INTER-LABORATORY VALIDATION STUDY OF THE SKIN^2 DERMAL MODEL ZK1100 AND MTT CYTOTOXICITY ASSAY KITS

Yuzo TANIGUCHI[1], Keiji SUZUKI[2], Keiya NAKAJIMA[3], Madoka NAKAJIMA[4], Yoshihisa MIWA[5], Yasushi YAMADA[5], Matoshi SATOH[6], Masahiro TAKEYOSHI[7], Yasuki AKIE[8], Matsuko MORIYASU[9], Hitoshi YAMAMOTO[10], Minoru KATAGIRI[11], Akemi KAZAMA[11] and Masami WATANABE[12]

[1]Drug Safety Research Laboratories, Research and Development Division, The Environmental Biological Life Science Inc., 555 Inaba, Ukawa, Minakuchi-cho, Koka-gun, Shiga 528, Japan

[2]Division of Radiation Biology, School of Medicine, Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa 236, Japan

[3]The Center of Japan Biological Chemistry Co., Ltd., 52 Fukue Kaizu-cho Kaizu-gun, Gifu 503-06, Japan

[4]Genetic Toxicology Group, Biosafety Research Center, Foods, Drugs and Pesticides, 582-2, Arahama Shioshinden, Fukuda-cho, Iwata-gun, Shizuoka 437-12, Japan

[5]Nihon Bioresearch Center, Inc., 6-104, Majima, Fukuju-cho, Hashima-shi, Gifu 501-62, Japan

[6]Safety Research Institute for Chemical Compounds Co., Ltd., 849, Kita 30-jo Nishi 11-chome, Kita-ku, Sapporo 001, Japan

[7]Hita Research Laboratories, Chemical Biotesting Center, Chemicals Inspection & Testing Institute, 3-822 Ishii-machi, Hita-shi, Oita 877, Japan

[8]Research & Development Group, Fuji Life Science Inc., 10211 Kobuchisawa-cho, Kitakoma-gun, Yamanashi 409-16, Japan

[9]Biochemical Laboratory, Panapharm Laboratories Co., Ltd., 1285, Kurisaki-cho, Uto-shi, Kumamoto 869-04, Japan

[10]Life Science Laboratory, 5-19, 2-chome, Nishihonmachi, Nishi-ku, Osaka 550, Japan

[11]Bioindustry Division, Oriental Yeast Co., Ltd., 10-11, Kodenma-cho, Nihonbashi, Chuo-ku, Tokyo 103, Japan

[12]Faculty of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852, Japan

(Received February 15, 1993 ; accepted December 27, 1993)

ABSTRACT — An inter-laboratory validation study was conducted to evaluate the potential of 4 chemicals to cause irritation with utilizing the Skin^2 Dermal Model ZK1100 kit developed by Advanced Tissue Sciences, Inc. (formerly Marrow-Tech, Inc., La Jolla, California, USA). The chemicals tested were sodium dodecyl sulfate (SDS), 1-n-hexadecyl-pyridinium chloride monohydrate (CC), ethanol (EtOH), and dimethyl sulfoxide (DMSO). Eleven Japanese insititutions participated in this validation re-

Correspondence : Yuzo TANIGUCHI at the above address.

search to evaluate the usefulness of the Skin^2 Model ZK1100 kit in accordance with an identical protocol. None of the participating laboratories had previously used the Skin^2 Model ZK1100 kit. The MTT-50 value obtained in the individual institutions was 42 to 91 μg / ml for SDS, 2.7 to 8.6 μg / ml for CC, 2.0 to 9.3% for EtOH, and 11.5 to 21.9% for DMSO. Reproducibility was reasonably good as noted when one test chemical was repetitively tested by the same investigator.

MTT-50 values obtained with the present method correlated with DS20 values obtained with Draize's method ($r=0.9881$) in one of the participant institutions.

The irritation study using the Skin^2 Model ZK1100 kit was easy to perform and generated quantitative data. When the test was repeated, reproducibility was demonstrated with a variation of less than 2σ. These data suggested that this newly developed *in vitro* method would be useful in toxicity screening studies in terms of both time and cost, and would serve as a useful alternative to the conventional methods of the eye irritation study.

KEY WORDS : the Skin^2 Dermal Model ZK1100, Human keratinocyte, Inter-laboratory varidation study, Cytotoxicity, Alternative to eye irritation test.

INTRODUCTION

Various irritation tests have been conducted to study irritation potential induced by drugs, pesticides, chemicals, or cosmetics to the eye or skin. The ability of these products to irritate the eye has historically been studied by using Draize's method. This widely used method, however, involves some uncertainty as has been pointed out since it had first been developed by Draize *et al.* (Draize *et al.*, 1944 ; Draize *et al.*, 1959). In recent years, as concerns preventing cruelty to animals, people have started movements against the use of rabbits to test eye irritation caused by chemicals. In developed countries including Japan, drug or cosmetic manufacturers are being urged to introduce non-animal testing methods, and efforts have been directed to the development of *in vitro* or non-animal methods for toxicity studies. In particular, the Draize method to test eye or skin irritation caused by chemicals has been exposed to extremely severe criticism, and needs to be replaced by an alternative without delay. The Skin^2 Model ZK1100 kit is a kit developed by Advanced Tissue Sciences, Inc. to test ocular irritation using cultured, three-dimensional human skin. It not only provides an alternative to the Draize method but also would be useful in drug efficacy studies and in the research and development of various medical materials (Triglia *et al.*, 1991). We designed a validation research program to confirm the usefulness of this Skin^2 Model ZK1100 kit in accordance with a protocol proposed by Oriental Yeast Co., Ltd. Eleven Japanese insititutions participated in this program. The irritation potential of four identical chemicals was tested by these institutions, twice for each chemical, by using the Skin^2 Model ZK1100 kit to define the reproducibility of results according to this method, intra-institution differences, and the usefulness of this Skin^2 Model ZK1100 kit in rank ordering the irritation potential of these chemicals.

In each institution, each chemical was tested twice by the same investigators, and the reproducibility of results was studied. The conventional Draize method was also performed in parallel with one of the participant institutions, and the possible correlation between DS20 and MTT-50 values was studied.

MATERIALS AND METHODS

Test chemicals : Four chemicals to be tested were chosen by Oriental Yeast Co., Ltd. Test samples of each chemical from one identical lot were distributed to the participant instititions, and each chemical was tested twice. The test chemicals used were 3.5 mM SDS (Lot No. 1129) attached to the testing kit as a positive control, CC produced by Kanto Chemical, Co., Ltd. (Lot No. 309P4107), EtOH produced by Kanto Chemical, Co., Ltd. (Lot No. 402B5512 ; purity

99.5%), and DMSO produced by Sigma Co., Ltd. (Lot No. 30H0608).

Cultured tissue: The Skin^2 Model ZK1100 kits (Advanced Tissue Sciences, Inc., La Jolla, California USA ; Lot Nos. 01398 and 01402) were supplied by Oriental Yeast Co., Ltd.

Animals: Male SPF Kbl : JW rabbits (10 weeks of age, 39 animals, body weight 1.5 to 3.0 kg) were purchased from Kitayama Labes Co., Ltd. After 3 days of quarantine and acclimatization, apparently healthy animals were chosen for the experiment.

Animals were individually housed in suspended aluminum cages (320-mm wide, 550-mm deep, 350-mm high) and placed in a room supplied with 12 air changes per hour and maintained at a temperature of 22±2℃, a relative humidity of 55±15%, and a light cycle of 12 hours light and 12 hours dark (lights were turned on 6:00 a.m. and off at 6:00 p.m.). Pellet diet (RC4, Oriental Yeast Co., Ltd.) and water were available *ad libitum*.

Toxicity study using cultured tissue

1) Preparation of test chemicals

The test chemicals were dissolved in accordance with the protocol supplied by Oriental Yeast Co., Ltd. Specifically, a 2.5-ml aliquot of 3.5-mM SDS solution, which had been attached to the Skin^2 Model ZK1100 kit as the positive control, was dissolved in 22.5 ml of the assay medium in the kit. The resultant SDS solution (100 μg / ml) was further diluted to the following test concentrations : 70, 50, 30, and 10 μg / ml. Twenty-five mg of CC, weighed on an electronic even balance, was dissolved in 25-ml of the assay medium, and solutions of the respective concentrations of 10, 7, 5, 3, and 1 μg / ml were prepared. A volume of 3.0-ml of EtOH was dissolved in 27.0-ml of the assay medium. The resultant 10% EtOH solution was further diluted to obtain test EtOH solutions of the respective concentrations of 7.0, 5.0, 3.0, and 1.0%. A volume of 6.0-ml of DMSO was diluted in 24.0-ml of the assay medium. The resultant 20% DMSO solution was further diluted to obtain 17.0, 10.0, 6.0, and 2.0% solutions. All these test solutions were prepared immediately before use.

2) *In vitro* test concentrations of test chemicals

The chemicals were tested at the following fixed concentrations :

SDS : 100, 70, 50, 30, and 10 μg/ml
CC : 10, 7, 5, 3, and 1 μg/ml
EtOH : 10.0, 7.0, 5.0, 3.0, and 1.0 v/v%
DMSO : 20.0, 17.0, 10.0, 6.0, and 2.0 v/v%

In vivo eye irritation study

1) Preparation of test chemicals

SDS, CC, EtOH, and DMSO were all dissolved in distilled water respectively for injection (Otsuka Pharmaceutical Co., Ltd.), and concentrations of the solutions were adjusted as follows :

SDS : 2, 5, and 10 w/v%
CC : 1, 2, and 5 w/v%
EtOH : 30, 50, and 70 v/v%
DMSO : 30, 50, 70, and 90 v/v%

Each of these solutions was sterilized by passing through a disposable sterile filter, and handled aseptically.

2) *In vivo* test concentrations of test chemicals

The test concentrations were originally fixed at 2, 5, and 10 w/v% for SDS, 1, 2, and 5 w/v% for CC, 30, 50, and 70 v/v% for EtOH, and 30, 50, 70 v/v% for DMSO. However, the highest concentration of DMSO was increased to 90 v/v% because this compound was not irritating even at 70 v/v%.

TESTING PROCEDURES

I. *In vitro* toxicity study using cultured tissue

Culture was appropriately initiated using the Skin^2 Model ZK1100 kit. After the test chemical solution prepared as mentioned above was added, the cultures were incubated for 18 to 24 hours in an incubator maintained at 37℃ with 5% CO_2 gas. The supernatant was removed. The MTT reagent was added and the culture was further incubated at 37℃ for 2 hours, while being gently shaken, in the CO_2 incubator. The excessive amount of MTT reagent was washed out, and then the remaining MTT was extracted with an MTT extractant solution contained in the Skin^2 Model ZA0014 kit (Advanced Tissue Sciences, Inc. ; Lot No. 1131). The absorbance of the liquid in each well was measured at 540 nm. Untreated and test chemical-treated cultures were both measured in this fashion, and MTT-50 values were read from a PC-9801 personal com-

puter (NEC, Japan ; Bliss, C. I., 1935, 1957).

In one institution where assays were not performed within 14 days of the production date, cells were later appropriately cultured, and assays were performed.

II. ***In vivo* eye irritation study**

This study using 39 rabbits was performed in accordance with the method described in the Federal Register (1972). Specifically, dose of 0.1-ml of the test chemical solution was applied into the left conjunctival sac, and the same volume of the distilled water for injection into the right. The cornea, iris, and conjunctiva were observed visually and by a slit-lump examination 1, 4, 24, 48, and 72 hours after the application. Typical features of the cornea, iris, and conjunctiva were photographed before the application and on each scheduled post-treatment observation.

After the observation at 72 hours, the animals were sacrificed under the sodium pentobarbital anesthesia.

Data evaluation was based on the Draize's criteria for eye-irritation reactions (Draize *et al.*, 1944) and Kay and Calandra's irritation criteria (Kay *et al.*, 1962).

III. **Statistics**

Results were represented as the mean±S. D. values. Statistical significance was assessed by the use of Student's t-test for unpaired samples, P values of less than 0.05 being considered significant.

RESULTS

I. ***In vitro* cytotoxicity study**

1. MTT-50 values

Cell viability curves in the presence of the test chemicals are shown in Fig. 1~4. MTT-50 values (the concentration that kills 50% of the cells in the tissues) were estimated from these curves, and listed in Table 1.

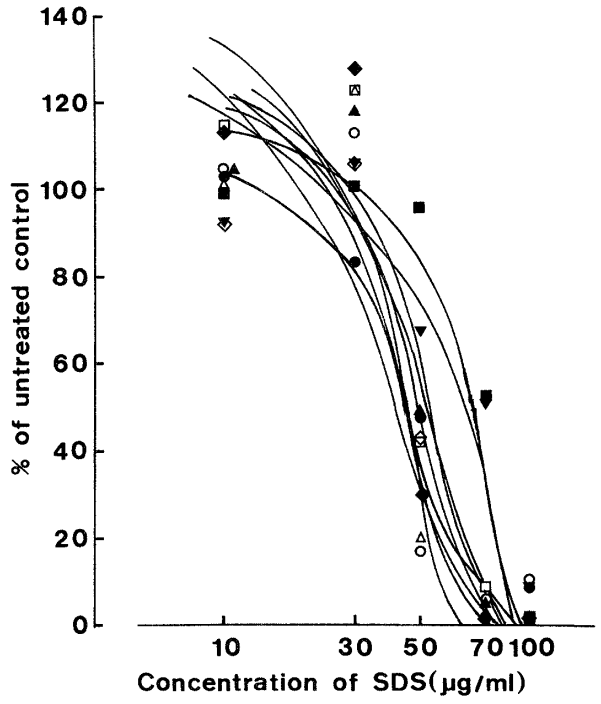


Fig. 1 Viability of cell treated with SDS.

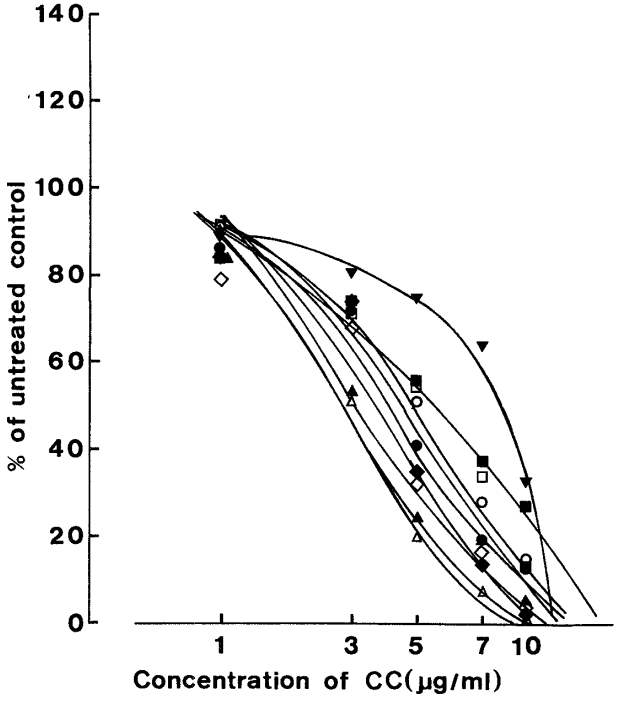
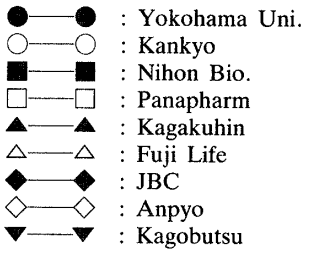
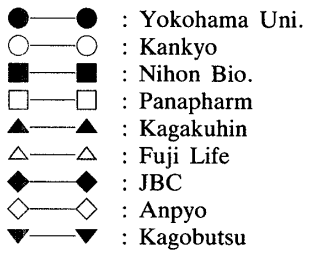


Fig. 2 Viability of cell treated with CC.





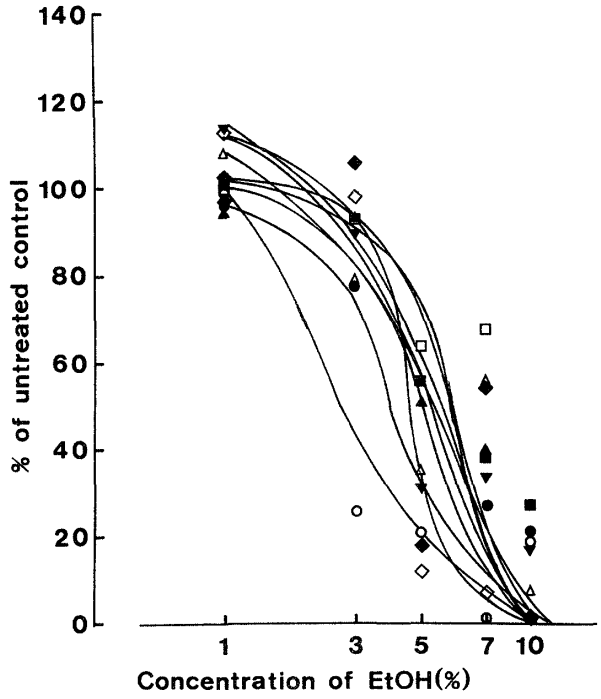


Fig. 3 Viability of cell treated with EtOH.

: Yokohama Uni.
: Kankyo
: Nihon Bio.
: Panapharm
: Kagakuhin
: Fuji Life
: JBC
: Anpyo
: Kagobutsu

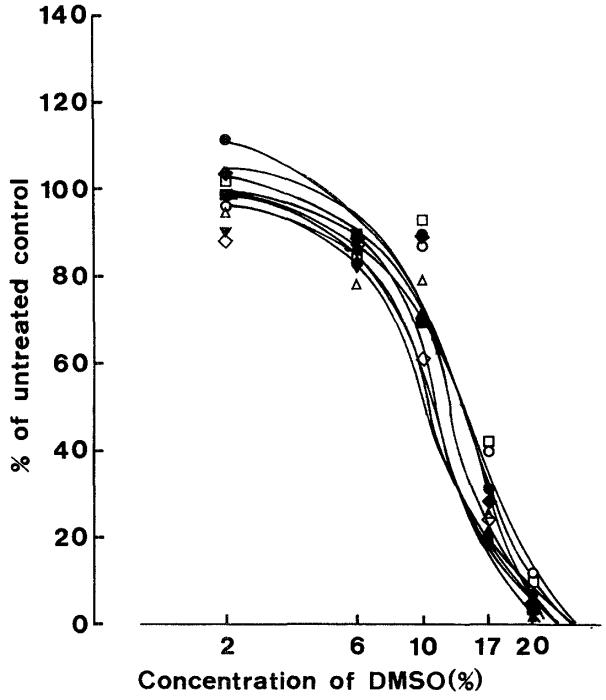


Fig. 4 Viability of cell treated with DMSO.

: Yokohama Uni.
: Kankyo
: Nihon Bio.
: Panapharm
: Kagakuhin
: Fuji Life
: JBC
: Anpyo
: Kagobutsu

Table 1. MTT-50 values from *in vitro* cytotoxicity studies.

Test agent	MTT-50 value [1) 2)]			
	n	X σ		C. V. (%)
SDS	9	51 ±8	μg/ml	17
CC	9	4.3±1.9	μg/ml	44
EtOH	9	4.9±1.0	V/V%	20
DMSO	9	13.9±5.8	V/V%	14

1) : Concentrations of the test agents extrapolated from the dose-survival curve giving 50% cell survival.
2) : Each of the test agents were assayed in quadruplicate at each of five concentration along with quadruplicate untreated controls.

MTT-50 values obtained on the first measurement at the individual institutions were 42 to 76 μg/ml (mean 49 μg/ml) for SDS, 3.1 to 10.9 μg/ml (mean 7.0 μg/ml) for CC, 3.5 to 7.2% (mean 3.9%) for EtOH, and 11.8 to 21.9% (mean 13.4%) for DMSO. The corresponding values obtained on the second measurement were 44 to 91 μg/ml (mean 54 μg/ml), 2.2 to 8.6 μg/ml (mean 2.4 μg/ml), 2.0 to 9.3% (mean 5.9%), and 7.5 to 17.0% (mean 10.0%), respectively. Averages of the first and second measurements were 51±8 μg/ml for SDS, 4.3± 1.9 μg/ml for CC, 4.9±1.0% for EtOH, and 11 ± 1.7% for DMSO. The coefficient of variation was 17% for SDS, 44% for CC, 20% for EtOH, and 14% for DMSO.

II. ***In vivo* eye irritation study**

The results are shown in Table 2.

All eyes treated with 5 or 10% SDS solutions showed diffuse conjunctival injection, conjunctival swelling, and increased conjunctival discharge from 1 hour post-treatment on, and diffuse corneal opacity from 24 hours on. These changes persisted until 72 hours. With 2% SDS solution, the conjunctivae in all treated eyes showed slight injection at 1 to 24 hours, but there was no abnormality in the cornea or iris.

All eyes treated with 1, 2, or 5% CC solutions showed diffuse conjunctival injection, conjunctival swelling, and increased conjunctival discharge from 1 hour post-treatment on. Corneal opacity was observed from 24 hours on in the eyes treated with 2 or 5% CC solutions. With 5% solution, iris swelling appeared at 24 hours, and persisted until 72 hours.

All eyes treated with 50 and 70% EtOH solutions showed diffuse conjunctival injection, conjunctival swelling, and increased conjunctival discharge from 1 hour post-treatment on. Diffuse corneal opacity was observed from 24 hours on in the eyes treated with 70% EtOH solution. EtOH at 30% did not induce any observable changes in the cornea, iris, or conjunctiva.

Two of three eyes treated with 90% DMSO solution showed injection and/or slightly increased conjunctival discharge from 1 hour post-treatment on, but these disappeared by 48 hours. DMSO at 30, 50, or 70% did not induce any identifiable changes in the cornea, iris, or conjunctiva.

The sum of all scores obtained for the cornea, iris, or conjunctiva at each time of inspection was calculated. The eye irritation was evaluated according to Key and Calandra (1962), and the results are shown in Table 2.

The respective mean scores of SDS at 5 and 10% were 14.2 and 13.2 points, and these treatments were evaluated as "minimally irritating." The respective results for other test chemicals were 2.4 points and "nonirritating" for 2% SDS ; 22.4 and 29.0 points and "mildly irritating" for 2 and 5% CC ; 14.8 points and "minimally irritating" for 1% CC ; 15.6 points and "mildly irritating" for 70% EtOH ; 8.2 points and "minimally irritating" for 50% EtOH ; and 1.6 points and "practically nonirritating" for 90% DMSO. The remaining 30% EtOH as well as 30, 50, and 70% DMSO were evaluated as "nonirritating."

Table 2. Result of *in vivo* eye irritation test (Draize method).

Test agent	Concentration (%)	Animal of treatment	Mean of total ocular irritation score Time after application (hrs) 1	4	24	48	72	Mean total score	Draize rank[1)]
SDS	2	3	5	5	1	1	0	2.4	Non
	5	3	15	13	18	16	9	14.2	Minimally
	10	3	13	13	17	14	9	13.2	Minimally
CC	1	3	17	16	16	15	10	14.8	Minimally
	2	3	18	18	21	24	31	22.4	Mildly
	5	3	20	18	37	36	34	29.0	Midly
EtOH	30	3	0	0	0	0	0	0	Non
	50	3	13	12	8	7	1	8.2	Minimally
	70	3	12	13	24	15	14	15.6	Mildly
DMSO	30	3	0	0	0	0	0	0	Non
	50	3	1	0	0	0	0	0.2	Non
	70	3	1	0	0	0	0	0.2	Non
	90	3	3	3	2	0	0	1.6	Non

[1)] : According to the scale of Kay and Calandra (1962).

Fig. 5 illustrates the relationship between the concentration of the test chemicals and the sum of all scores obtained for the cornea, iris, and conjunctiva. All chemicals tested showed a correlation between the two parameters : the higher the concentration, the higher the total score.

Fig. 6 illustrates the relation between MTT-50 values obtained from the test using the Skin^2 Model ZK1100 kit and DS20 values obtained from Draize test. The following correlation was noted between the two parameters obtained for the 4 test chemicals : $Y=0.2702X+2.9613$ ($r=0.9881$).

DISCUSSION

A large number of drugs, pesticides, and cosmetics are now on the market. The toxicity of those products has been studied in animal experiments. Parties for the prevention of cruelty to animals have severely criticized such experiments, and have requested testing methods which would not use animals. *In vitro* testing methods were recently introduced as an alternative. They are not cruel to animals, are less expensive, enable rapid precise evaluations, are easily standardized, and show better reproducibility of results. It is difficult to completely replace animal experiments with non-animal experiments. However, a step-by-step replacement procedure will improve the avenue of animal tester. Experiments without animals are the most favorable, and a decrease in the number of animals to be used is the second most favorable. The third choice is to reduce the animals' pain. Unfortunately, there is no established system to approve the extrapolation of the results of an *in vitro* study to an *in vivo* study.

As a result of reviewing the data, 2 of the 11 participanting institutions were excluded from statistical analysis : one institution did not prepare control groups, and the other tested some other chemicals which had not been designated as the test articles. Nine institutions tested the 4 test chemicals at the designated concentrations. Deviant data at one of these 9 institutions do not reflect an inter-institution difference, but reflects the fact that the second assay for EtOH and DMSO could not be done.

The mean MTT-50 value was 51 μg/ml for SDS, 4.3 μg/ml for CC, 4.9% for EtOH, and 11% for DMSO. Previously reported MTT-50 values for SDS were 60 and 46 μg/ml (Triglia *et al.*, 1991) or 62, 60, and 46 μg/ml (J. P. Weintraub). The presently obtained mean MTT-50 value was consistent with 46 μg/ml reported by J. P. Weintraub and 46 μg/ml reported by D. Triglia. According to J. P. Weintraub, the mean MTT-50 for SDS varied

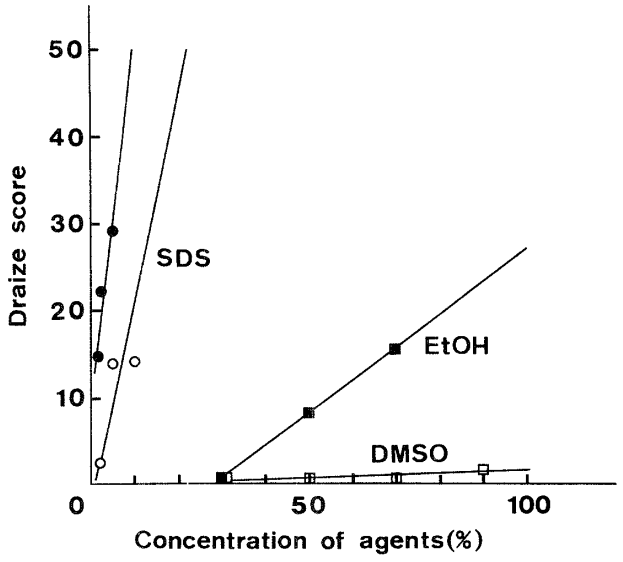


Fig. 5 Rank correlation of agent concentration and eye irritation in the Draize test (score).

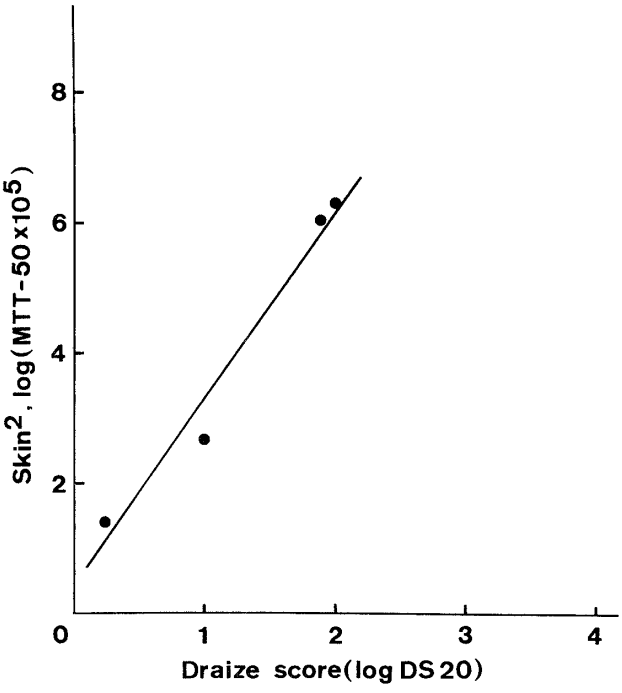


Fig. 6 Rank correlation of the cytotoxicities of cells *in vitro* and eye irritation by Draize test.

from 46 to 62 μg/ml when measured repetitively in one identical institution. EtOH was reported to have an MTT-50 value of 2.5% by D. Triglia (Triglia *et al.*, 1991). The presently obtained value of 4.9% was slightly higher.

In one institution, the MTT-50 values measured for SDS, DMSO, and EtOH were different between the first and second assays : the second values were about twice as high as the first. In the second assay, cells were incubated for 18 hours, and the incubation time was further prolonged. The reason why the second assay produced higher MTT-50 values is unknown. EtOH produced a biphasic curve in some cases but not in other cases although the concentrations tested were equal. It should be investigated whether this variation was ascribable to the assay system factors or technical factors. When repetitively tested by the same investigator in the same institution, the MTT-50 value for any of SDS, CC, EtOH, and DMSO was reasonably well reproduced with a variation of less than 2σ. The variation coefficient varied from 14% for DMSO to 44% for CC. These values were considered to be small when various factors of the individual institutions were taken into account.

The four test chemicals used in the joint research were also tested with the conventional Draize method in one institution. The mean sum of the scores obtained for the cornea, iris, and conjunctiva at each time of inspection correlated with the concentration of the test chemical. DS20 values obtained with Draize method were highly consistent with MTT-50 values obtained with the the Skin^2 Model ZK1100 kit ($r=0.9881$). From these results, we concluded that Skin^2 Model ZK1100 kit provide a rapid and useful quantitative method to test a chemical's capacity to irritate the skin and the eyes. The use of Skin^2 Model ZK1100 kits decreases the total number of testing days because it takes only 2 days to complete each test whereas the conventional Draize method requires 14 days of observation (including 10 days of quarantine) and 24 days oftesting. The testing expense can be cut by half by using the Skin^2 Model ZK1100 kit instead of Draize method. Thus, it was suggested that the method using cultured tissues is available as an alternative to the conventional method of the eye irritation study.

ACKNOWLEDGMENTS

The authors thank Mrs. Pam K. Logemann for reviewing the manuscript and checking the English. We are also grateful to Dr. Tagahiko Morinaga for their kindly providing with the computer program.

REFERENCES

Advanced Tissue Sciences Inc. (1992) : The Lab Partner, vol. 2, No. 11, pp.1–2.

Bliss, C. I. (1935) : The calculation of the dose-mortality curve. Ann. Appl. Biol., **22**, 134–167.

Bliss, C. I. (1957) : Some principles of bioassay. Am. Sci., **45**, 449–466.

Draize, J. H., Woodard, G. C., Calvery, H. O. (1944) : Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther., **82**, 377–390.

Draize, J. H. (1959) : "Dermal Toxicity", Appraisal of Safety of Chemicals in Foods, Drugs and Cosmetics, pp. 46–59, Association of Food & Drug officials of the United States, Topeka, Kansas.

Kay, J. H. (1962) : Interpretation of eye irritation of tests. J. Soc. Cosmetic Chemists., **13**, 281–289.

Marrow-Tech, Inc., Skin^2 Dermal Model Kit (CA).

Marrow-Tech, Inc. (now Advanced Tissue Sciences Inc.) (1991) : The Lab Partner, No. 4, pp. 2–4.

Triglia, D., Braa, S. S., Donnelly, T., *et al.*(1991) : A three dimensional human dermal model substrate for *in vitro* toxicological studies. *In* Alternative Methods in Toxicology, **8**, 351–362 A. M. Goldberg ed., MaryAnn Liebert, Inc., New York.

U. S. Federal Register (1972) : 191 ; pp. 8534–8535, April.