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In Vitro Cytotoxicity of 20 MEIC Chemicals with "Quantum-Type" Release of Rhodamine 123 Detected by Flow Cytometry

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Summary

We determined the *in vitro* cytotoxicity of 20 MEIC chemicals by flow cytometry. Suspension-cultured Daudi cells were pre-stained with rhodamine-123 and treated with MEIC chemicals for 3 hr. Rhodamine-123 localizing preferentially in mitochondria was detected quantitatively. Relative changes in cell size and intracellular structure were also determined simultaneously by monitoring the relative forward and side scattering fluorescence intensity, respectively, by flow cytometry. Different patterns of these 3 parameters were observed in cells treated with different chemicals. For example, acetylsalicylic acid decreased rhodamine-123 content at the 50% effective dose (ED_{50}) of 1.07 mM but did not affect the other 2 parameters. However, amitriptyline-HCl decreased all 3 parameters near ED_{50} values, while digoxin increased the side scattering light intensity suggesting that a dramatic change may have occurred in the intracellular structure. Based on these patterns, we classified toxicity of the MEIC chemicals into 5 types. ED_{50} values for the rhodamine-123 release caused by the test chemicals were highly correlated with those derived from MTT assay, LDH-release assay, and the image analysis assay which we developed previously. This simple flow cytometric assay provides a new sight in cytotoxicity assays of chemicals.

Key words: Cytotoxicity detected by flow cytometry

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Introduction

Due to the strong opposition to use of animals in toxicology and biomedical research and the practical advantages of *in vitro* alternatives to *in vivo* testing methods (i.e., simplicity, rapidity and low cost of screening chemical toxicity), many *in vitro* methods have been developed to predict chemical toxicity in animals and humans such as colony formation assay (1, 2), neutral red uptake (NR) assay (3, 4), MTT assay (3, 5, 6, 7, 8) and lactate dehydrogenase (LDH) release assay (9, 10, 11). These assays measure total changes of function of a cell population and take several days to yield results. In our previous study, we established a new assay to test toxicity of chemicals to mitochondria in living cells by a fluorescence-image processing system. This assay measures the residual fluorescence in mitochondria of individual cells *in situ* and requires only 3 hr for completion (12). This assay measures the

changes in mitochondrial function *in situ* of individual anchorage-dependent monolayer cultured cells under a fluorescence microscope, allowing the observation of only a small number of cells at one time.

FACScan is flow cytometery with which fluorescence intensity of fluorescent-labeled organelles, relative changes in cell size and intracellular structure of an individual cell can be measured simultaneously with fluorescence [FL1(515–545 nm), FL2(564–606 nm), FL3(over 650 m)], FSC (forward scattering) light and SSC (side scattering) light by excitation with LASER light at 488 nm (13, FACScan Manual, Becton Dickinson Immunocytometry Systems, USA). This piece of equipment can measure a large number of suspended cells in one minute. These parameters were utilized for characterization of specific types of cells (14).

It is now possible to stain mitochondria in living cells specifically with fluorescent lipophilic cations such as rhodamine dyes (15, 16), since there is a unique mitochondrial membrane potential which is dependent on a proton electrochemical gradient generated by proton pumps which in turn are driven by the respiratory electron transport chains. The amount of such dyes retained in mitochondria is dependent on the voltage of the membrane potential.

In the present investigation, we simultaneously observed the above 3 parameters, i.e. relative change in fluorescence intensity of rhodamine-123 in preloaded mitochondria, cell size and intracellular structures such as those of granules in a cell population caused by various MEIC chemicals.

Materials and Methods

Test chemicals Twenty of the 50 chemicals recommended for the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) by the Scandinavian Society for Cell Toxicology (7, 17) were tested. These chemicals, purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), are listed in Table 1. They were dissolved and serially diluted in RPMI-1640 medium supplemented with 10% fetal bovine serum just before being tested.

Table 1. ED50 values of MEIC chemicals determined by *in vitro* assays

MEIC Chemical		FACScan	Image processing		MTT assay		LDH release	
Code	Name	ED50(mM)	ED50(mM)	Image/FACScan	ED50(mM)	MTT/FACScan	ED50(mM)	LdH/FACScan
1	Paracetamol	12					7.10	0.59
2	Acetylsalicylic acid	1.07					4.40	4.11
5	Amitriptyline HCl	0.08					0.18	2.25
6	Digoxin	0.25					0.00	0.00
12	Phenol	7.5					9.60	1.28
13	Sodium chloride	102	35	0.34	31.6	0.31	150.6	1.48
14	Sodium fluoride	29	1.9	0.07	1.0	0.03	2.10	0.07
18	Nicotine	4.3	30	6.98	6.8	1.58	21.60	5.02
20	Lithium sulfate	40	47	1.18	44.1	1.10	36.50	0.91
23	Propranolol HCl	0.22					0.31	1.41
25	Paraquat dichloride	0.17					0.29	1.71
29	Thioridazine HCl	0.015					0.012	0.80
31	Warfarin	0.9					0.42	0.47
35	Isoniazide	7.3	24	3.29	17.5	2.40	49.2	6.74
39	Pentachlorophenol sodium	0.065					0.051	0.78
41	Chloroquine diphosphate	0.43	0.42	0.98	0.18	0.43	0.41	0.95
43	Quinidine sulfate	0.265	0.07	0.26	0.12	0.46	0.20	0.75
48	Caffeine	20	11	0.55	2.6	0.13	5.70	0.29
49	Atropine sulfate	3.1	2.6	0.84	1.2	0.38	2.90	0.94
50	Potassium chloride	62.5	20	0.32	29.0	0.46	61.7	0.99
	Average			1.48		0.73		1.58

Cell culture The human Burkitt's-lymphoma-derived leukemia cell line Daudi was cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum (18, 19).

Pre-loading of rhodamine-123 Daudi cells were stained with rhodamine-123 (1 μg/ml in RPMI1640–10%FBS medium) at 37°C in 5% CO_2-95% air for 30 min. After rinsing with the medium 3 times, they were subsequently used in the experiments (15, 16, 19, 20).

Cytotoxicity assay The pre-stained 1×10^5 Daudi cells in 0.5ml of RPMI1640–10%FBS medium were mixed with 0.5ml of test chemical solution dissolved and diluted in RPMI1640–10%FBS medium and incubated for 3 hr at 37°C. After this treatment, the cells were washed and suspended in calcium-and magnesium-free Dulbecco's phosphate buffered saline for use in FACScan™ (Becton Dickinson Immunocytometry Systems, U.S.A).

Flow cytometry Lysis II software program was used to acquire and analyze FACScan data. The detector voltage of FL1 was adjusted to measure the fluorescence of rhodamine-123 in the preloaded cells. FSC Gain was set to E00 and SSC detector was adjusted to measure the cellular particles and organelles. The threshold of FSC-H was set to 100 to exclude the dead cells from the count. For one sample, 25,000 cells were measured with 488nm LASER light excitation. Values were detected for FL1, FSC and SSC simultaneously.

Data analysis The intensity of the rhodamine-123 fluorescence released by cells treated with different concentrations of the MEIC chemicals, expressed as % Rhodamine-123, were calculated as follows:

$$\%\text{Rhodamine-123} = \frac{\text{FL1 values of treated cells}}{\text{FL1 values of untreated control cells}} \times 100$$

where FL1 values were read from the relative area of the M2 range as illustrated in Fig. 1A.

Percent changes in intensity of FSC (forward scattering) light and SSC (side scattering) light by excitation at 488 nm were defined similarly. Since changes in FSC and SSC

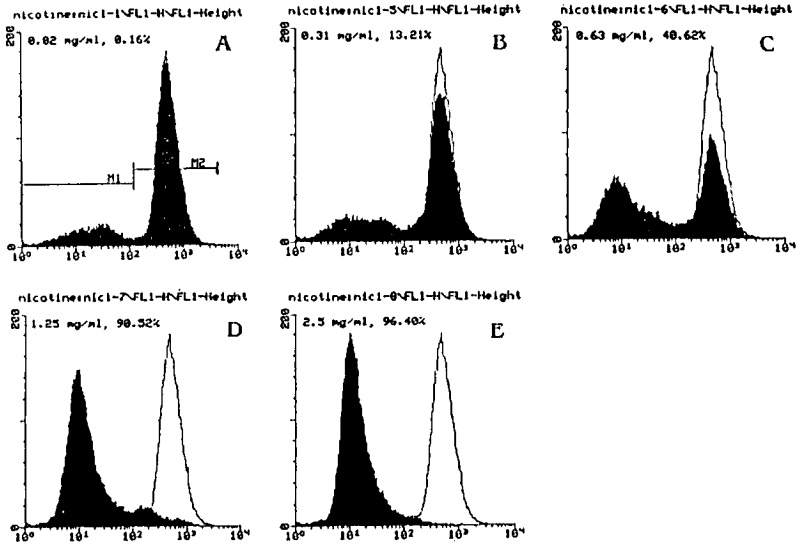

Fig. 1. Histograms of rhodamine-123 fluorescence intensity of Daudi cells treated with nicotine.

The empty peaks represents the distribution of control cells and the filled peaks were for those of treated cells. (A) Treated with nicotine at 0.12 mM, (B) at 1.93 mM, (C) at 3.85 mM, (D) at 7.70 mM and (E) at 15.41 mM. Note that percentages on the top of each figure indicate those lost in the M2 area compared to control cells.

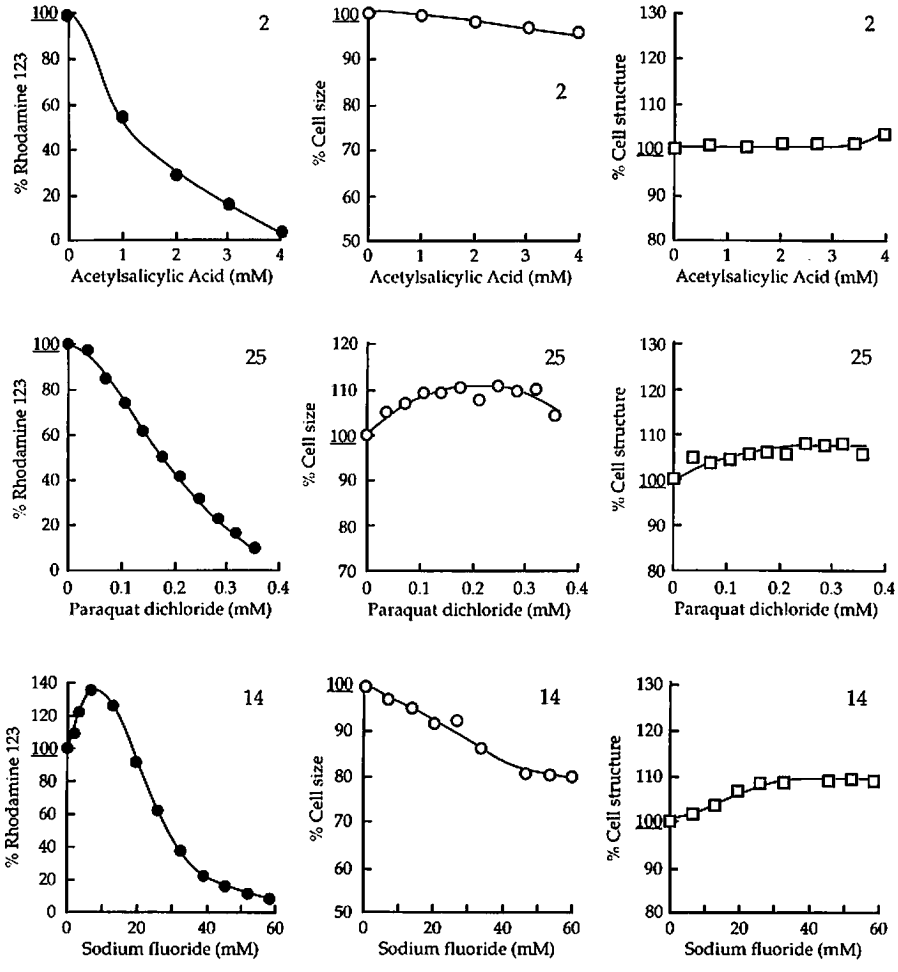

Fig. 2A. Dose-response curves of % rhodamine-123, % cell size, and % cell structure caused by the 20 MEIC chemicals.

The numbers appearing on the top right in each figure are the code numbers of MEIC chemicals. See definitions of % rhodamine-123, % cell size, and % cell structure in Materials and Methods.

correspond mainly to the changes in cell size and intracellular structure, respectively, these two parameters are expressed as "% Cell size" and "% Cell structure", respectively, in Fig. 2.

The 50% effective dose (ED_{50}) was defined as the concentration of test chemical which resulted in the 50% decrease of "% rhodamine-123". ED_{50} values were read directly from the curves shown in Fig. 2.

Results

"Quantum"-type release fo preloaded rhodamine-123 from cells

With nicotine as a representative chemical, we examined flow cytometric patterns of rhodamine-123 release from Daudi cells (Fig. 1). The cells were grouped into two ranges: one with a low-fluorescence peak (range M1 in Fig. 1A), and the other with a high-fluorescence peak (range M2 in Fig. 1A).

At a concentration of 0.12 mM, 99.8% of the cells retained their fluorescence for 3 hrs.

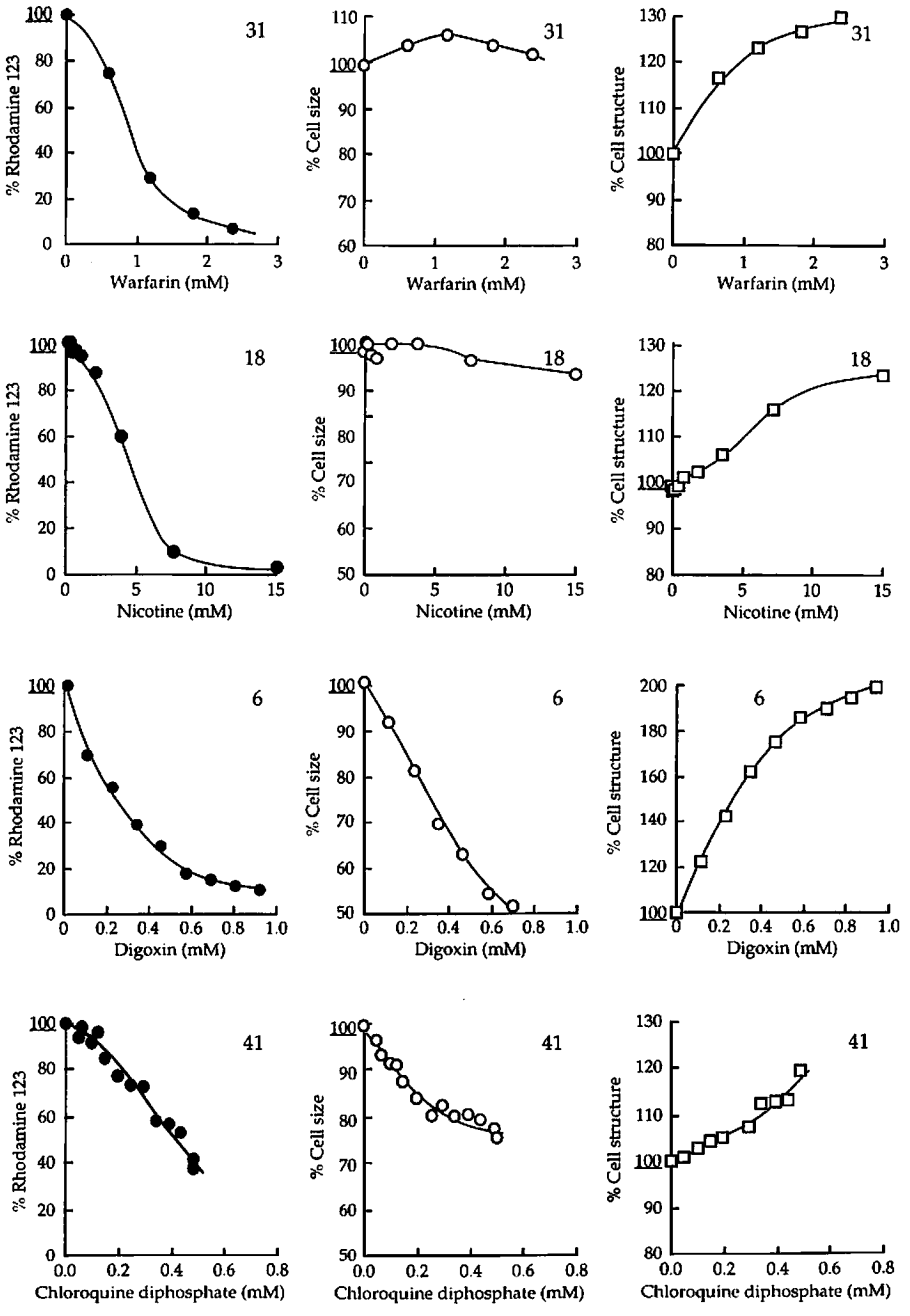

Fig. 2B.

Treatment of cells with higher concentrations of the chemical compelled more cells to release their rhodamine-123 in a "quantum"-type manner (Fig. 1B-E). At 15.4 mM, 96% of cells lost their fluorescence. The "quantum"-type release of rhodamine 123 was observed for all the chemicals tested in the present investigation.

Dose-response curves of rhodamine-123 release, and relative change in cell size and cell structure

Figure 2 shows dose-response curves for the 20 MEIC chemicals tested for "% rhodamine-

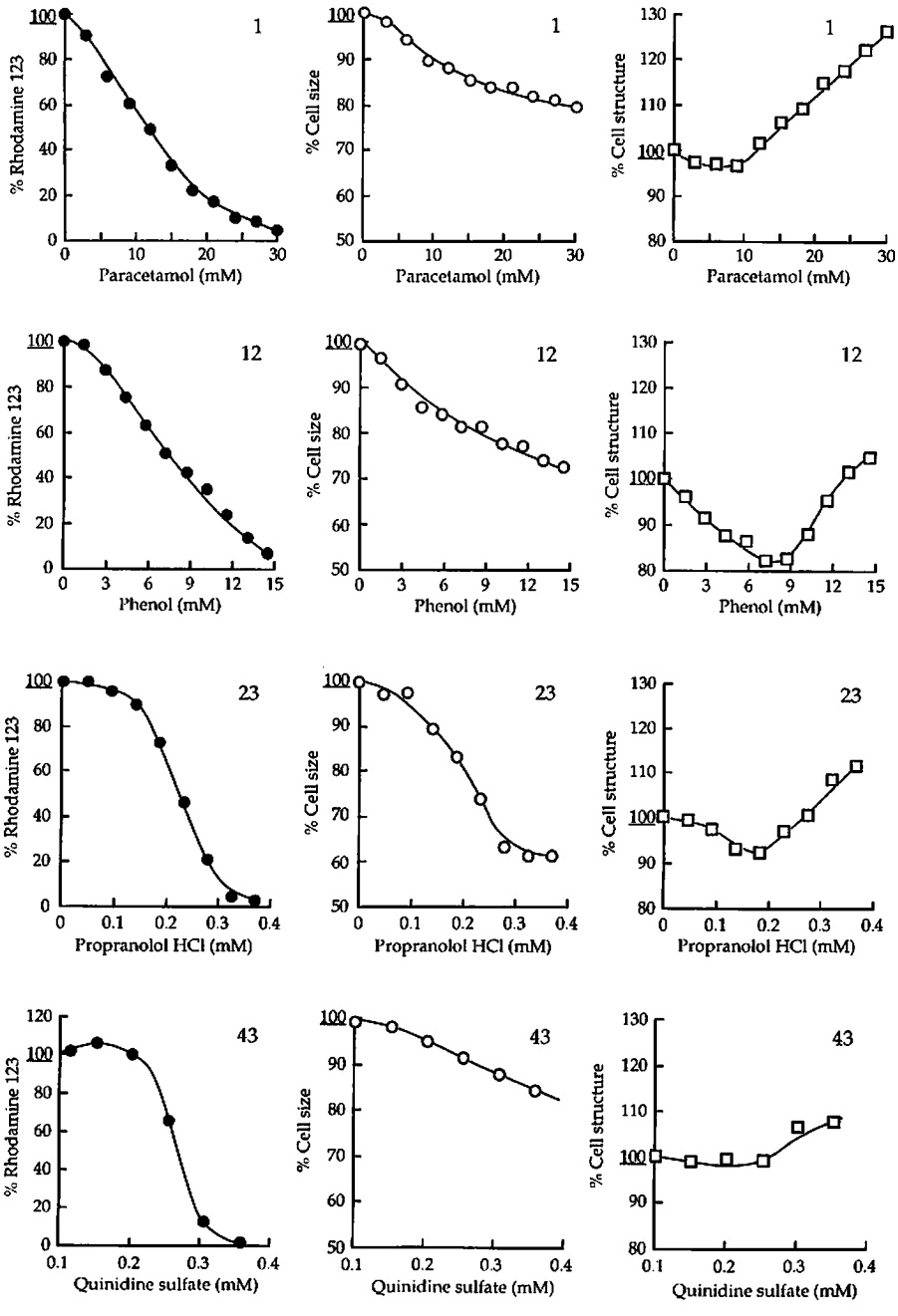

Fig. 2C.

123", "% cell size", and "% cell structure", each of which were defined as described in Materials and Methods. It should be noted that, although the curves for % rhodamine-123 generally decreased with increases in concentration of the test chemicals, those of sodium fluoride at 0–20mM (Fig. 2A), quinidine sulfate at 0.1–0.22mM (Fig. 2C), and caffeine at 0–10mM (Fig. 2E) were over 100%.

As shown in Fig. 2A, acetylsalicylic acid did not alter % cell size or % cell structure near ED_{50} value for % rhodamine-123. Paraquat dichloride caused a slight increase in % cell

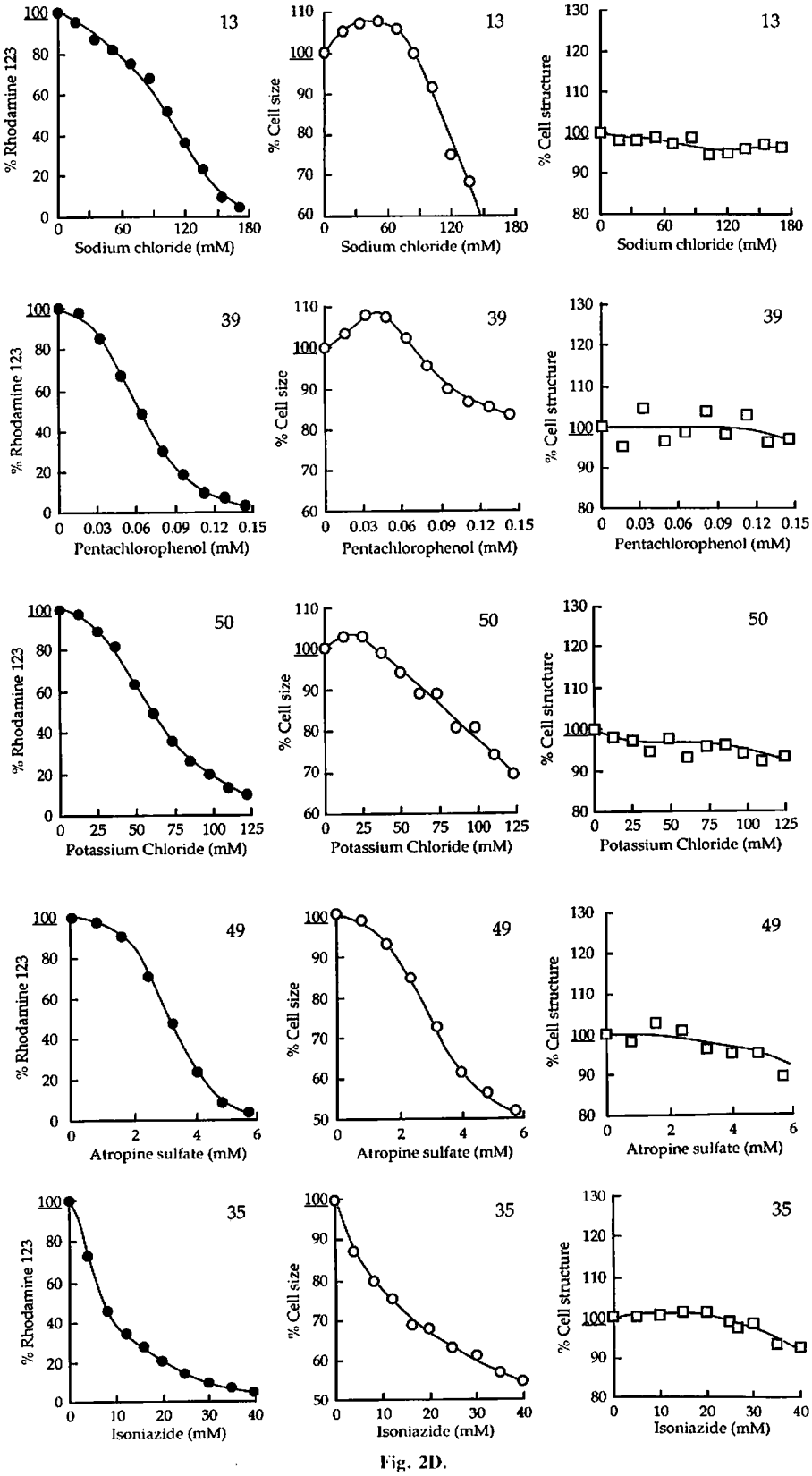
13
% Rhodamine 123
Sodium chloride (mM)
13
% Cell size
Sodium chloride (mM)
13
% Cell structure
Sodium chloride (mM)
39
% Rhodamine 123
Pentachlorophenol (mM)
39
% Cell size
Pentachlorophenol (mM)
39
% Cell structure
Pentachlorophenol (mM)
50
% Rhodamine 123
Potassium Chloride (mM)
50
% Cell size
Potassium Chloride (mM)
50
% Cell structure
Potassium Chloride (mM)
49
% Rhodamine 123
Atropine sulfate (mM)
49
% Cell size
Atropine sulfate (mM)
49
% Cell structure
Atropine sulfate (mM)
35
% Rhodamine 123
Isoniazide (mM)
35
% Cell size
Isoniazide (mM)
35
% Cell structure
Isoniazide (mM)

Fig. 2D.

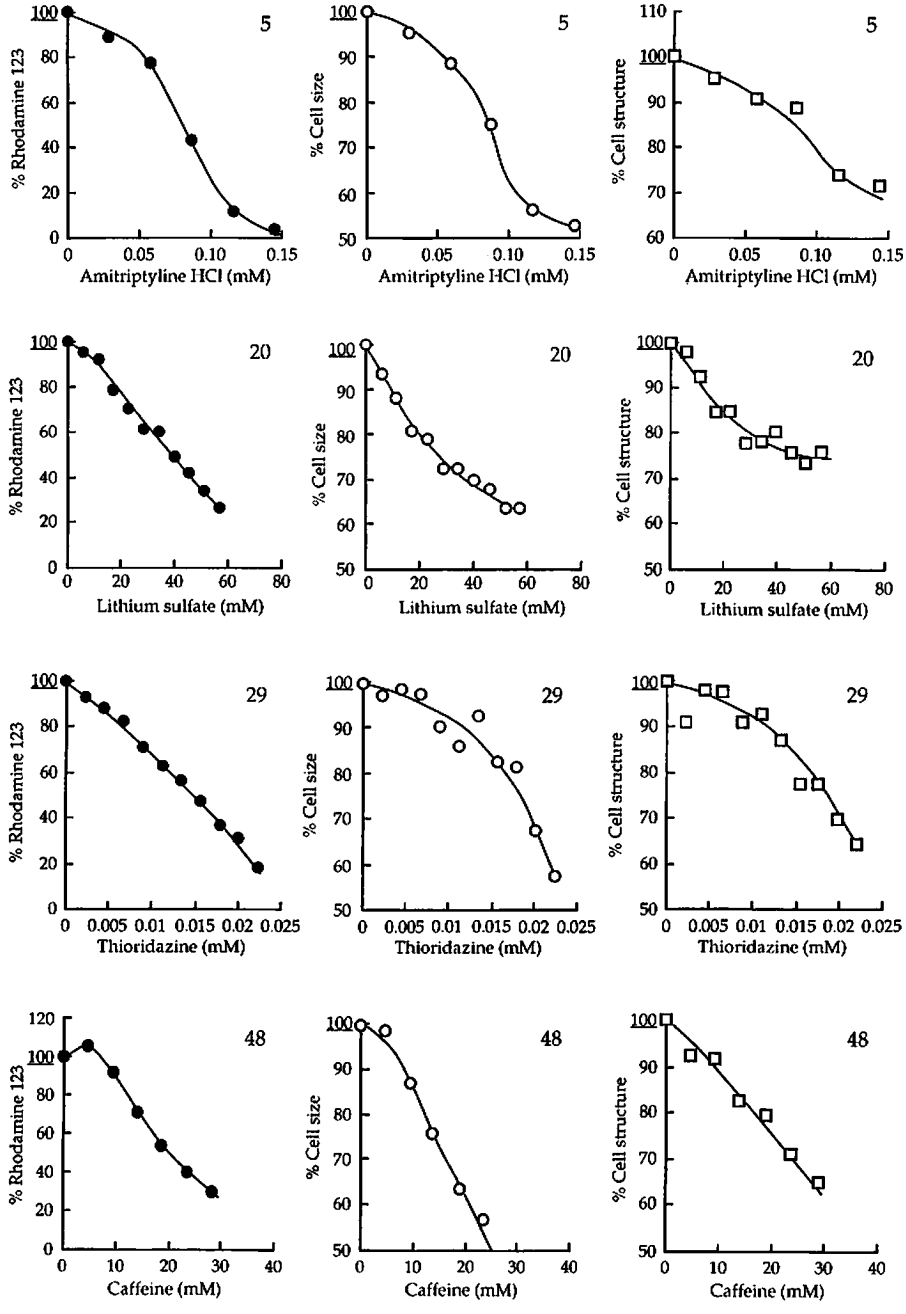

Fig. 2E.

size but not in % cell structure, while sodium fluoride caused a weak decrease in the former but a slight increase in the latter. However, compared to the other MEIC chemicals tested, these changes were relatively small. Thus, we tentatively classified these chemicals as Type A.

Compared to these 3 chemicals, warfarin, nicotine, digoxin, and chloroquine diphosphate caused clear increase in % cell structure, although the former two did not change % cell size and the latter two induced apparent decrease in this parameter. Tentatively we classified these 4 chemicals as Type B.

In contrast to these Type A and B chemicals, paracetamol, phenol, propranolol HCl, and quinidine sulfate showed "V"-shaped dose response curves of % cell structure accompanied with progressive decreases in % rhodamine-123 and % cell size (Fig. 2C). Percent cell structure initially decreased to values near those of ED_{50} dose for % rhodamine-123, and then increased. We classified this type of chemicals as Type C.

The five chemicals, sodium chloride, potassium chloride, pentachlorophenol, atropine sulfate and isoniazide, did not cause significant changes in % cell structure but strongly decreased % rhodamine-123 and % cell size. However, the former three chemicals resulted in an initial increase in % cell size in the dose range lower than that of ED_{50} dose for % rhodamine-123 (Fig. 2D). We classified these chemicals as Type D.

We classified amitriptyline HCl, lithium sulfate, thioridazine and caffeine as Type E, Since these chemicals simply decreased the three parameters in the dose range tested (Fig. 2E).

Correlations of the ED_{50} values from the present FACScan assay with those from image processing, MTT assay, and LDH release assay

Exact ED_{50} values for each chemical are shown in Table 1 together with the ED_{50} values obtained by the image processing method described in our previous paper (12), the MTT assay (12), and the LDH release assay (11). We tentatively named the present assay, "FACScan assay".

The 10 chemicals used in the present study were common with those analyzed by image processing and MTT assays, and 20 were common with the LDH release assay. The sensitivity of FACScan assay was compared with the previous 3 assays by calculating ratios of the ED_{50} values of the latter to those of the former. Sensitivity of the FACScan assay was close to that of the previous 3 assays as shown by the averages of the ratios for the common chemicals, i.e. 1.5, 0.7, and 1.6 on Image/FACScan, MTT/FACScan, and LDH/FACScan, respectively (Table 1).

However, markedly different ED_{50} values were observed in the case of digoxin in LDH release assay and FACScan assay giving a ratio of 0.002. In contrast, the ED_{50} value of sodium fluoride from FACScan assay was more than 10 times those from the previous 3 assays.

However, for some other chemicals such as nicotine, warfarin, isoniazide, and caffeine large variations in the ratios were observed. Correlation coefficients, which were determined based on the log ED_{50} values of the present FACScan assay and those from the previous 3 assays, were high; i.e. 0.803, 0.829 and 0.832 (Fig. 3A, B, and C, respectively).

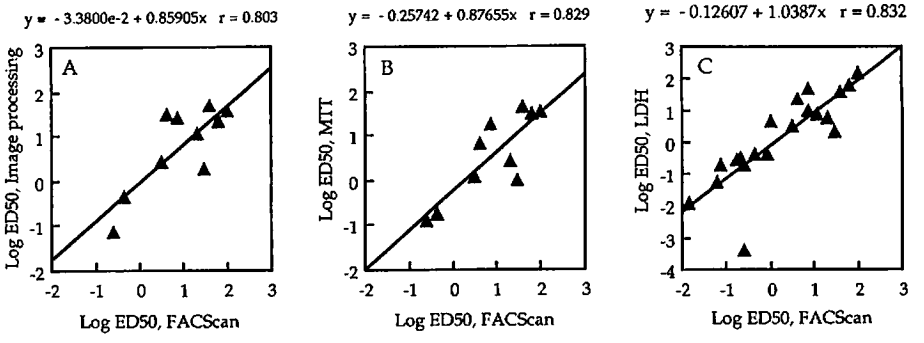

Fig. 3. Correlations between the ED_{50} values observed in the present FACScan assay and (A) those of the image processing assay, (B) MTT assay, and (C) LDH release assay.
ED_{50} values observed in the latter 3 assays were derived from our previous papers (11, 12).

Discussion

Flow cytometry is a powerful technique for analysis of cytotoxicity of chemicals and has been used as an effective tool in alternative researches (19). Compared with detection of the changes in cell populations by colony formation (3), LDH release assay (10, 11), MTT (5), neutral red uptake (3, 4), and crystal violet staining assays (21), the present FACScan assay measures changes in individual cells one by one in a short period of time. In the present experiments, we were able to examine a total of 25000 cells/sample in only 1–2 min, while the other cytotoxicity assays required 4 hr to 2 weeks to measure a parameter such as cell viability. Therefore, this rapid cytotoxicity assay is mostly restricted by the long incubation time of cells treated with toxic compounds to complete the process.

Although it is possible to measure the changes in the function of mitochondria by MTT assay or by the image processing assay as described in our previous paper (12), the FACScan assay enabled us to measure changes in multiple parameters in individual cells simultaneously. In the present experiments, we determined only 3 simple parameters, i.e. relative changes of rhodamine-123 release, cell size and cell structure by measuring fluorescence, FSC, and SSC, respectively, with 488 nm LASER light. With other detection parameters such as surface antigens labeled with fluorochrome-conjugated specific antibodies, this assay would provide data concerning many parameters simultaneously in individual cells within a population. However, the cells must be dissociated completely from each other and suspended in fluid for analysis by flow cytometry. FACScan assay is better applicable to suspension cultured cells, while the image processing assay is suitable to analyze changes in adherent cell monolayers since the cells are observed under a microscope.

As shown in Fig. 1, we observed the "quantum"-type release of rhodamin-123 from living cells. Probably, the massive release of rhodamine-123 which corresponds to removal of the mitochondrial membrane potential requires a certain "threshhold" of toxicity in a cell. This phenomenon has not, to our knowledge, been described in the literature (15, 16, 19, 20). We were not also unaware of this phenomenon in our previous investigation using the image processing assay, where a small number of cells were observed at one time under fluorescent microscopy and changes in relative fluorescent intesity of mitochondria and cytoplasm in the same cell were determined (12).

Although the dose-response curves for % rhodamine-123 decreased generally with increases in concentrations of test chemicals (Fig. 2), the dose response curves for sodium fluoride, quinidine sulfate, and caffeine initially increased beyond 100% and then declined to relatively lower concentrations (Fig. 2A, C, and E), suggesting that the dye was actively accumulated by the cells treated with low concentrations of these chemicals.

In the LDH release assay, sodium fluoride at about 1 mM was found to promote "cell growth" but quinidine sulfate and caffeine were not (22). In the present study, the low doses of these chemicals used caused very small relative changes in % cell size and % cell structure (Fig. 2A, C, and E). Therefore, we do not yet know how the increase of % rhodamine 123 caused by low doses of these chemicals correlates with stimulation of cellular metabolic activity.

The quantitative measurement of % cell size showed that, after treatment with toxic chemicals, cell size was generally reduced except at lower dose ranges of paraquat dichloride, warfarin, sodium chloride, pentachlorophenol, and potassium chloride. With more variety than the curves for % rhodamine-123 and % cell size, the dose-response curves for % cell structure calculated from SSC values increased for 8 chemicals (Fig. 2B and C), did not change much (but slightly

decreased) in 8 (Fig. 2A and D), and apparently decreased for 4 (Fig. 2E). The increased SSC values indicate increases in reflection of LASER light from cellular particles and organelles (13, 14, FACScan Manual, Becton Dickinson Immunocytometry Systems, USA).

These phenomena prompted us to classify the MEIC chemicals into 5 types (A to E), although this classification does not necessarily correspond to the 3 major types and the 2 subtypes depending on cell growth inhibition (CGI*) and cell killing (CK*) derived from the LDH release assay described previously (22). However, compiling efforts on the typing of cytotoxic actions will help determining the characteristics of toxic chemicals more rapidly than with precise investigations of the mechanisms of action of these chemicals.

ED_{50} values for % rhodamine-123 correlated closely with the ED_{50} values from the LDH release assay, the MTT assay, and the image processing assay (Fig. 3), suggesting that the present FACScan assay is useful as a rapid cytotoxicity assay and could serve as an alternative to *in vivo* toxicological tests, even though some extreme differences were found as described in Results. In general, the order of sensitivity of assays will be MTT > FACScan > image processing > LDH release as judged from the averages of the ratio of ED_{50} values from each assay to those from the present FACScan assay.

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