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New Method of Specimen Preparation as Cell Suspension for a Micronucleus Test

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A new specimen preparation method named ''cell suspension method'' for *in vivo* micronucleus test is proposed. Generally, a specimen of the micronucleus test is prepared by smearing the sample cells on a slide glass before staining with Giemsa or acridine orange (A.O.). In this new method, the sample cells are suspended with formalin, and stained with A.O., then put on a slide glass and examined under a microscope without smearing. This method is more convenient than the conventional method in consideration of preparation and observation of specimens. Furthermore, preparation is repeatable after the observation in this method, the specimen is suitable for a long storage, and this method is applicable to specimen preparation using peripheral blood.

Key words: micronucleus test, acridine orange, smeared method, cell suspension method, bone marrow, peripheral blood

Introduction

The *in vivo* micronucleus (MN) test has been widely used as a cytogenetic test to detect environmental mutagen chemicals. May-Grünwald Giemsa staining (1) or Giemsa single staining (2) has been routinely used for the MN test. The Giemsa methods, however, have some disadvantages that not only the MN but also intracellular materials containing RNA and other acidic materials stain dark blue by Giemsa, and it is often difficult to identify the MN. Acridine orange (A.O., 494-38-2) is metachromatic to acid polysaccharides and emits green fluoresces first and bright red fluorescence (3–5) later. With A.O., MN emits a yellowish-green fluorescence, polychromatic erythrocytes (PCEs) red fluorescence, and normochromatic erythrocytes (NCEs) almost no fluorescence or only dim outline (6). The staining characteristics of MN, PCEs, and NCEs are more distinctive with A.O. than with Giemsa, and resulted a more rapid and accurate count of micronucleated polychromatic erythrocytes (MNPCEs).

In this new method named ''cell suspension method,'' the cells obtained from bone marrow or peripheral blood are suspended with formalin, mixed and stained with A.O. upon observation, then, put on a slide glass and examined under a fluorescence microscope. This method is applicable to the cells obtained from both bone marrow and peripheral blood, and recommended as a simple, stable, and reliable method compared with the conventional methods.

Material and Methods

Chemical: Cyclophosphamide monohydrate (abbreviation: CP, CASRN 6055-19-2) obtained from Sigma-Aldrich Corporation (St. Louis, U.S.A.), was dissolved in water for injection just before use. Water for injection and 2 mg/mL CP solution were used for the dosing formulation of negative and positive controls, respectively.

Animals: Seven-week-old male Crl:CD(SD) rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) and BrlHan:WIST@Jcl(GALAS) rats (CLEA Japan Inc., Tokyo, Japan) were purchased and acclimated for 1 week. They were given commercial pellets and tap water ad libitum throughout the acclimatization and experimental periods and were subjected to a 12 h light/dark cycle. They were 8 weeks old at the start of the study. All procedures to be carried out on live animals were subject to the provisions of the Committee for Ethics in Animal Studies in the testing facility.

MN test: Bone marrow and peripheral blood MN test were performed simultaneously in the same animals by the double dosing regimen. Water for injection for Crl:CD(SD) rats or 0.5 w/v% Hydroxypropylmethylcellulose (0.5% HPMC) for BrlHan:WIST@Jcl (GALAS) rats, and CP at 20 mg/kg/day were administrated orally twice, 24 h apart to 5 male rats. Bone

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marrow and peripheral blood were collected, 24 h after the final dosing. The peripheral blood for cell suspension method was mixed with 10% neutral buffered formalin solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The ratio of the peripheral blood to 10% neutral buffered formalin solution was approximately 1: 2–3. The peripheral blood was mixed to avoid congealment, and stored at room temperature. The peripheral blood for another method for comparison (called conventional smeared method) was smeared to a clean slide directly and fixed with methanol. The bone marrow cells were collected by washing the cavity of femur with approximately 0.5 mL of phosphate buffered saline (available substitute for 10% neutral buffered formalin, handled appropriately to avoid droplet of formalin) for the cell suspension method. The cell suspension was mixed with approximately 0.5 mL of 10% neutral buffered formalin and then left for 5 min. The supernatant was further mixed with approximately 1 mL of 10% neutral buffered formalin and centrifuged at 1000 rpm (approximately $170 \times g$) for 5 min. The supernatant was discarded, and the precipitate was suspended again in a small amount of 10% neutral buffered formalin. The resulting bone marrow cell suspension was stored at room temperature. The bone marrow cells were collected by washing the cavity of the other femur with approximately 0.5 mL of fetal bovine serum (Invitrogen Co., California, USA) for the conventional smeared method, and smeared to a clean slide directly and fixed with the methanol. The cell suspension for the cell suspension method was mixed and stained with equal volumes of 0.05 w/v% aqueous solution of acridine orange (A.O.) (Wako Pure Chemical Industries, Osaka, Japan) just before the microscopic observation, and 10 μL of the stained cell suspension was dropped on the middle of a cover slide. The specimen was prepared by covering the cover glass of the stained cell suspension with a slide glass, and the specimen was turned and pressed slightly with paper to remove excess fluid, then it was observed after the stain conditions of the slide and dispersion of the cells were confirmed. The conventional smeared method was also stained with 0.05 w/v% A.O. just before observation.

Observation of specimens: Cell specimens were observed under a fluorescence microscope with B excitation filter. In use of erythrocytes in bone marrow, 1000 erythrocytes (500 cells per area × 2 areas of view), including both PCEs and NCEs each animal were examined to determine the ratio of PCEs among the total erythrocytes. A total of 2000 PCEs in 2 areas of view under the microscope (1000 PCEs per area) were examined for the number of MNPCEs. MN in the cytoplasm and the types of erythrocytes (PCEs or NCEs) were identified in accordance with the methods of Hayashi *et al*. (3). In use of erythrocytes in peripheral blood, 1000 erythrocytes (500 cells per area × 2 areas of view), including reticulocyte (RETs) and NCEs, each animal was examined to determine the ratio of RETs among the total erythrocytes. A total of 2000 RETs in 2 areas of view under the microscope (1000 RETs per area) were examined for the number of micronucleated cells (MNRETs). Micronuclei (MNs) in the cytoplasm and the types of erythrocytes (RETs or NCEs) were also identified in accordance with the methods of Hayashi *et al*. (3). Granules with yellowish-green fluorescence in the cytoplasm were recognized as MNs. Erythrocytes with red fluorescence in the cytoplasm were recognized

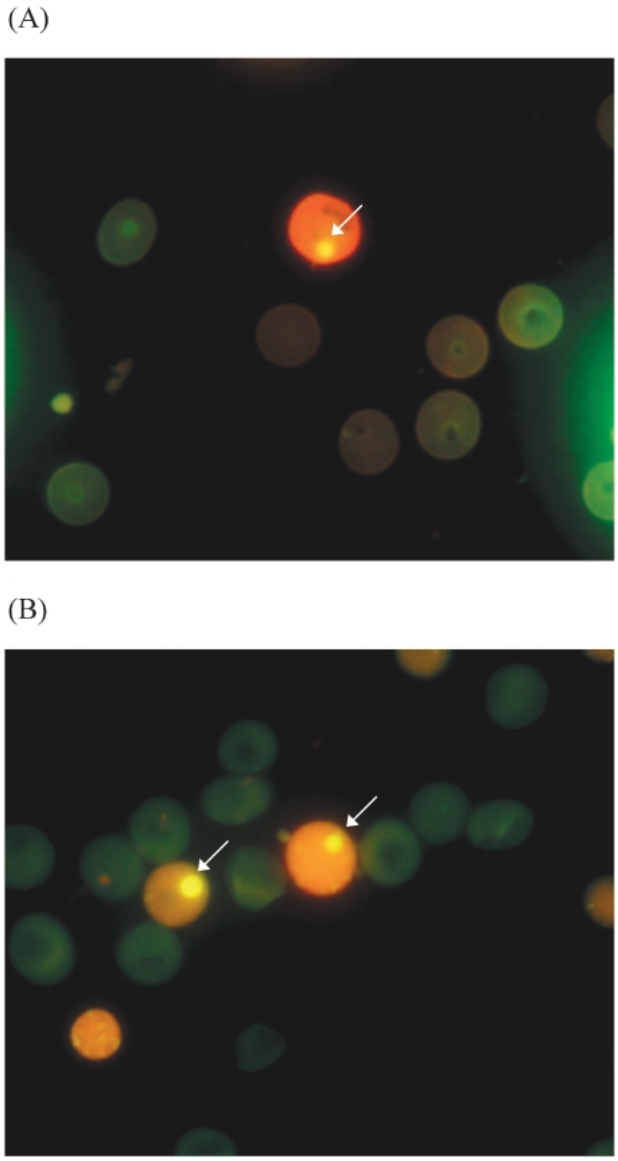

Fig. 1. The specimens of bone marrow cells using Crl:CD(SD) rats 24 h after the final dosing at 20 mg/kg cycrophosphamide monohydrate by cell suspension method (A) and conventional smeared method (B). Cell specimens were observed under a fluorescence microscope with B excitation filter. There are MNs in this field (arrows).

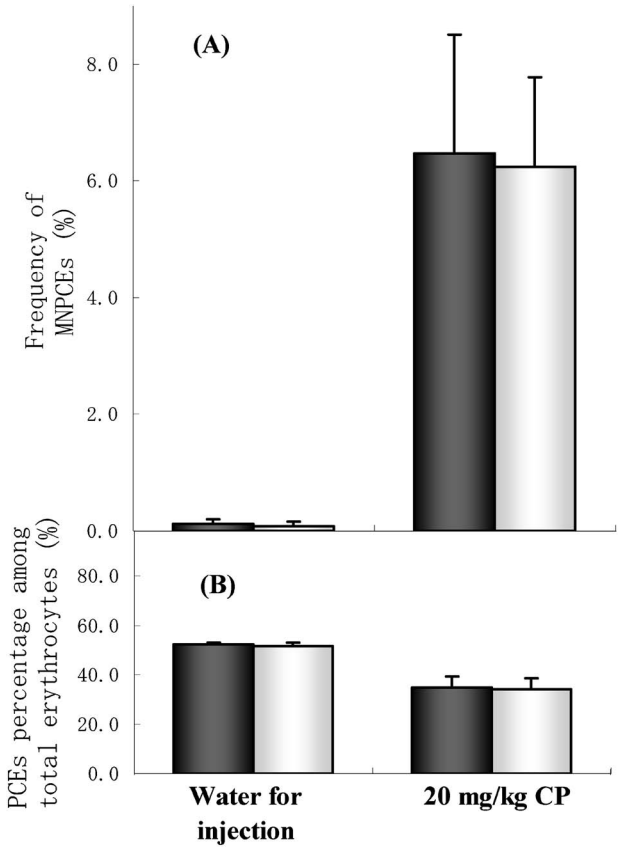

Fig. 2. Results of MN test using Crl:CD(SD) rat bone mallow cells. Water for injection or 20 mg/kg/day CP was administrated orally twice in 24 h apart to 5 male rats. Bone marrow was collected 24 h after the final dosing, and prepared the specimen by cell suspension method or conventional smeared method. The specimen was observed and the frequency of MNPCEs (A) and PCEs percentage among total erythrocytes (B) were calculated.
■: Cell suspension method
□: Conventional smeared method

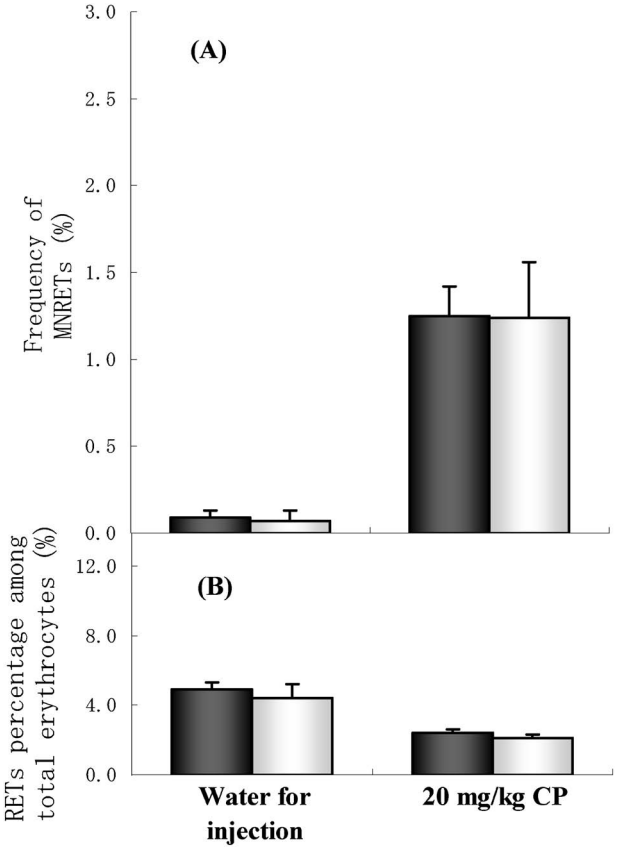

Fig. 3. Results of MN test using Crl:CD(SD) rat peripheral blood. Water for injection or 20 mg/kg/day CP was administrated orally twice in 24 h apart to 5 male rats. Peripheral blood was collected 24 h after the final dosing, and prepared the specimen by cell suspension method or conventional smeared method. The specimen was observed and the frequency of MNRETs (A) and RETs percentage among total erythrocytes (B) were calculated.
■: Cell suspension method
□: Conventional smeared method

as PCEs in bone marrow or RETs in peripheral blood, and erythrocytes without any fluorescence in the cytoplasm were recognized as NCEs.

Statistical analysis: Student's *t* test was applied as a statistical analysis to compare incidence of MNPCEs (MNRETs) and percentage of PCEs (RETs) in the cell suspension method with the conventional smeared method (two-tailed significance levels: 5% and 1%).

Results

Staining patterns: To determine validity of the cell suspension method comparing to the conventional smeared method, CP as a reliable positive control, and water for injection or 0.5% HPMC as a negative control were orally administered to Crl:CD(SD) and BrlHan:WIST@Jcl(GALAS) rats twice in 2 days. The bone marrow and peripheral blood were obtained from the rats and prepared into specimens after 24 h of the 2nd administration, which is the time period known to induce the positive result with CP. Fig. 1 shows typical microscopic images of the cell suspension method and the conventional smeared method. No significant difference was observed in configuration of the specimens. Cytoplasm of PCEs and RETs of the conventional smeared method stained in orange-red was slightly clearer in contrast, however, there was no difference for the determination of the MNPCEs or PCEs comparing to the cell suspension method. The microscopic image of the cell suspension method was appropriate and it was comparable to the specimen of the conventional smeared method.

MN test using Crl:CD(SD) rats: The Crl:CD strain is commonly used in MN tests and other toxicity tests using rats. In bone marrow of Crl:CD(SD) rats, the incidences of MN in the positive and negative controls were almost comparable between the cell suspension method

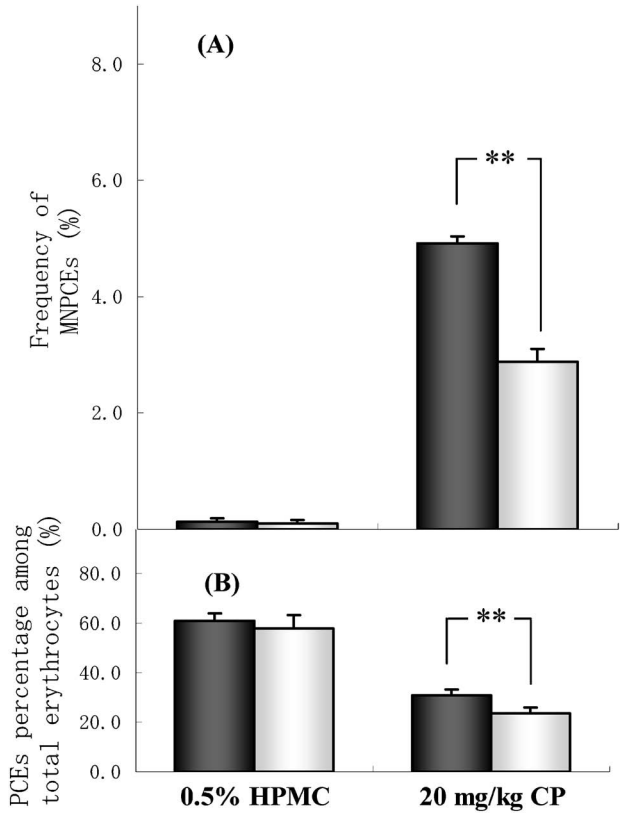

Fig. 4. Results of MN test using BrlHan:WIST@Jcl(GALAS) rat bone mallow cells. 0.5% HPMC or 20 mg/kg/day CP was administrated orally twice in 24 h apart to 5 male rats. Bone marrow was collected 24 h after the final dosing, and prepared the specimen by cell suspension method or conventional smeared method. The specimen was observed and the frequency of MNPCEs (A) and PCEs percentage among total erythrocytes (B) were calculated. **Significantly different from cell suspension method: $p<0.01$ by student's *t* test
■: Cell suspension method
□: Conventional smeared method

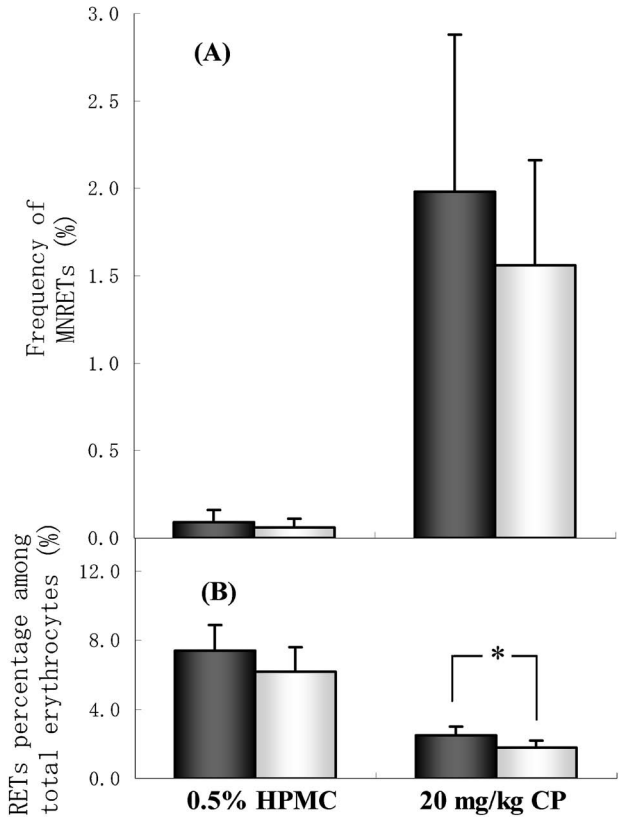

Fig. 5. Results of MN test using BrlHan:WIST@Jcl(GALAS) rat peripheral blood. 0.5% HPMC or 20 mg/kg/day CP was administrated orally twice in 24 h apart to 5 male rats. Peripheral blood was collected 24 h after the final dosing, and prepared the specimen by cell suspension method or conventional smeared method. The specimen was observed and the frequency of MNRETs (A) and RETs percentage among total erythrocytes (B) were calculated. *Significantly different from cell suspension method: $p<0.05$ by student's *t* test
■: Cell suspension method
□: Conventional smeared method

and the conventional smeared method, and no significant difference was observed. No significant difference in PCEs ratio was observed in the positive or negative control between the either methods (Fig. 2). Furthermore, in peripheral blood, there was no significant difference in any of the incidence of MN or RETs ratio between the either methods (Fig. 3).

MN test using BrlHan:WIST@Jcl(GALAS) rats: The BrlHan:WIST@Jcl strain has recently been investigated for its usefulness and convenience in toxicity tests using rats. Thus, this strain is expected to be used in the toxicity fields in the future. In bone marrow of BrlHan:WIST@Jcl(GALAS) rats, incidence of MN and PCEs ratio in the negative controls were comparable between the cell suspension method and the conventional smeared method, and there was no significant difference observed. However, in the positive control group, the appearance incidence of MN and PCEs ratio using the conventional smeared method were significantly lower than the cell suspension method (Fig. 4).

In peripheral blood, the result of the negative control was almost comparable in MN and RETs ratios and there was no significant difference. However, in the positive control, RETs ratio of the conventional smeared method was significantly lower than the cell suspension method (Fig. 5). In addition, although it was not statistically significant, the incidence of MN and PCEs/RETs ratio of the conventional smeared method was lower than the cell suspension method in both Crl:CD(SD) and BrlHan:WIST@Jcl(GALAS) rats.

Background data: We have been using the cell suspension method, and retained historical data concerning the negative controls regarding bone marrow cells of rats and bone marrow cells and peripheral blood of ICR mice (Table 1). These data are stable with very minor variations, and comparable to the studies conducted with the conventional smeared method (7–9).

Table 1. Historical data of micronucleus test in rats and mice

(A)

Species	Sampling specimens and time after the dosing	Strain	Compound	Dosing freqency and route	No. of animals and studies	Frequency of MNPCE or MNRET (%)			PCE or RET ratio among total erythrocytes (%)		
						Mean ± SD*	Maximum and minimum individual values	Maximum and minimum in mean of each study	Mean ± SD*	Maximum and minimum individual values	Maximum and minimum in mean of each study
Rat	Bone mallow 24 hr	Crl:CD(SD)	Negative control	—	449 95	0.13 ± 0.08	Max: 0.45 Min: 0.00	Max: 0.30 Min: 0.05	53.2 ± 2.9	Max: 60.7 Min: 33.4	Max: 58.1 Min: 46.9
			Positive control (CP, 10 mg/kg)	Once i.p.	345 71	2.64 ± 1.01	Max: 6.85 Min: 1.00	Max: 5.43 Min: 1.45	48.5 ± 4.9	Max: 59.0 Min: 30.2	Max: 56.8 Min: 38.5
			Positive control (CP, 20 mg/kg)	Twice p.o.	62 16	5.07 ± 1.56	Max: 9.00 Min: 2.30	Max: 6.87 Min: 3.00	28.2 ± 10.6	Max: 50.2 Min: 10.7	Max: 46.7 Min: 13.9
		BrlHan:WIST @Jcl(GALAS)	Negative control	—	40 8	0.12 ± 0.07	Max: 0.25 Min: 0.00	Max: 0.17 Min: 0.07	66.0 ± 10.2	Max: 90.3 Min: 45.9	Max: 73.2 Min: 53.5
			Positive control (CP, 20 mg/kg)	Twice p.o.	25 5	4.57 ± 0.63	Max: 5.65 Min: 2.90	Max: 4.92 Min: 4.03	22.1 ± 7.5	Max: 33.3 Min: 7.3	Max: 30.9 Min: 9.6
Mouse	Bone mallow 24 hr	Crlj:CD1 (ICR)	Negative control	—	298 64	0.12 ± 0.08	Max: 0.40 Min: 0.00	Max: 0.27 Min: 0.06	52.5 ± 2.6	Max: 62.1 Min: 42.2	Max: 56.9 Min: 47.6
			Positive control (CP, 40 mg/kg)	Once i.p.	270 57	2.65 ± 1.21	Max: 9.4 Min: 0.80	Max: 5.60 Min: 1.32	47.4 ± 4.5	Max: 61.8 Min: 33.7	Max: 54.9 Min: 40.1
			Positive control (CP, 80 mg/kg)	Twice p.o.	10 2	2.96 ± 0.74	Max: 3.85 Min: 1.35	Max: 3.19 Min: 2.73	11.1 ± 4.2	Max: 16.8 Min: 5.7	Max: 14.8 Min: 7.4
	Peripheral blood 48 hr	Crlj:CD1 (ICR)	Negative control	—	34 7	0.13 ± 0.08	Max: 0.30 Min: 0.00	Max: 0.20 Min: 0.06	5.08 ± 2.51	Max: 6.6 Min: 3.5	Max: 5.5 Min: 4.8
			Positive control (CP, 40 mg/kg)	Once i.p.	34 7	1.63 ± 0.81	Max: 3.80 Min: 0.40	Max: 2.47 Min: 1.12	2.85 ± 1.02	Max: 5.6 Min: 1.3	Max: 3.5 Min: 1.7
	Peripheral blood 72 hr	Crlj:CD1 (ICR)	Negative control	—	34 7	0.14 ± 0.06	Max: 0.30 Min: 0.00	Max: 0.20 Min: 0.08	5.07 ± 1.20	Max: 7.4 Min: 3.0	Max: 6.5 Min: 4.0
			Positive control (CP, 40 mg/kg)	Once i.p.	34 7	0.29 ± 0.18	Max: 0.80 Min: 0.05	Max: 0.44 Min: 0.18	2.95 ± 1.38	Max: 6.6 Min: 1.3	Max: 4.2 Min: 1.6

(B)

Species	Sampling specimens and time after the dosing	Strain	Compound	Dosing freqency and route	No. of animals and studies	Frequency of MNPCE or MNRET (%)			PCE or RET ratio among total erythrocytes (%)		
						Mean ± SD*	Maximum and minimum individual values	Maximum and minimum in mean of each study	Mean ± SD*	Maximum and minimum individual values	Maximum and minimum in mean of each study
Rat	Bone mallow 24 hr	Crl:CD (SD)	Negative control	—	55 11	0.13 ± 0.07	Max: 0.35 Min: 0.00	Max: 0.18 Min: 0.09	51.8 ± 3.4	Max: 58.8 Min: 44.9	Max: 57.9 Min: 49.0
			Positive control (CP, 10 mg/kg)	Once i.p.	50 10	2.17 ± 0.76	Max: 4.15 Min: 1.00	Max: 3.43 Min: 1.46	45.6 ± 6.6	Max: 54.9 Min: 25.6	Max: 51.6 Min: 34.4
			Positive control (CP, 20 mg/kg)	Twice p.o.	3 1	3.10 ± 0.74	Max: 3.95 Min: 2.60	Max: 3.10 Min: 3.10	15.5 ± 2.6	Max: 18.5 Min: 13.7	Max: 15.5 Min: 15.5
Mouse	Bone mallow 24 hr	Crlj:CD1 (ICR)	Negative control	—	10 2	0.10 ± 0.07	Max: 0.20 Min: 0.00	Max: 0.10 Min: 0.10	52.4 ± 2.7	Max: 55.4 Min: 47.5	Max: 54.1 Min: 50.7
			Positive control (CP, 40 mg/kg)	Once i.p.	10 2	2.20 ± 0.74	Max: 3.95 Min: 1.50	Max: 2.44 Min: 1.96	48.1 ± 4.2	Max: 54.6 Min: 38.5	Max: 50.1 Min: 46.1
	Peripheral blood 48 hr	Crlj:CD1 (ICR)	Negative control	—	10 2	0.10 ± 0.06	Max: 0.20 Min: 0.05	Max: 0.10 Min: 0.09	5.13 ± 0.83	Max: 6.4 Min: 4.2	Max: 5.2 Min: 5.1
			Positive control (CP, 40 mg/kg)	Once i.p.	34 7	1.33 ± 0.79	Max: 2.70 Min: 0.55	Max: 1.96 Min: 0.70	3.20 ± 1.06	Max: 5.4 Min: 1.8	Max: 3.5 Min: 2.9
	Peripheral blood 72 hr	Crlj:CD1 (ICR)	Negative control	—	10 2	0.10 ± 0.06	Max: 0.20 Min: 0.00	Max: 0.13 Min: 0.06	6.75 ± 1.60	Max: 8.9 Min: 4.5	Max: 7.4 Min: 6.1
			Positive control (CP, 40 mg/kg)	Once i.p.	34 7	0.15 ± 0.06	Max: 0.20 Min: 0.05	Max: 0.19 Min: 0.10	4.80 ± 1.49	Max: 7.3 Min: 2.5	Max: 6.0 Min: 3.6

Male (A) and female (B) of rats and mice were administrated at 7 or 8 weeks old. Positive control using cyclophosphamide monohydrate (CP) was administrated once or twice for 24 hr interval.
*: Calculated from individual data

Discussion

The specimen for *in vivo* MN test with bone marrow cells are commonly prepared smearing the sample before staining with Giemsa or A.O. However, due to the fact that the smear samples are inappropriate for a long-term preservation, re-preparation is difficult after the microscopic test, and preparation of adequate sample requires certain skills, a simple and reliable new method has been sought after.

According to the result of *in vivo* MN test using bone marrow cells, the conventional smeared method and the cell suspension method showed comparable results in both negative and positive control groups, and it was confirmed that the difference of the preparation method did not affect the results. In addition, our historical data obtained in MN tests using bone marrow cell of ICR mice and Crl:CD(SD) rats show steady and reliable results. Furthermore, when compared to the studies conducted with the conventional smeared method, the results are approximately equivalent. Therefore, the MN test with the specimen observation prepared by the cell suspension method was considered as an adequate alternative the conventional smeared method.

Moreover, this method is applicable to the MN test using peripheral blood. The results of these methods were almost same in both negative and positive controls, and it was confirmed that the difference of specimen preparation method did not affect the test results.

Generally, mice had been used widely in MN tests, but so as to share the information with the other toxicity studies, and to perform toxicokinetic study for exposure proof, the MN test using rats has been increased (10). However, there are still some disadvantages in MN test using rats. One of the concerns when peripheral blood was used for the test is elimination of MN erythrocyte in the spleen. In fact, in the positive group of this study, incidence of MNPCE was higher in the bone marrow sample than in peripheral blood sample. There is a study with rats reporting that colchicine, which induced positive result in the bone marrow sample, was negative in the peripheral blood sample (7). Therefore, it is considered appropriate to use bone marrow cells at using rats in MN test.

However, bone marrow cells of rats often show MN-like structure by mast-cell granules (11), and identifying them is very difficult in the Giemsa stained specimens, therefore staining with A.O. has been suggested. Preparing A.O. staining specimen is easier in the cell suspension method, and if the preparation of a slide glass specimen failed, it is possible to re-prepare. Regarding the preservation, since the specimen is preserved in formalin, as long as it is not dried, it is possible to preserve it for more than half a year. The cell suspension method is applicable to the MN test using peripheral blood, and it is recommended as a simple and accurate method for the MN test.

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