

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 PlanApoint ×63 oil objective
Litron Laboratories, Rochester, NY	L2	PB	FCM	BD-FACSort, BD-FACScan
Health Canada, Ottawa, Ontario, Canada	L3	PB	FCM	BD-FACSCalibur
National Institute of Health Sciences, Tokyo, Japan	L4	Coordinated SV-AO laboratories		BD-FACSCalibur
Nitto Denko Corporation, Osaka, Japan	L5	PB	SV-AO	Olympus AHB3-RPC (×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 μ l) 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 μ l heparin solution, blood was collected until a final volume of approximately 750 μ l was obtained; into a second tube containing 5 ml heparin solution, approximately 1 ml blood was collected. To tubes with the 750 μ l 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 μ l 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 μ l 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 1100 rpm for 5 min and then resuspended with approximately 600 μ l FBS. As with the peripheral blood, 5 μ l 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). Supravital 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

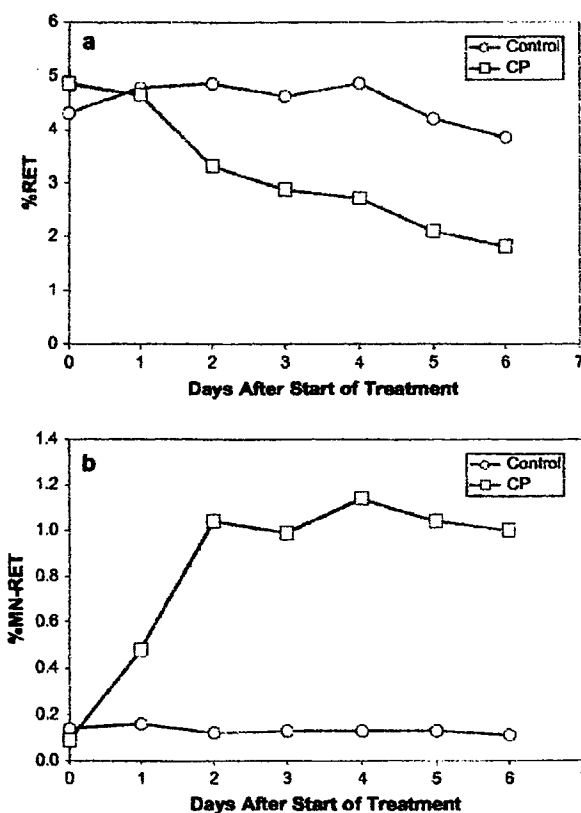


FIG. 1. The frequency of peripheral blood RETs (%RET, panel a) and peripheral blood micronucleated RETs (%MN-RET, panel b) as a function of time in the individual rats used to generate reference samples for analytical comparison. These data were acquired by flow cytometric analysis (laboratory L2) and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

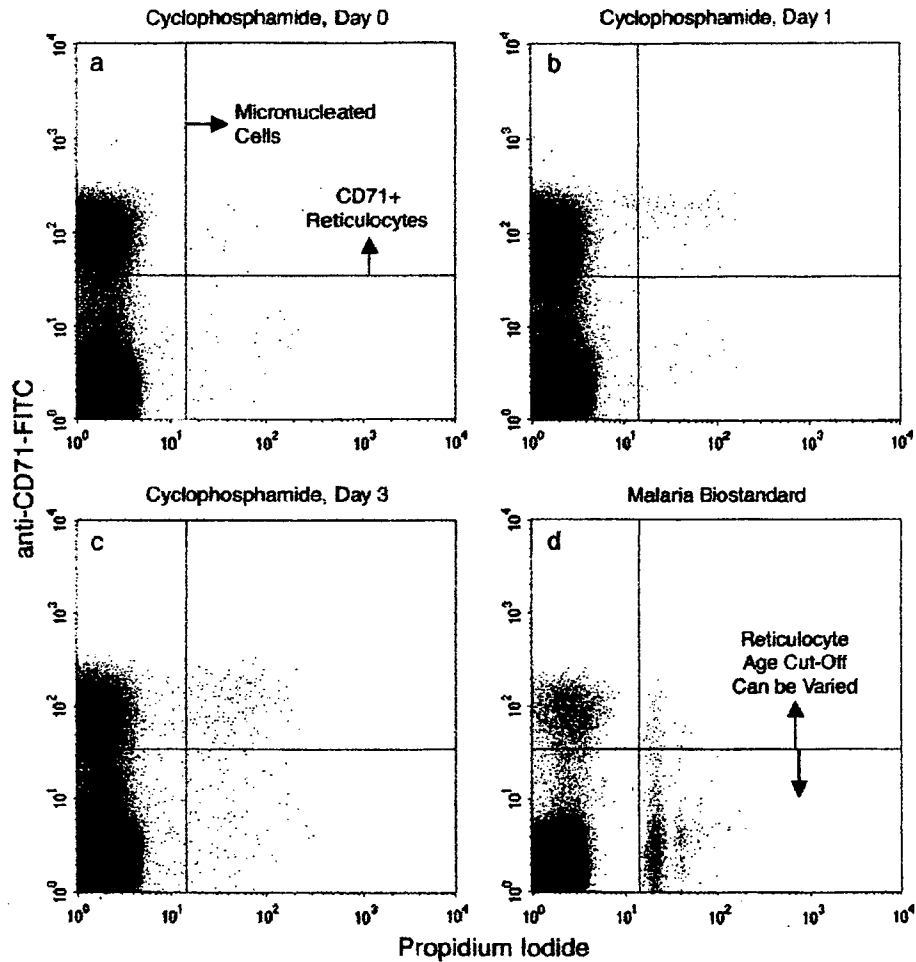


FIG. 2. Panels (a–c): Bivariate graphs illustrate the staining characteristics of rat blood specimens over the course of several days of CP treatment. Note the appearance of micronuclei at Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution 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
<i>Pooled^b L1, 9, 10, 11</i>			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
<i>Pooled L1, 9, 11</i>			Vehicle		6.9 ± 0.29	12.5	
			CP		5.4 ± 0.32	17.6	- 21.7
L5	SV-AO	PB	Vehicle	%Type I + II/III-IV	55.2 ± 1.95	6.1	
			CP		42.2 ± 0.12	0.5	- 24
L6	SV-AO	PB	Vehicle	%Type I/II + II	42.4 ± 2.8	11.5	
			CP		29.1 ± 2.1	12.4	- 31
L7	SV-AO	PB	Vehicle	%Type I + II/III-IV	52.3 ± 2.1	6.8	
			CP		34.8 ± 3.1	15.6	- 34
<i>Pooled^c L5, 7</i>			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
<i>Pooled L1, 2, 3</i>			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.

^aEach 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/III-IV = percentage of Type I and Type II RETs relative to total RETs; %Type I/II + 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.

^bValues 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.

^cOnly data from the two SV-AO laboratories that measured toxicity similarly (%Type I + II/III-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
<i>Pooled^a L1, 9, 10, 11</i>			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
<i>Pooled L1, 9, 11</i>			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
<i>Pooled L5, 6, 7</i>			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
<i>Pooled L1, 2, 3</i>			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.

^aValues 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,

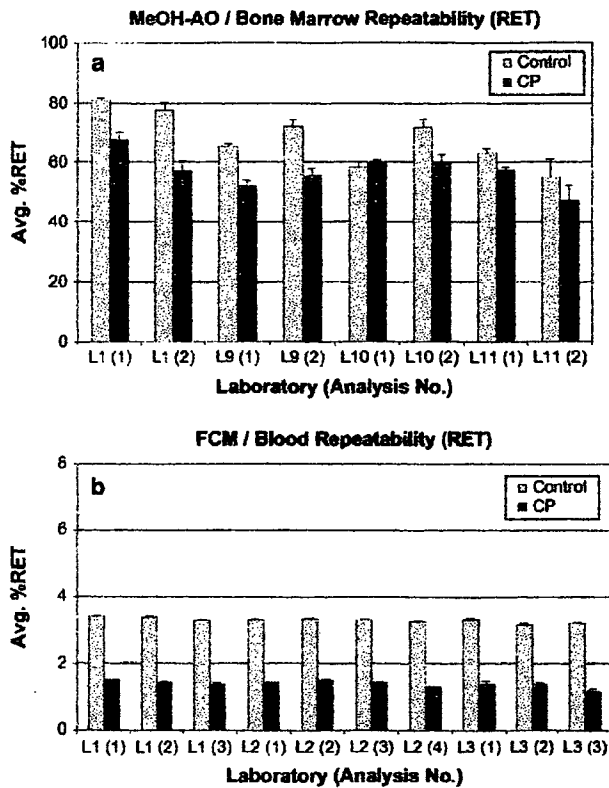


FIG. 3. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow RETs (%RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two separate occasions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

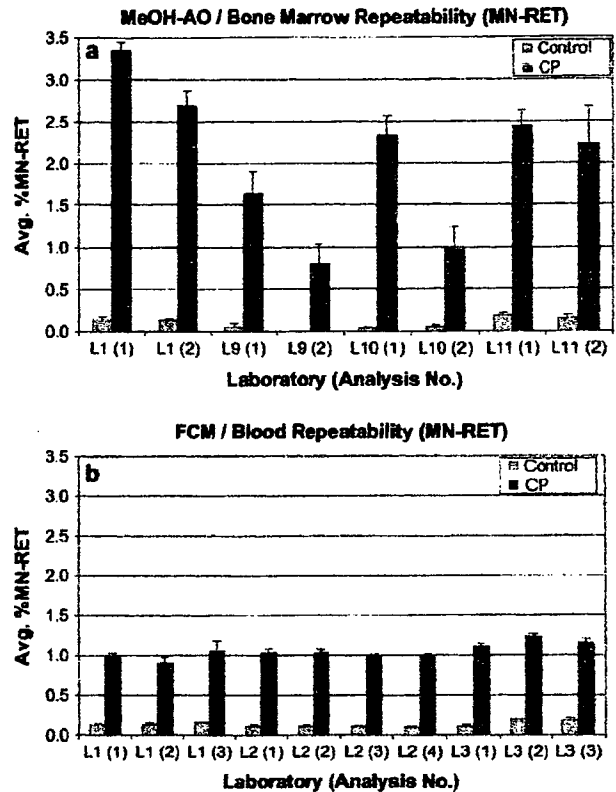


FIG. 4. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow micronucleated RETs (%MN-RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two 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 $0.11\% \pm 0.045$. 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).

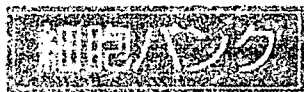
ACKNOWLEDGMENTS

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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

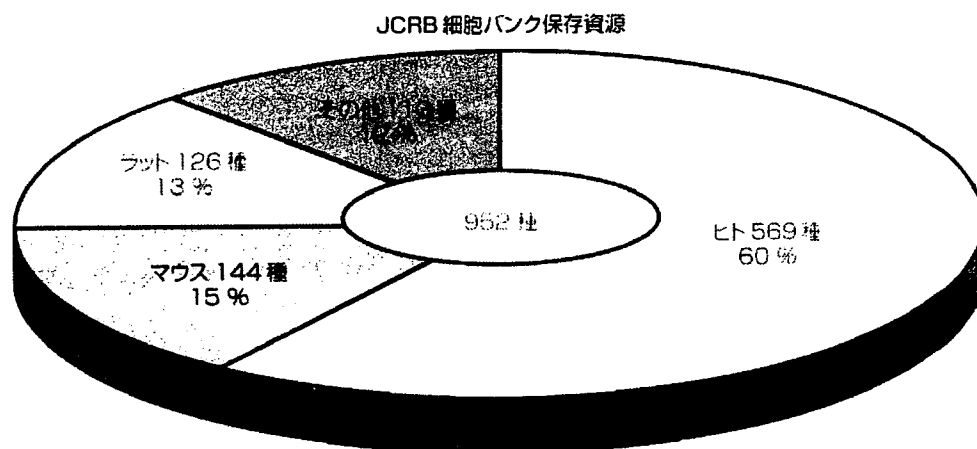
● はじめに

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 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。

*KOHARA Arihiro, MIZUSAWA Hiroshi/(独)医薬基盤研究所・生物資源研究部



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① JCRB 細胞バンクの資源保有数と分譲実績(筆者作成)

JCRB 細胞バンクが保有する細胞資源数は952種となっており、そのうち約60%がH19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HIS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①)。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②)。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、信頼されない研究に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

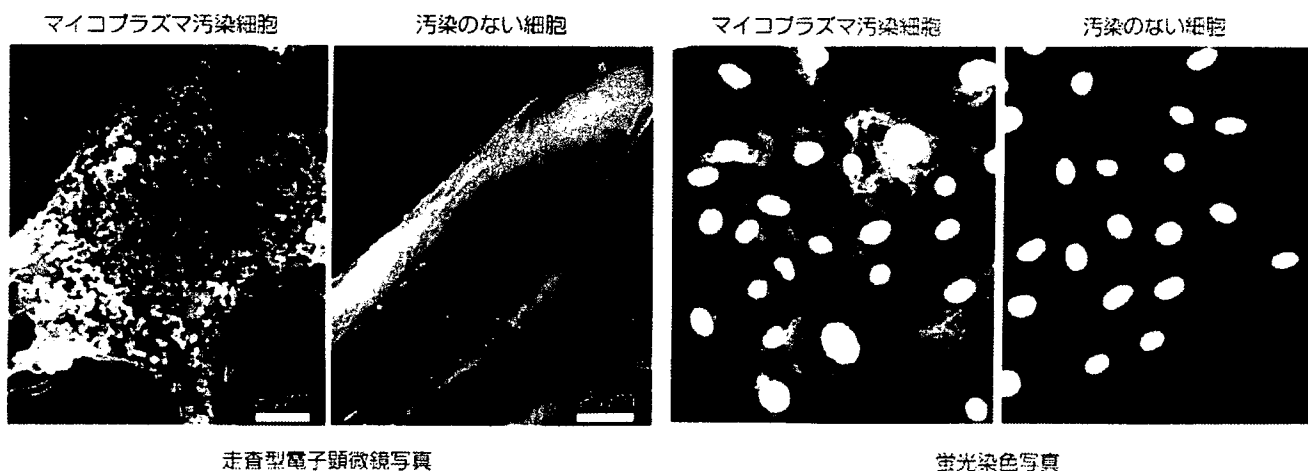


図2 マイコプラズマによる汚染

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ふ。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。

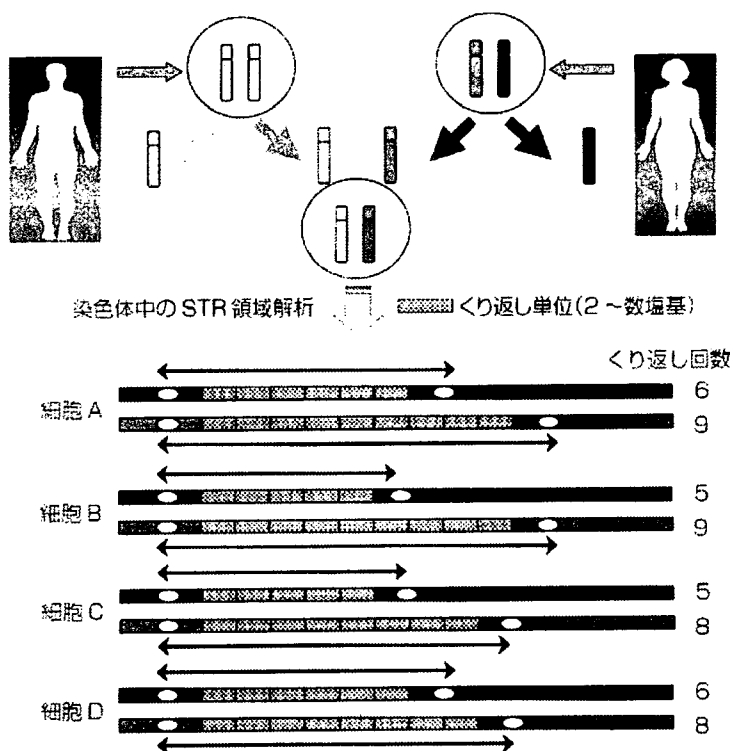
では、何が原因で汚染が広がってゆくのであろうか？その答えには三つあげられる。第一に培養実験室の環境からの汚染である。培地などをこぼしてしまったりしたとき、すぐにしっかりと拭き取らないと何らかの培養操作の過程でほかの細胞へ混入することがある。第二に培地の使い回しによる汚染があげられる。同じ培地を用いるのだから細胞が違っても大きなビンから同じ培地を使用するとか、ほかの研究者と培地を共有してしまうなどよく聞く話ではないだろうか？これが汚染の拡大に繋がっているのである。第三に人の唾液を通じての汚染が考えられる。マイコプラズマは人の上部気道や尿生殖器に常在する微生物である。培養する際のおしゃべりが汚染の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

③ 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。そこに問題を提起したのがGartler²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Jeffreys³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3)。

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

ヒト細胞を個体識別する STR-PCR 法 = DNA 個人識別
STR = Short Tandem Repeat (短鎖反復配列)



図③ 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 ~ 8 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 を含む 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(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の根底を揺るがす深刻な問題となりかねないものである。

4 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。

《細胞寄託、品質管理に関する問い合わせ》

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Present State of New Chiral Drug Development and Review in Japan

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The current situation of chiral drug development in Japan was investigated. The trend in the Japanese pharmaceutical development is increasingly moving towards the development of single isomers rather than racemates. The development of single-enantiomer drugs was made possible by the current technologies of asymmetric synthesis and chiral separation, and encouraged by the guidelines on the development of chiral drugs worldwide. Japan has not issued specific guidelines on the development of chiral drugs, however, the chiral drug development approached in Japan were essentially consistent with the approaches recommended by the U.S.A. and EU guidelines.

Key words — chiral drug, single-enantiomer drug, racemic drug, pharmaceutical development, guideline

INTRODUCTION

Pharmaceuticals with an asymmetric carbon (chiral center) are often referred to as chiral drugs. Chiral drugs were mainly presented as the racemate, which is a mixture of equal amounts of left- and right-handed enantiomers. Two enantiomers in a racemate show completely identical physical and chemical properties when they are in an achiral environment. However, in a chiral environment such as *in vivo*, they demonstrate different chemical, biochemical, and pharmacologic behaviors. In principle, therefore, enantiomers in a racemic drug should be treated as two different compounds.

Although single-enantiomer drugs have been thought to be preferable to racemic drugs, most chiral drugs were developed as racemates due to the lack of technologies that produce single-enantiomers until recently. Current technologies of asymmetric synthesis and chiral separation made it possible for pharmaceutical companies to develop single-enantiomer drugs. Lately, many single-enantiomer drugs have been approved and marketed broadly. The Japanese government approved single-

enantiomer drugs four times as much as racemic drugs in early 2000s, although there was not significant difference in the number of approved drugs between racemic and single-enantiomer drugs in early 1990s (Fig. 1). The trends in world development and approval of chiral drugs were similar; the worldwide market share of single-enantiomer drugs increased from 27% in 1996 to 39% in 2002.^{1–3)}

Pharmaceutical companies cannot market new drugs in Japan and in other regions until approved by the regulatory authorities, and they have to submit technical documents for new drug applications. The regulatory authorities evaluate the content of the technical documents to assure quality, efficacy and safety. In order to assure drug quality⁴⁾ in Japan, specifications and critical manufacturing processes are needed to be written in legally binding approval documentation. In contrast, almost all of subjects described in technical documents are considered as legally binding matters in other countries. As a result, approval matters on quality-related issues are often different between Japan and other countries.

We considered it important to research what kinds of data are to be collected during the development of chiral drugs and how the regulatory authorities evaluate these data. Additionally it is valuable to realize what kinds of issues are dealt with as legally binding matters. The findings can provide common understanding between pharmaceuti-

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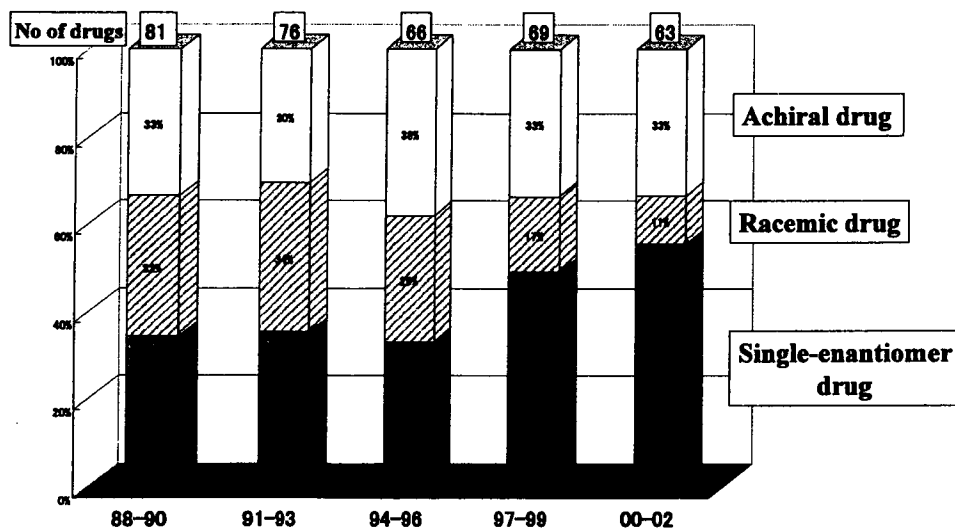


Fig. 1. Number of Drugs Approved in Japan during 1988–2002

The new active ingredients, which are chemical substances and were approved in Japan between 1988 and 2002, were classified according to steric structures.

cal companies and the regulatory authorities, and facilitate development of chiral drugs.

In this article, we analyzed data on manufacturing, quality, pharmacology, toxicology, and pharmacokinetics of single-enantiomer and racemic drugs that were approved by the Japanese government, and discussed the current state of development and approval of chiral drugs.

MATERIALS AND METHODS

The drugs that were approved from January 2001 to July 2003 were surveyed. The information sources were the data summaries [Module 2 of Common Technical Document (CTD) in the present system] that were submitted by the applicants for New Drug Application (NDA) and the approval documentations that were described specifications and test methods of drug substances. This information, especially quality parts, is not completely publicly available, although Module 2 of CTD and review reports available on the Internet.^{5,6)} Therefore, we did not disclose the individual substances' name.

In this article, the term of 'chiral drug' contains both a single-enantiomer and a racemic drug.

RESULTS

Classification of Approved New Chiral Drugs by Stereochemistry (Fig. 2)

There were 76 new active ingredients that were approved between January 2001 and July 2003 excluding biologics, antiseptics for medical devices and *in vivo* diagnostics.

We classified them into achiral drugs, racemic drugs and single-enantiomer drugs with one-chiral center or multi-chiral centers. The 76 new substances consisted of the 29 achiral drugs (black portion, 39%), the 23 single enantiomer drugs with multi-chiral centers drugs (strip portion, 30%), the 14 single enantiomers with one-chiral center (gray portion, 18%), and the 10 racemic drugs (white portion, 13%). The total number of single enantiomer was 37.

We investigated further details of the 37 single-enantiomer drugs and the 10 racemic drugs as shown in the following sections.

Single-enantiomer Drug Substances

Manufacturing Routes (Fig. 3): The 29 single-enantiomer drugs out of the 37 investigated drugs were produced from single enantiomeric starting materials (gray portion, 78%). In this research, the starting materials indicate starting compounds by manufacturing method in approval documentation and in good manufacturing practices (GMP) compliance.

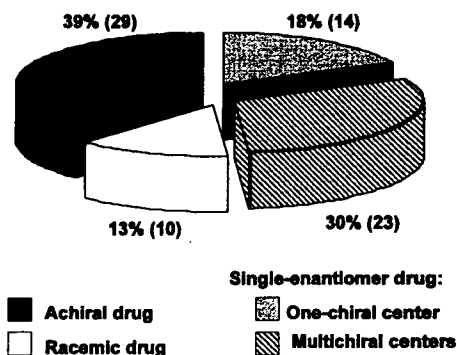


Fig. 2. Classification of New Chemical Drugs by Stereochemistry

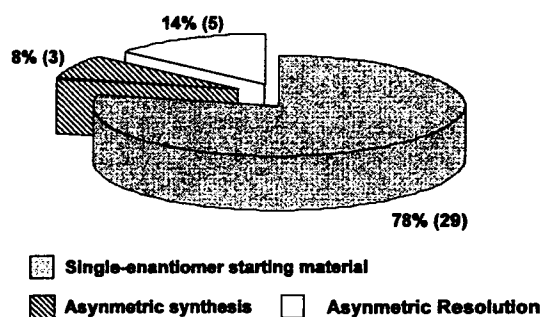


Fig. 3. Manufacturing Routes to Single-enantiomer Drug Substances

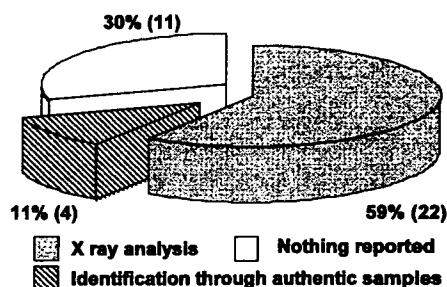


Fig. 4. Stereochemical Characterizations on Chirality of Single-enantiomer Drug Substances

Asymmetric synthesis was used for formation of single-enantiomers for the three single-enantiomers (stripe portion, 8%).

The five substances were isolated by asymmetric resolution (white portion, 14%). The four substances out of the five were purified by crystallization. The other one was purified by chromatographic resolution.

Stereochemical Characterizations on Chirality (Fig. 4): The stereochemical structures of 22

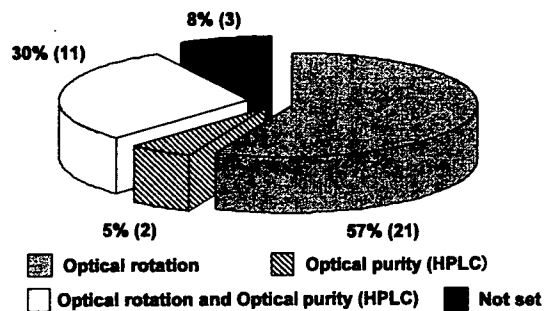


Fig. 5. Specifications for Assuring Chirality of Single-enantiomer Drug Substances

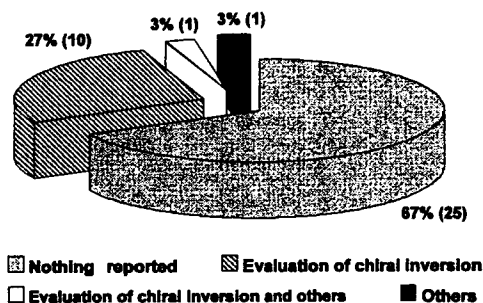


Fig. 6. Pharmacokinetic Studies on Chirality of Single-enantiomer Drug Substances

single-enantiomers were determined by X-ray crystal structure analysis (gray portion, 59%), and 4 single-enantiomers were confirmed by identification through the authentic samples (stripe portion, 11%). No information was reported on characterization of chirality for the remaining 11 single-enantiomers (white portion, 30%).

Specifications for Assuring Chirality (Fig. 5): Optical rotation was adopted as specifications for the 21 single-enantiomers (gray portion, 57%). The chromatographic methods for optical purity determination were chosen for the two enantiomers (strip position, 5%). Both optical rotation and optical purity were adopted as specifications for the 11 enantiomers (white portion, 30%).

No specification for assuring chirality was set for the three enantiomers (black portion, 8%).

Pharmacokinetic Studies on Chirality (Fig. 6): Pharmacokinetic studies were conducted for all of the single-enantiomer drugs, and some sort of pharmacokinetic evaluation relating to chirality was reported for the 12 single-enantiomers. Among them, chiral inversion was evaluated for the 10 single-enantiomers (strip portion, 27%). One out of the 10 single enantiomers indicated chiral inversion on

mouse, although the other 9 single enantiomers did not show any chiral inversion.

One of the other two single enantiomers was proved to generate an enantiomer-specific metabolite (black portion, 3%); and the other one, which did not show any chiral inversions, was additionally investigated on the chiral inversion in metabolite formation (white portion, 3%).

No pharmacokinetic study on chirality was reported in the data summaries of the 25 single-enantiomers (gray portion, 67%).

Racemic Drug Substances

Stereochemical Characterization (Fig. 7): All of the 10 racemic drugs were confirmed to be a racemic substance by some methods. Optical rotation was reported for all of the 10 racemates. Chiral HPLC analysis and X-ray analysis was reported for the nine and the four racemates, respectively. For the four racemate, both chiral HPLC analysis and X-ray analysis were performed.

Pharmacology of Individual Isomers (Fig. 8): Pharmacological activity of each enantiomer was investigated for all of the 10 racemic drugs. Little difference was observed in pharmacological activities of both enantiomers in the five racemates (stripe portion, 50%). For the four racemates, each enantiomer in the racemate indicated different pharmacological potency (gray portion, 40%). For the three of the four racemates, the enantiomers that had greater pharmacological potency were more toxic. The mechanisms of toxicities were same as those of pharmacological activities. In the other racemate, the one enantiomer indicated equivalent toxic potency to the racemate.

Different results were observed between both enantiomers depending on assay systems for the one racemate (white portion, 10%). *In vivo* assay the component isomers in this racemate did not show much difference.

Single-dose Toxicity of Individual Isomers (Fig. 9): Individual single-dose toxicity was reported for 7 drugs out of the 10 racemic drugs. For the three racemates, both enantiomers indicated different single-dose toxicity (gray portion, 30%), and the enantiomer that had higher pharmacological activity was more toxic. Therefore, the development of single-enantiomer drugs was not necessarily beneficial in terms of both safety and efficacy, and the applicants developed the drugs as racemates.

For the four racemates, the individual enantiomers were not different at single-dose toxicity

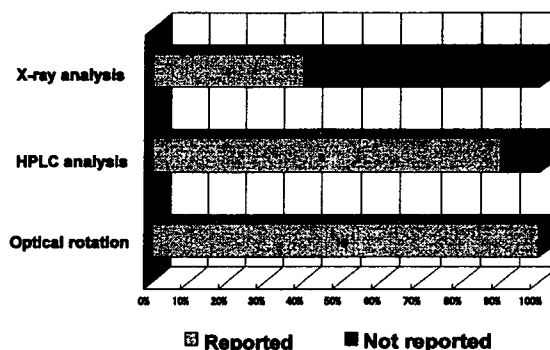


Fig. 7. Stereochemical Characterizations on Chirality of Racemic Drug Substances

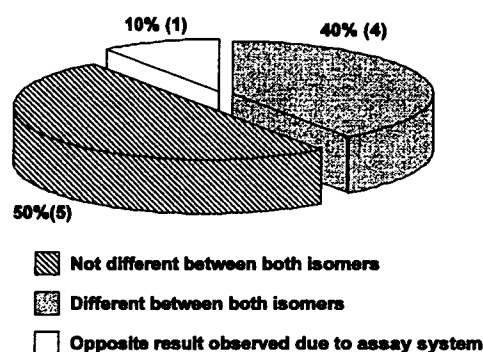


Fig. 8. Pharmacology of Individual Isomers in Racemic Drugs

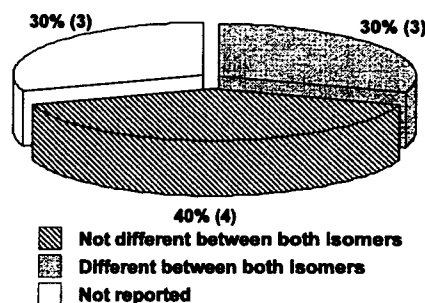


Fig. 9. Single-dose Toxicity of Individual Isomers in Racemic Drugs

(stripe portion, 40%).

Both enantiomers in the three racemates, which had no toxic information of individual isomers, indicated similar pharmacological activities.

Pharmacokinetic Study of Individual Isomers (Fig. 10): Pharmacokinetic evaluations of individual enantiomers were performed for nine racemates using experimental animals and/or human including human tissue-derived materials (Fig. 10). Chiral in-

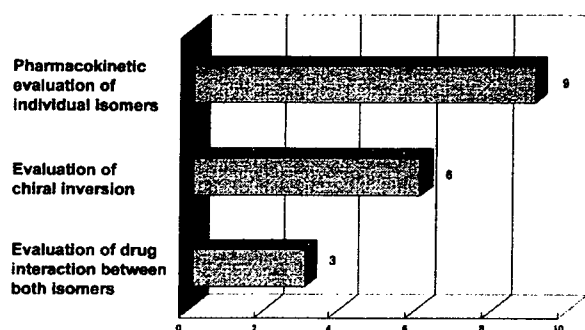


Fig. 10. Classification of Pharmacokinetic Studies on Chirality of Racemic Drugs

version was evaluated for the six racemates, all of which were not observed any chiral inversion. Drug interaction between both enantiomers of the three racemic drugs was investigated. In the other one racemic drug, no pharmacokinetic study relating to chirality was reported.

Pharmacokinetic profiles of enantiomers were investigated for the eight racemic drugs using experimental animals. The four racemic drugs indicated that the individual enantiomers have different profiles. However, there was no difference between both isomers in the rest of four racemates.

Pharmacokinetics was studied using human (healthy volunteers, human tissue-derived materials) for the seven racemates. Pharmacokinetic profiles were different between both enantiomers of the three racemic drugs. The enantiomers of the racemic drug demonstrated different results depending on experimental subjects.

For the four racemates of which each enantiomer indicated different pharmacological potency, both pharmacokinetic profiles of individual enantiomers and chiral inversion were investigated. Drug interaction between both enantiomers was evaluated in the two racemates out of the four.

DISCUSSIONS

Development of single-enantiomer drugs was made possible by the introduction of asymmetric synthesis and chiral separation technologies. In addition, the publication of several guidelines dealing with chiral drugs⁷⁻¹⁰ encouraged the development of single-enantiomer drugs for pharmaceutical manufacturers. The following problems of racemic drugs were parts of the reason for making those guidelines:⁷⁻⁹ One member of an enan-

tiomeric pair might be pharmacologically active and the other inactive; Enantiomers might have different concentration-response relationships for some property; Enantiomers might have completely different activities; Toxicity of a racemic drug might be linked to one member of enantiomeric pairs; Enantiomers might have different pharmacokinetic behavior.

North America^{7,8}) and Europe⁹) have their original guidelines on chirality that describe manufacturing, quality, pharmacology, toxicology, pharmacokinetics and so on. Additionally, quality of chiral drugs was stipulated by a guideline of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceutical Human Use (ICH). The guideline, entitled "Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances"¹⁰) and encoded as Q6A, recommends applicants, in case of development of single-enantiomer drugs, to consider the other enantiomer as an impurity and to set the identity tests capable of distinguishing both enantiomers and the racemic mixture.

Japan has not issued specific guidelines on the development of chiral drugs. ICH-Q6A was implemented officially in Japan on May 1, 2001 and came into effect on July 1, 2001.¹¹) There was a transitional period from the previous guideline until July 1, 2003. Although the drugs surveyed in this paper are not necessarily objects of ICH-Q6A, applicants could prepare to follow ICH-Q6A since it reached Step 4 in October 1999. Additionally, the Japanese regulatory authorities indicated that where the active ingredient is an optical isomer, a method of discriminating between enantiomers should be investigated and the ratio of enantiomers determined.¹²⁻¹⁵) Except for the quality point of view, 'Guidelines on Non-clinical Pharmacokinetic Studies'¹⁶) says that when the investigational substance is a racemate, sponsors should monitor the enantiomers individually to determine the pharmacokinetic profiles. The developments of chiral drugs in Japan must be affected by U.S.A. and/or EU guidelines because pharmaceutical developments parallel in worldwide.

The above guidelines do not prescribe a selection strategy to develop as a single enantiomer or a racemate. However, applicants would be expected to provide a scientifically based justification when a racemic drug had been developed instead of a single-enantiomer drug.

Single-enantiomer Drug Substances

For the 11 single-enantiomers, the stereochemical characterization was not reported (Fig. 4). It does not necessarily mean that the Japanese authorities did not receive any information, but it means that no stereochemical characterization was described in the data summaries. In Japan, reviewers use data summaries as a primary assessment document and technical reports as an additional tool, because significant issues are described in data summaries in the Japanese review system. On the other hand, reviewers directly assess individual technical reports on chemistry, manufacturing and control (CMC) in U.S.A.

The stereochemical characterization was reported for all the substances which were produced by asymmetric synthesis or asymmetric resolution, and hence we supposed that the stereochemical characterizations of the 11 single-enantiomers were regarded as less significant for the following reason: Those 11 substances were synthesized from single-enantiomeric starting materials containing multi-chiral centers such as sugars and steroids; Synthetic procedures of those substances were regarded as ensuring their stereochemistry.

ICH-Q6A guideline says that the identity tests should be capable of distinguishing both enantiomers and the racemic mixture for a drug substance developed as a single enantiomer. However, the specification for assuring chirality was not adopted for the three single-enantiomer drugs. One of them had one-chiral center, and the others had multi-chiral centers. Although the reason for rejected specifications is not clear from the data summaries, it might result from the fact that the NDA of three drugs were submitted during the transitional period for implementing Q6A, or those three substances might be regarded as retaining their starting material chirality, or the specification for assuring chirality might be considered to be meaningless.

Also, no pharmacokinetic study on chirality was reported in the data summaries of the 25 (67%) single enantiomers (Fig. 6), 19 of which had multi-chiral centers. In contrast, the chiral inversion was reported for the four single enantiomers with multi-chiral centers, however, less or equal two chiral centers were investigated in those enantiomers. For the single enantiomer with multi-chiral centers, their chiral inversion and isomer-specific metabolism tended to be considered to be complicated and unimportant.

Racemic Drug Substances

Based on the guidelines, we reassessed the justifications for developing the 10 racemates rather than a single enantiomer.

The pharmacologic activity and the pharmacokinetic profile of the individual enantiomers should be characterized, because rapid interconversion *in vivo* was not observed for all of the 10 racemic drugs. Although the principal pharmacological activity was characterized for the individual isomers of the 10 racemic drugs, the pharmacokinetic profiles of the individual enantiomers were not reported for the one. It might be one of the reasons for not reporting that this racemic drug has been a common therapeutic agent for long time in worldwide.

According to the guidelines, it is ordinarily sufficient to carry out toxicity studies on the racemate. The toxicity study of each isomer was not reported for the three racemic drugs, because both enantiomers of these three racemates indicated similar pharmacological activities.

Relating to stereochemical characterization, it is recommended to perform chromatographic tests in addition to optical rotatory tests for mixtures of optical isomers. The chiral HPLC analysis was not performed for the one racemic drug. This racemic drug has been a common therapeutic agent for long time in worldwide, and the Japanese authorities did not require additional stereochemical characterization.

In conclusion, the trend in the Japanese pharmaceutical development is increasingly moving toward the development of single isomers rather than racemates. The chiral development approaches approved in Japan were essentially consistent with the approaches recommended by the guidelines. The racemic drugs, which shared only 13% in the new chemical drug substances, had some rationale to be developed as racemates. Decline of racemic drugs development may continue in Japan as well as worldwide, because some studies need to be carried out with not only a racemic mixture but its component enantiomers.

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