

Development of an *in vitro* photosensitization assay using human monocyte-derived cells

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ARTICLE INFO

Article history:

Received 18 November 2008

Accepted 31 March 2009

Available online 7 April 2009

Keywords:

Photosensitization

CD86

CD54

THP-1 cells

In vitro

ABSTRACT

In this study, with the aim of developing a cell-based *in vitro* photosensitization assay, we examined whether changes of CD86 and CD54 expression on cells of a human monocytic cell line, THP-1, could be used to assess the photosensitizing potential of chemicals. First, we identified suitable conditions of UV-irradiation (irradiation dose; 5.0 J/cm², irradiation intensity; 1.7 mW/cm²) by investigating the effect of UV-irradiation on CD86 and CD54 expression on untreated or 6-methylcoumarin (a representative photoallergen)-treated THP-1 cells (irradiation method). However, acridine, a representative photo-irritant, augmented CD86 and CD54 expression on THP-1 cells, apparently via induction of reactive oxygen species (ROS). In order to abolish the effect of ROS, we examined CD86 and CD54 expression on THP-1 cells treated with pre-irradiated chemicals (pre-irradiation method). We found that UV-irradiated photoallergens, but not photo-irritants, enhanced CD86 and/or CD54 expression on the THP-1 cells. Finally, based on the results of irradiation, non-irradiation, and pre-irradiation with 18 test chemicals, we built a decision tree, which allows us to distinguish between photoallergens and photo-irritants. We suggest that this system may be useful for *in vitro* evaluation of the photoallergic potential of chemicals.

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1. Introduction

Evaluation of the safety of ingredients is an important part of the development of cosmetics and drugs. Photosensitization, which includes phototoxicity and photoallergy, is a health hazard arising from exposure of skin to normally harmless levels of light in the presence of a radiation-absorbing material (photosensitizer). Ultraviolet A (UVA) plays a key role (Tokura, 2005). Phototoxicity is the acute toxic response elicited after the first exposure of skin to photosensitizers, followed by irradiation with light. Phototoxicity is mediated by reactive oxygen species (ROS), in particular singlet oxygen (Inbaraj et al., 2005; Onoue and Tsuda, 2005). On the other hand, photoallergy is provoked as a consequence of immunotoxicological response to photosensitizers under light irradiation. Ingredients of cosmetics and drugs that have been reported to induce photocontact dermatitis include antibacterial agents (e.g., 3,3',4',5-tetrachlorosalicylanilide), sunscreen agents (e.g., *p*-aminobenzoic acid), fragrances (e.g., 6-methylcoumarin) and non-steroidal anti-inflammatory drugs (e.g., ketoprofen) (Rietchel and Fowler, 2001). Assessments of phototoxicological properties of materials have generally been performed in animal tests employing guinea pigs, rabbits, rats or mice. However, the development of alternative *in vitro* assays is

now required following the 7th amendment of the European Cosmetics Directive, published in 2003. For phototoxicity testing, the *in vitro* 3T3 NRU phototoxicity test has been adopted as an alternative to *in vivo* phototoxicity tests (Organization for Economic Cooperation and Development (OECD), 2004). Of course, *in vitro* replacements for the *in vivo* photoallergy test have also been sought. The mechanism of photoallergy involves the conjugation of photoallergens to epidermal cells under UVA irradiation. Subsequently, photoantigen-bearing epidermal Langerhans cells sensitize T cells in draining lymph nodes (Kurita et al., 2007). So far, two models of photoantigen formation, the photohapten model and the prohaptent model, have been reported (Tokura et al., 1996; Tokura, 2000). In the induction phase of photoallergy, photoallergens produce ROS and induce apoptosis in keratinocytes, but the use of ROS and apoptosis as biomarkers might result in detection of both photoallergy and phototoxicity (Onoue and Tsuda, 2005; Kurita et al., 2007). Lovell and Jones focused on the formation of photoantigen, and evaluated an *in vitro* test using the photobinding property of photoallergens to human serum albumin (Lovell and Jones, 2000). Barratt et al. reported an *in silico* approach for the prospective identification of photoallergens using a rule-based expert system (Barratt et al., 2000). There have been few reports on *in vitro* cell-based assays able to distinguish photoallergens and photo-irritants.

In our previous reports, we suggested that measuring CD86 and/or CD54 expression on THP-1 (human monocytic leukemia cell

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line) could be used as an *in vitro* skin sensitization assay (Ashikaga et al., 2002, 2006; Sakaguchi et al., 2006). CD86 and CD54 expression on Langerhans cells is increased by exposure to a photoallergen plus UVA irradiation (Nishijima et al., 1999). Therefore, CD86 and/or CD54 expression on THP-1 cells might be useful biomarkers for assessing photoallergic potency. In this study, we evaluated whether induction of CD86 and CD54 expression on THP-1 cells could be used to identify photoallergens and developed a new *in vitro* photosensitization test system based on the results.

2. Materials and methods

2.1. Cells and culture medium

THP-1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 4 mM L-glutamine (Invitrogen Corp.), and 1% penicillin/streptomycin/fungizone (Invitrogen Corp.).

2.2. Chemicals

Chlorpromazine hydrochloride (CPZ) was from Nacalai Tesque, Inc. (Kyoto, Japan). Benzophenone (2-hydroxy-4-methoxybenzophenone) (BP), *p*-phenylenediamine (PPD), ketoprofen, promethazine hydrochloride (PM), bithionol, *p*-aminobenzoic acid (PABA), piroxicam, enoxacin, chlorhexidine dihydrochloride (CHD), 2,4-dinitrochlorobenzene (DNCB), 5-methoxypsoralen (5-MOP), lactic acid (LA) and sodium lauryl sulfate (SLS) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). 3,3',4',5-Tetrachlorosalicylanilide (TCSA) was from Acros Organics N. V. (Geel, Belgium). 6-Methylcoumarin (6-MC) and anthracene were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acridine was from Lancaster Synthesis, Inc. (Windham, NH, USA). Distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), ethanol (Wako Pure Chemical Industries, Ltd.), or RPMI 1640 medium without phenol red (Invitrogen Corp.) (medium (–)) was selected as a solvent. FITC-labeled anti-CD86 antibody was purchased from BD Biosciences Pharmingen (Clone: Fun-1, San Diego, CA, USA). FITC-labeled anti-CD54 and anti-mouse IgG1 was purchased from DAKO (Glostrup, Denmark). Gamma-globulins from human blood were from Sigma-Aldrich Corp.

2.3. Irradiation

A SOL 500 Sun Simulator (Dr. Hönle AG, Gräfelfing, Germany) equipped with a 500 W metal halide lamp and a H1 filter to attenuate UVB was used for irradiation. A UVA-Meter (Dr. Hönle AG) was used to measure UVA intensity.

2.4. Test procedure

2.4.1. Irradiation and non-irradiation methods

For treatment, the test chemical was dissolved in distilled water, ethanol, or medium (–), diluted with medium (–), and dispensed into a 24-well plate. The final concentration of each solvent was 0.5%. THP-1 cells were washed with medium (–) and seeded in the aforementioned plate (1×10^6 cells/mL; 1 mL/well). Plates were irradiated (irradiation method) or kept in the dark (non-irradiation method).

2.4.2. Pre-irradiation method

In the pre-irradiation method, the irradiation was applied to a 24-well plate into which medium (–) had been dispensed and test chemical added. After incubation for 30 min at 37 °C, THP-1 cells suspended in medium (–) were seeded into the wells (1×10^6 cells/mL; 1 mL/well).

2.4.3. Flow cytometric analysis

After treatment for 24 h with chemicals under the conditions as described in Sections 2.4.1 and 2.4.2, the cells were washed twice with PBS containing 0.1% BSA, then treated with 0.01% globulins, Cohn fraction II, III (Sigma-Aldrich), for 10 min on ice to block FcR. The cells were then washed once with PBS containing 0.1% BSA. Staining was performed with FITC-conjugated anti-human CD86 antibody, anti-human CD54 antibody or IgG1 at 4 °C for 30 min. The expression of CD86 and CD54 on viable cells unstained with 0.5% propidium iodide solution (Sigma-Aldrich Corp.) was measured with a flow cytometer (EPICS XL/XL-MCL System II, Beckman Coulter, Inc., Harbor Boulevard, CA, USA). In total, 10,000 living cells were analyzed. In principle, cells were treated with test chemical at a concentration giving around 75% cell viability under the conditions used.

2.5. Calculation of CD86 and CD54 expression

RFI of CD86 and CD54 expression was used as an indicator of activation of THP-1 cells. RFI was calculated by use of the following formula: CD86 or CD54 expression (% of control) = $100 \times (\text{mean fluorescence intensity (MFI) of sample-treated or UV-irradiated}) / (\text{MFI of non-irradiated control})$.

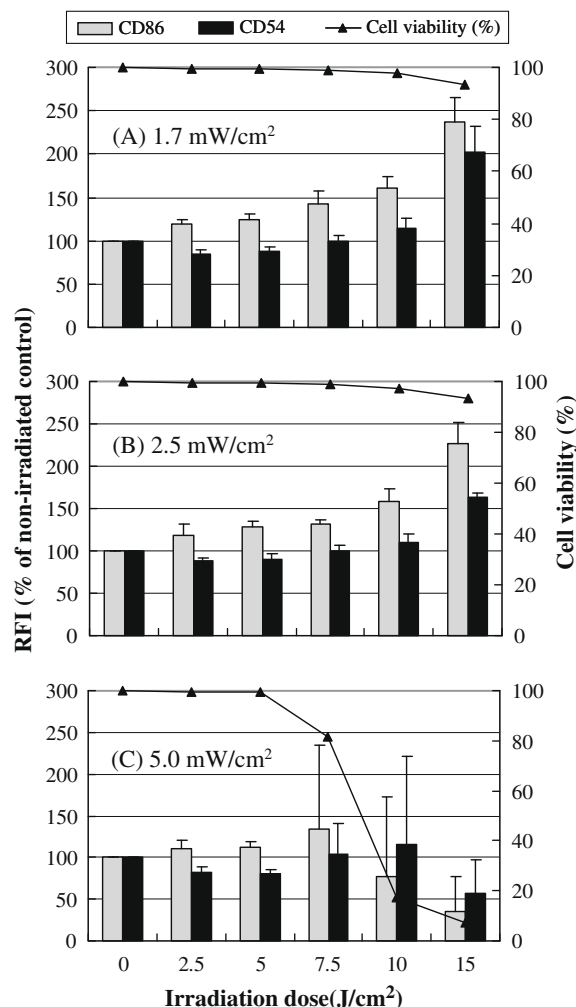


Fig. 1. Effect of intensity and total amount of irradiation on CD86 and CD54 expression and viability of na THP-1 cells. Cells were irradiated at the doses of (A) 1.7 mW/cm², (B) 2.5 mW/cm² and (C) 5 mW/cm². After irradiation, THP-1 cells were incubated for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs (% of non-irradiated control) were calculated. Mean values $\pm$ S.D. for at least three independent experiments are shown.

cells (anti-CD86 or CD54 antibody treated) – MFI of sample-treated or UV-irradiated isotype control cells (IgG1 treated))/(MFI of vehicle-treated or non-irradiated cells (anti-CD86 or CD54 antibody treated) – MFI of vehicle-treated or non-irradiated isotype control cells (IgG1 treated)).

2.6. Data analysis

Tests were performed at least three times on each chemical and the values of CD86 and CD54 expression and cell viability were calculated, each as the mean of at least three test results. If the cell viability was less than 50%, the data for the corresponding concentration of the test chemical were not used for the judgment.

3. Results

3.1. Effects of irradiation

We examined the effect of intensity and total amount of irradiation on THP-1 cells. Irradiation caused a dose-related increase of CD86 and CD54 expression on THP-1 cells, especially at the intensities of 1.7 and 2.5 mW/cm² (Fig. 1A and B). At the intensity of 5.0 mW/cm², cytotoxicity emerged around the irradiation dose of 7.5 J/cm² (Fig. 1C).

3.2. Effects of irradiation in the presence of a photoallergen

To ascertain the effect of irradiating THP-1 cells in the presence of a photoallergen, cells were treated with 6-methylcoumarin (6-MC), a representative photoallergen, under various conditions of irradiation. The maximum value of CD86 expression on THP-1 cells was little affected by irradiation (Fig. 2). However, CD54 expression was upregulated by irradiation and reached a maximum at the irradiation dose of 5.0 J/cm² at the intensity of 1.7 mW/cm². The cytotoxic effect of 6-methylcoumarin was dependent on the irradiation dose. The irradiation dose of 5.0 J/cm² at the intensity of 1.7 mW/cm² was chosen as an appropriate test condition to detect the effects of photoallergens on THP-1 cells.

3.3. Effects of irradiation in the presence of a photoirritant

The effects of a phototoxic material on THP-1 cells were confirmed under the optimized irradiation conditions. Acridine, a photo-irritant, increased the expression of CD86 and CD54 on THP-1 cells under irradiation (Fig. 3). To determine the mechanism of activation of THP-1 cells by phototoxic chemicals, the effect of hydrogen peroxide, a representative reactive oxygen species (ROS), was examined. Expression of both CD86 and CD54 was dose-dependently increased in THP-1 cells exposed to hydrogen peroxide (Fig. 4).

3.4. Effects of a pre-irradiated photoallergen or a pre-irradiated photoirritant

In order to eliminate the effect of ROS on CD86 and CD54 expression, we used the pre-irradiation method to investigate changes of CD86 and CD54 expression on acridine-treated THP-1 cells. Compared with the irradiation method, the extent of up-regulation was reduced when the pre-irradiation method was used (Fig. 5A). On the other hand, 6-MC, a representative photoallergen, induced CD86 expression to the extent of over 150% of RFI, which is the tentatively proposed cut-off value (Sakaguchi et al., 2006), in the pre-irradiation method (Fig. 5B).

3.5. Effects of a panel of test chemicals using non-irradiation, irradiation and pre-irradiation methods

To further evaluate the pre-irradiation method, we evaluated more chemicals using the non-irradiation, irradiation and pre-irradiation methods. Eighteen test chemicals were selected (LeVine, 1984; Hariya et al., 1993; Tokura, 1998; National Toxicology Program, 1999; Nishijima et al., 1999; Lovell and Jones, 2000; Landers et al., 2003) and evaluated (Table 1). Dose-setting was determined based on the CV₇₅ value (estimated dose of 75% cell viability) as a sub-toxic concentration, as described by Sakaguchi et al. (2009). Three arbitrary thresholds were selected: RFI values of 150%, 200% and 250%. The minimum concentrations of test chemicals which were judged as positive in terms of changes in CD86 or CD54

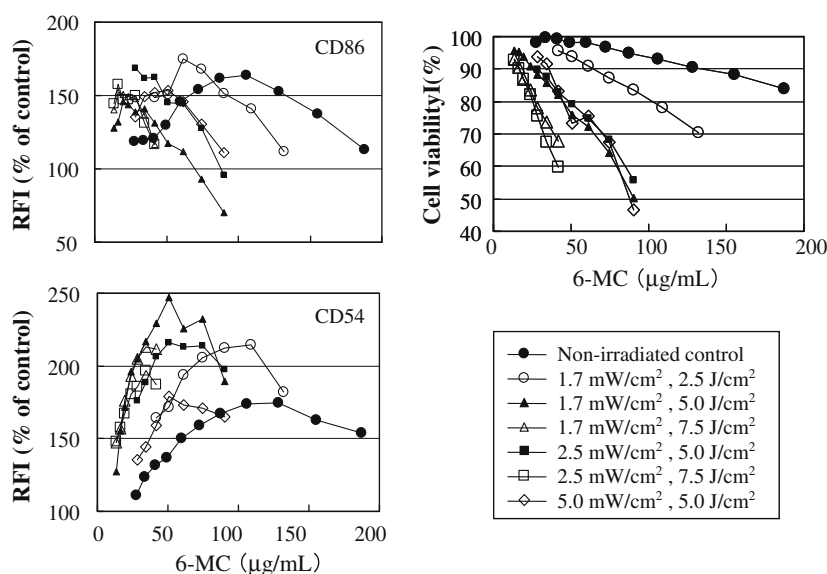


Fig. 2. Effect of intensity and total amount of irradiation on CD86 and CD54 expression and viability of 6-MC-treated THP-1 cells. Cells were irradiated at various intensities and total amounts of irradiation as shown, after treatment with various concentrations of 6-MC. After irradiation, cells were incubated for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFI were calculated. Mean values $\pm$ S.D. for at least three independent experiments are shown.

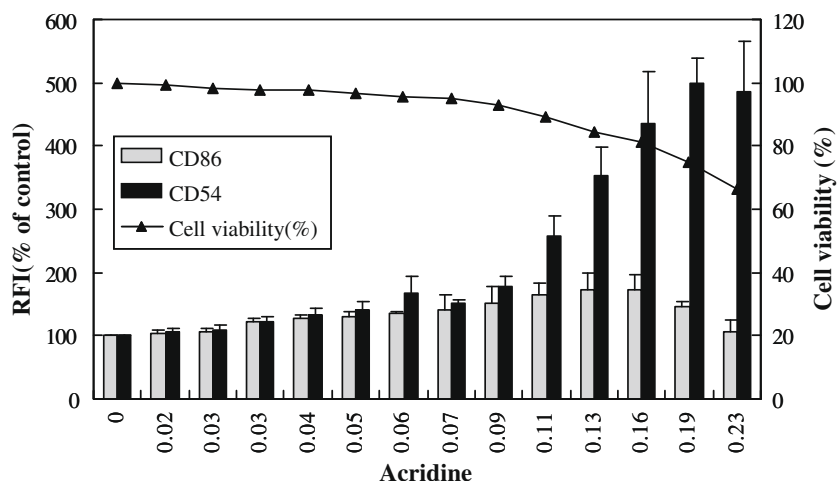


Fig. 3. Activation of THP-1 cells by acridine under irradiation. Cells were treated with various concentrations of acridine, and after irradiation, were incubated for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs were calculated. Mean values $\pm$ S.D. for at least three independent experiments are shown.

expression at each threshold were designated as EC150, EC200 and EC250, and the values obtained are summarized in Table 2.

3.6. Development of a decision tree for general cytotoxicity, phototoxicity, allergy and photoallergy

To identify photoallergens, photo-irritants, allergens and non-sensitizers using the non-irradiation, irradiation and pre-irradiation methods, we establish a tiered evaluation system to predict the photoallergic and phototoxic potential of substances (Fig. 6). Step 1: when the expression of both CD86 and CD54 in the non-irradiation and irradiation methods was judged as negative, the photoallergic, allergic and phototoxic potentials of chemical are considered to be negative. Step 2: when the ratio of EC200 in the non-irradiation

method to that in the irradiation method (EC200 (non-irradiation)/EC200 (irradiation) value) is below three or when the RFI value of CD86 or CD54 expression in the irradiation method is below 200% at all set doses, the photoallergic and phototoxic potentials of the chemical are considered to be negative but the allergic potential is considered to be positive. If the EC200 value of CD86 is different from that of CD54, the lower concentration is selected for calculation. Chemicals whose EC200 (non-irradiation)/EC200 (irradiation) value is over 3 are further tested using the pre-irradiation method. Step 3: when RFI of 150% (CD86 expression) or RFI of 250% (CD54 expression) is applied as the tentative criterion in the pre-irradiation method, chemicals which are judged as negative are considered to be phototoxic. Step 4: when chemicals judged as positive in the pre-irradiation method are judged as negative in the non-irradiation method, their photoallergic (not allergic) potential is considered to be positive. On the other hand, when a chemical is judged as positive in both the pre-irradiation and non-irradiation method, it is considered to show both photoallergic and allergic potential.

The result of evaluation of 18 chemicals are summarized in Table 3. In Step 1, among 18 chemicals, SDS, LA, PABA and piroxicam were judged as negative. In Step 2, EC200 (non-irradiation)/EC200 (irradiation) values of TCSCA, CHD and DNCB were below three. These chemicals were judged as allergenic. In Step 3, CD86 or CD54 expression of enoxacin, 5-MOP and acridine was not augmented in the pre-irradiation method. These chemicals were judged as phototoxic. In Step 4, CD86 or CD54 expression of 6-MC, bithionol and anthracene was not augmented in the non-irradiation method. These chemicals were judged as photoallergic. On the other hand, CPZ, BP, PPD, ketoprofen and PM, which were judged as positive in all steps, were evaluated as photoallergic/allergenic. Among 18 chemicals, 13 chemicals were judged correctly, compared with *in vivo* data (LeVine, 1984; Hariya et al., 1993; Tokura, 1998; National Toxicology Program, 1999; Nishijima et al., 1999; Lovell and Jones, 2000; Landers et al., 2003). Among five chemicals which were misjudged, TCSCA (*in vivo*; photoallergic, phototoxic and allergenic) was judged as allergenic, enoxacin (*in vivo*; photoallergic), was judged as phototoxic, anthracene (*in vivo*; phototoxic), was judged as photoallergic, and PABA and piroxicam (*in vivo*; photoallergic) were judged as negative.

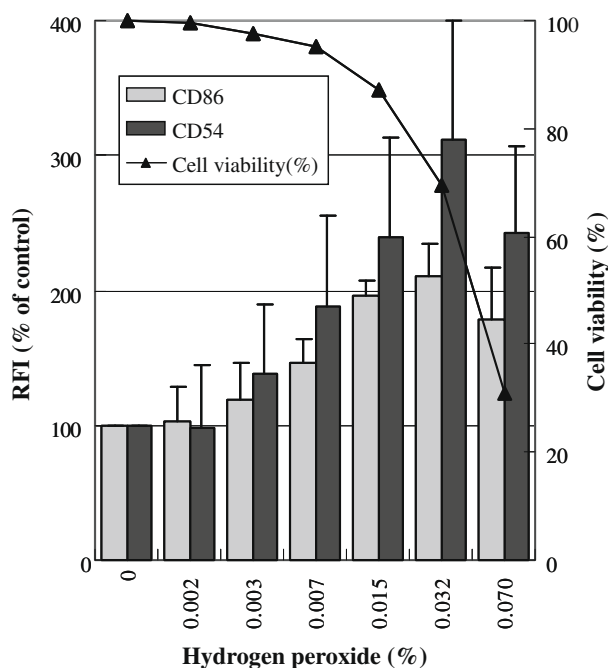


Fig. 4. Effect of hydrogen peroxide on THP-1 cells. Cells were treated with various concentrations of hydrogen peroxide for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs were calculated. Mean values $\pm$ S.D. for at least three independent experiments are shown.

4. Discussion

A flow cytometric method using human monocytic leukemia cell line, THP-1, has been developed and validated as an *in vitro*

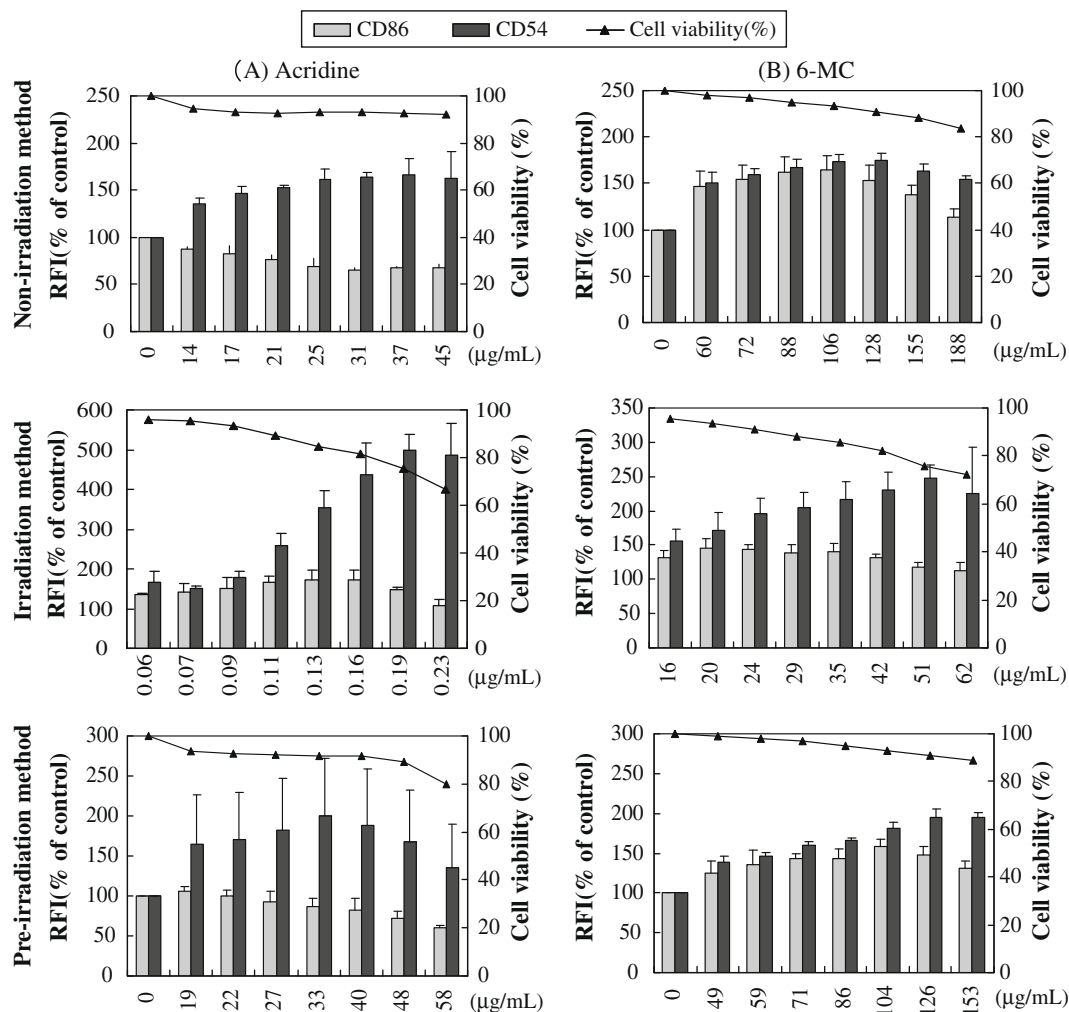


Fig. 5. Activation of THP-1 cells by acridine (a photo-irritant) and 6-MC (a photo-allergen) determined with the non-irradiation, irradiation and pre-irradiation methods. Cells were treated with various concentrations of acridine or 6-MC, then irradiated according to the irradiation and pre-irradiation methods described in Section 2, and incubated for 24 h at 37 °C. In the non-irradiation method, cells were incubated for 24 h at 37 °C without irradiation after treatment with acridine or 6-MC. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs were calculated. Mean values $\pm$ S.D. for at least three independent experiments are shown.

Table 1
CV₇₅ value of test chemicals.

Test samples		CV ₇₅ (µg/mL)			
		Non-irradiation method	Irradiation method	Pre-irradiation method	Solvent
CPZ	Chlorpromazine HCl	7.3	0.9	29	Saline
BP	Benzophenone	104	13	123	Ethanol
TCSA	3,3',4',5-Tetrachlorosalicylanilide	2.6	15	N.D.	Ethanol
PPD	p-Phenylenediamine	10	1.3	8.2	Saline
Ketoprofen	-	528	17	110	Ethanol
PM	Promethazine HCl	26	1.6	38	Ethanol
6-MC	6-Methylcoumarin	188	62	153	Ethanol
Bithionol	-	104	13	123	Ethanol
PABA	p-Aminobenzoic acid	2500	2066	2500	Ethanol
Piroxicam	-	100	100	N.D.	Ethanol
Enoxacin	-	100	28	100	Ethanol
CHD	Chlorhexidine HCl	23	8.3	N.D.	Ethanol
DNCB	2,4-Dinitrochlorobenzene	5.0	0.6	3.7	Ethanol
Anthracene	-	50	50	50	Ethanol
5-MOP	5-Methoxypsoralen	50	8.9	50	Ethanol
Acridine	-	45	0.2	58	Ethanol
SLS	Sodium lauryl sulfate	85	70	N.D.	Saline
LA	Lactic acid	2801	2844	N.D.	Medium

sensitization test (Ashikaga et al., 2002, 2006; Sakaguchi et al., 2006). THP-1 is used as a substitute for dendritic cells, which are

involved in skin allergic reaction, for the study of alternatives to animal experiments. Several studies have confirmed the usefulness

Table 2

Summary data for changes of CD86 and CD54 expression on THP-1 cells exposed to 18 chemicals according to the non-irradiation, irradiation and pre-irradiation methods. Relative fluorescence intensities (RFIs) of CD86 and CD54 on THP-1 were compared using three tentative criteria in the non-irradiation, irradiation and pre-irradiation methods. The minimum concentrations which were judged as positive for CD86 or CD54 expression under each threshold are indicated as EC150, EC200 and EC250. "N.D." means that EC150, EC200 or EC250 was not determined because CD86 or CD54 expression did not exceed the relevant criterion. "N.T." means that CD86 and CD54 expression was not tested in the pre-irradiation method.

Test samples	Non-irradiation method						Irradiation method						Pre-irradiation method					
	CD86			CD54			CD86			CD54			CD86			CD54		
	EC150	EC200	EC250	EC150	EC200	EC250	EC150	EC200	EC250	EC150	EC200	EC250	EC150	EC200	EC250	EC150	EC200	EC250
	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)
CPZ	3.1	7.2	N.D.	4.3	5.6	6.3	0.3	N.D.	N.D.	0.2	0.3	0.3	19	24	N.D.	12	17	18
BP	42	68	N.D.	38	67	N.D.	8.7	N.D.	N.D.	4.8	6.9	9.8	44	83	N.D.	<39	<39	48
TCSA	N.D.	N.D.	N.D.	<0.8	1.2	N.D.	N.D.	N.D.	N.D.	4.8	7.6	9.8	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
PPD	2.1	4.4	N.D.	6.9	N.D.	N.D.	0.9	N.D.	N.D.	0.8	1.0	1.2	4.0	7.2	N.D.	<3.8	6.0	8.1
Ketoprofen	N.D.	N.D.	N.D.	172	193	311	7.1	N.D.	N.D.	<5.4	6.0	6.8	<35	N.D.	N.D.	<35	37	60
PM	N.D.	N.D.	N.D.	8.4	10	12	1.0	N.D.	N.D.	<0.7	1.0	1.0	N.D.	N.D.	N.D.	12	15	18
6-MC	66	N.D.	N.D.	60	N.D.	N.D.	N.D.	N.D.	N.D.	16	26	N.D.	94	N.D.	N.D.	62	N.D.	N.D.
Bithionol	N.D.	N.D.	N.D.	<7.1	N.D.	N.D.	N.D.	N.D.	N.D.	11	14	17	N.D.	N.D.	N.D.	<27	43	45
PABA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	697	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Piroxicam	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Enoxacin	N.D.	N.D.	N.D.	65	N.D.	N.D.	<9.5	N.D.	N.D.	<9.5	21	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CHD	N.D.	N.D.	N.D.	<4.2	18	N.D.	N.D.	N.D.	N.D.	<2.6	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
DNCB	<0.67	1.2	1.4	0.7	1.3	2.2	0.2	0.5	N.D.	0.4	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Anthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	20	36	N.D.	20	28	35	<16	N.D.	N.D.	<16	<16	16
5-MOP	19	N.D.	N.D.	20	22	N.D.	1.4	2.8	N.D.	4.1	N.D.	N.D.	N.D.	N.D.	N.D.	24	N.D.	N.D.
Acridine	N.D.	N.D.	N.D.	19	N.D.	N.D.	0.09	N.D.	N.D.	0.05	0.10	0.11	N.D.	N.D.	N.D.	<19	33	N.D.
SLS	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
LA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

Table 3

Relationship between *in vivo* data and the results of the present *in vitro* photosensitization test based on the proposed decision tree. *In vivo* data were taken from the literature (LeVine, 1984; Hariya et al., 1993; Tokura, 1998; National Toxicology Program, 1999; Nishijima et al., 1999; Lovell and Jones, 2000; Landers et al., 2003). "(a)/(b)" means the ratio of EC200 value in the non-irradiation method (a) to that in the irradiation method (b), and was calculated for each of CD86 and CD54. "N.D." means that EC200 was not determined because CD86 or CD54 expression did not exceed the criterion (RFI > 200%). "N.C." means that "(a)/(b)" could not be calculated because the EC200 value in the irradiation method could not be determined. ">3" was assigned when CD86 or CD54 expression was above the criterion level in the irradiation method, but not the non-irradiation method. "N.T." means that CD86 and CD54 expression was not tested in the pre-irradiation method. +: positive, -: negative.

Test samples	In vivo data			In vitro test data										Judgment	References
	Photo-allergenicity	Photo-toxicity	Allergenicity	Non-irradiation method (a)		Irradiation method (b)		(a)/(b)		Pre-irradiation method					
				EC200 value (mg/mL)											
				CD86	CD54	CD86	CD54	CD86	CD54	CD86	CD54				
CPZ	+	+	+	7.2	5.6	N.D.	0.3	N.C.	19	+	+	Photoallergic/allergic	Lovell and Jones (2000)		
BP	+	—	+	68	67	N.D.	6.9	N.C.	10	+	+	Photoallergic/allergic	Landers et al. (2003)		
TCSA	+		+	N.D.	1.2	N.D.	7.6	N.C.	0.15	N.T.	N.T.	Allergic	Nishijima et al. (1999), National Toxicology Program (1999)		
PPD	+		+	4.4	N.D.	N.D.	1.0	N.C.	>3	+	+	Photoallergic/allergic	LeVine (1984), National Toxicology Program (1999)		
Ketoprofen	+	—	+	N.D.	193	N.D.	6.0	N.C.	32	+	+	Photoallergic/allergic	Lovell and Jones (2000)		
PM	+	+	+	N.D.	10	N.D.	1.0	N.C.	11	—	+	Photoallergic/allergic	Lovell and Jones (2000)		
6-MC	+	—	—	N.D.	N.D.	N.D.	26	N.C.	>3	+	—	Photoallergic	Lovell and Jones (2000)		
Bithionol	+	+		N.D.	N.D.	N.D.	14	N.C.	>3	—	+	Photoallergic	Lovell and Jones (2000)		
PABA	+	—	—	N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	Lovell and Jones (2000)		
Piroxicam	+			N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	Hariya et al. (1993)		
Enoxacin	+			N.D.	N.D.	N.D.	21	N.C.	>3	—	—	Phototoxic	Tokura (1998)		
CHD	—	—	+	N.D.	18	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Allergic	Lovell and Jones (2000)		
DNCB	—	—	+	1.2	1.3	0.5	N.D.	2.7	N.C.	N.T.	N.T.	Allergic			
Anthracene	—	+	—	N.D.	N.D.	36	28	>3	>3	+	+	Photoallergic	Lovell and Jones (2000)		
5-MOP	—	+	+	N.D.	22	2.8	N.D.	>3	>3	—	—	Phototoxic	Lovell and Jones (2000)		
Acridine	—	+	—	N.D.	N.D.	N.D.	0.10	N.C.	>3	—	—	Phototoxic	Lovell and Jones (2000)		
SLS	—	—	—	N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	Lovell and Jones (2000)		
LA	—	—	—	N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative			

of this cell line for *in vitro* sensitization tests. Here, we confirmed that it can be used to assess photoallergenicity, as well as allergenicity.

We found that the expression of CD86 and CD54 on THP-1 cells under irradiation was up-regulated dose-dependently. At high irradiation intensity, cytotoxicity was observed. Ultraviolet light

irradiation *in vivo* is known to induce immunosuppression, and this is attributed to the sensitivity of several types of cells in the skin to ultraviolet light. The immunosuppression involves perturbed expression of several co-stimulatory molecules, including CD86 and CD54 on dendritic cells (Denfeld et al., 1998; Rattis et al., 1998; Dittmar et al., 1999; Iwai et al., 1999; Nakagawa et al., 1999,

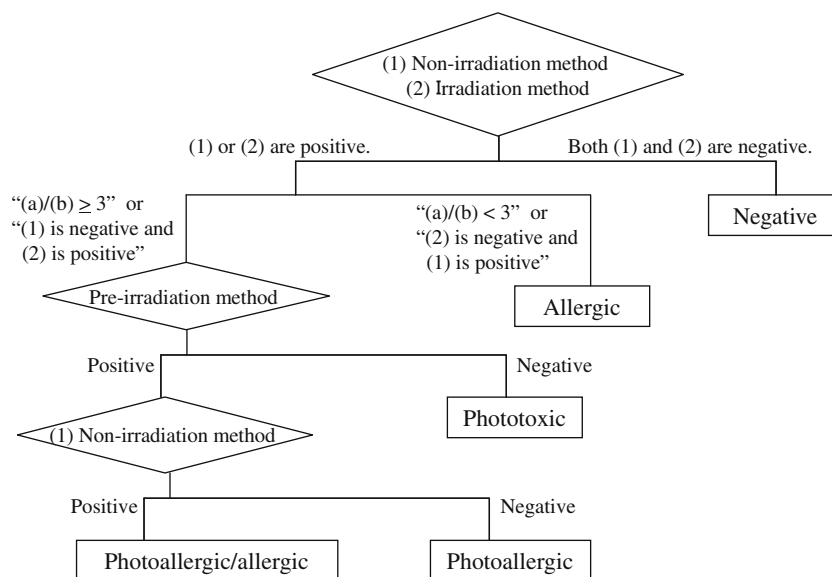


Fig. 6. Proposed decision tree for predicting photoallergenicity, phototoxicity, and allergenicity. If the RFI of either CD86 or CD54 exceeds the criterion, the chemical is positive. If the RFIs of CD86 and CD54 are below the criterion, the chemical is negative. In the (1) non-irradiation and (2) irradiation methods, RFI of 200% (CD86 and CD54 expression) was applied as a tentative criterion. In the pre-irradiation method, RFI of 150% (CD86 expression) or RFI of 250% (CD54 expression) was applied. “(a)/(b)” indicated the ratio of EC200 in the non-irradiation method (a) to that in the irradiation method (b).

2004; Laihia and Jansen, 2000; Mittelbrunn et al., 2005). Ultraviolet light also induced apoptosis of dendritic cells (Rattis et al., 1998; Aubin, 2003; Nakagawa et al., 2004; Timares et al., 2008). These facts imply that at least some of the photodynamic mechanisms that occur *in vivo* are replicated under the test conditions *in vitro*. Suitable irradiation conditions for the test (1.7 mW/cm², 5 J/cm²), which minimized cytotoxicity, were determined using 6-MC as a representative photoallergen. The irradiation conditions are identical to those of the 3T3 NRU PT, for which the same irradiation apparatus is used. Interestingly, a fixed intensity of irradiation, 5 J/cm², has been indicated to be necessary and sufficient for eliciting photocontact allergic reactions in the photopatch test (Hasan and Jansen, 1996).

Under the test conditions used for the irradiation method, photo-irritants, as well as photoallergens, induced CD86 and CD54 expression on THP-1 cells. ROS including hydrogen peroxide, which is generated by UV-activated photo-irritants, appear to be involved (Fig. 4). Indeed, dendritic cells are activated by ROS, especially hydrogen peroxide (Caceres-Dittmar et al., 1995; Rutault et al., 1999). To discriminate photoallergens from photo-irritants, we examined the pre-irradiation method, in which THP-1 cells were treated with irradiated test chemicals, in addition to the non-irradiation and irradiation methods. Hino et al. reported a similar approach to examine the prohaptenic ability of chemicals (Hino et al., 2008). The augmentation of CD86 expression by 6-MC, but not acridine, in the pre-irradiation method might be explained by photo-induced binding of test substance or photo-decomposition products, but not short-lived ROS, to THP-1 cells. CD86 and/or CD54 expression on THP-1 cells is thus upregulated by photoallergens, but not photo-irritants.

Based on the results of the non-irradiation, irradiation and pre-irradiation methods using 18 chemicals, we prepared a decision tree to distinguish between photoallergens and photo-irritants (Fig. 6). The photoallergenicity of seven out of 11 photoallergic test chemicals was correctly assessed by using CD86 and CD54 as biomarkers. It has already been reported that the extents of increase of CD86 and CD54 expression are not necessarily the same (Sakaguchi et al., 2006). Furthermore, our data indicated that there were some chemicals that induced either CD86 or CD54 expression in

the non-irradiation, irradiation and pre-irradiation methods. Thus, CD86 and CD54 were considered to be complementary biomarkers. This decision tree appears to be suitable in part for identifying photoallergens. However, the *in vitro* 3T3 NRU phototoxicity test (OECD, 2004) seems to be more suitable for detecting phototoxic potential only, because the phototoxicity of chemicals which were judged in that test as “photoallergic and allergenic” or “photoallergenic” such as CPZ, PM and bithionol was not detected using our decision tree. So, a battery system combining this decision tree and the *in vitro* 3T3 NRU phototoxicity test is an interesting possibility. In our decision tree, TCSA, which is photoallergic and allergenic *in vivo*, was only assessed as allergenic. TCSA is known to bind non-covalently to one major site on human serum albumin and to form characteristic photoconjugates. The special role of albumin as a carrier protein in TCSA photoallergy has been reported (Barratt et al., 1987; Pendlington and Barratt, 1990). Thus, it may be important to consider the role of carriers in the pre-irradiation method. In addition, PABA and piroxicam were classified as negative. Both showed low cytotoxicity towards THP-1 cells under the test conditions. Cytotoxicity of test chemicals towards THP-1 cells is required for detecting sensitizing potential in *in vitro* sensitization tests (Yoshida et al., 2003). The cytotoxic effect of these chemicals on Langerhans cells should be verified.

In conclusion, we have developed a new *in vitro* photosensitization using a cultured cell line, THP-1, and confirmed its utility to predict the photoallergic potential of various chemicals. A small number of individuals who develop photoallergic contact dermatitis following exposure to photosensitizing chemicals retain a persistent reactivity to light after all exposure to the chemicals has ceased (persistent light reaction; PLR) (Rietchel and Fowler, 2001). Thus, identification of chemicals with photosensitizing potential may reduce the risk of PLR, as well as being important for immunology and dermatotoxicology research. Further development of this method, perhaps as part of a battery system, is expected to provide a useful tool for predicting photoallergenicity.

Acknowledgment

We thank Richard Steele for critical review of the manuscript.

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