MN-RET as measured by the flow cytometric (FCM) technique are graphed (with SEM bars). These data were collected on three or four separate occasions.

and 48.2%, respectively, and 29.1% for MeOH-AO scored bone marrow.

Fold difference values based on each laboratory's average MN-RET frequencies, as well as for like-method pooled data, are also presented (Table 3). It was somewhat surprising that the fold difference in MN-RETs between vehicle and CP-associated blood specimens, as well as absolute MN-RET frequencies, were no higher with the flow cytometric or SV-AO techniques than with the conventional MeOH-AO method as it has been reported that restriction of MN analysis to an immature RET cohort based on RNA content or CD71 expression levels could reduce, if not eliminate, the influence of the spleen's erythrophagocytotic activity (Abramsson-Zetterberg *et al.*, 1999; Hayashi *et al.*, 1992). Splenic activity and its effects on assay sensitivity for blood-based analyses have been investigated thoroughly, and these data are discussed in a companion paper that appears in this issue (MacGregor *et al.*).

Intralaboratory Variability Across Time

In addition to the inter- and intralaboratory analyses, an evaluation of scoring reproducibility over time was studied. This was accomplished by having flow cytometry laboratories analyze coded peripheral blood specimens on three or four different occasions, while triplicate vehicle and CP bone marrow slides were submitted to L9, L10, and L11 laboratories for analysis on two separate occasions. Reagents were prepared separately for each day of analysis. The resulting repeat-analysis RET data are presented in Figure 3 and demonstrate higher reproducibility for the multiple flow cytometric analyses compared to MeOH-AO.

As with RET enumeration, repeat-analysis MN-RET microscopy data were also quite variable. For instance, laboratories using the MeOH-AO method reported average CP-induced values that differed from their original mean reading by 19.4,

50.9, 58.4, and 8.6% (L1, L9, L10, and L11, respectively; see Fig. 4). Repeat-analysis MN-RET data generated by the flow cytometric technique were considerably more reproducible as average values were all within 11% of the originally reported mean frequencies. It should also be noted that the fourth flow cytometric analyses by L2 was performed more than 2 years after blood fixation, demonstrating this procedure's compatibility with long-term storage of fixed blood specimens.

CONCLUSIONS

Distribution of replicate bone marrow and blood specimens obtained from single rats that were first shown to exhibit steady-state spontaneous or genotoxicant-induced MN-RET frequencies were used to assess inter- and intralaboratory scoring variability using two widely used microscopic and one flow cytometric procedure. These results demonstrate that the quantification of MN-RETs benefits from an objective flow cytometry–based method of data acquisition. The flow cytometric method provides better reproducibility, and the high throughput capability allows interrogation of tens of thousands of RETs per specimen. Enhanced scoring precision is important as it is necessary to offset the spleen-dependent loss of dynamic range observed in peripheral blood relative to bone marrow—a phenomenon that was observed in this as well as other reports (MacGregor *et al.*, this issue; Wakata *et al.*, 1998). A recent report by Torous *et al.* (2006) delineates the consequential improvements to assay power when the number of cells scored per specimen is increased and supports this view.

Beyond overcoming lower genotoxicant-induced MN-RET frequencies in blood relative to bone marrow, further incentive for automating rat peripheral blood MN-RET measurements comes from a recent recommendation of the *In Vivo* MN Assay Expert Group of the International Working Group on Genetic Toxicology Testing (IWGT; Hayashi *et al.*, in press). Specifically, IWGT has recommended that a sufficient number of RETs should be scored to ensure that the MN-RET counting error is kept below the level of interanimal variability. This allows the sensitivity of the experiment to be limited by the variability of spontaneous MN-RET frequency among animals, rather than being limited by the statistical variation of count. Based on the flow cytometric scoring of 20,000 peripheral blood RETs from each of the 15 control animals from the three experiments reported in the MacGregor *et al.* companion paper in this issue (laboratory L2, the reference laboratory), we find that the mean incidence of MN-RET ± 1 SD is 0.11% ± 0.045. This is a 41% CV. Poisson distribution theory allows us to calculate that 6 MN-RETs per animal must be scored to limit counting error to this level of variation (SD of the Poisson count = $\sqrt{\text{absolute count}}$). At a spontaneous MN-RET frequency of approximately 0.1%, this means that an average of 6000 RETs per individual need to be scored for micronuclei in order to achieve a CV that is at or below the interanimal variance. This is a significantly higher number of RETs per animal than required to be scored under the current OECD MN assay guideline (which recommends scoring 2000 RETs per animal) and is difficult to achieve by manual microscopic scoring.

In conclusion, the data presented herein and in the companion paper that follows support the growing consensus that rat peripheral blood can be used to perform *in vivo* MN tests more effectively than the standard bone marrow–based assay. The ability of the described automated scoring procedure to greatly enhance the precision of MN-RET measurements overcomes the somewhat attenuated genotoxicant-induced frequencies observed in peripheral blood relative to bone marrow. This conclusion is supported by experiments described in the accompanying paper whereby intact and splenectomized rats were treated with diverse genotoxicants (MacGregor *et al.*, this issue).

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Early Pregnancy Failure Induced by Dibutyltin Dichloride in Mice

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ABSTRACT: In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0–3 or days 4–7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0–3, the rate of non-pregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0–3 at 15.2 mg/kg and higher and on days 4–7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0–3 or days 4–7. A decline in the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure. © 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 44–52, 2007.

Keywords: dibutyltin dichloride; organotin; pregnancy failure; early embryonic loss; progesterone

INTRODUCTION

Organotin compounds are chemicals widely used in agriculture and industry. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during the melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). Wide-spread use of organotin compounds has caused increasing amounts to be released into environment. The most important route of entry of organotin compounds as nonpesticides into the environment is through the leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and its use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991). The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms (Sasaki et al., 1988; Lau, 1991) and marine products (Suzuki et al., 1992) has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment (Seligman et al., 1988; Stewart and de Mora, 1990). Food chain bioaccumulation of butyltin in oysters (Waldock and Thain, 1983), mud crabs (Evans and Laughlin, 1984), marine mussels (Laughlin et al., 1986), Chinook salmon (Short and Thrower, 1986), and dolphin, tuna, and shark (Kannan et al., 1996) has been reported. These findings indicate that butyltins accumulate in the

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food chain and are bioconcentrated, and that humans can be exposed to butyltins via food.

Organotins possesses toxic effects on reproduction and development in experimental animals (Ema and Hirose, 2006). We previously reported that dibutyltin dichloride (DBTCl) by gavage throughout the period of organogenesis resulted in a significant increase in the incidence of fetal malformations in rats (Ema et al., 1991) and that rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on day 7 and day 8 of pregnancy (Ema et al., 1992). Tetrabutyltin (TeBT) is metabolized to TBT, DBT, and monobutyltin (MBT) derivatives (Fish et al., 1976; Kimmel et al., 1977). The TBT compound is metabolized to DBT and MBT derivatives and DBT is metabolized to MBT derivatives (Iwai et al., 1981). The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by TeBT, TBT, and MBT (Ema et al., 1995, 1996). Tributyltin chloride (TBTCl) (Harazono et al., 1996, 1998ab) and DBTCl (Ema and Harazono, 2000ab) during early pregnancy produced pregnancy failure in rats. In rats, the predominant adverse effects on reproduction and development of TBTCl and DBTCl on days 0–3 of pregnancy were a decrease in the pregnancy rate and an increase in the incidence of preimplantation embryonic loss, and TBTCl and DBTCl on days 4–7 of pregnancy mainly caused postimplantation embryonic loss (Harazono et al., 1998b; Ema and Harazono, 2000ab). The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Ema and Harazono, 2000b). Thus, the possibility exists that DBTCl and/or metabolites participate in the induction of early embryonic loss due to TBTCl.

The reproductive and developmental effects of organotin compounds, including DBT, were extensively investigated in rats (Ema and Hirose, 2006). We are unaware of any studies in which the adverse effects of DBT on initiation and maintenance of pregnancy have been assessed in mice. Studies in mice would be of great value in evaluating the reproductive and developmental toxicity of DBT. The present study was therefore conducted to determine the adverse effects on the initiation and maintenance of pregnancy of maternal exposure to DBTCl during early pregnancy in mice.

MATERIALS AND METHODS

Animal Husbandry and Maintenance

Male and female Crlj:CD1(ICR) mice at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, (Yokohama, Japan). The mice were acclimatized to the laboratory for 11 days prior to the start of the experiment. Male and female mice found to be in good health were selected for use. Female mice were caged with male mice and checked the following morning for signs of successful mating by examining vaginal plugs. The day when vaginal plugs were detected was considered to be day 0 of pregnancy. Successfully mated females were distributed into eight groups of 12 mice each and housed individually. Animals were reared on a γ-irradiated basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and filtered tap water *ad libitum*, and maintained in an air-conditioned room at 22°C ± 3°C, with a relative humidity of 50% ± 20%, under a controlled 12 h light/dark cycle, and ventilation with 10–15 air changes/hour. This study was performed in 2005 at the Safety Research Institute for Chemical Compounds. (Sapporo, Japan) in compliance with the "Law for the Humane Treatment and Management of Animals" (Ministry of the Environment, Japan, 1973), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Prime Minister's Office, Japan, 1980) and "Guidance for Animal Care and Use of the Safety Research Institute for Chemical Compounds, Co."

Chemicals and Dosing

DBTCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The DBTCl used in this study was 99.5% pure, and it was kept in a dark and cool place. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The female mice were dosed once daily by gastric intubation with DBTCl at a dose of 7.6, 15.2, or 30.4 mg/kg bw (25, 50 or 100 μmol/kg bw) on days 0–3 of pregnancy or on days 4–7 of pregnancy. The dosage levels were determined based on the results of our previous studies, in which increases in the incidence of pre- and postimplantation embryonic loss were caused in female rats gavaged with DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 and days 4–7 of pregnancy, respectively (Ema and Harazono, 2000ab) and our dose-finding study in which no adverse effects on embryonic survival at 15.2 mg/kg bw/day and lower, increased embryonic loss at 30.4 mg/kg bw/day, and one death and three pregnancy failure in four females at 60.8 mg/kg bw/day were found in mice gavaged with DBTCl on days 0–3 of pregnancy. The volume of each dose was adjusted to 5 mL/kg of body weight based on the daily body weight. The control mice received olive oil only on days 0–3 or days 4–7 of pregnancy. All DBTCl solutions were prepared fresh daily.

Observations

All mice were observed for clinical signs of toxicity twice a day during the administration period and daily during the nonadministration period. Females showing a moribund condition were euthanized under ether anesthesia. Maternal

TABLE I. Maternal findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	1	0	0
Moribund condition (euthanized)	0	1	1	1
Vaginal discharge	0	1	0	0
Jaundice	0	2	7*	10*
Decreased locomotor activity	0	2	1	1
Hypothermia	0	1	1	1
Soil of perigenital fur	0	0	1	0
Initial body weight (g)[a]	27.4 ± 2.0	27.2 ± 2.1	27.2 ± 2.4	27.2 ± 2.1
Body weight gain (g)[a]				
Days 0–4	1.7 ± 1.1	0.6 ± 1.2	1.2 ± 1.6	0.3 ± 0.9*
Days 4–8	2.9 ± 1.5	2.5 ± 2.6	2.1 ± 2.0	1.6 ± 1.5
Days 8–18	20.1 ± 9.1	21.3 ± 12.4	13.6 ± 12.2	8.6 ± 12.2
Adjusted weight gain[b]	8.9 ± 3.4	9.9 ± 3.8	7.9 ± 4.8	5.3 ± 5.0
Food consumption (g)[a]				
Days 0–4	18.2 ± 1.8	15.0 ± 1.9*	16.7 ± 3.2	14.8 ± 2.3*
Days 4–8	22.9 ± 4.9	22.0 ± 2.7	21.7 ± 3.5	20.9 ± 3.5
Days 8–18	71.7 ± 10.1	71.0 ± 12.5	64.6 ± 13.3	57.8 ± 13.4*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

body weight was recorded daily, and food consumption was recorded on days 0, 4, 8, 12, and 18 of pregnancy. The females were euthanized by exsanguination under ether anesthesia on day 18 of pregnancy. The uterus was weighed and the number of corpora lutea was recorded. The numbers of implantations, live and dead fetuses, and of resorptions were counted. The uteri were placed in 10% ammonium sulfide for confirmation of the dam's pregnancy status (Salewski, 1964). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. The placental weight was also measured.

Analysis of Serum Steroids Hormone Levels

Blood samples were collected from the abdominal aorta under ether anesthesia on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl at 0 or 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The serum was separated and stored at −80°C for later assay of steroid hormones. Serum progesterone and 17β-estradiol were measured by Teizo Medical (Kawasaki, Japan) using the liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of serum progesterone and 17β-estradiol were 10.0 and 0.25 pg/mL, respectively. The intra- and interassay coefficients of variation for 17β-estradiol were below 6.4% and 8.9%, respectively. The intra- and interassay coefficients of variation for progesterone were below 9.0% and 7.9%, respectively.

Statistical Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, food consumption, numbers of corpora lutea, implantations, embryonic/fetal loss and live fetuses, fetal weight, and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney U test. The incidences of pre- and postimplantation embryonic loss and fetuses with external malformations were analyzed using Wilcoxon's rank sum test. The incidence of clinical signs in dams, pregnancy, nonpregnancy, and litters with fetal malformations, and the sex ratio of live fetuses were analyzed using Fisher's exact test. The levels of serum progesterone and 17β-estradiol were analyzed by Student's *t*-test. The 0.05 level of probability was used as the criterion for significance.

TABLE II. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	3	4	7*
No. of pregnant females	11	9	8	5*
No. of implantations per female[a,b]	9.5 ± 5.1	9.8 ± 7.1	8.3 ± 7.0	5.4 ± 6.7
Pre-implantation loss per female (%)[a,b]	9.7	29.7[c]	34.0	58.3*
No. of pregnant females surviving until scheduled sacrifice	11	8	7	4
No. of litters totally resorbed	0	0	1	1
No. of corpora lutea per litter[a,d]	10.5 ± 4.3	13.1 ± 4.9	12.4 ± 4.4	13.3 ± 1.3
No. of implantations per litter[a,d]	10.4 ± 4.3	12.6 ± 4.9	12.3 ± 4.4	13.3 ± 1.3
Pre-implantation loss per litter (%)[d,e]	1.5	3.3	1.1	0
No. of post-implantation loss per litter[a,d]	1.0 ± 1.0	1.1 ± 1.5	4.1 ± 3.2	4.0 ± 5.4
Post-implantation loss per litter (%)[d,f]	10.1	14.1	41.3*	32.2
No. of live fetuses per litter[a,d]	9.4 ± 4.2	11.5 ± 5.3	8.1 ± 5.0	9.3 ± 6.2
Sex ratio of live fetuses (male / female)	50/53	47/45	30/27	21/16
Body weight of live fetuses (g)[a]				
Male	1.54 ± 0.19	1.30 ± 0.12*	1.14 ± 0.22*	1.12 ± 0.10*
Female	1.42 ± 0.15	1.28 ± 0.20	1.08 ± 0.26*	1.01 ± 0.11*
External examinations of fetuses				
No. of fetuses (litters) examined	103 (11)	92 (8)	57 (6)	37 (3)
No. of fetuses (litters) with anomalies	1 (1)	0	1 (1)	0
Cleft palate	1	0	1	0
Kinked tail	0	0	1	0
Placental weight (mg)[a]	125 ± 56	116 ± 15	120 ± 17	119 ± 16

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

RESULTS

Administration of DBTCl on Days 0–3 of Pregnancy

Table I shows the maternal findings in mice given DBTCl on days 0–3 of pregnancy. One death was observed at 7.6 mg/kg bw/day, and one female each showed a moribund condition at 7.6, 15.2, and 30.4 mg/kg bw/day, and was euthanized. The female mice in the DBTCl-treated groups showed vagina discharge, jaundice, decreased locomotor activity, hypothermia and/or soiled perigenital fur, and the incidence of females showing jaundice was significantly increased at 15.2 mg/kg bw/day and higher. A significantly decreased body weight gain on days 0–4 was noted at 30.4 mg/kg bw/day. Food consumption on days 0–4, days 4–8, and days 8–18 in the DBTCl-treated groups were reduced, and significantly decreased food consumptions on days 0–4 at 7.6 and 30.4 mg/kg bw/day and on days 8–18 at 30.4 mg/kg bw/day were observed.

The reproductive and developmental findings in mice given DBTCl on days 0–3 of pregnancy are shown in Table II. The total absence of any implantation site, i.e., nonpregnancy, was found in one, three, four, and seven of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively. In the successfully mated females, the pregnancy rate was significantly decreased, and the incidence of preimplantation embryonic loss per females was significantly increased at 30.4 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, the number of corpora lutea per litter, implantations per litter, live fetuses per litter, the incidence of litters totally resorbed and of preimplantation loss per litter, and the sex ratio of live fetuses were not significantly different between the control and DBTCl-treated groups. The incidence of postimplantation loss per litter was increased in the DBTCl-treated groups, and a significant increase was observed at 15.2 mg/kg bw/day. A significantly lower fetal weight was found in males at 7.6 mg/kg bw/day and in both sexes at 15.2 and 30.4 mg/kg bw/day. One fetus with cleft palate in the control group and one fetus with a cleft palate and kinked tail in the 15.2 mg/kg bw/day group were observed. The placental weight in the DBTCl-treated

TABLE III. Maternal findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	0	1	0
Moribund condition (euthanized)	0	0	0	1
Vaginal discharge	0	0	4	4
Jaundice	0	0	2	6*
Decreased locomotor activity	0	0	0	1
Hypothermia	0	0	0	1
Initial body weight (g)[a]	28.1 ± 1.8	28.1 ± 1.8	28.1 ± 1.8	28.2 ± 1.7
Body weight gain (g)[a]				
Days 0–4	1.6 ± 1.0	1.9 ± 0.8	1.2 ± 1.2	1.6 ± 0.9
Days 4–8	3.1 ± 1.1	1.9 ± 1.6	0.5 ± 1.8*	−0.3 ± 2.1*
Days 8–18	24.9 ± 9.1	14.9 ± 8.9*	2.9 ± 6.3*	2.4 ± 2.4*
Adjusted weight gain[b]	8.3 ± 3.5	8.1 ± 4.3	3.2 ± 5.3*	3.8 ± 3.2*
Food consumption (g)[a]				
Days 0–4	18.5 ± 1.9	18.9 ± 2.4	18.4 ± 2.7	18.8 ± 1.3
Days 4–8	21.8 ± 1.9	19.2 ± 2.6	16.4 ± 3.3*	15.6 ± 3.5*
Days 8–18	74.5 ± 12.1	67.7 ± 9.9	55.2 ± 12.6*	57.2 ± 6.2*

[a] Values are given as mean ± SD.
[b] Adjusted weight gain refers to body weight gain excluding the uterus.
* Significantly different from the control, $P < 0.05$.

groups was not significantly different from that in the control group.

Administration of DBTCl on Days 4–7 of Pregnancy

Table III shows the maternal findings in mice given DBTCl on days 4–7 of pregnancy. One death was observed at 15.2 mg/kg bw/day, and one female that showed a moribund condition at 30.4 mg/kg bw/day was euthanized. The female mice in the DBTCl-treated groups showed vaginal discharge, jaundice, decreased locomotor activity, and/or hypothermia, and the incidence of females with jaundice was significantly increased at 30.4 mg/kg bw/day. The body weight gain on days 4–8 and adjusted weight gain, which indicates the net weight gain of female mice, at 15.2 mg/kg bw/day and higher, and on days 8–18 at 7.6 mg/kg bw/day and higher were significantly decreased. Food consumption on days 4–8 and days 8–18 was significantly lowered at 15.2 mg/kg bw/day and higher.

The reproductive and developmental findings in mice given DBTCl on days 4–7 of pregnancy are presented in Table IV. Although nonpregnancy was found in one, two, and one of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively, no significant decrease in the pregnancy rate was noted in the DBTCl-treated groups. In the successfully mated females, the number of implantations per female was significantly decreased at 15.2 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, totally resorbed litters were found in 2 of the 11 females at 7.6 mg/kg bw/day, 8 of the 9 females at 15.2 mg/kg bw/day, and 10 of the 10 females at 30.4 mg/kg bw/day. At 30.4 mg/kg bw/day, no live fetuses were obtained. The numbers of corpora lutea per litter, implantations per litter, and preimplantation loss per litter, and the sex ratio of live fetuses in the DBTCl-treated groups were not significantly different from those in the control group. A significant increase in the number and incidence of postimplantation loss per litter, and a decrease in the number of live fetuses were found in the DBTCl-treated groups. The weights of male and female fetuses were significantly lowered at 7.6 mg/kg bw/day. One fetus with omphalocele, and one fetus with exencephaly and open eyelids were observed at 7.6 mg/kg bw/day. The placental weight was not significantly different between the control and the DBTCl-treated groups.

Serum Progesterone and 17β-Estradiol Levels

The serum progesterone and 17β-estradiol levels are shown in Figure 1. A significant reduction in the serum progesterone levels was noted in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Although higher levels of serum 17β-estradiol were observed after the administration of DBTCl on days 4–7 of pregnancy, no statistically significant difference in 17β-estradiol levels were detected between the control and DBTCl-treated groups.

DISCUSSION

The present study was designed to evaluate the adverse effects of DBTCl on the initiation and maintenance of