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Flow Cytometric Analysis of Micronuclei in Peripheral Blood Reticulocytes: II. An Efficient Method of Monitoring Chromosomal Damage in the Rat

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We have evaluated a flow cytometric method that allows assessment of micronucleated reticulocytes (MN-RETs) in microliter quantities of peripheral blood and compared results using this assay with those of established microscopic methods of scoring bone marrow and peripheral blood from rats treated with well-characterized genotoxic agents. Young reticulocytes (RETs) are labeled with FITC–anti-CD71 (transferrin receptor) and micronuclei with propidium iodide (with RNase treatment). Red blood cells parasitized with *Plasmodia* serve as a calibration standard for DNA content. Microscopic scoring used acridine orange (AO) staining of methanol-fixed slides or supravital AO staining. The effect of the rat spleen on the parameters evaluated was determined by comparing age- and sex-matched normal and splenectomized rats treated with cyclophosphamide, *cis*-platin, or vinblastine under treatment conditions that established a steady-state frequency of MN-RETs in the bone marrow and peripheral blood compartments. The data demonstrate the sensitivity and reproducibility of the flow cytometric assay in the Sprague-Dawley rat, and comparative studies using identical blinded samples at multiple laboratories show that inter- and intra-laboratory reproducibility is much higher with the flow method than with the microscopic methods currently employed for regulatory studies. A significant effect of splenic selection against genotoxicant-induced MN-RETs was observed with each of the three scoring methodologies, despite the fact that the flow and supravital AO techniques restrict analysis to the youngest fraction of RETs. The high precision of flow-based measurements also demonstrated a slight but statistically significant level of selection against spontaneously arising MN-RET. Despite these spleen effects, assay sensitivity for blood-based analyses was maintained by the flow method as it was shown to have superior counting statistics, lower variability, and higher sensitivity than manual scoring. The data suggest that flow cytometric assessment of micronucleus induction can be integrated into routine toxicity testing, eliminating the need for a separate bioassay.

Key Words: flow cytometric analysis; micronucleated reticulocytes; erythrocytes; cyclophosphamide, *cis*-platin; vinblastine.

The erythrocyte micronucleus assay is widely used as a regulatory assay and research tool for assessment of chromosomal damage *in vivo* (D'Arcy and Harron, 1998; Hayashi *et al.*, 1994, 2000; Heddle *et al.*, 1983, 1991; Müller *et al.*, 1999; Organization for Economic Co-operation and Development, 1997; U.S. Food and Drug Administration, 2003). Traditionally, this assay has been conducted in a rodent species, most often the mouse, with microscopic scoring of the frequency of micronucleated reticulocytes (MN-RETs) in bone marrow samples obtained at appropriate times following treatment (Hayashi *et al.*, 1994, 2000; MacGregor *et al.*, 1987; U.S. Food and Drug Administration, 2003). Among the improvements in the bone marrow assay originally described by Schmid (1976) and Heddle *et al.* (1991) is the demonstration by MacGregor and coworkers (1980; 1983) that peripheral blood could be used to obtain suitable target cells in the mouse. This allowed multiple sampling in the mouse without the necessity of sacrificing the animals. Further, MacGregor *et al.* (1990) demonstrated that the efficiency of the assay was increased by using a multiple treatment protocol to achieve a steady-state frequency of micronucleated cells, thus allowing sampling at a single time (Hayashi *et al.*, 2000) and opening the possibility that micronucleus measurements could be integrated into repeated-dose toxicology studies (MacGregor *et al.*, 1995; Witt *et al.*, 2000). However, concern over selective removal of micronucleated red blood cells from the peripheral circulation by the spleen has limited application of peripheral blood measurements in most species, including rats (Schlegel and MacGregor, 1984), dogs (MacGregor *et al.*,

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1992), and humans (Schlegel *et al.*, 1986). Although a comprehensive collaborative study organized by the Japanese Environmental Mutagen Society (Hamada *et al.*, 2001; Wakata *et al.*, 1998) has shown good correlation between bone marrow and peripheral blood for damage induced in the rat by a wide array of genotoxic agents when scoring young reticulocytes (RETs) in peripheral blood using a supravital staining method that allows identification of RETs of different ages (Hayashi *et al.*, 1990), the lack of comprehensive information about the possible influence of alterations in spleen function on values observed in peripheral blood has led expert bodies to recommend that bone marrow should be the tissue used for definitive conclusions about weak responses (Hayashi *et al.*, 2000).

More recently, flow cytometric scoring methods have been introduced that have increased the efficiency and reproducibility of the mouse peripheral blood assay greatly (Abramsson-Zetterberg *et al.*, 1999, 2000; Dertinger *et al.*, 1996, 2000; Torous *et al.*, 2001, 2005). Three methods (Abramsson-Zetterberg, 1999, 2000; Offer *et al.*, 2005; Torous *et al.*, 2000, 2003) have been described that score only the very youngest erythrocytes in peripheral blood, and it has been suggested that this makes possible measurements in peripheral blood of even species that are strong selectors against circulating micronucleated cells, such as the human (Dertinger *et al.*, 2002, 2003; Everson *et al.*, 1988; Grawé *et al.*, 2005; Schlegel *et al.*, 1986; Stopper *et al.*, 2005).

These advances suggest the possibility of replacing the traditional bone marrow assay. If measurements of the frequency of MN-RETs in peripheral blood, using methods that require only microliter quantities of sample, can be shown to reflect the frequencies that occur in bone marrow RETs, then it would be possible to monitor micronucleus frequency routinely during most regulatory studies, and it would not be necessary to conduct a separate experiment for the purpose of evaluating chromosomal damage. Rather, an assessment of chromosomal damage could be made using samples obtained during the toxicology, pharmacokinetics, and metabolism series of studies conducted for regulatory submissions. In most cases, this would provide much more comprehensive data from both sexes of at least two different species exposed by routes and for durations that are directly relevant to the proposed use of the product and would allow the use of important pharmacokinetic and metabolic information that under current practice is often not available under the conditions employed in the micronucleus assay.

However, validation of this approach as a regulatory method requires the following:

1. data to establish the validity of the flow cytometric scoring method in species other than the mouse (the only species for which a multilaboratory validation has been conducted; Torous *et al.*, 2001, 2005),

2. determination of the kinetics of the appearance and elimination of micronucleated cells in the peripheral blood cell population monitored (young RETs) in those species in which measurements will be made, and

3. data to confirm that the frequency of micronuclei in the circulating RET population scored accurately reflects the frequency in the primary bone marrow cell population from which they derive (including determination of the effect of spleen function on the values obtained).

We present here interlaboratory data that support the validity of the flow cytometric measurement using peripheral blood of Sprague-Dawley rats treated with well-characterized agents known to induce micronuclei by each of the two major mechanisms of micronucleus formation, chromosome breakage (cyclophosphamide and *cis*-platin) and anaphase chromosome lag (vinblastine). The flow cytometric method of Dertinger *et al.* (1996, 2004; commercially available as "MicroFlow^{PLUS} Kits") was compared with conventional microscopic scoring of methanol-fixed blood or bone marrow smears stained with acridine orange (MeOH-AO; Hayashi *et al.*, 1983) and also with peripheral blood samples stained supravitally with acridine orange (SV-AO; Hayashi *et al.*, 1990). To determine the influence of selective removal of micronucleated cells from the peripheral circulation by the spleen, we have compared the frequencies of MN-RETs in bone marrow and blood in normal and age-matched splenectomized animals under exposure conditions that result in a steady-state frequency of MN-RETs in the bone marrow and peripheral blood compartments.

The key comparative samples obtained at steady state were analyzed independently at a minimum of three different laboratories using each of the three scoring methods evaluated, including analysis of bone marrow at three contract laboratories highly experienced in conducting regulatory micronucleus studies for regulatory submissions. Based on the results obtained, we suggest that the potential to induce micronucleated erythrocytes resulting from chromosomal damage or mitotic spindle disruption can be assessed by integration of the flow cytometric endpoint into general toxicology studies and that it is not necessary to conduct an independent study for this purpose.

METHODS

Participating laboratories. Participating laboratories, the types of analyses conducted by each, and key features of the instrumentation and equipment employed at each laboratory are summarized in a companion paper that appears in this issue: Dertinger *et al.*, Table 1. For each of the three scoring methods evaluated, one laboratory was designated as the "reference laboratory" on the basis of extensive experience with that method. In each case, a senior investigator involved in the development of that method oversaw the training of technical personnel at that site. The reference laboratories were: National Center for Toxicological Research (NCTR) for microscopic scoring of methanol fixed, AO stained, slides of bone marrow and peripheral blood; Litron Laboratories for flow cytometric analysis; and Nitto Denko Corporation for microscopic scoring of peripheral blood samples stained supravitally with AO. Data from the reference laboratories are shown in Figures 7–9 to illustrate key findings, and the data from all participating laboratories are given in the data tables presented.

TABLE 1
Absolute and Relative Increases in the Frequency of MN-RETs in Normal and Splenectomized Rats at Steady State

		AO microscopy					Flow cytometry			SV-AO microscopy	
		%MN-RET		% of BM	% Splenic ↓		%MN-RET	% Splenic decrease		%MN-RET	% Splenic decrease
Treatment	Laboratory	Bone marrow	Peripheral blood	Peripheral blood	Peripheral blood	Laboratory	Peripheral blood	Peripheral blood	Laboratory	Peripheral blood	Peripheral blood
Cyclophosphamide											
N control											
	L1	0.09 ± 0.03	0.15 ± 0.03	167		L1	0.12 ± 0.01		L5	0.21 ± 0.07	
	L9	0.04 ± 0.01	0.10 ± 0.04	250		L2	0.13 ± 0.02		L6	0.19 ± 0.04	
	L10	0.09 ± 0.04	0.05 ± 0.03	56		L3	0.16 ± 0.01		L7	0.18 ± 0.02	
	L11	0.22 ± 0.08	0.18 ± 0.06	82							
SX control											
	L1	0.15 ± 0.02	0.16 ± 0.03	107		L1	0.15 ± 0.02		L5	0.39 ± 0.07	
	L9	0.12 ± 0.03	0.13 ± 0.03	108		L2	0.17 ± 0.02		L6	0.22 ± 0.03	
	L10	0.20 ± 0.04	0.09 ± 0.03	45		L3	0.19 ± 0.02		L7	0.31 ± 0.05	
	L11	0.32 ± 0.08	0.14 ± 0.04	44							
N treated											
	L1	3.42 ± 0.28	1.62 ± 0.33	47	50	L1	1.15 ± 0.15	43	L5	2.11 ± 0.38	43
	L9	1.52 ± 0.12	0.88 ± 0.25	58	35	L2	1.39 ± 0.24	36	L6	2.33 ± 0.31	29
	L10	1.92 ± 0.36	0.41 ± 0.12	21	32	L3	1.39 ± 0.22	42	L7	1.23 ± 0.34	32
	L11	2.74 ± 0.40	1.72 ± 0.37	62	32						
SX treated											
	L1	3.34 ± 0.29	3.31 ± 0.40	99		L1	2.03 ± 0.18		L5	3.74 ± 0.50	
	L9	1.39 ± 0.15	1.37 ± 0.17	99		L2	2.19 ± 0.26		L6	3.35 ± 0.24	
	L10	1.95 ± 0.33	0.61 ± 0.13	31		L3	2.43 ± 0.25		L7	1.84 ± 0.37	
	L11	3.06 ± 0.60	2.56 ± 0.42	84							
Cis-platin											
N control											
	L1	0.13 ± 0.03	0.08 ± 0.01	62		L1	0.09 ± 0.01		L5	0.17 ± 0.08	
	L9	0.08 ± 0.03	0.09 ± 0.02	113		L2	0.08 ± 0.02		L6		
	L10	0.07 ± 0.04	0.05 ± 0.04	71		L3	0.09 ± 0.01		L7		
	L11	0.17 ± 0.03	0.20 ± 0.02	118							
SX control											
	L1	0.14 ± 0.03	0.17 ± 0.03	121		L1	0.15 ± 0.02		L5	0.15 ± 0.02	
	L9	0.11 ± 0.01	0.12 ± 0.01	109		L2	0.16 ± 0.02		L6		
	L10	0.06 ± 0.02	0.22 ± 0.04	367		L3	0.16 ± 0.02		L7		
	L11	0.17 ± 0.07	0.29 ± 0.07	171							

N treated										
	L1	1.11 ± 0.24	0.65 ± 0.14	59	27	L1	0.48 ± 0.09	33	L5	0.85 ± 0.26	3
	L9	0.32 ± 0.08	0.41 ± 0.12	128	4	L2	0.53 ± 0.14	25	L6		
	L10	0.33 ± 0.10	0.28 ± 0.11	85	68	L3	0.48 ± 0.12	33	L7		
	L11	0.62 ± 0.17	0.88 ± 0.38	142	− 31						
SX treated											
	L1	1.06 ± 0.25	1.12 ± 0.17	106		L1	0.90 ± 0.14		L5	1.11 ± 0.23	
	L9	0.38 ± 0.13	0.54 ± 0.11	142		L2	0.89 ± 0.18		L6		
	L10	0.52 ± 0.12	1.12 ± 0.20	215		L3	0.91 ± 0.19		L7		
	L11	1.05 ± 0.30	0.85 ± 0.05	81							
Vinblastine											
N control											
	L1	0.10 ± 0.02	0.13 ± 0.03	130		L1	0.16 ± 0.01		L5	0.34 ± 0.07	
	L9	0.06 ± 0.02	0.07 ± 0.02	117		L2	0.10 ± 0.01		L6		
	L10	0.18 ± 0.05	0.08 ± 0.01	44		L3	0.11 ± 0.02		L7		
	L11	0.15 ± 0.03	0.24 ± 0.03	160							
SX control											
	L1	0.12 ± 0.01	0.21 ± 0.03	175		L1	0.20 ± 0.02		L5	0.35 ± 0.10	
	L9	0.04 ± 0.01	0.04 ± 0.02	100		L2	0.17 ± 0.03		L6		
	L10	0.11 ± 0.03	0.09 ± 0.02	82		L3	0.20 ± 0.02		L7		
	L11	0.26 ± 0.08	0.30 ± 0.12	115							
N treated											
	L1	1.46 ± 0.18	0.57 ± 0.09 ($n = 3$)	39	29	L1	0.47 ± 0.07 ($n = 3$)	55	L5	0.65 ± 0.20 ($n = 2$)	49
	L9	0.65 ± 0.13	0.43 ± 0.15 ($n = 3$)	66	− 39	L2	0.43 ± 0.08 ($n = 3$)	44	L6		
	L10	0.51 ± 0.11	0.11 ± 0.05	22	− 32	L3	0.41 ± 0.05 ($n = 3$)	58	L7		
	L11	0.70 ± 0.13	0.32 ± 0.06 ($n = 3$)	46	34						
SX treated											
	L1	0.87 ± 0.24	0.60 ± 0.20 ($n = 3$)	69		L1	0.78 ± 0.22		L5	0.95 ± 0.53 ($n = 3$)	
	L9	0.34 ± 0.16	0.23 ± 0.10	68		L2	0.57 ± 0.19		L6		
	L10	0.51 ± 0.15	0.13 ± 0.03	25		L3	0.72 ± 0.18		L7		
	L11	0.75 ± 0.25	0.36 ± 0.08	48							

Note. Values are means of five animals ± SEM, except for vinblastine SX control rats where $n = 4$; other instances of $n < 5$ are due to bone marrow toxicity and are noted. L1–L9 to left of each column are the laboratories performing each scoring; L1 is FDA/NCTR, L2 is Litron Laboratories, and L3 is Health Canada. Splenic ↓ = 100 − [(%MN-RET in peripheral blood of normal / %MN-RET in peripheral blood of SX) × (%MN-RET in bone marrow of SX / %MN-RET in bone marrow of normal) × 100]. Values for % splenic decrease are normalized to the normal/splenectomized bone marrow (BM) ratios based on pooled bone marrow scores of all laboratories. N = normal (eusplenic) animals; SX = splenectomized animals.

Chemicals and other reagents. Cyclophosphamide (CAS No. 6055-19-2), vinblastine sulfate (CAS No. 143-67-9), and *cis*-platin (CAS No. 15663-27-1) were purchased from Sigma, St Louis, MO. AO-coated slides used for supravital staining were prepared at Toyobo (Osaka, Japan) according to the method of Hayashi *et al.* (1990). Flow cytometry reagents including fixed malaria-infected rat blood (malaria biostandard) were from Rat MicroFlow^{PLUS} Kits (provided by Litron Laboratories, Rochester, NY; available from Litron and BD Biosciences PharMingen, San Diego, CA).

Animals and treatment regimens. All animal studies were 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. Sprague-Dawley rats 4- to 5-weeks old were purchased from Charles River Laboratories, Wilmington, MA. Animals were housed two per cage and assigned randomly to treatment groups. The animals were acclimated for approximately 2 weeks before experiments were initiated. Food and water were available *ad libitum* throughout the acclimation and experimentation periods. Splenectomies were carried out by Charles River Laboratories approximately 2–3 weeks before initiation of treatment.

The time course of MN-RET appearance and disappearance following a single dose of each of the three test agents was determined in both male and female rats. For these experiments, four animals per group were treated once: via oral gavage with distilled water or 5 or 10 mg cyclophosphamide/kg; injected ip with 0.9% saline or 0.125, 0.25, or 0.50 mg vinblastine/kg; or injected with isotonic phosphate-buffered saline or 1.0 or 2.5 mg *cis*-platin/kg, ip. A low-volume blood sample (see below) was collected prior to dosing and then daily at 24-h intervals after dosing.

To compare frequencies of MN-RET in bone marrow and peripheral blood at steady state and to determine the effect of the spleen on these values, splenectomized and normal (eusplenic) male Sprague-Dawley rats were treated with cyclophosphamide, vinblastine, or *cis*-platin for 5 days to attain a steady-state frequency of MN-RET in bone marrow and peripheral blood. For these experiments, five animals per group were treated via oral gavage with distilled water or 10 mg cyclophosphamide/kg/day, injected with 0.9% saline or 0.125 mg vinblastine/kg/day, or injected with isotonic phosphate-buffered saline or 1.0 mg *cis*-platin/kg/day for 5 consecutive days. A low-volume blood sample (see below) was collected prior to the first dosing and then daily until 24 h after the last dosing. On the sixth day after initiation of dosing, a large volume blood sample and a homogeneous bone marrow sample were collected from each animal (see below).

Blood sample collection and storage. Each day before vehicle or genotoxicant treatment, low-volume blood samples (approximately 100 μl) were collected from the tail vein of each animal after a brief warming period under a heat lamp. These samples were fixed for flow cytometric enumeration of micronucleus and RET frequencies according to procedures described in the Rat MicroFlow^{PLUS} manual (v020213 and v031230, Litron Laboratories). Fixed samples were stored at − 80°C until analysis. Approximately 24 h after the last administration of vehicle or test article, high-volume blood and bone marrow (femur) samples were collected from each animal. The high-volume blood collection was via heart puncture into a heparinized syringe. Bone marrow samples were prepared by flushing one femur with 1 ml of fetal bovine serum (FBS), centrifuging at 1000 rpm for 5 min, resuspending the pellet in approximately 300 μl fresh FBS, and spreading a small drop on a standard microscope slide. A fraction of the blood was added to an equal volume of heat-inactivated FBS, and replicate supravital AO slides were prepared for each animal. These slides were frozen and were shipped to collaborating laboratories on dry ice. Replicate blood and bone marrow smears were also prepared, allowed to air dry, and then fixed with absolute methanol for 10 min. These slides were stored in a slide box until they were shipped to collaborating laboratories for standard AO staining and scoring according to their standard operating procedures. The remaining heparinized blood suspension was fixed with ultra-cold methanol in order to preserve the cells for flow cytometric scoring. These cell suspensions were stored at − 80°C until analysis at NCTR or shipment on dry ice to collaborating laboratories for flow cytometric analysis.

Microscopic scoring of methanol-fixed slides with AO staining (MeOH-AO). Blood and bone marrow smears, air dried and fixed for 10 min in absolute methanol, were scored at the Food and Drug Administration (FDA)-NCTR laboratory and three contract testing laboratories using the MeOH-AO technique as described in the accompanying paper (Dertinger *et al.*, this issue). The contract laboratories 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.

Microscopic scoring of slides stained supravitally with AO (SV-AO). Laboratories L5, L6, and L7 scored peripheral blood samples using the SV-AO scoring technique as described in the accompanying paper (Dertinger *et al.*, this issue; also see Hayashi *et al.*, 1990, 1992).

Flow cytometric scoring. Peripheral blood samples were washed to remove fixative and labeled for flow cytometric analysis according to procedures described in the Rat MicroFlow^{PLUS} Kit (v020213 and v031230, Litron Laboratories). Data acquisition was performed with 488 nm capable instruments (FACSCalibur, FACSort, and FACScan, all from Becton Dickinson, San Jose, CA). Anti-CD71–FITC, anti-CD61–PE, and propidium iodide fluorescence signals were detected in the FL1, FL2, and FL3 channels, respectively. Calibration of the flow cytometers for the micronucleus scoring application, as well as other details of MN-RET and RET enumeration, are described in the companion paper (Dertinger *et al.*, this issue).

RESULTS AND DISCUSSION

Kinetics of Appearance and Loss of MN-RET in Peripheral Blood

The time courses of appearance and disappearance of MN-RET in peripheral blood following a single dose of test article, and the time required to establish a steady-state frequency of MN-RETs with repeated daily treatments, were determined at the NCTR laboratory by flow cytometric analysis of daily samples obtained from the lateral tail vein.

Figures 1–3 show the frequency of MN-RETs and RETs as a function of time after a single dose of cyclophosphamide, *cis*-platin, or vinblastine, respectively. The kinetics of MN-RET induction and disappearance were similar for all three agents, at doses of cyclophosphamide and *cis*-platin that caused minimal to moderate bone marrow toxicity and doses of vinblastine ranging from minimal to severe toxicity (based on %RETs). In each case, the maximum frequency of MN-RETs was observed in the sample obtained at 48 h after treatment. The percentage of MN-RET decreased dramatically by 72 after treatment and was at or near spontaneous baseline values by 96 h after treatment. The animals treated with vinblastine are shown individually because the responses of individual animals to vinblastine were much more variable within a given dose group than with cyclophosphamide or *cis*-platin, with animals varying from no response to severe toxicity or death. Although all but one animal treated with vinblastine showed the greatest frequency of MN-RET at the 48-h sampling time, it is likely that the maximum frequency of MN-RET occurs slightly earlier with vinblastine than with cyclophosphamide or *cis*-platin because this agent exerts its effect later in the cell cycle. However, for practical

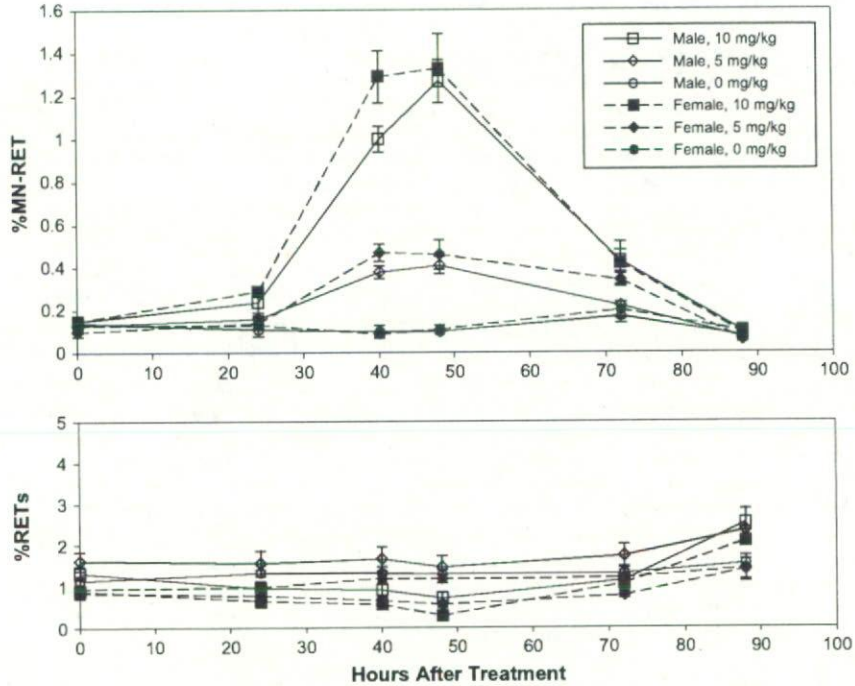

FIG. 1. Kinetics of appearance and loss of MN-RETs following a single oral dose of 5 or 10 mg/kg cyclophosphamide. Upper panel: Frequency of MN-RETs. Lower panel: Percent RETs among total erythrocytes. Solid lines are males, and dashed lines are females. Values are means ± SEM of the four animals in each sex/treatment group.

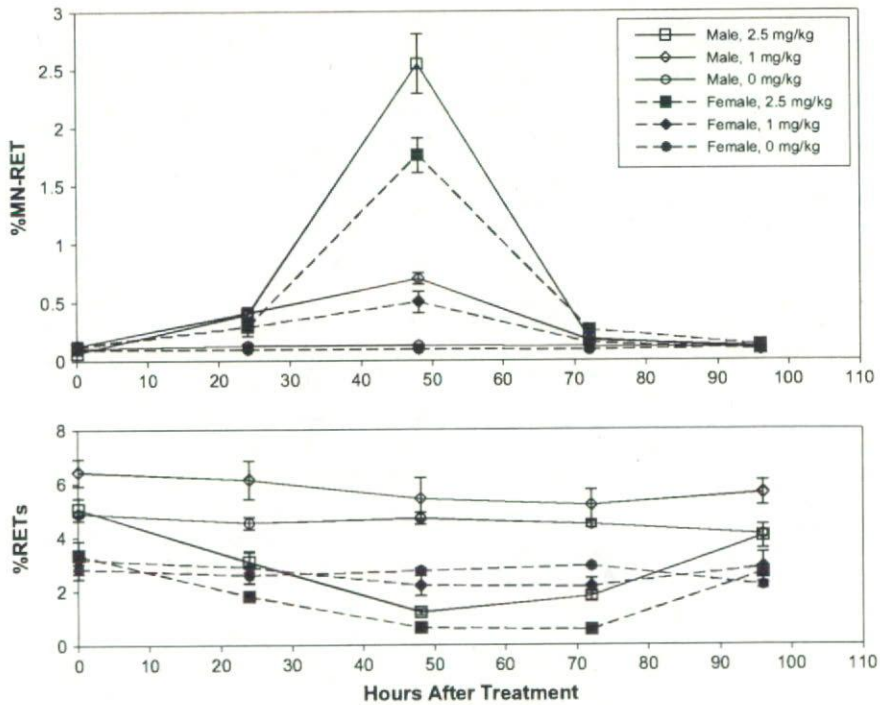

FIG. 2. Kinetics of appearance and loss of MN-RETs following a single *i.p.* dose of 1.0 or 2.5 mg/kg *cis*-platin. Upper panel: Frequency of MN-RETs. Lower panel: Percent RETs among total erythrocytes. Solid lines are males, and dashed lines are females. Values are means ± SEM of the four animals in each sex/treatment group.

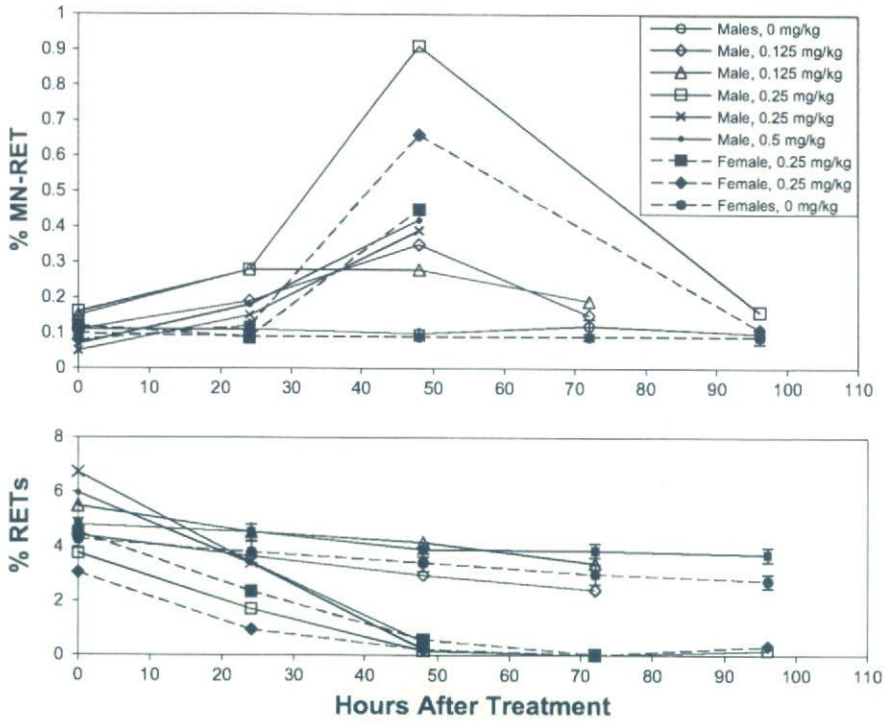

FIG. 3. Kinetics of appearance and loss of MN-RETs following a single *i.p.* dose of 0.125, 0.25, or 0.50 mg/kg vinblastine. Upper panel: Frequency of MN-RETs. Lower panel: Percent RETs among total erythrocytes. Treated animals are shown individually. The means and SEM of the (N) control animals are also shown. Solid lines are males, and dashed lines are females.

purposes, this would not significantly alter the design of experiments conducted for routine regulatory testing.

With repeated daily treatments with doses of cyclophosphamide, *cis*-platin, or vinblastine that cause minimal to moderate bone marrow toxicity (based on %RET in bone marrow and peripheral blood), an approximate steady-state frequency was reached between 2 and 4 days of daily treatment with cyclophosphamide or *cis*-platin, and the frequency of MN-RETs remained at a constant level thereafter (Figs. 4 and 5). With vinblastine, individual animal responses were more variable due to the higher toxicity of this agent, but the general kinetics was similar (Fig. 6). This kinetic behavior is expected from the responses observed following single treatments.

The kinetics of appearance and loss of MN-RET after single doses of agents that cause DNA strand breakage or spindle damage, and the kinetics of attainment of steady state with repeated daily treatments, are very similar to those previously reported in the mouse (MacGregor *et al.*, 1990). Thus, appropriate treatment and sampling regimens used in studies with either Swiss-Webster mice or Sprague-Dawley rats would not differ significantly.

Effect of Splenectomy and Scoring Method on Genotoxicant-Induced MN-RETs in Bone Marrow and Peripheral Blood

The effects that spleen function and scoring method have on the frequency of genotoxicant-induced MN-RETs in peripheral blood were examined by comparing values in peripheral blood with bone marrow under steady-state conditions established by repeated daily treatments of normal or age-matched splenectomized sprague dawley rats with cyclophosphamide, *cis*-platin, or vinblastine. In the absence of spleen function, frequencies of MN-RET in bone marrow and peripheral blood are expected to be approximately equal at steady state, as has previously been demonstrated in the mouse (MacGregor *et al.*, 1990) and F344 rat (Schlegel *et al.*, 1984). For the current study, attainment of steady state was examined in each individual rat by flow cytometric analysis at the NCTR laboratory of daily samples during 5 consecutive days of treatment (Figs. 4–6 show group means, but data from individual animals were also examined). Twenty-four hours after the last dose, large sample volumes of peripheral blood and bone marrow were obtained, and replicate samples were prepared for scoring at participating laboratories using each of the three methods evaluated. Data obtained at each of the participating laboratories are presented in Tables 1 and 2. Results from the three reference laboratories, NCTR (MeOH-AO scoring), Litron Laboratories (flow cytometric scoring), and Nitto Denko Corporation, Osaka, Japan (SV-AO scoring), are presented in Figures 7–9 and illustrate key features of the behavior of the rat system.

Distribution of coded bone marrow and blood specimens demonstrated the general robustness of the *in vivo* micronucleus assay as treatment with each of the three genotoxicants

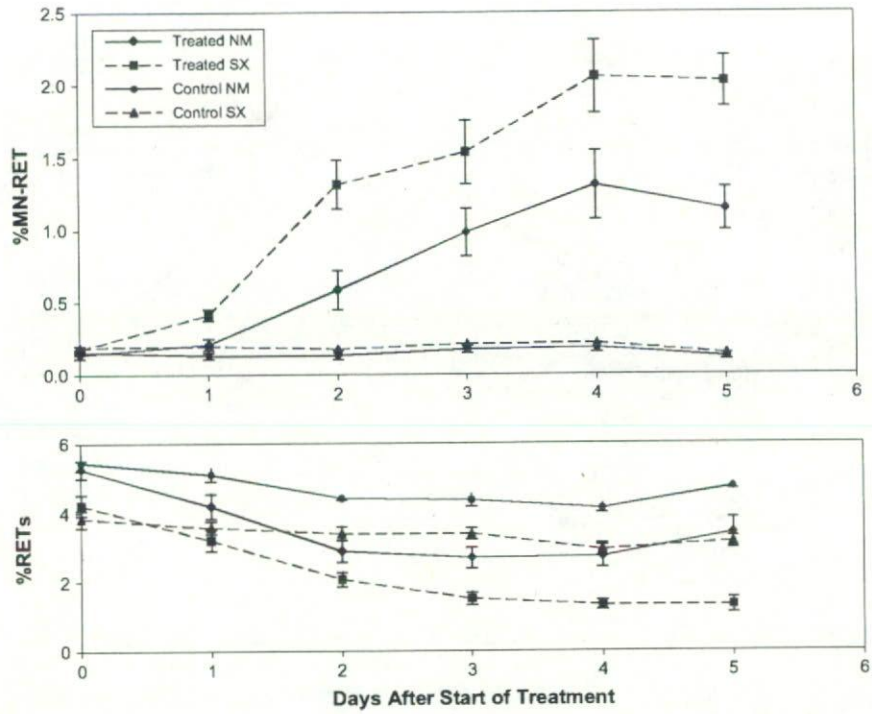

FIG. 4. Approach to steady state during daily treatments with 10 mg/kg/day cyclophosphamide, po. Upper panel: Frequency of MN-RETs. Lower panel: Percent RETs among total erythrocytes. Values are means ± SEM of the five animals in each sex/treatment group.

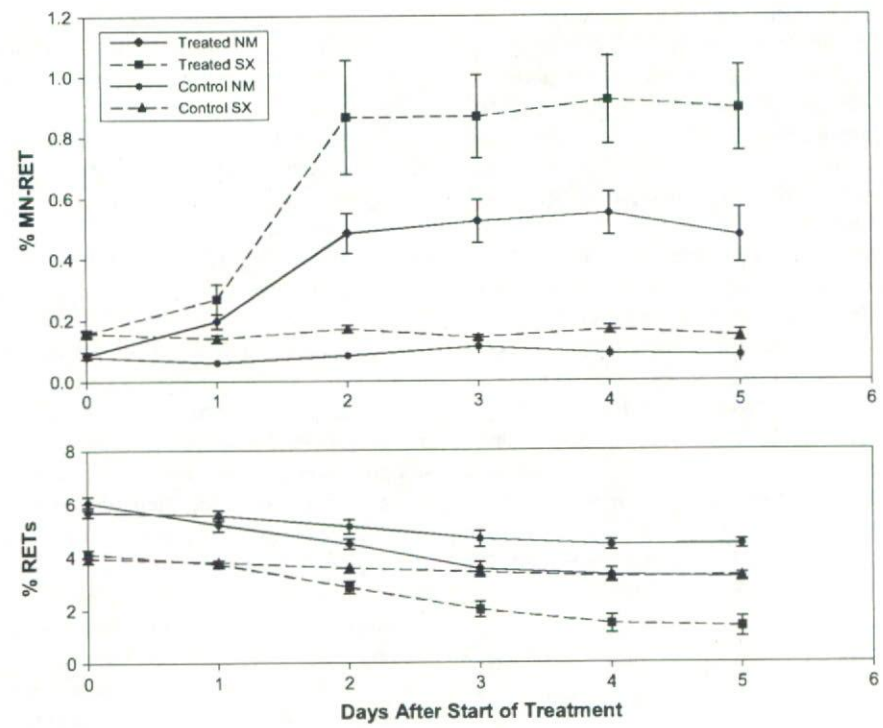

FIG. 5. Approach to steady state during daily treatments with 1.0 mg/kg/day *cis*-platin, ip. Upper panel: Frequency of MN-RETs. Lower panel: Percent RETs among total erythrocytes. Values are means ± SEM of the five animals in each sex/treatment group.

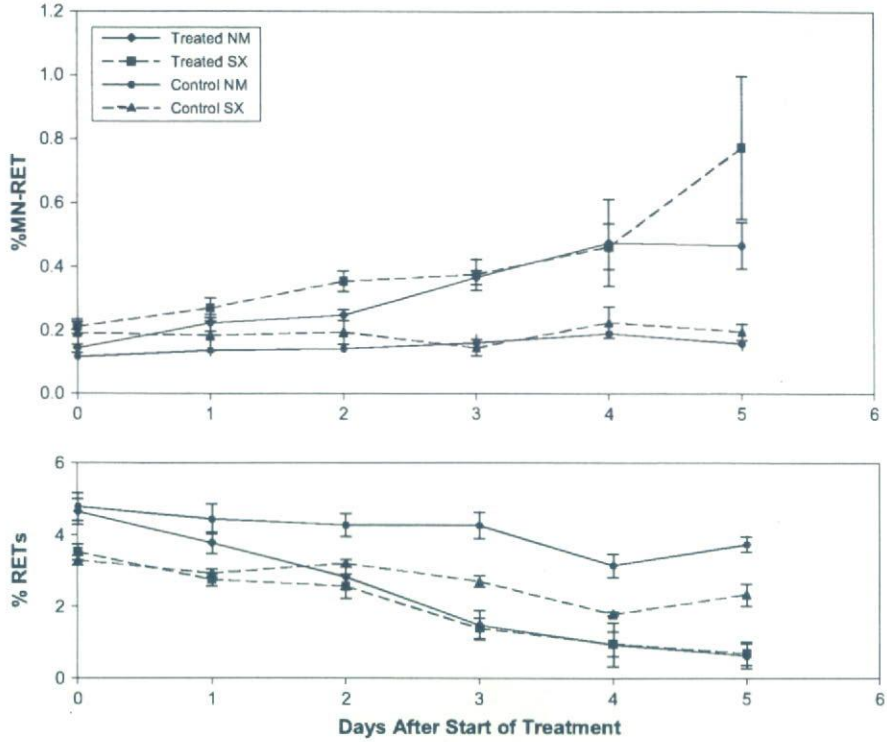

FIG. 6. Approach to steady state during daily treatments with 0.125 mg/kg/day vinblastine, ip. Upper panel: Frequency of MN-RETs. Lower panel: Percent RETs among total erythrocytes. Values are means + SEM. Only animals that achieved an approximate steady-state frequency of MN-RETs, and were subsequently sampled for comparative scoring, are shown.

studied resulted in mean MN-RET frequencies that were elevated in both the bone marrow and peripheral blood compartments of normal and splenectomized rats. These increases were observed for each of the scoring methods studied and at each of the participating laboratories (Table 1).

These intra- and interlaboratory data demonstrate a significant degree of splenic selection against cyclophosphamide- and *cis*-platin–induced MN-RET, as evidenced by reduced peripheral blood MN-RET values for intact rats relative to parallel frequencies observed in the bone marrow and also in the blood of splenectomized rats (see Figs. 7 and 8). The effect is less obvious in the vinblastine graph (Fig. 9), which can be explained by the higher MN-RET bone marrow response observed for the intact animals relative to the splenectomized group. For instance, Table 1 shows MN-RET frequencies in peripheral blood of eusplenic rats ranged from 22 to 66% of the values observed in bone marrow observed with microscopic scoring, suggesting that the relative efficiency of the splenic filtration for vinblastine-induced MN-RETs is similar to that observed with cyclophosphamide and *cis*-platin. As RETs containing the relatively larger micronuclei derived from whole chromosomes behave similarly to those derived from chromosomal fragments, the effects of agents causing whole chromosome loss should be readily detectable by analysis of peripheral blood.

Somewhat surprisingly, splenic selection against genotoxicant-induced MN-RET was evident for each of the three scoring methods studied. For example, considering the reference laboratory data, the percentage difference in the cyclophosphamide-induced MN-RET frequency in peripheral blood between the normal and splenectomized rats was only slightly less with flow cytometric scoring than with microscopic scoring (value from the blood of normal animals was 49% of the value from splenectomized animals using MeOH-AO scoring and 63% with flow cytometric scoring). The overall values from all laboratories, normalized for the difference in bone marrow frequency between the two groups and expressed as the percentage reduction caused by the spleen, were as follows: 50, 32, 35, and 32% reduction by MeOH-AO scoring of peripheral blood smears; 43, 36, and 42% reduction by flow cytometric scoring; and 43, 29, and 32% reduction by the SV-AO method (Table 1).

Thus, contrary to our original expectation and previously published suggestions that methods that score the youngest fraction of RETs reduce the degree of splenic selection against circulating RETs, we found in our present study with Sprague-Dawley rats that scoring the younger fraction of RETs with the flow cytometric method does not significantly reduce the effect of splenic selection against MN-RETs on the scores observed in the peripheral circulation relative to that observed with

TABLE 2
Frequency of RETs in Normal and Splenectomized Rats at Steady State

	AO microscopy			Flow cytometry		SV-AO microscopy	
	%RET			%RET		%RET	
Treatment	Laboratory	Bone marrow	Peripheral blood	Laboratory	Peripheral blood	Laboratory	Peripheral blood
Cyclophosphamide							
N control							
	L1	82.6 ± 1.36	4.8 ± 0.67	L1	4.75 ± 0.07	L5	53.5 ± 1.54
	L9	59.4 ± 1.92	7.9 ± 0.74	L2	2.92 ± 0.12	L6	54.7 ± 2.63
	L10	71.0 ± 2.18	11.0 ± 0.99	L3	3.70 ± 0.08	L7	52.6 ± 1.14
	L11	54.7 ± 4.44	3.9 ± 0.60				
SX control							
	L1	85.0 ± 1.15	8.1 ± 0.60	L1	3.17 ± 0.15	L5	48.6 ± 3.02
	L9	58.7 ± 1.44	6.0 ± 0.36	L2	2.00 ± 0.14	L6	58.4 ± 1.70
	L10	67.1 ± 1.31	9.9 ± 1.11	L3	2.49 ± 0.18	L7	53.0 ± 2.13
	L11	61.0 ± 7.02	3.7 ± 0.36				
N treated							
	L1	80.0 ± 2.68	8.4 ± 1.13	L1	3.42 ± 0.44	L5	46.5 ± 1.98
	L9	58.2 ± 1.74	7.4 ± 0.97	L2	2.06 ± 0.32	L6	58.3 ± 1.48
	L10	70.6 ± 2.29	8.5 ± 0.91	L3	2.72 ± 0.40	L7	52.5 ± 1.89
	L11	66.7 ± 6.38	1.9 ± 0.51				
SX treated							
	L1	72.7 ± 2.07	10.7 ± 1.03	L1	1.34 ± 0.21	L5	37.7 ± 6.43
	L9	53.5 ± 1.30	3.7 ± 0.54	L2	0.72 ± 0.11	L6	48.2 ± 3.16
	L10	61.8 ± 2.24	6.3 ± 0.93	L3	0.98 ± 0.16	L7	47.5 ± 1.84
	L11	57.7 ± 4.51	3.9 ± 0.60				
Cis-platin							
N control							
	L1	83.6 ± 1.68	5.3 ± 0.30	L1	4.46 ± 0.17	L5	47.6 ± 2.36
	L9	55.8 ± 0.89	9.1 ± 0.69	L2	3.60 ± 0.13		
	L10	73.4 ± 1.83	4.6 ± 0.29	L3	3.50 ± 0.13		
	L11	48.0 ± 4.10	5.8 ± 0.56				
SX control							
	L1	84 ± 0.96	4.2 ± 0.40	L1	3.27 ± 0.16	L5	54.0 ± 2.26
	L9	58.1 ± 1.48	5.5 ± 1.03	L2	2.75 ± 0.18		
	L10	72.1 ± 1.83	3.2 ± 0.28	L3	2.37 ± 0.16		
	L11	55.7 ± 1.82	4.9 ± 0.10				

TABLE 2—*Continued*

		AO microscopy			Flow cytometry		SV-AO microscopy
		%RET			%RET		%RET
Treatment	Laboratory	Bone marrow	Peripheral blood	Laboratory	Peripheral blood	Laboratory	Peripheral blood
N treated							
	L1	79.3 ± 1.10	4.1 ± 0.26	L1	3.21 ± 0.15	L5	40.6 ± 1.42
	L9	62.9 ± 1.67	8.7 ± 0.93	L2	2.42 ± 0.15		
	L10	70.7 ± 1.63	4.0 ± 0.26	L3	2.87 ± 0.17		
	L11	62.0 ± 2.21	4.9 ± 0.63				
SX treated							
	L1	74.3 ± 2.94	2.0 ± 0.41	L1	1.35 ± 0.36	L5	31.5 ± 5.09
	L9	56.2 ± 0.64	2.2 ± 0.70	L2	0.92 ± 0.24		
	L10	68.0 ± 1.62	2.0 ± 0.38	L3	1.00 ± 0.26		
	L11	54.0 ± 1.62	3.2 ± 0.54				
Vinblastine							
N control							
	L1	79.5 ± 1.09	7.68 ± 0.47	L1	3.75 ± 0.22	L5	38.6 ± 2.63
	L9	58.2 ± 2.87	7.06 ± 0.35	L2	3.44 ± 0.19		
	L10	63.2 ± 1.51	11.7 ± 1.63	L3	4.38 ± 0.24		
	L11	61.3 ± 0.09 ($n = 2$)	3.78 ± 0.44				
SX control							
	L1	79.6 ± 2.66	5.87 ± 0.21	L1	2.34 ± 0.31	L5	38.5 ± 4.10
	L9	56.7 ± 1.88	5.44 ± 0.22	L2	2.21 ± 0.24		
	L10	63.4 ± 2.73	11.15 ± 2.03	L3	2.93 ± 0.26		
	L11	67.8 ± 4.74 ($n = 2$)	3.32 ± 0.64				
N treated							
	L1	40.0 ± 15.39	1.95 ± 1.04	L1	0.64 ± 0.34	L5	18.2 ± 8.38 ($n = 2$)
	L9	38.1 ± 8.54	1.77 ± 0.47	L2	0.52 ± 0.28		
	L10	58.3 ± 4.8	5.64 ± 1.53	L3	0.73 ± 0.40		
	L11	ND	0.85 ± 0.36				
SX treated							
	L1	58.2 ± 7.53	2.32 ± 0.96	L1	0.70 ± 0.32	L5	18.6 ± 4.92 ($n = 3$)
	L9	49.3 ± 6.76	3.23 ± 1.18	L2	0.66 ± 0.30		
	L10	51.8 ± 2.48	6.86 ± 0.76	L3	0.83 ± 0.36		
	L11	50.7 ± 3.65 ($n = 3$)	2.14 ± 0.48				

Note. Values are means of five animals ± SEM, except for vinblastine SX control rats where $n = 4$; other instances of $n < 5$ are due to either excessive toxicity and/or considerations regarding the quality of staining. ND = not determined (considerations regarding the quality of staining). %RET for SV-AO labs = frequency of Type I and Type II RETs among total RETs (L5 and L7) or the frequency of Type I RETs among Type I and Type II RETs (L6). N = normal (eusplenic) animals; SX = splenectomized animals.

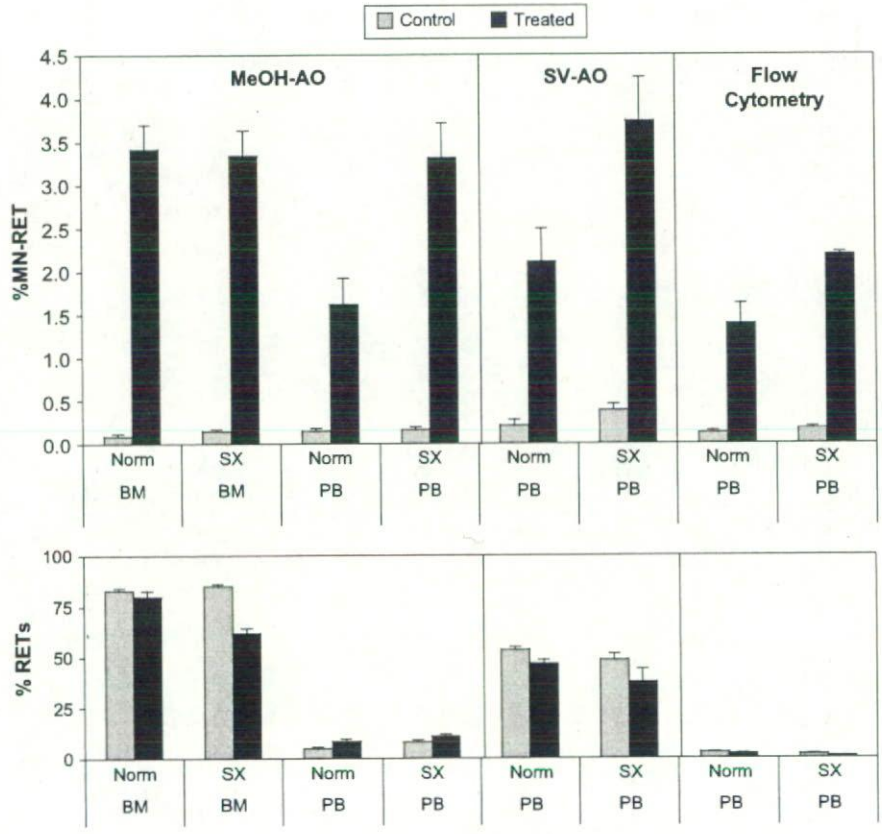

FIG. 7. Frequency of MN-RETs in bone marrow and peripheral blood of normal and splenectomized rats at steady state after five daily treatments with 10.0 mg/kg cyclophosphamide. Upper panels: Frequency of MN-RETs. Lower panels: Percent RETs among total erythrocytes or for SV-AO scoring % Type I RETs among total RETs. Values are means ± SEM of the data from the three reference laboratories. BM, bone marrow; PB, peripheral blood; Norm, normal (eusplenic) animals; SX, splenectomized animals.

MeOH-AO microscopic scoring. This was confirmed with all three agents studied at all three laboratories using the flow cytometric procedure and also at the laboratories using the SV-AO staining method (which also scores a younger fraction of RETs than the MeOH-AO method) with samples from the cyclophosphamide and vinblastine experiments.

Effect of Splenectomy and Scoring Method on Spontaneous MN-RETs in Bone Marrow and Peripheral Blood

The presence of the spleen had little or no effect on spontaneously occurring MN-RET frequencies according to microscopy-based measurements (Table 1). However, the slight differences that would be expected between these groups are beyond the power of detection of methods that consider 2000 RETs per rat. The high precision of flow cytometry–based analyses (based on the interrogation of 20,000 RETs per animal) was able to detect an apparent splenic selection against spontaneously arising MN-RET. For instance, pooling reference laboratory (L2) vehicle control data, we find that the mean MN-RET values for normal rats ($n = 15$) versus splenectomized rats ($n = 14$) were 0.11 and 0.17%, respectively ($p = 0.00091$, two-tailed Student's t-test). This finding could be interpreted as a cause for concern as it suggests the possibility of a nongenotoxic agent inhibiting the splenic removal of spontaneously occurring MN-RETs to a degree that causes an increase in the observed frequency, an increase that could erroneously be interpreted as a genotoxic effect.

There are several reasons why the possibility of false positives due to spleen toxicity is of limited concern. Firstly, as discussed further below, rat blood–based MN-RET analyses will most appropriately be integrated with other studies, especially toxicology studies, where histopathology will be available. Congestion or other changes to splenic architecture that might affect filtration function will be recorded and available to help interpret MN-RET results. Secondly, the flow cytometric procedure described herein is able to provide the frequency of micronucleus-containing mature (CD71 negative) erythrocytes simultaneously with MN-RET measurements. This endpoint of splenic filtration function was elevated by more than an order of magnitude for all the splenectomized rats studied (data not shown). Thus, a quantitative and concurrent means of assessing the effect of treatment on spleen function exists that can aid data interpretation. Thirdly, beyond

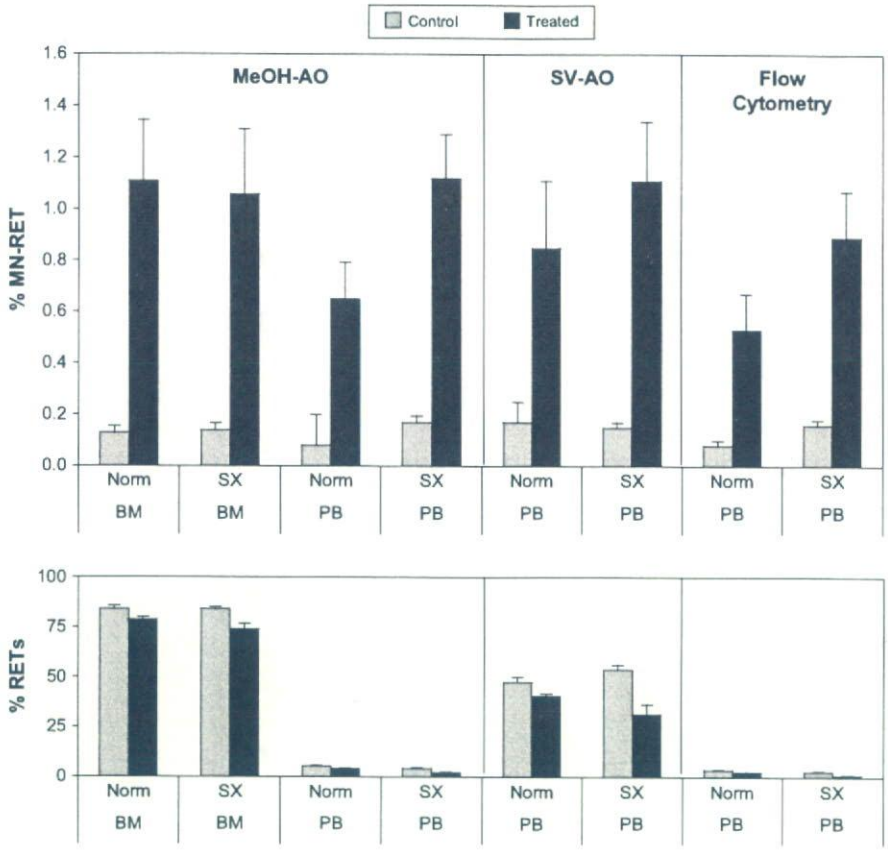

FIG. 8. Frequency of MN-RETs in bone marrow and peripheral blood of normal and splenectomized rats at steady state after five daily treatments with 1.0 mg/kg *cis*-platin, ip. Upper panels: Frequency of MN-RETs. Lower panels: Percent RETs among total erythrocytes or for SV-AO scoring % Type 1 RETs among total RETs. Values are means ± SEM of the data from the three reference laboratories. BM, bone marrow; PB, peripheral blood; Norm, normal (eusplenic) animals; SX, splenectomized animals.

evaluating flow cytometry–based MN-RET data for statistical significance, it would be simple and scientifically justifiable to require positive responses to exceed a threshold MN-RET frequency that corresponds to the values observed in bone marrow of normal, or in the peripheral blood of splenectomized, rats.

Sensitivity of Blood-Based Assays

The hypothesis that restricting micronucleus scoring to the youngest fraction of RETs can significantly reduce or even negate the influence of splenic selection against micronucleated erythrocytes was not supported by this investigation. Even so, an effective solution to the issue was demonstrated. Specifically, the high throughput nature of flow cytometric scoring enables the analysis of tens of thousands of RETs per specimen, an advantage that overcomes the somewhat attenuated responses observed in blood. For instance, examination of spontaneous MN-RET frequencies for intact rats suggests that the mean values of about 0.1% were common for the microscopy-based methods (Table 1). Assuming the microscopy-based measurements are collected by inspecting 2000 RETs for micronuclei per animal (5 rats per group) and that the resulting data are Poisson distributed, a 3.3-fold increase or a 4.75-fold increase would be necessary to detect the effect with 90% power at significance levels of 0.05 and 0.01, respectively. On the other hand, for normally distributed flow cytometry data (20,000 RETs analyzed for micronuclei per rat) with a mean (experimentally determined) spontaneous MN-RET frequency of 0.1%, 1.81- and 2-fold increases would be detected at the 0.05 and 0.01 significance levels, respectively. This enhanced detection capability more than offsets the splenic filtration effects exhibited herein. Note that for those microscopy-based laboratories that reported lower spontaneous frequencies in the range of 0.05% (e.g., L10), the situation is exacerbated. For instance, 4.5- and 6.8-fold increases would be necessary to detect a response with 90% confidence at 0.05 and 0.01 significance levels, respectively, when the spontaneous incidence is scored at 0.05%. With flow cytometic scoring, interanimal MN-RET frequencies are normally distributed, and conventional statistical analysis of the frequencies of MN-RETs based on the normal distribution can be applied to determine significant responses and the statistical power of test designs.

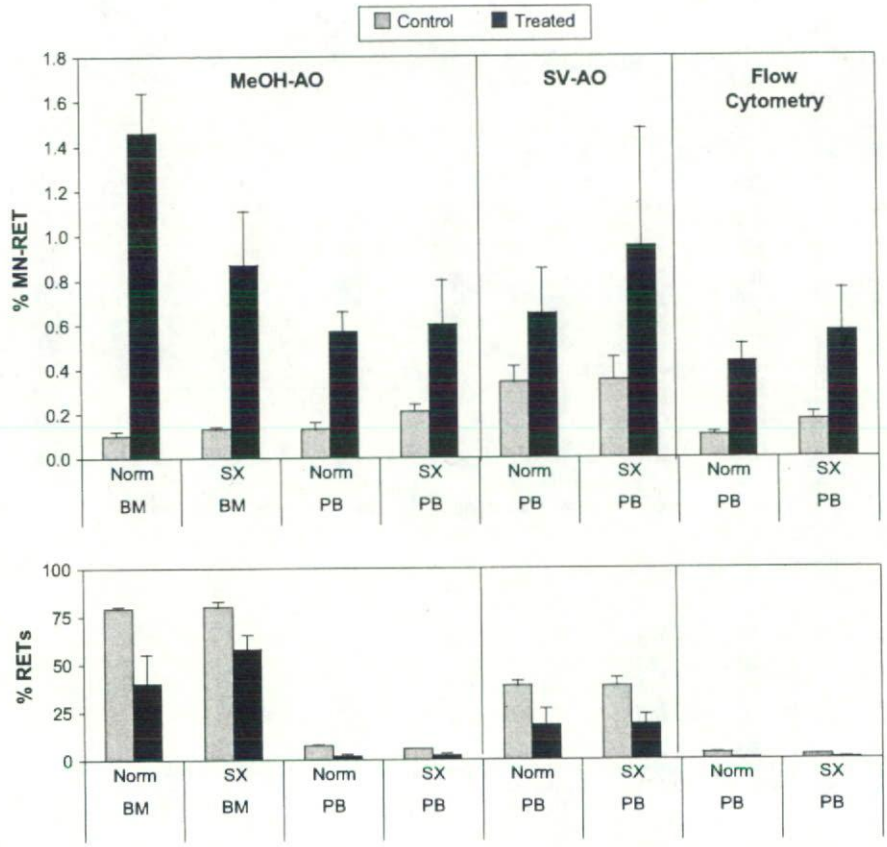

FIG. 9. Frequency of MN-RETs in bone marrow and peripheral blood of normal and splenectomized rats at approximate steady state after five daily treatments with 0.125 mg/kg vinblastine. Upper panels: Frequency of MN-RETs. Lower panels: Percent RETs among total erythrocytes or for SV-AO scoring % Type I RETs among total RETs. Values are means ± SEM of the data from the three reference laboratories. BM, bone marrow; PB, peripheral blood; Norm, normal (eusplenic) animals; SX, splenectomized animals.

The major limitations of flow cytometric analysis are the current lack of suitable methods for analysis of bone marrow and the fact that appropriate instrumentation may not be available in some laboratories. There is also some potential for fluorescent artifacts, and samples should be examined microscopically whenever a previously uncharacterized positive response is obtained in order to confirm the characteristics of the micronucleated cells induced.

CONCLUSIONS

Although spleen-dependent reduction in circulating MN-RET frequency resulting from genotoxic exposures attenuates the magnitude of MN-RET frequencies in peripheral blood relative to that in bone marrow, the analytical and statistical advantages of the flow cytometric measurement in peripheral blood more than offset the higher frequencies observed for bone marrow smears analyzed by MeOH-AO. The reproducibility of the three scoring approaches investigated are discussed in detail in the companion paper (Dertinger *et al.*, this issue), but simple inspection of the results in Table 1 shows that interlaboratory reproducibility is much higher, and within-laboratory variability is lower with the flow cytometric method than with the current regulatory method of microscopic evaluation of bone marrow. Important factors that provide this higher analytical performance are the higher cell counts obtained with the flow cytometric procedure, the objective nature of the flow cytometric fluorescence measurements relative to microscopic scoring, and, importantly, the inclusion of a DNA calibration standard (Tometsko *et al.*, 1993) in the flow cytometric method that allows different laboratories to standardize instrument settings. As discussed in the companion paper, the sensitivity of microscopic scoring to small increases in the spontaneous frequency is limited by the counting error in determining the frequency of MN-RET in each individual animal, whereas the greater counts obtained with flow cytometry allow the sensitivity limit of the assay to be determined by the actual inter-animal variability of the spontaneous MN-RET frequency.

In addition to the analytical advantages of the flow cytometric method, the ready accessibility of the small (microliter quantity) blood samples required for the flow assay

make possible the integration of the micronucleus frequency assessment with routine toxicology studies. Thus, rather than conducting a separate experiment for the purpose of evaluating chromosomal damage, the flow cytometric method using peripheral blood allows the routine evaluation of chromosomal damage via monitoring the MN-RET frequency in peripheral blood samples obtained at appropriate times during the course of studies conducted for other toxicological evaluations. Implementation of MN-RET measurement as a routine endpoint in general toxicology studies would provide more comprehensive information because both sexes are often studied under treatment regimens of differing duration and more relevant information because the results of the micronucleus evaluation can be compared directly with the metabolic and toxicological information being evaluated in the same studies, than is provided under current practice. The cost of obtaining this more extensive information would be lower because the conduct of separate animal studies can be eliminated, and the use of animals for the purpose of conducting micronucleus studies during product development can be eliminated because the required samples can be obtained from experiments being conducted for other purposes. The data show that microscopic scoring of peripheral blood is equally valid but is limited by higher variability and a more laborious scoring requirement than the flow cytometric method.

Thus, we suggest that it is not necessary to conduct separate *in vivo* studies for the purpose of evaluating the potential for chromosomal damage in rodent bone marrow but that more reliable information can be obtained by flow cytometric analysis of peripheral blood samples obtained from rodent studies during the course of routine toxicological evaluations.

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Combined Repeated Dose and Reproductive/ Developmental Toxicity Screening Test of the Nitrophenolic Herbicide Dinoseb, 2-*sec*-Butyl-4,6-Dinitrophenol, in Rats

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ABSTRACT: In a combined repeated dose toxicity study with reproduction/developmental toxicity screening test, Crj:CD(SD)IGS rats were dosed with dinoseb, 2-*sec*-butyl-4,6-dinitrophenol, by gavage at 0 (vehicle), 0.78, 2.33, or 7.0 mg/kg bw/day. Six males per group were dosed for a total of 42 days beginning 14 days before mating. Twelve females per group were dosed for a total of 44–48 days beginning 14 days before mating to day 6 of lactation throughout the mating and gestation period. Recovery groups of six males per group and nonpregnant six females per group were dosed for 42 days followed by a 14-day recovery period. No deaths were observed in males of any dose group or in females of the recovery groups. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. Hematocrit was significantly higher at 0.78 mg/kg bw/day and above in the main group males at the end of administration period. Reduction in extramedullary hematopoiesis in the spleen was significant at 2.33 mg/kg bw/day in the main group females. Sperm analysis revealed a decrease in sperm motility and an increase in the rates of abnormal sperm, abnormal tail, and abnormal head at 7.0 mg/kg bw/day. A number of dams delivered their pups and of dams with live pups at delivery was significantly lowered in the 7.0 mg/kg bw/day group. Based on these findings, the LOAEL for males and NOAEL for females were 0.78 mg/kg bw/day, and the NOAEL for reproductive/developmental toxicity was considered to be 2.33 mg/kg bw/day. © 2008 Wiley Periodicals, Inc. Environ Toxicol 23: 169–183, 2008.

Keywords: dinoseb; nitrophenolic herbicide; 2-*sec*-butyl-4,6-dinitrophenol; repeated dose toxicity; reproductive and developmental toxicity; screening test; testis toxicity; rat

INTRODUCTION

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7), was approved for sale as a nitrophenolic herbicide in the United States in 1948, and it is used in soybeans, vegetables, fruits, nuts, citrus, and other field crops for the selective control of grass and broadleaf weeds. It is also used as an insecticide in grapes and as a seed crop drying agent (EXTOXNET, 1996). Although the use of dinoseb as a pesticide was banned in the United States in 1986 and in Europe in 1991, based on the potential risk of birth defects and other adverse health effects in humans (EXTOXNET,

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1996; Rotterdam Convention, 2006), it is reported that dinoseb is a high volume chemical with production or importation exceeding 1000 ton/year in Organisation for Economic Cooperation and Development (OECD) member countries and still widely used (OECD, 2004; PAN, 2006). Dinoseb and dinoseb salts are banned in Japan but consented to import (PAN, 2006). It is estimated that the volume of dinoseb imported to Japan is 110 ton from April 2005 to March 2006 (NITE, 2007).

Dinoseb is well absorbed by the oral route in mice (Gibson and Rao, 1973) and the dermal route in rats (Shah et al., 1987; Hall et al., 1992) and can pass through the placenta into the fetus of mice (Gibson and Rao, 1973). In a dermal toxicity study, dinoseb was more absorbed in adult female rats than in young rats (Shah et al., 1987). Dinoseb shows strong acute toxicity with the dermal LD_{50} of 40 mg/kg in rabbits (US EPA, 2003). The inhalation LC_{50} is 33–290 mg/m^3 for 4-h exposure in rats (US EPA, 2003). The basic mechanism of toxicity is thought to be stimulation of oxidative metabolism in cell mitochondria by the uncoupling of oxidative phosphorylation (Leftwich et al., 1982). Toxicity of dinoseb is enhanced by physical activity and high ambient temperature such as in an outdoor agricultural environment (Leftwich et al., 1982; US EPA, 2007). Early symptoms of dinoseb exposure include hyperthermia, sweating, headache, and confusion. Other signs and symptoms include dyspnea, pulmonary edema, nausea, vomiting, abdominal pain, malaise, dehydration, and tachycardia. Severe exposure may result in restlessness, seizures, coma, and death (Leftwich et al., 1982; US EPA, 2006, 2007).

As for developmental toxicity, many studies were conducted in experimental animals in the 1970s and the 1980s. Dinoseb was reported to be teratogenic in mice when administrated intraperitoneally or subcutaneously (Gibson, 1973; Preache and Gibson, 1975a,b), but not by gavage administration (Gibson, 1973). Dietary administered dinoseb was also reported to induce several adverse effects on reproduction and development including teratogenic effects in rats (McCormack et al., 1980; Spencer and Sing, 1982; Giavini et al., 1986; Daston et al., 1988). In a rabbit teratology study, Chinchilla rabbits were exposed by oral gavage to dinoseb at levels of 0, 1, 3, or 10 mg/kg bw/day on days 6–18 of gestation. Teratogenic effects were observed at 10 mg/kg bw/day without maternal toxicity (Research and Consulting Company, 1986). This study, conducted by a laboratory in Switzerland, became a main trigger for the cancellation of dinoseb. A male reproductive toxicity study in rats showed decreased sperm counts and increased atypical spermatozoa when receiving 9.1 mg/kg bw/day and above of dinoseb in feed (Linder et al., 1982). This result is in concordance with a recent study by Takahashi et al. (2004) in which reduced sperm motility and increased incidence of tailless sperm were found.

Exposure to dinoseb may occur by direct contact, ingestion, and inhalation for users and producers, but potential indirect exposure to dinoseb via the environment for consumers is also anticipated. Dinoseb is not strongly adsorbed on most agricultural soils. Microbial breakdown of dinoseb is demonstrated on soils, but dinoseb persists for about 2–4 weeks after application. Dinoseb was reported to be detected in water supplies in Canada and the United States (Health Canada, 1991). The US FDA examined 70 food items in 1985 and 1986 for dinoseb residues. Although no residues were detected in most of crops treated with dinoseb, a positive result was obtained in one cotton meal sample (Health Canada, 1991). Dinoseb is listed in the most recent OECD List of High Production Volume (HPV) chemicals to be investigated for environment and human health effects (OECD, 2004). Although many studies had been conducted for developmental toxicity of dinoseb, these reports could be determined to be inadequate for the initial assessment of the chemical in the OECD HPV Chemicals Programme, because these studies were non-Good Laboratory Practice (GLP) studies or did not totally comply with a specific testing guideline (Klimisch et al., 1997; OECD, 2005). No studies on female reproductive performances were evaluated. Therefore, dinoseb was selected as a target substance for the Safety Examination of Existing Chemicals in Japan (MHLW, 2005) to obtain reliable information on the possible effects on reproduction and development in compliance with the OECD Test Guideline and in accordance with the principles for GLP. The present article reports the result of combined repeated dose and reproductive/developmental toxicity screening test of dinoseb in rats.

MATERIALS AND METHODS

This study was performed in 2003–2004 at the Nihon Bioresearch (Hashima, Japan) in compliance with OECD Guideline 422 Combined Repeated Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Test (OECD, 1996) and in accordance with the OECD Principles for GLP (OECD, 1998) and Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances (EA, 1984). All animals were treated in accordance with the law governing the protection and management of animals (MOE, 1973), the guidelines for experiments using animals (JALAS, 1987), and the Regulations of the Committee for the Ethical Treatment of Animals (Hashima Laboratory, Nihon Bioresearch).

Animals

International Genetic Standard (Crj: CD(SD)IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and

historical control data are available. Males and females at 7 weeks of age were purchased from Hino Breeding Center, Charles River Japan, (Yokohama, Japan). The rats were quarantined for 5 days and acclimatized to the laboratory for 16 or 17 days before the start of the experiment. Male and female rats found to be in good health were selected for use. The vaginal smears of each female were recorded, and only females showing a 4-day or 5-day estrous cycle were used in the experiment. Male and female rats were distributed into four groups on a random basis. Each group consisted of six males and 12 females as main groups and six males and six females as recovery groups. Rats were housed individually except during the mating period. From day 18 of pregnancy to day 3 of lactation, individual dams and litters were reared using wooden chips as bedding (Sunflake®; Charles River Japan).

Animals were reared on a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and water *ad libitum* and maintained in an air-conditioned room at a room temperature of 20–26°C, a relative humidity of 40–70%, a 12-h light/dark cycle, and 12 air changes per hour.

Chemicals and Dosing

Dinoseb, yellowish crystals with pungent odour, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The dinoseb (Lot no. RWN9641) used in this study was 96% pure, and was stored under refrigeration before use. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with dinoseb at a dose of 0 (control), 0.78, 2.33, or 7.0 mg/kg bw. The dosage levels were determined based on a previous dose-finding study in which no effects were seen at 5 mg/kg bw/day, but deaths and lower body weights were found at 10 and 20 mg/kg bw/day in rats given dinoseb by gavage at 0, 2.5, 5, 10, or 20 mg/kg bw/day for 14 days. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. Twelve males per group were dosed for a total of 42 days beginning 14 days before mating. After the administration period, 6 of 12 males per group were reared for 14 days without administration of dinoseb as the recovery groups. The main group females were dosed for a total of 44–48 days beginning 14 days before mating to day 6 of lactation throughout the mating and gestation period. The recovery group females were given dinoseb for a total of 42 days, followed by a recovery period of 14 days. The first day of dosing was designated as day 0 of administration and the day after the final dose was designated as day 0 of the recovery period. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight. The stability of formulations was confirmed after storage under refrigeration in the dark for 7 days. During use, the formulations were maintained under such conditions for up to 7 days, and the concentration of each preparation was within the acceptable range (91.3–96.4%).

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in males and females of the recovery groups, and twice a week during the premating period, on days 0, 7, 14, and 21 of pregnancy and on days 0, 4, 6, and 7 of lactation in females of the main groups. Food consumption was recorded twice a week in males and in the recovery group females, and twice a week during the premating period, on days 2, 9, 16, and 20 of pregnancy and on day 2 of lactation in the main group females. Functional observation battery (FOB) in all animals was recorded once a week during the administration period for 1 h following administration, as follows: (i) posture, biting behaviour, eyelid closure, and convulsion, (ii) ease of removal from cage and handling, muscle tone, fur condition, lacrimation, salivation and respiration, and (iii) rearing frequency, grooming frequency, ambulation, palpebral closure, arousal, behavioural abnormality, and righting reflex.

Six animals in each group, with the exception of one surviving female in the 7.0 mg/kg bw/day main group, were subjected to the following observations and examinations unless noted otherwise. Sensory reactions for pupillary reflex, approximation reflex, behavioural abnormality, tactile reflex, auditory reflex and pain reflex, and grip strength of fore and hind limbs were tested in the main group males on day 40 of administration and in the main group females on day 3 of lactation. Spontaneous motor activity, ambulation, and rearing were recorded (Activity Monitor, Med Associate, Vermont) after administration for 1 h at intervals of 10 min on day 39 of administration in the main group males and on day 4 of lactation in the main group females. Fresh urine was sampled from animals using a urine-collecting cage under fasting and watering conditions and collected for 24 h.

The main group rats were euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 7 of lactation in females, and the recovery group rats were euthanized on the day after completion of the recovery period. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta. The brain (cerebrum, cerebellum, and medula oblongata), pituitary gland, thyroid, thymus, heart, liver, spleen, kidney, adrenal glands, testis, epididymis, tail of the epididymis, ovary, and uterus were isolated and weighed. The lung, trachea, pancreas, salivary gland (sublingual gland and submandibular gland), esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, lymph nodes (mandibular lymph nodes and