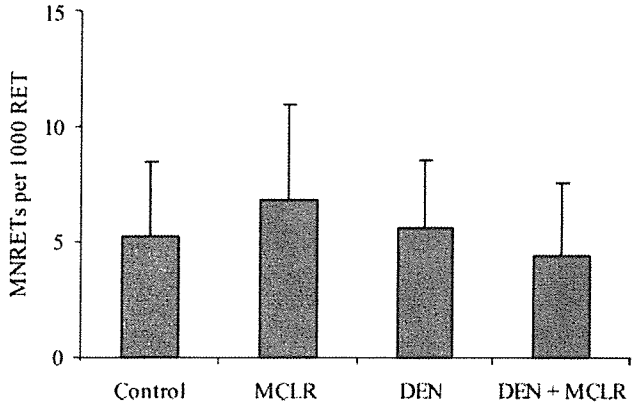

Fig. 1. Incidence of MNRET in the peripheral blood of Muta™Mouse 48 h following treatment with MCLR (1 mg/kg), DEN (25 mg/kg) and DEN (25 mg/kg) + MCLR (1 mg/kg).

duced and 46 DEN-induced mutants together with 42 spontaneous mutants from the liver were subjected to sequence analysis. The mutation spectra are summarized in Table 2. Spontaneous mutations consisted mainly of base substitutions (37/42). Among them, G:C to A:T transitions (21/26) predominated and most of them (17/21) occurred at CpG sites. DEN-induced mutations also consisted mainly of base substitutions (42/46). Compared to the control, G:C to A:T transitions were decreased in DEN treated group (50% versus 24% respectively) while A:T to T:A transversions were increased (2% versus 28%, respectively). However no obvious change was observed for incidences of mutations induced by MCLR including transitions (62% versus 62%) and transversions (27% versus 21%).

Table 1. MFs in the *lacZ* and *cII* gene from liver and lung of Muta™Mouse treated with MCLR (1 mg/kg), DEN (25 mg/kg) and DEN (25 mg/kg) + MCLR (1 mg/kg)

Organ	Treatment	*lacZ*				*cII*			
		Total plaques	Mutants	MF ($\times 10^{-6}$)	Mean ± SD	Total plaques	Mutants	MF ($\times 10^{-6}$)	Mean ± SD
Liver	Control	3311250	138	41.7	43.8 ± 11.7	3486000	69	19.8	20.5 ± 8.2
	MCLR	4053750	173	42.7	40.3 ± 13.7	4282500	94	21.9	21.1 ± 3.5
	DEN	3175000	963	261.7	268.4 ± 62.4*	3,495,000	788	225.5	226.6 ± 54.2*
	DEN + MCLR	2122500	472	222.4	206.9 ± 83.4†	2,149,500	391	181.9	176.4 ± 77†
Lung	Control	3823750	144	28.8	32.1 ± 13.9	4305000	110	25.6	25.7 ± 4.3
	MCLR	3823750	134	35.0	35.7 ± 4.89	2468250	93	37.7	36.9 ± 21.3
	DEN	3622500	416	114.8	117.5 ± 17.2*	2874000	332	115.5	118.1 ± 10.1*
	DEN + MCLR	2576250	264	102.5	109.9 ± 44.7†	1,136,250	141	124.1	132.2 ± 20.6†

*Compared to the control group $P < 0.05$
†Compared to the DEN-treated group $P > 0.05$

Table 2. Summary of *cII* mutations in the liver of control, MCLR- and DEN-treated Muta™Mice

Mutation class	Liver					
	Control	CpG	MCLR (%)	CpG	DEN (%)	CpG
Base	37 (89)		28 (82)		42 (91)	
Transitions	*26 (62)*		*21 (62)*		*20 (43)*	
G:C to A:T	21 (50)	17 (40)	20 (59)	17 (52)	11 (24)	6 (13)
A:T to G:C	5 (12)		1 (3)		9 (20)	
Transversions	*11 (27)*		*7 (21)*		*22 (48)*	
A:T to T:A	1 (2)		1 (3)		13 (28)	
A:T to C:G	4 (10)		2 (6)		2 (4)	
G:C to T:A	4 (10)		4 (12)		7 (15)	
G:C to C:G	2 (5)		0 (0)		0 (0)	
−1 Frameshift	1 (2)		2 (6)		1 (2)	
+1 Frameshift	3 (7)		4 (12)		0 (0)	
Deletion	0 (0)		0 (0)		1 (2)	
Insertion	0 (0)		0 (0)		0 (0)	
Complex	1 (2)		0 (0)		2 (4)	
Total	42 (100)		34 (100)		46 (100)	
MF ($\times 10^{-6}$)	43.8		40.3		268.4	

Discussion

The occurrence of toxic cyanobacterial blooms found in eutrophic, municipal, and residential water supplies is an increasing public health problem. Frequent deaths of domestic and wild animals are caused by drinking water contaminated by lethal toxins produced by cynobacteria. MCLR is the most commonly encountered and among the most toxic algal cyclic peptide hepatotoxins. Epidemiological studies have indicated a close relationship between primary liver cancer in human and cynobacteria contaminated drinking water (15,16). While there are several reports showing the *in vitro* genotoxicity of MCLR (21,22) or cyanobacterial extract (18,19), the evidence for the *in vivo* genotoxicity of this toxin is less convincing. Therefore, the main objectives of this study were to assess the *in vivo* genotoxicity of MCLR (if any) and its role in potentiation of DEN induced mutations for its suggested tumor promoting effects. To meet out these objectives male Muta^{TM}Mouse were administered with MCLR alone or in combination with DEN and examined for two end points- point mutation in transgenes, and micronucleus induction in peripheral blood cells. Considering the strong correlation between organ specific genotoxicty and organ specific carcinogenicity, the assessment of genotoxicity in multiple organs *in vivo* may indicate its target organ in humans and provide useful information for the evaluation of chemical safety. In the present research, hence, two target organs -liver and lungs- were examined for the evidence for mutagenicity.

Intraperitoneal injection of the raw cyanobacterial extracts containing several other microcystins besides MCLR induced micronucleus in the mouse bone marrow cells (19) and degradation and fragmentation of DNA in the liver cells (30). In the present study a pure MCLR (1 mg/kg = 1/10 of LD_{50}) was used, but no mutagenicity was observed. In another study, neoplastic nodule formation has been observed in the livers of mice received 100 intraperitoneal injections of sublethal doses of MCLR (20 μg/kg) over a period of 28 weeks (31). In the same study, oral administration of relatively higher doses of MCLR (80 μg/kg) under similar experimental conditions did not induce characteristic chronic injuries. Similarly, as suggested by the authors, fragmentation of DNA observed in hepatocytes of mice treated with the extract or MCLR (0.5-2.0 folds of LD_{50} doses) might be a consequence of endonucleolytic DNA degradation associated with cytotoxicity, rather than by a direct toxin-DNA interaction (30). In support to this, recently Zegura *et al.* (32) have reported that the genotoxicity of MCLR could be mediated by reactive oxygen species. So it may be inferred that some other mutagenic toxins present in the extracts or different routes of administration might be responsible for the positive results observed in those studies. However, MCLR treatment caused enhanced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in a time- and dose-dependent manner *in vitro* in primary cultured hepatocytes and *in vivo* in rat liver cells that could involve in the formation of hepatic tumors during long-term exposure to this cyanobacterial hepatotoxin (33). In contrast, in our study, under present experimental conditions, MCLR failed to induce mutation in both target genes (*lacZ* and *cII*) in liver and lungs of TG mouse. The *in vivo* micronucleus test in peripheral blood cells also yielded negative results. These results indicate that MLCR is capable of inducing neither point mutation nor chromosomal breakage *in vivo* in mouse organs.

It is widely believed that MCLR has tumor promoting effect (5,6). To test the possible potentiating effect of MCLR on mutagenicity of DEN, in our study, mice were simultaneously treated with DEN (25 mg/kg) and MCLR (1 mg/kg) once a week for four weeks. Relative to control mice, no significant increase in micronucleus frequency was observed either in DEN- or DEN+ MCLR-treated mice. This is in consistent with the negative results observed with DEN as previously reported (24). Further, simultaneous administration of MCLR with DEN did not increase MF caused by DEN in either of the target genes, although DEN treatment resulted in a significant increase in MFs in both *lacZ* and *cII* genes from liver and lungs. This indicates that the tumor promoting effects of MCLR is independent of mutagenicity of DEN. Because MCLR is known as an inhibitor of protein phosphatase 1 and 2A (5,34), the tumor promoting activity might be exerted by a disturbance of protein phosphorylation. Okadaic acid, which is known as a tumor promoter and a strong inhibitor of protein phosphatases (35), has similar mutagenic properties as MCLR (non-mutagenic in Salmonella and mutagenic in mammalian cells (36,37)). It is possible that tumor promoting activity of both compounds has a common mechanism through the inhibition of protein phosphatases.

In conclusion, pure MCLR has no *in vivo* genotoxicity as it is failed to induce gene mutation and micronucleus in transgenic mouse. Also lack of potentiation of DEN induced mutations in transgenes, as observed in the present study, indicates that the tumor promoting effects of MCLR is independent of its interaction to DNA.

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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 interlaboratory 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

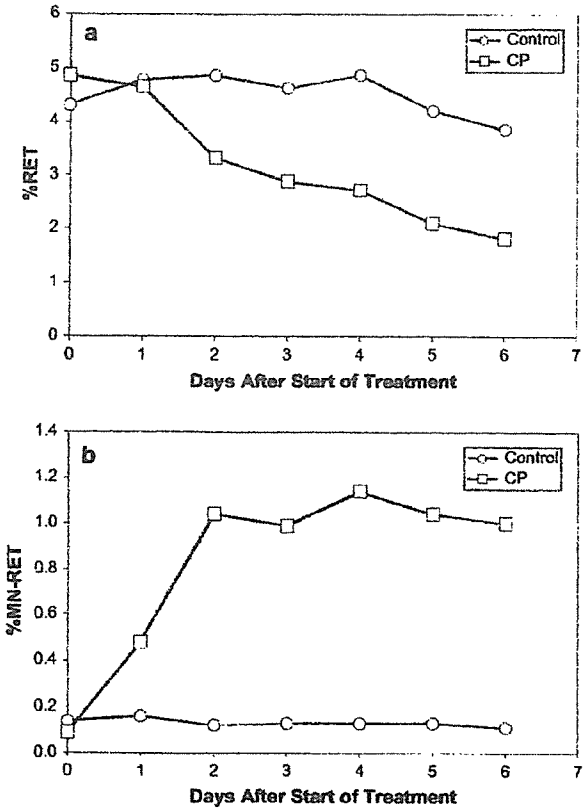

FIG. 1. The frequency of peripheral blood RETs (%RET, panel a) and peripheral blood micronucleated RETs (%MN-RET, panel b) as a function of time in the individual rats used to generate reference samples for analytical comparison. These data were acquired by flow cytometric analysis (laboratory L2) and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

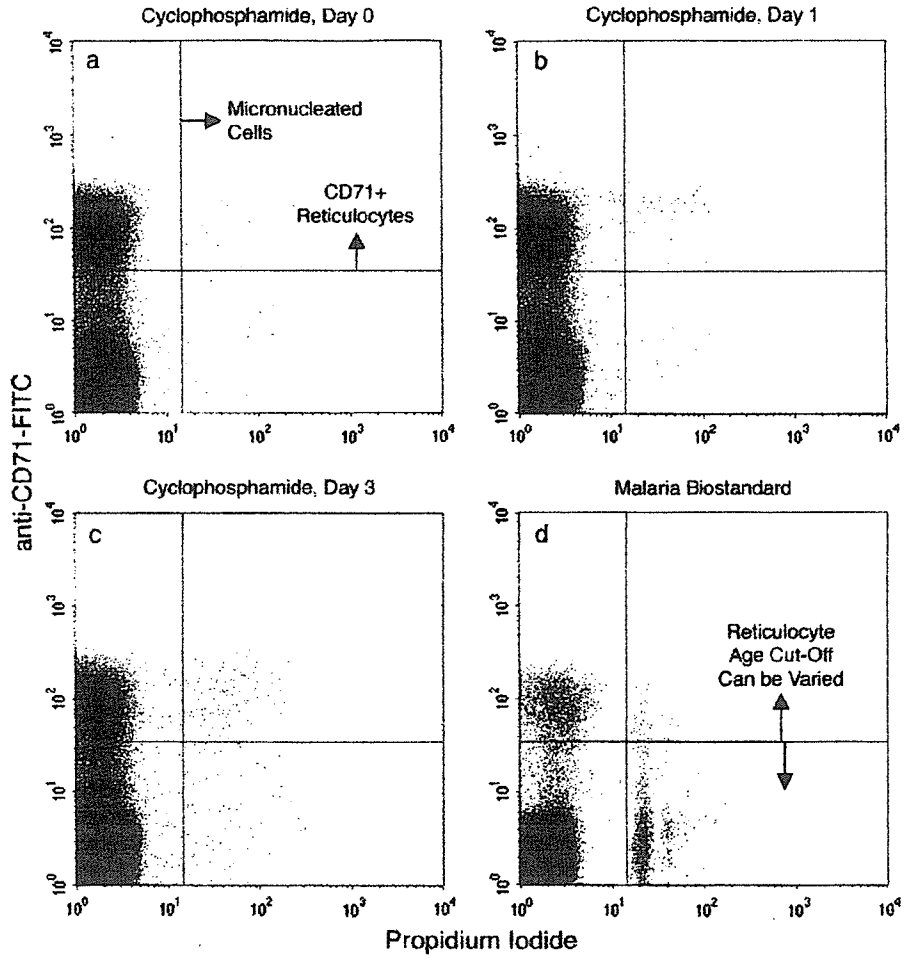

FIG. 2. Panels (a–c): Bivariate graphs illustrate the staining characteristics of rat blood specimens over the course of several days of CP treatment. Note the appearance of micronuclei at Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution 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,

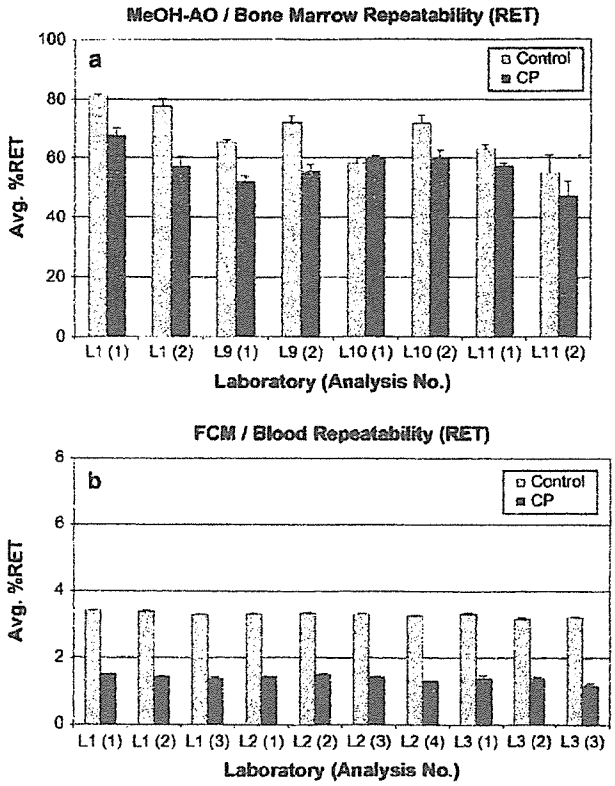

FIG. 3. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow RETs (%RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two separate occasions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

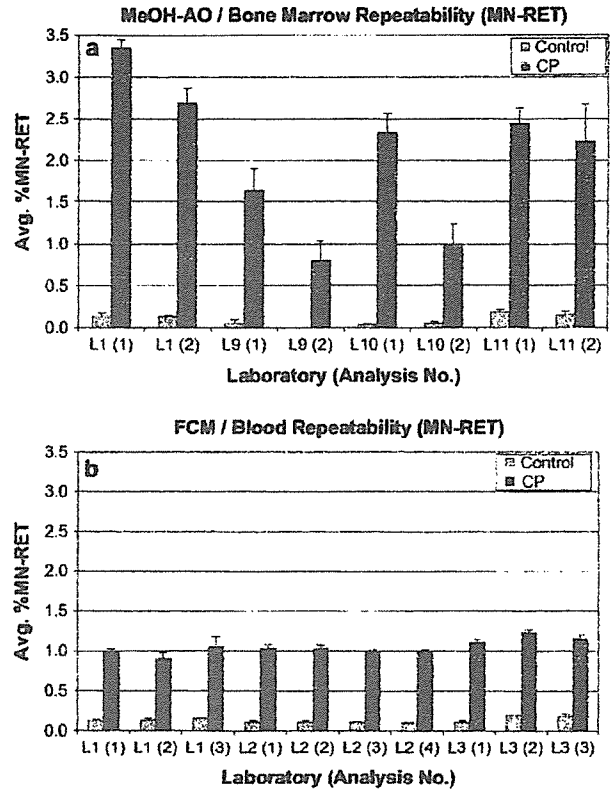

FIG. 4. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow micronucleated RETs (%MN-RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two 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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細胞バンク

第4回

JCRB細胞バンク事業の概要

小原有弘* 水澤 博*

Point

1 厚生労働省によって設立されたJCRB細胞バンクは生命科学研究の研究支援として高品質な細胞を研究者に提供している.
2 研究に使用している細胞のマイコプラズマ汚染の現状は深刻で,研究への悪影響を知らずに研究利用している研究者が多い.
3 STR-PCR法による細胞個体識別はクロスカルチャーコンタミネーションの発見に非常に有用である.
4 クロスカルチャーコンタミネーションの問題は生命科学研究の根底を揺るがしかねない大きな問題である.
5 ウイルス汚染検査の確立,ES細胞・体性幹細胞の供給,細胞特性解析などJCRB細胞バンクの今後の役割は大きい.

Key Words /JCRB細胞バンク,マイコプラズマ汚染,細胞個体識別,
STR-PCR法,クロスカルチャーコンタミネーション

● はじめに

1985年,わが国最初の細胞バンクとして厚生労働省(当時厚生省)によってJCRB細胞バンク(http://cellbank.nibio.go.jp/)が誕生した.当時すでに,ヒトに由来する培養細胞は生命科学研究に欠かせない研究材料となっており,対がん10ヵ年総合戦略(1984年～1993年)における,研究の基盤整備の一環として,日本がん研究資源バンク(Japanese Cancer Research Resources Bank:JCRB)の整備が進められた.そのなかで「培養細胞研究資源」に関するものとして,国立医薬品食品衛生研究所(当時国立衛生試験所,変異遺伝部)に設立されたのがJCRB細胞バンクである.

こうしてがん研究の支援をおもな目的として細胞バンクがスタートし,生命科学研究全体の発展とともに細胞バンク事業も発展してきた.現在は(財)ヒューマンサイエンス振興財団(HS財団)との協力体制をとり,JCRB細胞バンクが細胞の収集,品質管理,長期安定保存を担当し,HS財団が細胞分譲に関する業務を担い,迅速な分譲体制を維持・確立している.また,2005年4月1日,国立医薬品食品衛生研究所にあったJCRB細胞バンクは大阪府北部の新しい街「彩都(茨木市)」の一角に建設された独立行政法人医薬基盤研究所に移転して新たなスタートを切ることになった.

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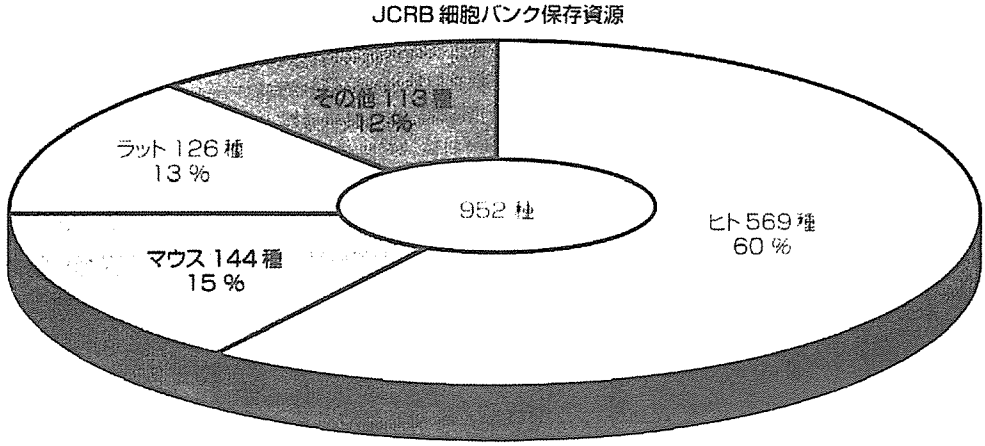


分譲実績(2002年1月～2005年11月)

年	海外	国内企業	研究機関	合計
2002				2406
2003	222	682	2095	2999
2004	294	727	2152	3173
2005	190	684	1863	2737

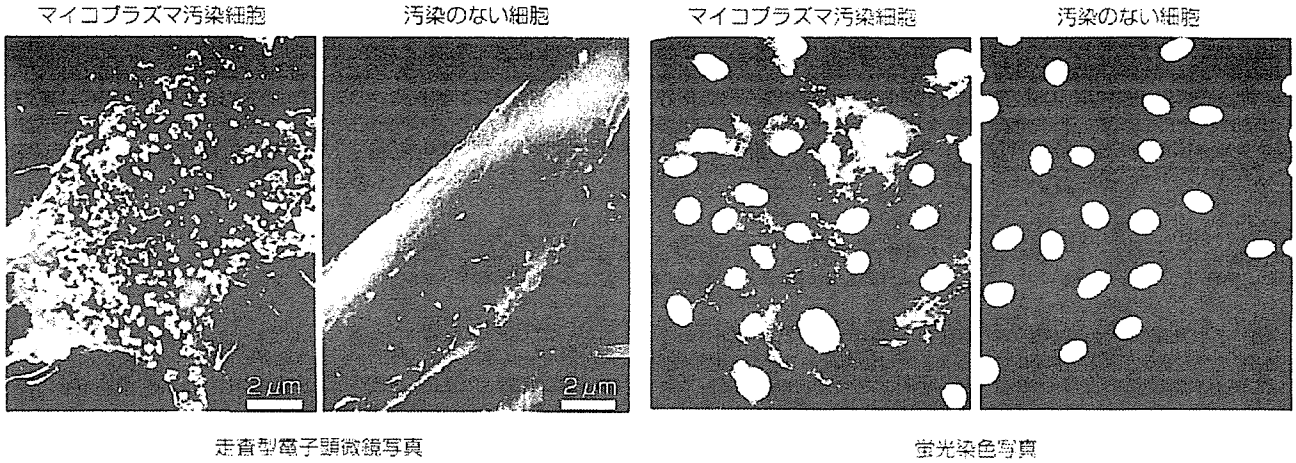
図❶ JCRB細胞バンクの資源保有数と分譲実績 筆者作成
JCRB細胞バンクが保存する細胞資源数は952種となっており，そのうち約60%がヒト由来の細胞である．分譲は年間3000アンプルを越え徐々に増加している．

1 細胞バンクの概要

JCRB細胞バンクの事業内容は，1研究に有用な培養細胞研究資源の収集，2収集した培養細胞研究資源の品質管理，3十分な数の細胞アンプルの長期安定的保存，4培養細胞に関連する新しい研究資源の開発および資源化に関する研究開発，5培養細胞研究資源の品質管理手法の開発研究，6培養細胞研究資源に関連する情報の収集と提供，7分譲を担当するHS財団への新規細胞株の分譲用細胞の提供，となっている．収集・登録した細胞は毎年40株程度ずつ増加しており，現在では952種となっている．また，年間に分譲するアンプル数も3000を越えて年々増加する傾向にある(図❶)．品質管理に関しては汚染検査，細胞個体識別，細胞性状確認を実施しており，後述するマイコプラズマ非汚染で，しかも細胞個体識別を確認した培養細胞を提供していることは，JCRB細胞バンクが研究者に提供する研究資源の品質の高さを示すものであり，研究者より好評を得ている．

2 マイコプラズマ汚染は古くて新しい課題である

培養細胞を用いて研究している研究者のなかでもマイコプラズマ汚染による研究への悪影響を知らない研究者は多いようである(図❷)．マイコプラズマは自己増殖能をもつ細菌の1/10ぐらいの大きさの微生物であり，培養細胞と共存して増殖するが，汚染しても培地が濁ったりしないので混入に気づきにくい．もし，自分が研究に使っている細胞がマイコプラズマ汚染されていたらどうであろうか？その意味は十分考える必要がある．マイコプラズマ汚染のためせっかくおこなった研究に再現性がなく，信頼されない研究となってしまうかもしれない．われわれ細胞バンクには培養の専門家から多くの細胞が寄託されるが，その約20%にマイコプラズマ汚染が見つかるのが現状である．専門家とよばれる研究者が使用していた細胞にマイコプラズマ汚染率が高いことを考え



図❷　マイコプラズマによる汚染

マイコプラズマ汚染された細胞の走査型電子顕微鏡像（ヒューマンサイエンス研究資源バンク：吉田東歩博士撮影）とヘキスト33258で染色される細胞の蛍光染色像．マイコプラズマにはいろいろな種類が存在するが，培養細胞では細胞表面に付着して細胞と共生する．走査型電子顕微鏡像では細胞表面の粒子状のものがマイコプラズマである．ヘキストによる核酸染色は培養細胞の核だけではなく，マイコプラズマの核酸も染色されるのでマイコプラズマ汚染が検出できる．

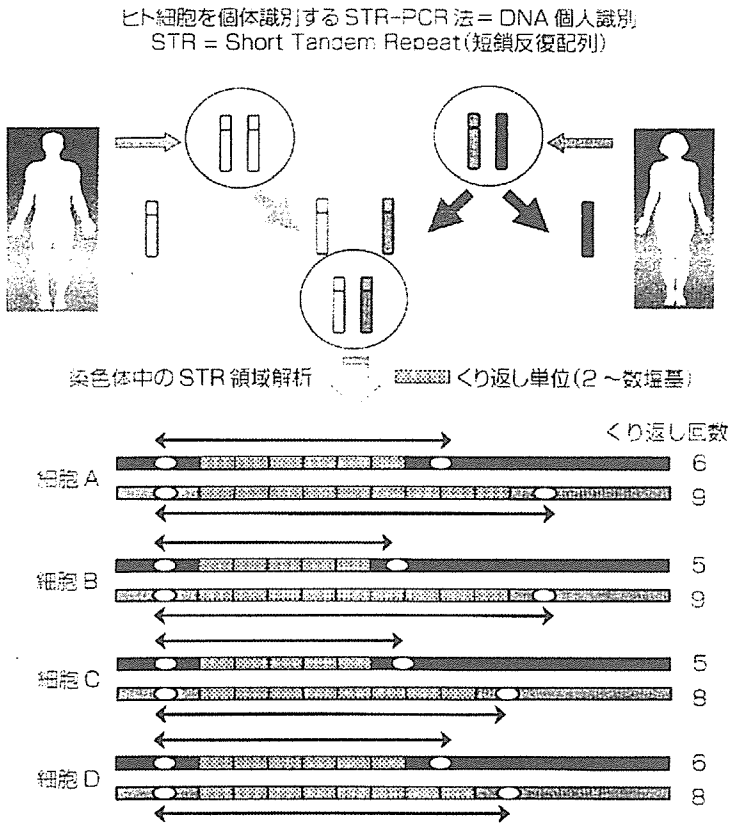
ると，広く普及した培養細胞の汚染率は相当高いと容易に予測できる．一度汚染した細胞は通常の培養方法で用いる抗生物質ではなかなか除去することは難しく増殖を抑える程度となってしまう．また，凍結保存しても細胞と同様にマイコプラズマも生き残ってしまう．培養細胞を研究に利用されている場合には，是非一度自分の細胞を調べてみることを勧めたい．

では，何が原因で汚染が広がってゆくのであろうか？その答えには三つあげられる．第一に培養実験室の環境からの汚染である．培地などをこぼしてしまったりしたとき，すぐにしっかりと拭き取らないと何らかの培養操作の過程でほかの細胞へ混入することがある．第二に培地の使い回しによる汚染があげられる．同じ培地を用いるのだから細胞が違っても大きなビンから同じ培地を使用するとか，ほかの研究者と培地を共有してしまうなどよく聞く話ではないだろうか？これが汚染の拡大に繋がっているのである．第三に人の唾液を通じての汚染が考えられる．マイコプラズマは人の上部気道や尿生殖器に常在する微生物である．培養する際のおしゃべりが汚染の原因の一つとも考えられている．これらのことから考えると汚染を拡大しないようにするには，培養環境を清潔に保つ，培地は「1培地－1細胞」，おしゃべりは厳禁（マスク着用）が原則となる．

❸ STR-PCR法はヒト由来細胞株を個体識別する

1951年にHeLa細胞が樹立されたことが刺激となり，多くの研究者が続々と新しいヒト由来の培養細胞の樹立に成功した[1]．そこに問題を提起したのがGartler[2]であり，アイソザイムの分析からHeLa以降樹立されたヒト細胞の多くがHeLa細胞ではないかと疑ったのだった．1900年代の後半には英国のJeffreys[3]らがDNAフィンガープリント法を報告し，ヒトをDNAレベルで識別することが可能だと紹介した．その後，この方法は犯罪捜査への応用という視点から急速に研究が進み，現在ではShort Tandem Repeat-Polymerase Chain Reaction（STR-PCR）法として迅速かつ精密な分析法として定着しつつある．STR-PCR法とはゲノム中に存在する2～数個の塩基からなるくり返し配列[(CAG)n，(GC)nなど]のくり返し回数に個人差があることから，その出現回数を分析することによって個体識別する方法である（図❸）．

JCRB細胞バンクではこの方法を1999年末から培養細胞に取り入れて，収集したヒト由来の培養細胞に関する調査を実施してデータを蓄積し，『ヒト培養細胞識別データベース』を構築した．このデータベースを利用しておこなった多種類のヒト細胞の比較で明らかに



図❸ STR-PCR法の原理(筆者作成)
細胞Aから細胞Dの4種類の細胞に存在するSTR領域の短鎖反復配列の構造を模式的に示した．高等動物の染色体は母方と父方に由来するものがペアになっており，STR(短鎖反復配列)領域も対になって2つ存在する．STRは2～数塩基程度のきわめて短い塩基配列が反復している構造であり，この領域の外側に適当なPCRプライマーを設定してSTRを挟む100～300塩基程度の長さのDNA鎖をPCR法によって増幅してその反応産物をジェネティックアナライザーによって解析する．

なったことは，ヒト培養細胞には意外と多くのクロスカルチャーコンタミネーション(細胞の入れ替わり)が発生していたということである．これまでJCRB細胞バンクで収集したヒトに由来する培養細胞数は560種(全体の60％)であるが，そのうち32種(約6％弱)にクロスカルチャーコンタミネーションが見つかったのである．この結果は詳細な実験手法を含めてJCRB細胞バンクのホームページで公開しているので是非参考にしていただきたい(http://cellbank.nibio.go.jp 中，『JCRB Cell Bank』のCell IDの欄に公開している)．

最近，JCRB細胞バンクで判明した一例を挙げてみたい．唾液腺がん由来の細胞としてわが国で樹立された細胞が歯学領域の研究者の間で広く研究利用されており，ヨーロッパの細胞バンクにも登録されていた．JCRB細胞バンクにこの細胞のSTR-PCR解析の依頼があり，調べた結果HeLa細胞と同一のヒトから樹立されたもの，つまりはHeLa細胞の入れ替わりと判定された．その後広く普及したこの細胞をヨーロッパの細胞バンクや日本国内の研究者より数種集めて再解析をおこなったが，結果はHeLa細胞由来であることを示した．細胞の入れ替わりがどの時点で起こったのか明らかではないが，現在その細胞を利用して研究している研究者は研究成果をどのように発表してよいのか苦悩している．このようにクロスカルチャーコンタミネーションは生命科学研究の根底を揺るがす深刻な問題となりかねないものなのである．

❹ JCRB細胞バンクの今後の役割は大きい

以上紹介したようにJCRB細胞バンクは，細胞の収集と品質管理を重視した運営をおこなってきたが，近年，ヒトゲノムの全塩基配列が決定されて個人単位の遺伝子解析が容易に実施できるようになった．その結果個人の遺伝情報が垂れ流しになるのではないかという心配から保護をする必要性が話題になり，それに関連して生命倫理という新たな課題が浮上している．もちろんこれまでも倫理問題はなかったわけではないが国内ではあまり関心をもたれなかった．そこで，細胞バンクで重視している研究材料がヒトに由来する細胞であることを考える

と，研究倫理を研究課題として細胞バンクでも独自に調査研究する必要があるのではないだろうかと考えはじめたところである．

また，培養細胞の品質管理については，①マイコプラズマ汚染の有無，②細菌・真菌汚染の有無，③アイソザイム分析による由来動物種の確認，④STR-PCR法によるヒト細胞の個体識別を実施する体制を確立してきた一方，まだ導入していない品質管理の課題としてはウイルスによる汚染検査の問題がある．再生医療や細胞治療に向けて非常に多くの研究開発が進んでいるが，細胞を用いた医療の実現にはウイルス汚染検査が必須である．JCRB細胞バンクでもウイルス汚染検査体制の確立が急務であるとして研究開発に着手した．

収集する細胞に関しても非常に多様かつ有用な細胞が次々と樹立されている．とくに近年ではES細胞や体性幹細胞とよばれる多分化能をもった増殖性の細胞の研究利用が進んでいる．これはヒトの正常な(がんではない)状態を *in vitro* で模倣できる非常に有用な研究材料であり，これらの研究材料を供給することにJCRB細胞バンクも積極的に取り組んでいる．

研究者が研究に利用する細胞を選択するには細胞特性の情報が非常に重要であり，これまでにもJCRB細胞バンクではそれらの特性を解析する研究を実施している．今後も染色体の詳細な解析，遺伝子発現情報の付加，細胞増殖過程の動画記録などの情報提供に取り組み，研究者の支援に努めていきたいと考えている．

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Chromosomal Instability in Human Mesenchymal Stem Cells Immortalized with Human Papilloma Virus E6, E7 and hTERT Genes

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Keywords; Human cord blood mesenchymal stem cell. Long-term culture. Karyotype analysis. mFISH. CGH. Differentiation.

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Running Title; Chromosomal instability of hMSCs

SUMMARY

Human mesenchymal stem cells (hMSCs) are expected to be an enormous potential source for future cell therapy, because of their self-renewing divisions and also because of their multiple lineage differentiation. The finite lifespan of these cells, however, is a hurdle for clinical application. Recently, several hMSC lines have been established by immortalized human telomerase reverse transcriptase gene (hTERT) alone or with hTERT in combinations with human papillomavirus type 16 E6/E7 genes (E6/E7) and human proto-oncogene, Bmi-1, but have not so much been characterized their karyotypic stability in detail during extended lifespan under in vitro conditions. In this report, the cells immortalized with the hTERT gene alone exhibited little change in karyotype, while the cells immortalized with E6/E7 plus hTERT genes or Bmi-1, E6 plus hTERT genes were unstable regarding chromosome numbers, which altered markedly during prolonged culture. Interestingly, one unique chromosomal alteration was the preferential loss of chromosome 13 in three cell lines, observed by fluorescence *in situ* hybridization (FISH) and comparative-genomic hybridization (CGH) analysis. The four cell lines all maintained the ability to differentiate into both osteogenic and adipogenic lineages, and two cell lines underwent neuroblastic differentiation. Thus, our results were able to provide a step forward toward fulfilling the need for a sufficient number of cells for new therapeutic applications, and substantiate that these cell lines are a useful model for understanding the mechanisms of chromosomal instability and differentiation of hMSCs.