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# First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (9) Evaluation of Cytotoxicity Tests on HeLa and CHL/IU Cells

Hajime Kojima[1,2], Junko Ohuchi[1,3], Yutaka Kasai[1,3], Joshin Okada[1,3], Katsuyoshi Tsukumo[1,4], Hiroshi Kakishima[1,4], Eriko Miyai[1,5], Junichi Akiyama[1,5], Yuuko Okamoto[1,6], Mayumi Kotani[1,7], Kaori Inoue[1,8], Michio Shibata[1,8], Hidenobu Okumura[1,9], Masaki Arashima[1,9], Takamasa Atsumi[1,9], Ikuyo Makino[1,10], Katsuyoshi Chiba[1,10], Yasuo Ohno[11], and Akira Takanaka[11]

*[1] Japan Cosmetic Industry Association (JCIA), 4th florr, Hatsumei Bldg., 9–14 2-chome Toranomon, Minato-ku, Tokyo 105, Japan, [2] Biochemical Research Institute, Nippon Menard Cosmetic Co. Ltd., 4–66 Asakusa, Ohgaki-shi, Gifu 503, Japan, [3] KAO Corp.-Tochigi. Biological Sciences, Akabane, Ichikai, Haga, Tochigi, 321–34 Japan, [4] Kanebo Cosmetic Laboratory, 3–28 5-chome, Kotobuki-cho, Odawara-shi, Kanagawa 250, Japan, [5] Kaminomoto Co. Ltd., 3–3–25 Kumochibashidori, Chuo-ku, Kobe 651, Japan, [6] Div. Fundamental Research, KOSÉ Corp., 1–18–4 Azusawa, Itabashi-ku, Tokyo 174, Japan, [7] SUNSTAR Inc., 3–1 Asahi-machi, Takatsuki-shi, Osaka 569, Japan, [8] Shiseido Safety & Analytical Research Center, 1050 Nippa-cho, Kohoku-ku, Yokohama 223, Japan, [9] Shiga Central Research Laboratory, NOEVIR Co., Ltd., 112–1 Okada-cho, Yokaichi-shi, Shiga 527, Japan, [10] Safety Research Center, Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi-shi, Tokyo 186, Japan, [11] Div. Pharmacol. National Institute of Health Sciences (NIHS), 1–18–1 Kamiyoga, Setagya-ku, Tokyo 158, Japan*

## SUMMARY

The first phase inter-laboratory validation of cytotoxicity tests using established cell lines was conducted by the collaboration of 7–8 laboratories for the purpose to evaluate the test for their relevance as alternative methods to the Draize rabbit eye irritation test (Draize test). The methods studied were the MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide) assay in HeLa cells (HeLa-MTT assay) and crystal violet staining assay in CHL/IU cells (CHL/IU-CVS assay). We tested nine surfactants and isotonic sodium chloride solution (phosphate saline) under the common standard operating procedure (SOP) and determined the concentrations of test chemicals that showed 50% absorbance reduction as compared to control (the median effective concentration : $EC_{50}$).

Inter-laboratory coefficients of variation of the $EC_{50}$ values obtained for the surfactants obtained were below 0.50 except for polyoxyethylene octylphenylether (10 E.O.: Triton X-100). The correlation coefficients of these $EC_{50}$ values with maximal average Draize total score (MAS) were -0.902 and -0.817 for the HeLa-MTT and CHL/IU-CVS assays respectively. The cytotoxicity potentials ($EC_{50}$) of test substances were similar between these two assay methods.

We conclude that the cytotoxicity tests in HeLa-MTT and CHL/IU-CVS assays are promising as alternative methods to the

Correspondence: H. Kojima, Biochemical Research Institute, Nippon Menard Cosmetic Co., Ltd., 4–66, Asakusa, Ohgaki-shi, Gifu 503, Japan
Tel.: +(81)584 89 5659
Fax.: +(81)584 89 8359

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Draize eye test for surfactants. Because surfactants constitute only a minor part of total cosmetic ingredients, these tests should be evaluated with a wider range of chemicals used as cosmetic ingredients.

## INTRODUCTION

Cytotoxicity tests using cultured cell lines have been studied as alternatives to the Draize rabbit eye irritation test (Draize test)[1-27]. Among them, the methods using human cervix derived HeLa cells and Chinese hamster lung derived cells seemed to be promising because of the wide availability of those cells, ease of culture, and correlation to Draize scores[11,17-19,21]. For the purpose of introducing appropriate methods for the evaluation of eye irritation potential for cosmetic ingredients, we conducted a first phase inter-laboratory validation of two different cytotoxicity tests using established cell lines, the MTT assay method in human cervix derived HeLa cells (HeLa-MTT) and the crystal violet staining assay in Chinese hamster lung derived CHL/IU cells (CHL/IU-CVS) to evaluate them as alternative methods to the Draize test. We selected nine surfactants and isotonic sodium chloride solution (physiological saline) as test substances.

This is a part of the Ministry of Health and Welfare (MHW) project entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics"[28].

## MATERIALS AND METHODS

*Test substances*

Name of the ten test substances used are listed in Table I. They are composed of one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants and physiological saline[29]. They were from the Japanese standards of cosmetic ingredients[30,31]. They were supplied from the Japan Cosmetic Industry Association (JCIA) to the National Institute of Health Science (NIHS). The coded substances were distributed to each laboratory to enable us to get objective information about the methods and intra-/inter-laboratory variation. The test substances, except sodium hydrogenated tallow-L-glutamate (HT-glutamate) and benzalkonium chloride, were prepared in culture medium and filtered before use. The medium consisted of Eagle's MEM (Gibco BRL.) supplemented with 10% calf serum (CS: Gibco BRL:culture medium). All laboratories used the same lot of the culture medium. HT-glutamate and benzalkonium chloride were insoluble in the culture medium. Phosphate-Buffered Saline {PBS(−)}, DMSO, or ethanol used for treating cells. The final PBS(−) concentration in

Table I. Test substances

| Samle number | Name | Abbreviation | Classification |
|---|---|---|---|
| S-1 | Isotonic Sodium Chloride Solution | Physiological saline | - |
| S-2 | Polyoxyethylene Hydrogenated Caster Oil(60 E.O.) | POE hydrogenated caster oil | Nonionic |
| S-3 | Polyoxyethylene Sorbitan Monolaurate (20 E.O.) | Tween 20 | Nonionic |
| S-4 | Polyethyleneglycol Monolaurate (10 E.O.) | PEG monolaurate | Nonionic |
| S-5 | Sodium N-Lauroyl Sarcosinate (30% solution) | Lauroyl sarcosinate | Anionic |
| S-6 | Sodium Hydrogenated Tallow - L- glutamate | HT- glutamate | Anionic |
| S-7 | Sodium Lauryl Sulfate | SLS | Anionic |
| S-8 | Sodium Polyoxyethylene Laurylether Sulfate (2 E.O. : 27% solution) | POE laurylether sulfate | Anionic |
| S-9 | Polyoxyethylene Octylphenylether (10 E.O.) | Triton X-100 | Nonionic |
| S-10 | Benzalkonium Chloride | Benzalkonium chloride | Cationic |

the culture medium did not exceed 10% and the concentrations of DMSO and ethanol were less than 0.5%.

We also used sodium lauryl sulfate (SLS) from Wako Pure Chemical Industries, Sigma or Tokyo Chemical Industry as positive control.

*1) HeLa-MTT assay*[32,33]

Eight laboratories participated in this study. HeLa cells were distributed by the Japanese Cancer Research Resources Bank (JCRB) to each laboratory and cytotoxicity in the cultured cells was evaluated by MTT (Sigma or Tokyo Chemical Industry) reduction assay[33]. Passage numbers of cell lines used in each laboratory were from 112 to 118 after getting P110 cell lines of HeLa cells. A check for mycoplasma was performed in advance and a few laboratories also checked after the experiments and demonstrated lack of contamination. The doubling time of cells obtained from all laboratories was from 22.0 to 27.2 h. The colony formation rates obtained from three laboratories was from 40.5 to 84.0%, the modal chromosome number obtained from three laboratories was from 79 to 82, and two laboratories detected the human A type isozyme (The Authenti Kit™ system: Innovative chemistry). One hundred $\mu$l of cell suspension ($4\times10^4$ cells/ml) in the culture medium was added to each well of 96 well-plates (Falcon: Becton Dickinson labware; Same lot in every laboratory) and cells were incubated for 3 days at 37°C in a 5% $CO_2$ incubator. For each test chemical, solutions of 10 different concentrations of chemical including non-treatment level were prepared in culture medium. One hundred $\mu$l of the solution was added to each well and 6 wells per concentration were used.

After 48 h, solutions were aspirated, cells were rinsed with PBS(+) and 200 $\mu$l of MTT (500 $\mu$g/ml in culture medium) was added to each well. After 2 h of incubation, black and fuzzy crystals of MTT formazan produced in viable cells were extracted with 200 $\mu$l of isopropanol. After 1 h, the absorbance was measured with a microplate reader at 590 nm. The ratio of MTT formazan formed in each well to that from non-treated wells was calculated for each concentration and the median effective concentration, i.e., $EC_{50}$, was calculated from two concentration-response curves obtained for each test substance.

*2) CHL/IU-CVS assay*[34]

Seven laboratories participated in this study. Chinese hamster lung (CHL/IU) cells were distributed by JCRB and cytotoxicity in the cultured cells was evaluated by a crystal violet staining method[7]. The passage numbers of cell lines used in each laboratory were from 7 to 16 after getting P6 cell lines of CHL/IU cells. The check of mycoplasma was performed in advance and a few laboratories also checked after experiments and demonstrated lack of contamination. The doubling time of cells obtained from all laboratories was from 12.0 to 18.5 h. The colony formation rate obtained in six laboratories was from 48.0 to 96.0%, the modal chromosome number obtained from five laboratories was 25, and two laboratories detected the Chinese hamster like Nucleoside phosphorylase, Lactate dehydrogenase and Glucose-6-phosphate isozymes. All laboratories used same lot of culture medium as for the HeLa-MTT assay. One hundred $\mu$l of $2\times10^3$ cells/ml suspension was added to each well of 96 well-plate. The cells were incubated in culture medium for 3 days. For each test chemical, solutions of 10 graded concentrations including non-treated control were prepared, one hundred $\mu$l of a solution was added to each well and 6 wells per concentration were used. After 48 h, solutions were aspirated, cells were rinsed with PBS (+) and the cells attached to the wells were fixed with methanol. Then, the cells were stained with 0.1% crystal violet solution for 15 min. After washing with water and drying, the absorbance was measured with microplate reader at 590 nm. The ratio of crystal violet stained cells in each well to those

from non-treated wells was calculated for each concentration and the median effective concentration, i.e. $EC_{50}$, was calculated from two concentration-response curves obtained for each test substance.

## RESULTS

The two $EC_{50}$ values for the 10 test substances reported from each laboratory and their averages, SD and coefficient of variations (CV) from the HeLa-MTT and CHL/IU-CVS tests are indicated in Table II and Table III, respectively. The differences in $EC_{50}$ values obtained in each laboratory for each test substance were small. The CVs among the data obtained from each laboratory were below 0.5, except for polyoxyethylene octylphenylether (10 E.O.; Triton X-100) for which those were 0.759 and 0.516 in the HeLa-MTT and CHL/IU-CVS tests, respectively. HT-glutamate and benzalkonium chloride responses should no differences despite use of different solvents across laboratories. Figs. 1 and 2 also show median, maximum, minimum and quartile results for the inter-laboratory results for each test substance, except physiological saline for which $EC_{50}$ values were not obtained. The HeLa-MTT and CHL/IU-CVS results show similar

Table II. The median effective concentration of MTT assay on HeLa cells.

| Labo. | A | | B | | C | | D | | E | | F | | G | | H | | (μg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment number | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | Ave. | SD | CV |
| S-1* | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >50,000 | >50,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | | |
| S-2 | 5,650.0 | 5,400.0 | 4,000.0 | 3,800.0 | 4,800.0 | 4,600.0 | 4,500.0 | 5,000.0 | 2,000.0 | 1,700.0 | 3,600.0 | 3,200.0 | 4,000.0 | 5,000.0 | 4,600.0 | 4,600.0 | 4,153.0 | 1,108.0 | 0.267 |
| S-3 | 560.0 | 510.0 | 580.0 | 580.0 | 500.0 | 490.0 | 440.0 | 420.0 | 580.0 | 580.0 | 480.0 | 420.0 | 670.0 | 780.0 | 460.0 | 680.0 | 551.0 | 104.0 | 0.189 |
| S-4 | 266.0 | 266.0 | 230.0 | 260.0 | 190.0 | 200.0 | 250.0 | 255.0 | 245.0 | 250.0 | 250.0 | 232.0 | 316.0 | 285.0 | 360.0 | 340.0 | 262.0 | 45.3 | 0.173 |
| S-5 | 400.0 | 410.0 | 320.0 | 325.0 | 450.0 | 400.0 | 340.0 | 360.0 | 320.0 | 320.0 | 440.0 | 310.0 | 365.0 | 380.0 | 420.0 | 400.0 | 373.0 | 46.4 | 0.125 |
| S-6 | 98.0 | 67.0 | 130.0 | 125.0 | 105.0 | 92.0 | 83.0 | 84.0 | 91.0 | 88.0 | 61.0 | 65.0 | 93.0 | 89.0 | 89.0 | 79.0 | 87.4 | 20.9 | 0.239 |
| S-7 | 213.0 | 167.0 | 155.0 | 160.0 | 130.0 | 124.0 | 149.0 | 145.0 | 160.0 | 185.0 | 182.0 | 130.0 | 200.0 | 200.0 | 180.0 | 160.0 | 166.0 | 26.8 | 0.161 |
| S-8 | 710.0 | 620.0 | 550.0 | 580.0 | 510.0 | 510.0 | 490.0 | 460.0 | 290.0 | 300.0 | 530.0 | 420.0 | 700.0 | 810.5 | 720.0 | 680.0 | 563.0 | 148.4 | 0.264 |
| S-9 | 69.0 | 71.0 | 6.0 | 7.0 | 85.0 | 87.0 | 48.0 | 44.0 | 1.8 | 2.0 | 52.0 | 22.0 | 52.0 | 41.5 | 12.0 | 11.0 | 37.0 | 28.1 | 0.759 |
| S-10 | 8.9 | 12.0 | 13.0 | 10.5 | 5.7 | 4.8 | 5.3 | 4.8 | 2.8 | 2.6 | 9.8 | 7.1 | 12.0 | 14.5 | 13.0 | 11.0 | 8.6 | 3.9 | 0.453 |
| SLS (Posi. con.) | 175.0 | 171.0 | 160.0 | 160.0 | 123.0 | 128.0 | 160.0 | 175.0 | 150.0 | 150.0 | 182.0 | 128.0 | 225.0 | 220.0 | 159.0 | 164.0 | 165.0 | 28.6 | 0.174 |

* Sample names show in Table I.

Table III. The median effective concentration of crystal violet staining assay on CHL/IU cells.

| Labo. | A | | B | | C | | D | | E | | F | | G | | (μg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment number | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | Ave. | SD | CV |
| S-1* | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 | | |
| S-2 | 1,890.0 | 2,470.0 | 2,100.0 | 1,650.0 | 2,500.0 | 2,750.0 | 1,100.0 | 960.0 | 1,520.0 | 1,440.0 | 2,380.0 | 2,350.0 | 2,200.0 | 2,210.0 | 1,965.0 | 551.0 | 0.280 |
| S-3 | 300.0 | 320.0 | 130.0 | 160.0 | 178.0 | 195.0 | 120.0 | 150.0 | 99.0 | 144.0 | 235.0 | 250.0 | 250.0 | 290.0 | 202.0 | 72.3 | 0.359 |
| S-4 | 220.0 | 220.0 | 75.0 | 90.0 | 270.0 | 270.0 | 140.0 | 170.0 | 147.0 | 143.0 | 195.0 | 195.0 | 310.0 | 240.0 | 192.0 | 68.9 | 0.359 |
| S-5 | 340.0 | 400.0 | 310.0 | 270.0 | 425.0 | 450.0 | 280.0 | 340.0 | 223.0 | 287.0 | 240.0 | 380.0 | 460.0 | 470.0 | 348.0 | 83.5 | 0.240 |
| S-6 | 50.0 | 48.0 | 50.0 | 43.0 | 81.0 | 78.0 | 53.0 | 49.0 | 30.0 | 39.0 | 40.0 | 67.0 | 52.0 | 65.0 | 52.9 | 14.5 | 0.274 |
| S-7 | 160.0 | 190.0 | 175.0 | 170.0 | 190.0 | 205.0 | 175.0 | 175.0 | 147.0 | 163.0 | 245.0 | 245.0 | 210.0 | 180.0 | 186.0 | 29.4 | 0.158 |
| S-8 | 690.0 | 660.0 | 550.0 | 620.0 | 785.0 | 750.0 | 540.0 | 560.0 | 470.0 | 500.0 | 680.0 | 870.5 | 760.0 | 710.0 | 668.0 | 133.0 | 0.199 |
| S-9 | 31.0 | 44.0 | 22.5 | 24.0 | 72.5 | 65.5 | 18.0 | 20.0 | 19.4 | 19.3 | 28.0 | 29.5 | 56.0 | 41.0 | 35.1 | 18.1 | 0.516 |
| S-10 | 18.0 | 22.0 | 17.5 | 19.0 | 27.8 | 27.1 | 14.0 | 12.0 | 12.7 | 15.5 | 18.0 | 29.5 | 23.0 | 26.0 | 20.2 | 5.8 | 0.287 |
| SLS (Posi. con.) | 200.0 | 180.0 | 160.0 | 175.0 | 190.0 | 172.0 | 170.0 | 165.0 | 134.0 | 162.0 | 230.0 | 210.0 | 180.0 | 168.0 | 178.0 | 23.6 | 0.132 |

* Sample names show in Table I.

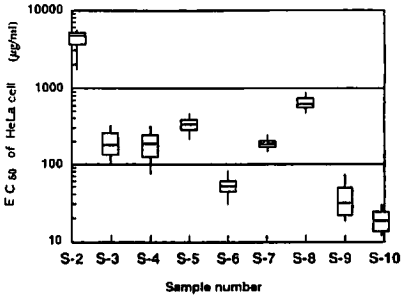



Figure 1. Summary statistics of MTT assay on HeLa cells.
Sample names show in Table I.

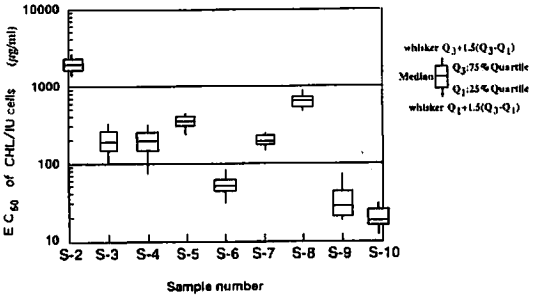



Figure 2. Summary statistics of crystal violet staining assay on CHL/IU cells.
Sample names show in Table I.

Table IV. Results of the Draize rabbit eye irritation test on 10% solution of 10 test substances.

| Sample number | Maximum average score | | | | 24 hrs score | | | | Area under the curve*:% | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Cornea | Iris | Conjuctiva | Total | Cornea | Iris | Conjuctiva | Total | Cornea | Iris | Conjuctiva |
| S-1** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S-2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S-3 | 0.7 (1)*** | 0.0 | 0.0 | 0.7 (1) | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.1 |
| S-4 | 3.3 (1) | 0.0 | 0.0 | 3.3 (1) | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.2 |
| S-5 | 10.3 (48) | 8.3 (48) | 0.0 | 8.0 (1,4) | 8.3 | 5.0 | 0.0 | 3.3 | 3.4 | 1.9 | 0.0 | 1.5 |
| S-6 | 26.7 (24) | 16.7 (24-72) | 1.7 (72-168) | 12.0 (4) | 26.7 | 16.7 | 0.0 | 10.0 | 14.9 | 10.7 | 0.8 | 3.5 |
| S-7 | 15.0 (4) | 8.3 (48,72) | 0.0 | 10.0 (4) | 14.7 | 6.7 | 0.0 | 8.0 | 7.1 | 4.2 | 0.0 | 3.0 |
| S-8 | 10.0 (4) | 3.3 (48) | 0.0 | 10.0 (4) | 2.7 | 0.0 | 0.0 | 2.7 | 2.0 | 0.7 | 0.0 | 1.4 |
| S-9 | 41.3 (72) | 30.0 (72) | 5.0 (168) | 10.0 (4,48) | 24.7 | 15.0 | 1.7 | 8.0 | 26.9 | 18.4 | 2.3 | 6.3 |
| S-10 | 78.0 (24) | 66.7 (24) | 5.0 (96-168) | 14.7 (96) | 78.0 | 66.7 | 0.0 | 11.3 | 57.3 | 43.9 | 2.5 | 10.9 |

* The area ratio under the curve means the ratio (%) of the area under the line connecting scores at each observation period to those based on theoretical maximum of Draize total score until 7 days after treatment.

** Sample names show in Table I.

*** Thses values in parenthesis are the time (hour) when the scores became maximum.

Table V. Correlation and Spearman rank correlation matrix between MTT assay on HeLa cells and Draize rabbit eye irritation scores.

| | | Correlation* coefficients | Rank correlation coefficients |
|---|---|---|---|
| Maximal average Draize total scores (MAS) | Total | - 0.902 | 0.930 |
| | Cornea | - 0.870 | 0.791 |
| | Iris | - 0.835 | 0.482 |
| | Conjuctiva | - 0.808 | 0.809 |
| Scores of 24 h after | Total | - 0.860 | 0.794 |
| | Cornea | - 0.817 | 0.770 |
| | Iris | - 0.358 | 0.152 |
| | Conjuctiva | - 0.812 | 0.761 |
| Area under the curve(AUC): %** | Total | - 0.883 | 0.891 |
| | Cornea | - 0.861 | 0.806 |
| | Iris | - 0.843 | 0.491 |
| | Conjuctiva | - 0.903 | 0.891 |

* Logarithmic transformed $EC_{50}$ values used.

**AUC indicates the area under the line connecting scores plotted at each observation period. The parameter used in this study was ratio of AUC of test substances to those based on theoretical maximum of the Draize score, cornea, iris and conjuctiva score, respectively.

Table VI. Correlation and Spearman rank correlation matrix between crystal violet staining assay on CHL/IU cells and Draize rabbit eye irritation scores.

| | | Correlation* coefficients | Rank correlation coefficients |
|---|---|---|---|
| Maximal average Draize total scores (MAS) | Total | - 0.818 | 0.894 |
| | Cornea | - 0.784 | 0.742 |
| | Iris | - 0.817 | 0.482 |
| | Conjuctiva | - 0.683 | 0.785 |
| Scores of 24 h after | Total | - 0.777 | 0.752 |
| | Cornea | - 0.730 | 0.733 |
| | Iris | - 0.408 | 0.152 |
| | Conjuctiva | - 0.753 | 0.718 |
| Area under the curve(AUC): %** | Total | - 0.804 | 0.842 |
| | Cornea | - 0.785 | 0.764 |
| | Iris | - 0.816 | 0.491 |
| | Conjuctiva | - 0.813 | 0.842 |

* Logarithmic transformed $EC_{50}$ values used.

**AUC indicates the area under the line connecting scores plotted at each observation period. The parameter used in this study was ratio of AUC of test substances to those based on theoretical maximum of the Draize score, cornea, iris and conjuctiva score, respectively.

patterns for all test substances.

Table IV shows Draize scores for 10% solution of the 10 test substances[38]. Maximal average Draize scores, scores at 24 h after application, and area under the curve (AUC) obtained for cornea, iris, conjunctiva, and the sum of these values are shown in Table IV. These were used to compare with the results of the HeLa-MTT and CHL/IU-CVS tests. The correlation coefficients and Spearman rank correlation coefficients between Draize scores and logarithmic transformed values of $EC_{50}$ are given in Tables V and VI. Figs. 3 and 4 show correlation diagrams between the maximal average Draize total score (MAS) and logarithmic transformed $EC_{50}$ values. Correlation coefficients between them were

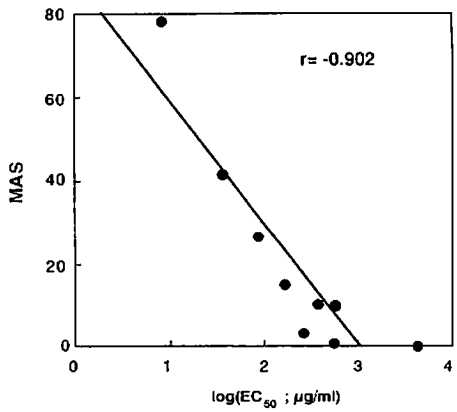



Figure 3. Correlation between maximal average Draize total scores (MAS) and MTT assay on HeLa cells.

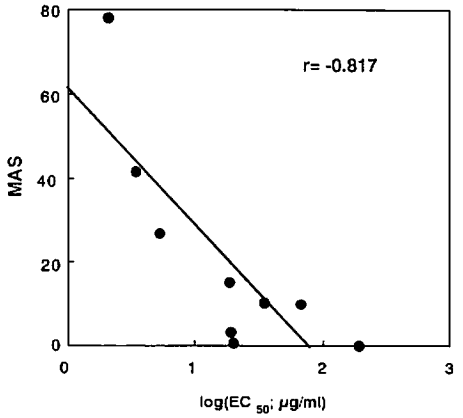
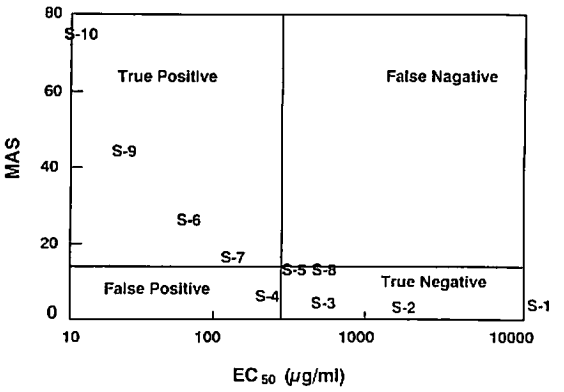
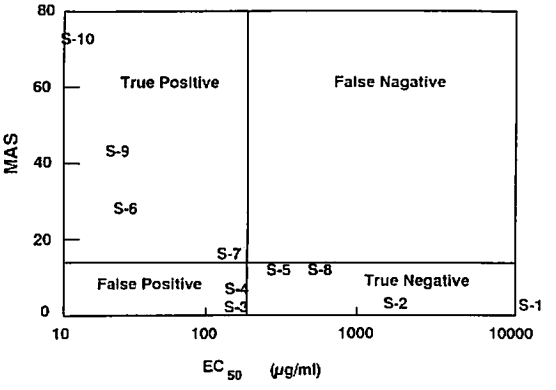



Figure 4. Correlation between maximal average Draize total scores (MAS) and crystal violet staining assay on CHL/IU cells.




Simple regression: log($EC_{50}$)= - 0.027MAS+2.87

Figure 5. Reproducibility of MTT assay on HeLa cells to maximal average Draize total scores (MAS). For a discrimination points of MAS 15, the $EC_{50}$ of MTT assay on HeLa cells is 288.0 μg/ml.
Sample names are shown in Table I.




Simple regression: log($EC_{50}$)= - 0.020MAS+2.65

Figure 6. Reproducibility of crystal violet staining assay on CHL/IU cells to maximal average Draize total scores (MAS).
For the discrimination points of MAS 15, the $EC_{50}$ of CVS assay on CHL/IU cells is 220.0 μg/ml.
Sample names are shown in Table I.

higher than 0.683 except for the case of iris. The correlation with MAS and with AUC of conjunctiva for the HeLa-MTT test was especially high (−0.902 and −0.903, respectively). Rank correlation coefficients between *in vivo* and *in vitro* results were also high except for the scores of iris after 24 h. We consider that the lack of a range of iris scores to compare to the *in vitro* data is the reason the correlations were low.

Predictability of these cell culture methods was assessed by linear regression lines and the MAS for the ten test chemicals. The results are shown in Figs. 5 and 6. When the discrimination point between positive and negative irritants was set at MAS 15, corresponding $EC_{50}$s for the HeLa-MTT and CHL/IU-CVS tests were 288.0 and 220.0 μg/ml. There were no false negative substances by both methods, but polyoxyethylene sorbitan monolaurate (20 E.O.: Tween 20) was indicated to be a false positive by HeLa-MTT, and Tween 20 and polyetyleneglycol monolaurate (10 E.O.: PEG monolaurate) by CHL/IU-CVS. We consider these cell culture methods to have good correspondence between *in vitro* data and MAS.

We compared the present results with those obtained by other cultured cell tests like Statens seruminstitut rabbit cornea (SIRC)-CVS, SIRC-NR[35] and colony formation assay on Chinese hamster V79 cells (V79-colony: unpublished data), in which serum was supplemented in the culture medium. Table VII shows very high correlation coefficients above 0.914 among these assays.

## DISCUSSION

Mammalian cell lines have been used to establish alternative methods to the Draize test. These include SIRC cells[1-7] and mouse fetal derived fibroblasts, Balb/c 3T3[5,8-14],

Table VII. Correlation coefficient among the cytotoxicity tests.

| | HeLa -MTT | CHL/IU -CVS | SIRC -CVS | SIRC -NR | V79-colony |
|---|---|---|---|---|---|
| HeLa-MTT | 1.000 | | | | |
| CHL/IU-CVS* | 0.961 | 1.000 | | | |
| SIRC-CVS | 0.990 | 0.949 | 1.000 | | |
| SIRC-NR** | 0.986 | 0.932 | 0.998 | 1.000 | |
| V79-colony | 0.924 | 0.947 | 0.917 | 0.914 | 1.000 |

CVS* :Crystal violet staining assay
NR** :Neutral red uptake assay

P815 murine mastocytoma cells[3,5], murine macrophage cell RAW 264.7[11], mouse epithelium derived cells, MEL/30[15], hamster derived fibroblasts, BHK-21/Cl3[16], human cervix epithelial cells, HeLa cells[17-19], human liver epithelium cells, Hep2[17], HepG2[11,20] and Chinese hamster lung derived cells, V79 cells[11,21], and so on. Normal cells like human keratinocytes[5,22,23], human dermal fibroblasts[24], and rabbit corneal epithelial cells[3,5,11,25-27] have also been used to compare responses with *in vivo* Draize scores. Endpoints to detect the cytotoxicities of test substances were neutral red uptake[5,6,9,12,23], growth inhibiton[14,15,18,21,22,24], colony formation[1,2,5,19,27] and $^3$H-uridine uptake[5,8,20], and so on. Results for surfactants, alcohols, aldehydes and other chemicals have been compared with those from *in vivo* tests. There were a few chemicals in each study reported that did not give results correlating with *in vivo* Draize tests. However, relatively good correlations were obtained for most of the chemicals. In addition, the cytotoxicities observed in various methods using serum-supplemented culture medium correlated each other regardless of cell types and endpoints of cytotoxicity. Results from the present study also showed high correlation with MAS and corneal scores. In addition, we confirmed by intra-/interlaboratory studies that these cultured cell methods gave reproducible results. From these results, we consider that cytotoxicity tests using mammalian cells cultured in serum supplemented medium, including the HeLa and CHL/IU tests, are useful as one of the alternative methods to Drize test.

While these results on surfactants are promising, a second phase validation of these methods using wider range of cosmetic ingredients is planned.

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