AATEX 2, 183–191 (1994)

In Vitro Assays to Predict Phototoxicity of Chemicals: (I) Red Blood Cell Hemolysis Assay

Mariko Sugiyama, Hiroshi Itagaki, Takeshi Hariya, Noriko Murakami and Shinobu Kato

Shiseido Safety & Analytical Research Center, 1050 Nippa-cho, Kohoku-ku, Yokohama, Japan, 223

Summary

Photohemolysis testing of red blood cells (RBC), a phenomenon based on photodynamic reaction of the cell membrane, was validated as a possible alternative method to predict phototoxicity of chemicals after optimization of various factors. The data from phototoxicity test results in guinea pigs for 24 chemicals (8 fragrances, 5 UV absorbers, 4 drugs, 4 antimicrobials and 3 dyes) were compared. The sensitivity, specificity, positive predictive value, negative predictive value and equivalence of the *in vitro* test were 67%, 73%, 60%, 79% and 73%, respectively. Four chemicals were identified as false positive compounds, and two psoralens, of which the phototoxic mechanism is different from cell membrane damage, were identified as false negative compounds in the photohemolysis test. This *in vitro* method is expected to be useful as a prescreening tool for evaluating phototoxic potential of chemicals, and should be an effective means of reducing the number of animals required for phototoxicity testing.

Introduction

Phototoxicological risk of chemicals has been assessed by testing in animals such as guinea pigs[1–5], rabbits[2,3] and mice[6–9]. However, from the viewpoint of animal welfare, it is desirable to develop reliable *in vitro* techniques instead. There have been various reports regarding *in vitro* methods for prediction of phototoxicity. Daniels[10] proposed a simple microbiological method evaluating damage to DNA or cell organelles. He investigated plants or plant materials possessing psoralen-type photosensitizing properties and found that they showed positive reactions. A photohemolysis test with red blood cells (RBC) developed by Kahn and Fleischaker[11] detects impairment of the function and integrity of the cell membrane; they evaluated 26 chemicals including well-known phototoxic chemicals, and suggested that this method may be a useful tool for screening drugs. However, both approaches failed to demonstrate phototoxicity of some well-known phototoxic chemicals such as Rose Bengal in the microbiological method and psoralens in the RBC photohemolysis method.

To develop a reliable alternative method with which to predict phototoxicity, we focused on photohemolysis which is considered to reflect membrane damage. Photohemolysis methods have been described in excellent reviews on *in vitro* methods for phototoxicity testing[12–14]. Hethering and Johnson[15] reported the detailed procedure and rationale of the photohemolysis technique. Several investigators have reported studies of the mechanisms involved[16], especially oxygen-dependent membrane impairment[17,18].

Key Words: *in vitro* test, photohemolysis, phototoxicity.

Correspondence to: Mariko Sugiyama
Shiseido Safety & Analytical Research Center, 1050, Nippa-cho, Kohoku-ku, Yokohama, Japan, 223
Tel: (Japan) 045–542–1339
Fax: (Japan) 045–546–4456

Although various methods for assessing photoxicity have been reported thus far, there have been few reports from the viewpoint of finding alternatives to animal testing.

In the present study, after optimizing UV source, dose and RBC source, we used the RBC photohemolysis test to assess the phototoxic potential of 24 chemicals. The data were compared to *in vivo* data in order to evaluate the feasibility of using photohemolysis testing for predicting phototoxicity of chemicals.

Materials and Methods

1. Test chemicals and in vivo test

1) Test chemicals

All test chemicals were obtained commercially and used without purification. Twenty-four chemicals (Table 1), consisting of 8 fragrance materials, 5 UV absorbers, 4 drugs, 4 antimicrobials and 4 dyes, including well-known phototoxic chemicals, all of which exibit UV absorption, were evaluated. These chemicals (reagent grade) were from Nakalai Tesque, Inc. (Kyoto, Japan), Sigma Chemical Co. (St. Louis, U.S.A.), Aldrich Chemical Company, Inc. (Milwaukee, WIS, U.S.A.), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Eastman Kodak Company (N.Y., U.S.A.). UV absorbers were obtained from Givaudan-Roure Corp. (N.J., U.S.A.), Shonan Kagaku Kogyo Ltd. (Hiratuka, Japan) and Van Dyke & Company, Inc. (N.J., U.S.A.). Some of the fragrance materials were obtained from perfumeries.

2) In vivo test

Our *in vivo* data were used for comparison with the *in vitro* data, so that no further animal testing was performed for the purpose of the present study. The technique used in the *in vivo* study was basically the same as the common phototoxicity test reported by Morikawa et al.[3]: Hartley-strain albino guinea pigs weighing 450–550 g were used, and each chemical was tested in at least five animals. Their dorsal area was shaved and depilated 24 h before exposure. A 0.02 ml aliquot of the test material was topically applied onto two sites on the back. One of these sites was covered with aluminum foil, and the other was exposed to UV irradiation (320–400 nm) from six fluorescent lamps (Toshiba 40-BLB fluorescent lamp) through a glass filter excluding wavelengths below 320 nm. The distance between the lamps and skin was 10 cm. The total energy of UV irradiation was 14.0 J/cm^2. After irradiation, the animals were returned to individual cages, and their skin reaction was assessed 24, 48 and 72 h after exposure according to the standard scoring system (erythema, 0–3; edema, 0–2). To compare *in vivo* data with *in vitro* data, reactions in guinea pigs were classified to three grades. Average scores at the time of maximal response were calculated, and the phototoxicity of chemicals was assessed according to the classification by maximal score: (−, 0–0.05; ±, 0.6–1.2; +, 1.3–5.0).

2. Photohemolysis of RBC

A modification of the photohemolysis technique described by Kahn and Fleischaker[11] was used in the present study. Red blood cells (RBC) were obtained from healthy human volunteers or guinea pigs, and sheep RBC were purchased from Nippon Bio-supp. Center Co., Ltd. (Tokyo, Japan). RBC were washed in physiological saline and centrifuged for 10 min at 3,000 rpm (900 g). This procedure was repeated three times, and the RBC were used within 40 h thereafter to avoid spontaneous hemolysis. The RBC were suspended in Na-Veronal buffer (3.1 mM barbital, 1.8 mM barbital sodium, 145 mM NaCl, pH 7.4) to make 2.5% (v/v) suspension.

Then, 990 μl aliquots of RBC suspensions were dispensed into each well of a 24-well microplate (Becton, Dickinson & Co., Lincoln Park, NJ, USA), 10 μl of test chemical solution prepared by serial five-fold dilution with solvent was added to each well and the plate was shaken for 30 sec. In cases where the

Table I. Test chemicals and *in vivo* data in guinea pigs.

Chemicals	Supplier	Concentration of chemical (%)	Phototoxic reaction Guinea pigs
Fragrance materials (8)			
Musk Ambrette	10	20.0	−
Musk Ketone	10	20.0	−
Musk Xylene	10	20.0	−
Phantolid	10	20.0	+
Galaxolide (50% in diethyl phthalate)	10	70.0	−
8-Methoxy Psoralen (8-MOP)	1	0.02	−
5-Methoxy Psoralen (5-MOP)	2	0.025	−
6-Methyl Coumarin (6-MC)	4	1.0	+
UV Absorbers (5)			
4-t-Butyl-4-methoxydibenzoylmethane (Parsol 1789)	7	10.0	−
2-Ethylhexyl-p-methoxycinnamate (Parsol MCX)	7	20.0	−
2-Hydroxy-4-methoxybenzophenone (ASL-24)	8	10.0	−
2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid (ASL-24S)	8	5.0	−
2-Ethylhexyl-p-dimethylaminobenzoate (Escalol 507)	9	20.0	±
Drugs (4)			
Sulfanilamide	1	20.0	−
Indomethacin	2	1.0	−
Piroxicam	2	5.0	−
Chlorpromazine HCl (CPZ)	1	50.0	+
Antimicrobials (4)			
3,4,4'-Trichlorocarbanilide (TCC)	3	5.0	−
Bithionol	2	1.0	−
3,4',5-Tribromosalicylanilide (TBS)	5	1.0	−
3,3',4',5-Trichlorosalicylanilide (TCSA)	6	1.0	+
Dyes (3)			
Rose Bengal	1	1.0	−
Acridine	2	1.0	+
Anthracene	1	0.5	+

1: Nakalai Tesque, Inc.
2: Sigma Chemical Co.
3: Aldrich Chemical Company, Inc.
4: Wako Pure Chemical Industries, Ltd.
5: Tokyo Kasei Kogyo Co., Ltd.
6: Eastman Kodak Company.
7: Givaudan-Roure Corp.
8: Shonan Kagaku Kogyo, Ltd.
9: Van Dyke & Company, Inc.
10: Fragrance materials were obtained from perfumeries.

test compounds were insoluble in water, they were dissolved in methanol, ethanol, acetone or dimethyl sulfoxide. None of these solvents *per se* had any effect on hemolysis.

The microplates were then irradiated with long wavelength ultraviolet light (UVA) or medium wavelength ultraviolet light (UVB) at room temperature. The radiation source was a transilluminator (Vilber Lourmat, France), and the emission spectrum was from 320 nm to 400 nm with a peak at 365 nm for UVA (T-15L) and from 270 nm to 330 nm with a peak at 312 nm for UVB (T-15M). The output of these lamps was set at 2.9 mW/cm^2 (UVA) or at 0.9 mW/cm^2 (UVB) at the 24-well microplate as measured with a UV radiometer

(Topcon, Japan). The non-irradiated control was kept in the dark at room temperature.

After irradiation, the microplates with samples were centrifuged for 15 min at 2000 rpm (400 g), and 100 μl of supernatants were transferred into the wells of a 96-well microplate (Becton, Dickinson & Co., Lincoln Park, NJ, USA). The absorbance at 540 nm, the wavelength typical of hemoglobin, was measured with an EIA reader system (BIO-RAD Model 3550) as a indicator of hemolysis.

Calculation

Percent hemolysis was calculated with respect to 100% hemolyzed solution by using the following equation.

Photohemolysis (%)=

$$\frac{[\text{Irradiated group}]}{} \quad \frac{[\text{Dark control}]}{}$$

$$\frac{(HC1-HS1)\times 100}{(T-HB1)} - \frac{(HC2-HS2)\times 100}{(T-HB2)}$$

where:

HC1: Absorbance of hemoglobin released by chemicals with irradiation.

HC2: Absorbance of hemoglobin released by chemicals without irradiation.

HS1: Absorbance of hemoglobin released by solvent with irradiation.

HS2: Absorbance of hemoglobin released by solvent without irradiation.

HB1: Absorbance of hemoglobin released by buffer with irradiation.

HB2: Absorbance of hemoglobin released by buffer without irradiation.

T: Absorbance of total hemoglobin in completely hemolyzed solution with or without irradiation.

Classification

To correlate photohemolytic activity with *in vivo* phototoxicity data, the grade of photohemolysis of each chemical was classified into one of 3 classes as follows.

+: photohemolysis≧10%.

±: 10%>photohemolysis≧5%.

−: 5%>photohemolysis.

Results and Discussion

1. In vivo study

The guinea pig phototoxicity test results are shown in Table I. The numbers of chemicals classified into the positive, equivocal and negative reaction categories were 7, 1 and 16, respectively.

2. Photohemolysis of RBC

1) Effect of wavelength

Photohemolysis with UVA (30 J/cm^2) or UVB (1.0 J/cm^2) was compared in the presence of anthracene, chlorpromazine HCl (CPZ), 3,3',4',5-trichlorosalicylanilide (TCSA) or sulfanilamide (Fig. 1). The final concentration of test chemical was 0.8 100 μg/ml for anthracene or TCSA, and 4–800 μg/ml for CPZ or sulfanilamide.

Photohemolysis was obviously greater with UVA than with UVB at all concentrations of anthracene or TCSA. In contrast, photohemolysis was almost equal with UVA and UVA in the presence of 500 μg/ml and 100 μg/ml CPZ, but was higher with UVA than with UVB at the lower concentrations of 4 μg/ml and 20 μg/ml. Photohemolysis was hardly observed with sulfanilamide under UVA or UVB irradiation.

In general, the optimum wavelength of irradiation is considered to depend on the absorption spectra of chemicals, but the majority of phototoxic chemicals are active with UVA[19]. Also, our accumulated *in vivo* phototoxicity data were acquired under UVA. Furthermore, it is reported that a high dose of UVB alone can cause hemolysis[15,20] even with non-phototoxic chemicals.

For a screening test of chemicals, it may be best to use both UVA and UVB, because each chemical has a specific action spectrum. However, because clearly positive photohemolysis was observed with 3 well-known phototoxic chemicals, we selected UVA irradiation for the present study.

2) Effect of UVA dose

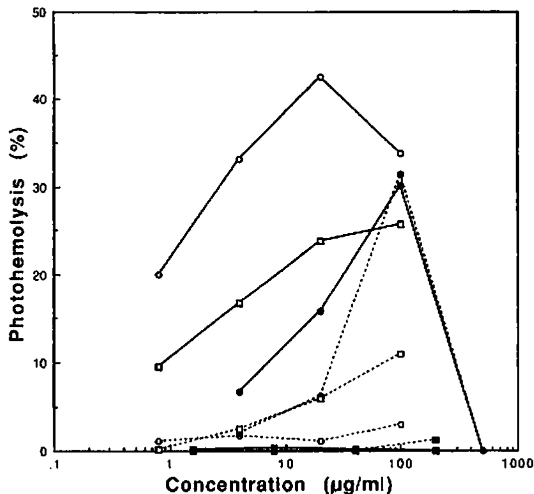

Fig. 1. Dose-response curves for phtohemolysis with UVA or UVB, in the presence of anthracene, CPZ, TCSA or sulfanilamide.
Anthracene (-○-: UVA 30 J/cm², --○--: UVB 1 J/cm²), CPZ (-●-: UVA 30 J/cm², --●--: UVB 1 J/cm²), TCSA (-□-: UVA 30 J/cm², --□--: UVB 1 J/cm²) and sulfanilamide (-■-: UVA 30 J/cm², --■--: UVB 1 J/cm²)

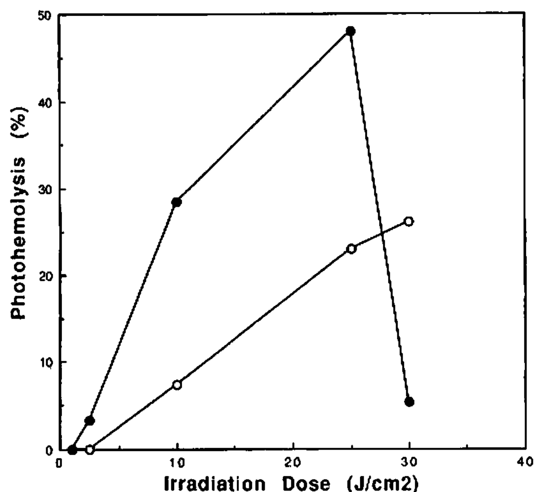

Fig. 2. Dose-response curves for photohemolysis with UVA (1–30 J/cm²), in the presence of anthracene (○) or CPZ (●).

Doses of 2.5, 10, 25 and 30 J/cm² of UVA were applied in the presence of anthracene or CPZ to find the optimum dose (Fig. 2). The final concentration of test chemicals was 100 μg/ml.

Photohemolysis with anthracene was increased by UVA dose-dependently. Photohemolysis with CPZ was also increased up to

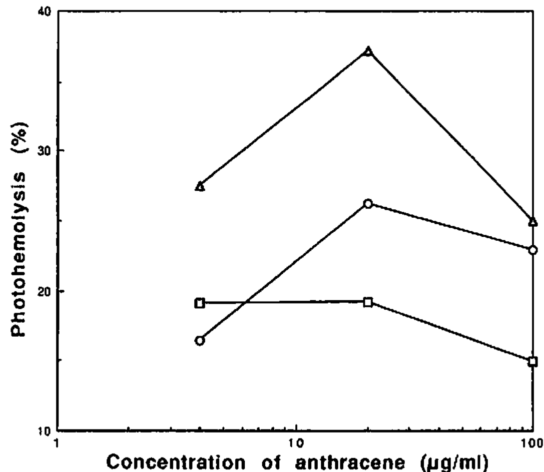

Fig. 3. Dose-response curves for photohemolysis of RBC obtained from human (○), guinea pig (△) and sheep (□), in the presence of anthracene.

25 J/cm², but declined at 30 J/cm², possibly because of methemoglobin formation. Under UV light, hemoglobin (λ max=540 nm) is known to be degraded to methemoglobin, which has a reddish-brown color (λ max=630 nm). Thus, it is important to select the appropriate irradiation energy so that the degradation of hemoglobin does not cause false results. We selected a 25 J/cm² irradiation dose for our screening test, because sufficient photohemolysis was observed.

3) Effect of RBC sources

RBCs were obtained from different animal species and photohemolysis in the presence of anthracene was examined (Fig. 3). RBC were obtained by venipuncture from healthy human volunteers and guinea pigs, and RBC samples from sheep were purchased. Before examination, we confirmed that photohemolysis fluctuated little between individuals, or with ages, sex and lot of RBC. These RBCs were irradiated with UVA (25 J/cm²) in the presence of anthracene (4–100 μg/ml).

Guinea pig RBC showed the highest photohemolysis at all test concentrations. Photohemolysis of human RBC was less than that of guinea pig RBC, but greater than that of

sheep RBC at 20 μg/ml and 100 μg/ml of anthracene. Kumar and Joshi[21] studied the differences of photohemolysis among different animal species with UVB, and reported that erythrocytes from different animals showed various photohemolytic characteristics. They also found that photohemolysis of human RBC was greater than that of sheep RBC.

We selected human RBC for our screening test because they are easily available, can be obtained without anesthesia, and show sufficient response to UV exposure.

4) Photohemolysis of RBC by 24 chemicals

The photohemolysis results, classification and *in vivo* data are summarized in Table II. For the purpose of comparison of phototoxic abilities predicted from the photohemolysis data and phototoxic reactions in guinea pigs, the results were arranged as shown in Table III. To evaluate the *in vitro* method, 5 parameters reported by Balls et al.[22], were used. The calculation method is shown in Table IV and Table V.

The photohemolysis technique had a sensitivity of 67%, a specificity of 73%, a positive predictive value of 60%, a negative predictive value of 79% and an equivalence of 73% (Table IV).

Two chemicals (8-methoxy psoralen [8-MOP] and 5-methoxy psoralen [5-MOP]),

Table II. Photohemolysis of RBC induced by 24 chemicals.

Chemicals	(Solvent)	Photohemolysis (%) (final concn; μg/ml)	Phtotoxic reaction *in vitro* RBC	*in vivo* Guinea pigs
Fragrance materials (8)				
Musk Ambrette	(Acetone)	14.5 (200)	+	−
Musk Ketone	(Acetone)	0 (1000)	−	−
Musk Xylene	(Acetone)	1.9 (600)	−	−
Phantolid	(Acetone)	33.5 (200)	+	+
Galaxolide	(Acetone)	15.9 (400)	+	+
8-MOP	(Ethanol)	0.8 (100)	−	+
5-MOP	(Ethanol)	0.3 (100)	−	+
6-MC	(Ethanol)	3.6 (1000)	−	−
UV Absorbers (5)				
Parsol 1789	(Ethanol)	0.6 (1000)	−	−
Parsol MCX	(Ethanol)	0.7(10000)	−	−
ASL-24	(Ethanol)	2.3 (500)	−	−
ASL-24S	(Distilled water)	0.3 (500)	−	−
Escalol 507	(Ethanol)	4.1 (2000)	−	+
Drugs (4)				
Sulfanilamide	(Methanol)	0.3 (500)	−	−
Indomethacin	(Ethanol)	0.3 (100)	−	−
Piroxicam	(Acetone)	0.3 (200)	−	−
CPZ	(Distilled water)	47.9 (100)	+	+
Antimicrobials (4)				
TCC	(Acetone)	0.5 (200)	−	−
Bithionol	(Acetone)	44.8 (80)	+	−
TBS	(Acetone)	10.4 (400)	+	−
TCSA	(Acetone)	22.7 (100)	+	+
Dyes (3)				
Rose Bengal	(Dimethyl sulfoxide)	33.5 (100)	+	−
Acridine	(Acetone)	33.4 (40)	+	+
Anthracene	(Acetone)	21.8 (100)	+	+

All samples were irradiated with UvA 25 J/cm[1]. The RBC were obtained from several human volunteers.

Table III. Comparison of phototoxic ability predicted from photohemolysis with the phototoxic reactions observed in guinea pigs.

in vitro \ *in vivo*	+	±	−
+	Phantolid Galaxolide CPZ TCSA Acridine Anthracene	None	Musk Ambrette Bithionol TBS Rose Bengal
±	None	None	None
−	8-MOP 5-MOP	Escalol 507	Musk Ketone Musk Xylene 6-MC Parsol 1789 Parsol MCX ASL-24 ASL-24S Sulfanilamide Indomethacin Piroxicam, TCC

which produced severe phototoxicity in guinea pigs, did not induce positive photohemolysis (false negative). On the other hand, 4 chemicals (Musk Ambrette, Rose Bengal, 3,4',5-tribromosalicylanilide [TBS] and Bithionol) showed positive reactions *in vitro*, while they were classified as negative in the *in vivo* test (false positive).

TCSA, Musk Ambrette, CPZ, Bithionol and TBS were positive in our present study, in accordance with the findings reported previously by Freeman[26], Kahn and Fleischaker[11] and Johnson et al.[13]. Our negative results with 8-MOP and 5-MOP confirmed the results of previous investigations. Since photohemolysis is based on damage to the cellular membrane, the negative result obtained with psoralen compounds suggests that their *in vivo* phototoxicity is mediated by a different mechanism. Musajo et al.[25] reported that psoralen combines with DNA to produce its phototoxic effect. Sulfanilamide, which was negative in our system, was reported to show 30% photohemolysis by Kahn and Fleischaker[11], although the conditions of photohemolysis were the same (pH, solvent and buffer system) and the irradiation energy was lower than that in the present study. Freeman[26] reported previously the same result for sulfanilamide as that obtained in our present study. Ljunggren[20] reported that indomethacin and piroxicam caused slight

Table IV. Terms used to describe screening tests and screening programs.

Parameter	Meaning	Equation*	Value
Sensitivity	The ratio of the number of positive and equivocal measurements by the *in vitro* method to the total number of phototoxic and equivocal chemicals.	$(a+b+d+e)/(a+b+d+e+g+h)$	67%
Specificity	The ratio of the number of negative measurements by the *in vitro* method to the total number of non-phototoxic chemicals.	$i/(c+f+i)$	73%
Positive predictive Value	The ratio of the number of phototoxic and equivocal chemicals to the total number of positive and equivocal measurements *in vitro*.	$(a+b+d+e)/(a+b+c+d+e+f)$	60%
Negative predictive Value	The ratio of the number of non-phototoxic chemicals to the total number of negatives *in vitro*.	$c/(g+h+i)$	79%
Equivalence	The ratio of the number of chemicals classified into the same classification, positive, equivocal or negative, *in vitro* and *in vivo*, to the total number of chemicals.	$\frac{\{1.0(a+e+i)+(0.5(b+d+f+h)\}\times 100}{a+b+c+d+e+f+g+h+i}$	73%

*a-i: The numbers of chemicals classified as shown in Table V.

Table V. The relationship between *in vivo* and *in vitro* results.

		In vito (guinea pigs) +	±	−
in vitro	+	a	b	c
	±	d	e	f
	−	g	h	i

a-i: The numbers of chemicals falling into the indicated classifications in the Table.

photohemolysis with UVB but not with UVA, which is in accordance with our findings. It appears that the result of photohemolysis can depend on the irradiation source.

The chemicals identified as false positive compounds in the photohemolysis test (Musk Ambrette, Rose Bengal, TBS and Bithionol) seem to induce photodynamic reactions. That is, photohemolysis is a phenomenon based on photodynamic reaction of chemicals, but the test does not allow us to distinguish between phototoxicity and photoallergenicity. Musk Ambrette, TBS and Bithionol have previously been reported to be photoallergens[23]. Rose Bengal and Bithionol showed no phototoxicity in guinea pigs, although they shown to induce phototoxicity in rabbits[3,24].

The results of the present *in vitro* method correlated reasonably well with those of the *in vivo* test, and the procedure is both simple and reproducible. Moreover, the endpoint is based on the mechanism of phototoxicity. In conclusion, the results suggest that photohemolysis testing may be useful as an alternative to animal experiments for predicting the phototoxicity of chemicals. By combining techniques to predict damage to the cellular membrane such as the photohemolysis test with those to estimate the effects on cell organelles, it should become possible to predict phototoxicity with considerable accuracy.

Acknowledgment

The authors are grateful to Mr. Masato Hatao for his advice.

Recieved: December 20, 1993; accepted: June 15, 1994

References

1) Sam, W.N., (1966) The experimental production of drug phototoxicity in guinea pigs. *Arch. Dermatol.*, **94**, 773–777.
2) Marzulli, F.N. and Maibach, H.I. (1970) Perfume phototoxicity, *J. Soc. Cosmet. Chem.*, **21**, 695–715.
3) Morikawa, F., Nakayama, Y., Fukuda, M., Hamano, M., Yokoyama, Y., Nagura, T., Ishihara, M. and Toda, K. (1974) Techniques for evaluation of phototoxicity and photoallergy in laboratory animals and man. in Sunlight and Man. Edited by M.A. Pathak, L.C. Harber, M. Seiji and A. Kikuta; Consulting editor, T.B. Fitzpatrick. pp. 529–557, University of Tokyo Press, Tokyo.
4) Kobayashi, F., Wada, Y., and Mizuno, N. (1974) Comparison studies on phototoxicity of chemicals. *J. Dermatol.*, **1**, 93–98.
5) Kochevar I.E., Hoover K.W. and Gamienowski, M. (1984) Benoxaprofen photosensitization of cell membrane disruption. *J. Invest. Dermatol.*, **82**, 214–218.
6) Ison, A.E. and Blank, H. (1967) Testing drug phototoxicity in mice. *J. Invest. Dermatol.*, **49**, 508–511.
7) Barth, J. (1978) Mouse screening test for evaluating protection to longwave ultraviolet radiation. *Br. J. Dermatol.*, **99**, 357–360.
8) Ljunggren, B. (1985) The mouse tail phototoxicity test. *Photodermatology*, **1**: 96–100.
9) Sambuco, C.P. and Forbes, P.D. (1984) Quantitative assessment of phototoxicity in the skin of hairless mice. *Fd. Chem. Toxicol.*, **22**, 233–236.
10) Daniels, F. (1965) A simple microbiological method for demonstrating phototoxic compounds. *J. Invest. Dermatol.*, **44**, 256–267.
11) Kahn, G. and Fleischaker, B. (1971) I. Red blood cell hemolysis by photosensitizing compounds. *J. Invest. Dermatol.*, **56**, 85–90.
12) Gabriel, K.L., Forbes, D.P. and Daniels, R.E. (1985) Phototoxicity. *J. Toxicol.-Cut. & Ocular Toxicol.*, **4**, 185–191.
13) Johnson, B.E., Walker, E.M. and Hetherington, A.M. (1986) *In vitro* models for cutaneous phototoxicity. in Skin Models. Edited by R. Marks and G. Plewig. pp 264–281, Springer—Verlag, Berlin Heidelberg.
14) Maurer, T. (1987) Phototoxicity testing—*in vitro* and *in vivo*. *Fd. Chem. Toxicol.*, **25**, 407–414.
15) Hethering, A.M. and Johnson, B.E. (1983) Photohemolysis. *Photodermatology*, **1**, 255–260.
16) Kochevar, I.E. and Hom, J. (1983) Photoproducts of chlorpromazine which cause red blood cell lysis. *Photochem. Photobiol.*, **37**, 163–168.
17) Cook, J.S. and Blum, H.F. (1959) Dose relationships and oxygen dependence in ultraviolet and photodynamic hemolysis. *J. Cell Comp. Physiol.*, **58**, 41–60.
18) Hayashi, K., Matsuno, I., Fujita, H. and Ohkido, M. (1987) Thiazide phototoxicity: Evidence for participa-

tion of singlet oxygen in photohemolysis. in The Biological Role of Reactive Oxygen Species in Skin. Edited by O. Hayaishi, S. Imamura and Y. Miyauchi. pp 101–106, University of Tokyo Press, Tokyo.

19) Ljunggren, B. and Bjellerup, M. (1986) Systemic drug photosensitivity. *Photodermatology*, **3**, 26–35.

20) Ljunggren, B., Bjellerup, M. and Moller, H. (1983) Experimental studies on the mechanism of benoxaprofen photoreactions. *Arch. Dermatol. Res.*, **275**, 318–323.

21) Kumar, S. and Joshi, P.C. (1992) Hemolysis by ultraviolet B of Red blood cells from different animal species. *Toxicol. in Vitro*, **6**, 345–347.

22) Balls, M., Blaauboer, B., Brusick, D., Frazier, J., Lamb, D., Pemberton, M., Reinhardt, C., Roberfroid, M., Rosenkranz, H., Schmid, B., Spielmann, H., Stammati, A., Walum, E. (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA.*, **18**, 313–337.

23) Maurer, T. (1983) Photobiology and Dermatology in Contact and Photocontact Allergens. Edited by C.D. Calnan, and H.I. Maibach. pp 125–140, Marcel Dekker, Inc., New York and Basel.

24) Takase, Y. (1968) ***SHIGAISEN TO HIFU TOKUNI TARUKEI SHIKISO TO KODOKUSYO***. (Japanese) Journal of J.C.C.A. Special Issue, 135–144.

25) Musajo, L., Rodighiero, G. and Dall's Acqua, F. (1965) Evidence of photoreaction of the photosensitizing furocoumarins with DNA and pyridine nucleosides and nucleotides. *Experientia*, **21**, 24–26.

26) Freeman, R.G. (1970) Interaction of phototoxic compounds with cells in tissue culture. *Arch. Dermatol.*, **102**, 521–526.