

RESEARCH ARTICLE

New approach to predict photoallergic potentials of chemicals based on murine local lymph node assay

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Abstract

Photoallergic dermatitis, caused by pharmaceuticals and other consumer products, is a very important issue in human health. However, S10 guidelines of the International Conference on Harmonization do not recommend the existing prediction methods for photoallergy because of their low predictability in human cases. We applied local lymph node assay (LLNA), a reliable, quantitative skin sensitization prediction test, to develop a new photoallergy prediction method. This method involves a three-step approach: (1) ultraviolet (UV) absorption analysis; (2) determination of no observed adverse effect level for skin phototoxicity based on LLNA; and (3) photoallergy evaluation based on LLNA. Photoallergic potential of chemicals was evaluated by comparing lymph node cell proliferation among groups treated with chemicals with minimal effect levels of skin sensitization and skin phototoxicity under UV irradiation (UV+) or non-UV irradiation (UV-). A case showing significant difference ($P < .05$) in lymph node cell proliferation rates between UV- and UV+ groups was considered positive for photoallergic reaction. After testing 13 chemicals, seven human photoallergens tested positive and the other six, with no evidence of causing photoallergic dermatitis or UV absorption, tested negative. Among these chemicals, both doxycycline hydrochloride and minocycline hydrochloride were tetracycline antibiotics with different photoallergic properties, and the new method clearly distinguished between the photoallergic properties of these chemicals. These findings suggested high predictability of our method; therefore, it is promising and effective in predicting human photoallergens.

KEYWORDS

local lymph node assay, photoallergy, photosafety, skin phototoxicity, skin sensitization

1 | INTRODUCTION

Photoallergic dermatitis describes type IV allergic reactions that occur when the photoallergen is applied orally or dermally and subsequently exposed to ultraviolet (UV) radiation, and it is sometimes caused by cosmetic and pharmaceutical products following dermal application, which is a very important health issue for humans.

To predict the photoallergic potential of chemicals applied by the dermal route, guinea pig prediction methods such as the Adjuvant and Strip method (Sato, Katsumura, Ichikawa, Kobayashi, & Nakajima,

1980) and Herber method (Herber, 1969), have been generally used. Prediction methods using mice, including those based on ear thickness change in the elicitation phase of photoallergic responses (Gerberick & Ryan, 1990a, b) and cytokine release in skin-draining lymph node cells by modified local lymph node assay (LLNA) (Ulrich, Homey, & Vohr, 1998), have also been reported.

However, these existing photoallergy tests using rodents require a relatively large number of animals and long experimental periods, because they evaluate the skin reaction in the elicitation phase. In addition, the proposal of the International Conference on

Harmonization (ICH) guideline S10 (2013) mentioned that these existing photoallergy tests are not recommended to predict photoallergens exposed by the dermal route because of their unknown predictability for humans and lack of official validations.

The detection of photoallergic potentials of chemicals is important for human health; however, effective prediction methods for photoallergy cannot be assigned in a safety evaluation strategy for chemicals. Therefore, an effective and reliable photoallergic test is needed to improve chemical safety evaluation strategies.

In this study, we tried to develop a reliable photosafety evaluation method, which can detect photoallergic chemicals with high accuracy for humans. For this purpose, we employed LLNA, which is currently used as the standard in vivo skin sensitization test for chemicals.

2 | MATERIALS AND METHODS

2.1 | Animals

Female CBA/Ca mice (Japan SLC, Inc., Shizuoka, Japan) in good health were randomly allocated into groups, two mice per groups for skin phototoxicity evaluation or four mice per groups for sensitization or photoallergy evaluation. All mice were housed in polycarbonate cages (215W × 320D × 150H mm) placed in an animal chamber (Toyoriko Co., Ltd., Tokyo, Japan), which was maintained at a temperature of $23 \pm 2^\circ\text{C}$ and relative humidity of $55\% \pm 15\%$. Daily, the chamber was ventilated at a frequency of 10–15 cycles h^{-1} and artificially lit for 12 hours. The mice were allowed free access to a laboratory diet (MF; Oriental Yeast Co., Tokyo, Japan) and tap water and were aged 8–10 weeks at the start of chemical application. All mice were treated in compliance with the Regulation for Animal Experiments at Chemicals Evaluation and Research Center, CERl based on the Act on Welfare and Management of Animals (Ministry of Education, Culture, Sports, Science and Technology, 2005) and Guidelines for

Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (2006).

2.2 | Chemicals

Thirteen chemicals consisting of seven positive chemicals known as photoallergens, and six expected negative chemicals, were used in this study (Table 1). Ketoprofen (KP; Cardoso, Canelas, Goncalo, & Figueiredo, 2009; The European Multicentre Photopatch Test Study Taskforce, 2012; Onoue et al., 2017; Tokura, 2009), piroxicam (Px; Cardoso et al., 2009; EMCPTS Taskforce, 2012; Onoue et al., 2017; Tokura, 2009), doxycycline hydrochloride (DOC; Onoue et al., 2017; Tokura, 2009), promethazine hydrochloride (PMZ; Cardoso et al., 2009; Onoue et al., 2017; Scalf, Davis, Rohlinger, & Cannolly, 2009; Tokura, 2009), 8-methoxypsoralen (8-MOP; Bonamonte, Foti, Lionetti, Rigano, & Angelini, 2010; Onoue et al., 2017), 3,3',4',5-tetrachlorosalicylanilide (TCSA; Gao et al., 2014; Matsuo, 2002; Scalf et al., 2009) and chlorpromazine hydrochloride (CPZ; Gao et al., 2014; Onoue et al., 2017; Scalf et al., 2009; Tokura, 2009) were selected as positive chemicals because these chemicals have abundant human evidence of photoallergy. Ethanol, propylene glycol, 1,3-butylene glycol, 2-propanol, 2-ethylhexyl methacrylate (2-EHMA) and minocycline hydrochloride (MIC) were selected as expected negative chemicals because these chemicals are non-UV absorbers and have no evidence of human photoallergy. MIC was also used as an expected negative chemical because the chemical has no evidence of photoallergy despite the UV absorber (Tokura et al., 2001).

As for the vehicles used in this study, methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a vehicle for UV absorption spectrum measurement in step 1 and *N,N*-dimethylformamide (Wako), 70% ethanol (EtOH; Wako; water/EtOH = 3:7) and methylethyl ketone (Wako) were used in other steps because these chemicals have no UVA absorption.

TABLE 1 Chemicals used in this study and photoallergy information

	Abbreviation	Supplier	CAS no.	Photoallergy information	References
Ethanol	EtOH	Wako	64-17-5	EN	-
Propylene glycol	PG	Wako	57-55-6	EN	-
1,3-Butylene glycol	1,3-BG	TCI	107-88-0	EN	-
2-Propanol	IPA	Wako	67-63-0	EN	-
2-Ethylhexyl methacrylate	2-EHMA	Wako	688-84-6	EN	-
Ketoprofen	KP	Wako	22071-15-4	P	1, 2, 3, 4
Piroxicam	Px	TCI	36322-90-4	P	1, 2, 3, 4
Doxycycline hydrochloride	DOC	MP Biomedicals, LLC	10592-13-9	P	2, 4
Minocycline hydrochloride	MIC	TCI	13614-98-7	EN	5
Promethazine hydrochloride	PMZ	TCI	58-33-3	P	1, 2, 4, 6
8-Methoxypsoralen	8-MOP	Sigma-Aldrich Co. LLC	298-81-7	P	4, 7
3,3',4',5-Tetrachlorosalicylanilide	TCSA	Kanto chemical	40743-51	P	6, 8, 9
Chlorpromazine hydrochloride	CPZ	TCI	69-09-0	P	2, 4, 6, 9

-, no information; EN, excepted negative; P, positive; TCI, Tokyo Chemical Industry Co., Ltd.

(1) Cardoso et al. (2009); (2) Tokura (2009); (3) The European Multicentre Photopatch Test Study Taskforce (2012); (4) Onoue et al. (2017); (5) Tokura et al. (2001); (6) Scalf et al. (2009); (7) Bonamonte et al. (2010); (8) Matsuo, 2002; (9) Gao et al. (2014).

2.3 | Ultraviolet light source

An irradiation apparatus with six parallel black light lamps (FL40S BLB; Toshiba Corporation, Tokyo, Japan) was used as the light source, with peak emission at 350–355 nm UVA radiation (320–400 nm). A glass filter was used to minimize the light in the UVB range.

In this strategy, we selected the UVA-based irradiation method to avoid the influence of UVB and simplify the handling procedures.

2.4 | Step 1: ultraviolet absorption

A flow diagram for photoallergy evaluation is shown in Figure 1. First, the UV absorption spectrum within the wavelength range of 290–400 nm were recorded using a spectrophotometer (BioSpec mini; Shimadzu, Kyoto, Japan). Then, the molar extinction coefficient (ϵ) of each chemical was calculated using the following formula: $\epsilon = A/(c \times l)$, where A , absorbance; c , concentration; l , path length.

Chemicals with $\epsilon \geq 1000 \text{ l mol}^{-1} \text{ cm}^{-1}$ at any wavelength were considered UV absorption-positive and were further subjected to Step 2. Chemicals with $\epsilon < 1000 \text{ l mol}^{-1} \text{ cm}^{-1}$ at any wavelength were considered UV absorption-negative. In this study, all chemicals, despite some being UV absorption-negative, were assessed using all the three steps to confirm the effectiveness of each step.

2.5 | Step 2: skin phototoxicity evaluation based on local lymph node assay

LLNA was conducted in accordance with OECD test guideline 429 (OECD, 2010). Maximum doses are decided from the results in a pre-screen test for systemic toxicity and primary irritation described for OECD test guideline 429. Consequently, maximum doses for step 3 of EtOH, propylene glycol, 1,3-butylene glycol, 2-propanol, 2-ethylhexyl methacrylate, KP, Px, DOC, MIC, PMZ, 8-MOP, TCSCA and CPZ were decided as 100, 100, 100, 100, 100, 1, 1, 10, 5, 25, 10, 0.02 and 10, respectively. Test solutions were applied to the dorsum of both the ears of each mouse for 3 consecutive days, and then, 20 μCi of ^3H -methyl thymidine (3H-TdR; Moravak Biochemicals, Brea, CA, USA) was injected into all the mice via the tail vein 3 days after the final application of chemicals. Five hours after the 3H-TdR

injection, auricular lymph nodes were removed after killing the mice. A single-cell suspension of lymph node cells was prepared in phosphate-buffered saline (MP Biomedicals, LLC., Santa Ana, CA, USA) using gentle mechanical separation through a 200-mesh stainless steel mesh. Lymph node cells were washed twice with an excess of phosphate-buffered saline. Next, 5% trichloroacetic acid (Wako) was added to the cell suspension, followed by incubation at 4°C for 18 hours, after which the pellets were resuspended in 1 ml of trichloroacetic acid and transferred to 10 ml of a scintillant (Ecolume™; MP Biomedicals). The incorporation of 3H-TdR was then measured using a liquid scintillation counter (Tri-Carb 3110TR; Perkin Elmer, Inc., Waltham, MA, USA) in terms of disintegrations per minute (DPM) per mouse in each experimental group. For each mouse, a stimulation index (SI) relative to the mean DPM of the concurrent vehicle-treated group was derived, and cases showing $\text{SI} \geq 3$ were considered positive.

The estimated concentration of a chemical required to induce an SI value of 3 (EC3) was calculated for chemicals that were LLNA positive. EC3 was also calculated if applicable using the following formula (Ryan et al., 2007):

$$\text{EC3} = c + \{(3-d)/(b-d)\} \times (a-c)$$

where (a,b) and (c,d) represent the coordinates (dose, SI) of the data points lying immediately above and below the SI value of 3 on the LLNA dose–response curve, respectively.

The concentration corresponding to the EC3 value in the positive case and the maximum concentration in the negative case in LLNA were selected for the maximum concentration used for the skin phototoxicity evaluation.

The test chemical was applied to the dorsum of the mouse ear, followed by irradiating the mouse with 10 J cm^{-2} of UVA using the irradiation apparatus. A similar treatment was repeated for 3 consecutive days.

The phototoxic effects were evaluated by measuring the ear thickness and body weight at day 0 and after 6 days of chemical application. The case showing more than 25% increase of ear thickness without more than 5% body weight loss compared with the values at day 0 were decided as skin phototoxicity positive.

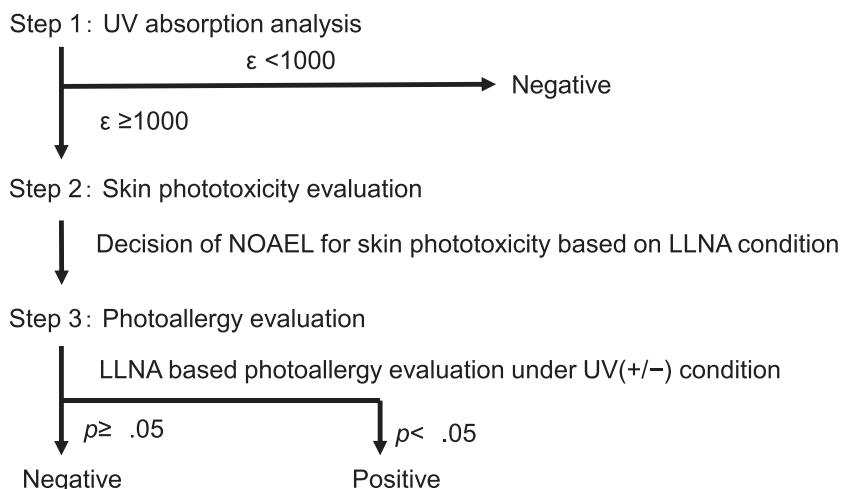


FIGURE 1 Flow diagram for photoallergy evaluation. Step 1: UV absorption analysis using spectrophotometer; chemical with ϵ of $\geq 1000 \text{ l mol}^{-1} \text{ cm}^{-1}$ at any wavelength is considered as UV absorption-positive. Step 2: Determination of NOAEL for phototoxicity under LLNA test condition. Step 3: Evaluation of photoallergic potential of chemical by comparing responses between UV+ and UV− groups applied with test chemical in selected dose expected to cause no or minimal sensitization potential (equal or less than EC3) without phototoxic response under standard LLNA protocol decided in Step 2. LLNA, local lymph node assay; NOAEL, no-observable-adverse-effect level; UV, ultraviolet

TABLE 2 Results of step 1: UV absorption analysis

Chemicals	UV absorption analysis		
	$\lambda_{\max}$ (nm)	MEC (l mol ⁻¹ cm ⁻¹)	P/N
Ethanol	-	-	N
Propylene glycol	-	-	N
1,3-Butylene glycol	-	-	N
2-Propanol	-	-	N
2-Ethylhexyl methacrylate	-	-	N
Ketoprofen	290	2200	P
Piroxicam	325	20 540	P
Doxycycline hydrochloride	350	13 490	P
Minocycline hydrochloride	340	12 910	P
Promethazine hydrochloride	300	6570	P
8-methoxypsoralen	299	13 700	P
3,3',4',5'-Tetrachlorosalicylanilide	325	8260	P
Chlorpromazine hydrochloride	308	4860	P

-, no UV absorption; MEC, molar extinction coefficient; N, negative; P, positive; UV, ultraviolet.

2.6 | Step 3: photoallergy evaluation

Mice were allocated into two groups: UV irradiation (UV+) and non-UV irradiation (UV-). The test concentration of each chemical was decided based on the results of the no-observable-adverse-effect level (NOAEL) for skin sensitization and skin phototoxicity in step 2. A given concentration of the chemical was applied to the dorsum of the mouse ear in both the groups. Next, the mice in the UV+ group were irradiated for 3 consecutive days as described in step 2. The mice in the UV- group received similar treatment, except for UV irradiation.

A 3H-TdR injection and subsequent treatments were performed according to LLNA.

The photo SI (pSI), corresponding to the SI value in standard LLNA, was calculated independently for the UV+ and UV- groups by dividing the mean DPM of the chemically treated group by the mean DPM of the concurrent vehicle control group.

An unpaired t-test was performed using pSI values for each chemical in the UV+ and concurrent UV- control group. The chemical was considered as photoallergy positive if a significant difference was found between groups ($P < .05$).

TABLE 3 Results of step 2: skin phototoxicity evaluation based on LLNA

	LLNA		Skin phototoxicity			
				Increase rate of ear thickness (%)		
Chemical	P/N	EC3 (%)	Concentration (%)	Mean (left)	Mean (right)	P/N
Ethanol	N	Not calculated	100	2.7	3.5	N
Propylene glycol	N	Not calculated	100	0.9	2.8	N
1,3-Butylene glycol	N	Not calculated	100	2.0	4.4	N
2-Propanol	N	Not calculated	100	0.2	0.1	N
2-Ethylhexyl methacrylate	P	6.5	6.5	3.1	3.6	N
Ketoprofen	N	Not calculated	0.1	7.7	6.0	N
			0.25	1.1	10.4	
			0.5	3.2	13.8	
			1	10.9	11.3	
Piroxicam	P	0.7	0.1	0.4	2.1	N
			0.25	1.0	0.3	
			0.5	-	-	
			0.7	-	-	
Doxycycline hydrochloride	N	Not calculated	2.5	11.4	9.3	P
			5	19.3	17.3	
			10	25.0	25.1	
Minocycline hydrochloride	N	Not calculated	2.5	8.0	7.3	N
			5	6.4	8.5	
Promethazine hydrochloride	N	Not calculated	25	10.4	12.1	P
			40	25.0	25.3	
8-Methoxypsoralen	N	Not calculated	0.01	20.7	16.4	P
			0.025	24.3	22.4	
			0.05	41.8	39.6	
			0.1	-	-	
			1.0	-	-	
			10	-	-	
3,3',4',5'-Tetrachlorosalicylanilide	P	0.006	0.006	1.8	3.0	N
Chlorpromazine hydrochloride	P	2.4	0.1	12.3	9.2	P
			0.25	27.4	23.3	
			0.5	33.2	30.7	
			1.0	55.4	60.6	
			2.4	-	-	

LLNA, local lymph node assay; N, negative; P, positive; -, data were excluded because of excessive irritation.

Concentration indicated in **bold type** was the selected concentration for step 3.

3 | RESULTS

3.1 | Step 1: ultraviolet absorption

The results of UV absorption analysis are shown in Table 2. Five chemicals (EtOH, propylene glycol, 1,3-butylene glycol, 2-propanol and 2-EHMA) tested negative because they had UV absorption of $<1000 \text{ l mol}^{-1} \text{ cm}^{-1}$ at all the wavelengths tested.

The other eight chemicals (KP, Px, DOC, MIC, PMZ, 8-MOP, TCSA and CPZ) had UV absorption of $\geq 1000 \text{ l mol}^{-1} \text{ cm}^{-1}$ at all the wavelengths tested, and ϵ values were calculated as 2200, 20540, 13490, 12910, 6570, 13700, 8260 and 4860, respectively.

3.2 | Step 2: skin phototoxicity

The results of LLNA are shown in Table 3. Four chemicals (2-EHMA, Px, TCSA and CPZ) were evaluated as positive for skin sensitization, with EC3 (%) values of 6.5, 0.7, 0.006 and 2.4, respectively. The other nine chemicals tested negative for skin sensitization.

The results of skin phototoxicity evaluation are shown in Table 3. The ear thickness within the UV+ group treated with DOC, PMZ, 8-MOP or CPZ increased by approximately 25% compared with day 0; therefore, these four chemicals were considered phototoxic. NOAELs for these chemicals were 5%, 25%, 0.025% and 0.1%, respectively. The remaining nine chemicals were found to cause no

change in the ear thickness, erythema and general condition of the mice and were thus considered non-phototoxic.

3.3 | Step 3: photoallergy

The results are shown in Table 4. pSI values were 2.4 ± 0.2 (UV-) and 4.1 ± 0.4 (UV+) for KP (1.0%), 1.8 ± 0.2 and 3.6 ± 0.6 for Px (0.25%), 1.9 ± 0.1 and 3.0 ± 0.4 for DOC (5%), 1.3 ± 0.2 and 12.7 ± 2.0 for PMZ (25%), 1.4 ± 0.1 and 3.9 ± 0.6 for 8-MOP (0.025%), 1.6 ± 0.3 and 3.5 ± 0.3 for TCSA (0.006%), and 1.3 ± 0.2 and 13.3 ± 2.3 for CPZ (0.1%), respectively. The other chemicals did not show marked differences.

Significant differences ($P < .05$) were noted in seven chemically treated groups (KP, Px, DOC, PMZ, 8-MOP, TCSA and CPZ), whereas no significant differences were observed in the remaining six chemically treated groups. Taken together, seven chemicals (KP, Px, DOC, PMZ, 8-MOP, TCSA and CPZ) were found to be photoallergens, whereas the other six were negative for photoallergy.

4 | DISCUSSION

Photoallergic dermatitis is caused by cosmetics, quasi-drugs and pharmaceutical products and is an important health issue in humans; however, existing photoallergy prediction methods have not been recommended in ICH (2013) due to their unknown predictability in

TABLE 4 Results of step 3: photoallergy evaluation

Chemical	Concentration (%)	UV +/-	pSI		Photoallergy P/N
			Mean	SE	
Ethanol	100	-	0.8	0.1	N
		+	0.6	0.0	
Propylene glycol	100	-	1.0	0.2	N
		+	0.7	0.1	
1,3-Butylene glycol	100	-	1.0	0.1	N
		+	0.7	0.1	
2-Propanol	100	-	0.8	0.1	N
		+	0.7	0.1	
2-Ethylhexyl methacrylate	6.5	-	3.2	0.2	N
		+	3.7	0.3	
Ketoprofen	1	-	2.4	0.2	P
		+	4.1 **	0.4	
Piroxicam	0.25	-	1.8	0.2	P
		+	3.6 **	0.6	
Doxycycline hydrochloride	5	-	1.9	0.1	P
		+	3.0 **	0.4	
Minocycline hydrochloride	5	-	1.8	0.4	N
		+	1.5	0.2	
Promethazine hydrochloride	25	-	1.3	0.2	P
		+	12.7 **	2.0	
8-Methoxypsoralen	0.025	-	1.4	0.1	P
		+	3.9 **	0.6	
3,3',4',5-Tetrachlorosalicylanilide	0.006	-	1.6	0.3	P
		+	3.5 **	0.3	
Chlorpromazine hydrochloride	0.1	-	1.3	0.2	P
		+	13.3 **	2.3	

N, negative; P, positive; UV, ultraviolet.

**Significantly different from the concurrent UV- control group (unpaired t-test, $P < .01$).

TABLE 5 Summary of results in three-step approach

Chemicals	UV absorption	Skin sensitization	Skin phototoxicity	Photoallergy
Ethanol	N	N	N	N
Propylene glycol	N	N	N	N
1,3-Butylene glycol	N	N	N	N
2-Propanol	N	N	N	N
2-Ethylhexyl methacrylate	N	P	N	N
Ketoprofen	P	N	N	P
Piroxicam	P	P	N	P
Doxycycline hydrochloride	P	N	P	P
Minocycline hydrochloride	P	N	N	N
Promethazine hydrochloride	P	N	P	P
8-Methoxypsoralen	P	N	P	P
3,3',4',5-Tetrachlorosalicylanilide	P	P	N	P
Chlorpromazine hydrochloride	P	P	P	P

N, negative; P, positive; UV, ultraviolet.

humans. To address this issue, we applied LLNA to develop a new photoallergy prediction method with higher predictability for humans.

Our new method consists of three steps: (1) UV spectrum analysis; (2) skin phototoxicity evaluation based on LLNA to determine NOAEL; and (3) photoallergy evaluation based on LLNA.

All assay results are summarized in Table 5. Consequently, seven tested positive and six with no evidence of photoallergic dermatitis or UV absorption tested negative. With regard to the predictability of this method, five well-known human photoallergens, such as KP, Px, DOC, PMZ and CPZ, could clearly be evaluated as positive. In addition, we tested structurally similar chemicals, DOC and MIC. These are both tetracycline antibiotics; DOC is a known photoallergen in humans (Onoue et al., 2017; Tokura, 2009), while MIC seems to be a non-photoallergen in spite of being a UV absorber because its phototoxic nor photoallergic effects have not been reported in humans (Tokura, 2001). Although no animal test data for photoallergic potentials was available for MIC, our method clearly distinguished the photoallergic properties of these chemicals, suggesting a high predictability when utilized for photoallergens in humans. To the best of our knowledge, this report is the first case to show the predictability of photoallergic potential against these similar chemicals in animal tests. Therefore, our method was suggested as very useful for photosafety evaluation in humans.

The current strategy of our method is to predict the photoallergic response qualitatively by comparing the dermal response between the UV+ and UV- groups applied with the test chemical in selected dose expected to cause no or minimal sensitization potential (equal or less than EC3) without a phototoxic response under the standard LLNA protocol. Schumann et al. (2014) reported a photosafety evaluation approach based on multiple dose groups using the lowest-observed-adverse-effect level and NOAEL. This approach may achieve the quantitative evaluation of the photoallergic potential of chemicals. The application of the lowest-observed-adverse-effect level- and NOAEL-based approach to our method may contribute to the development of more reliable and quantitative prediction methods for human photoallergens.

In this study, we used two mice per group to determine NOAEL for skin phototoxicity, and it seems to work well for the purpose. However, considering experimental variations of in vivo studies and applicability of the statistical approach, at least three mice per group would be desirable. Anyway, based on the outcomes of this study, our method would be a promising candidate for animal prediction tests for human photoallergens.

CONFLICT OF INTEREST

The authors did not report any conflict of interest.

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