

Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency

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ABSTRACT

The human Cell Line Activation Test (h-CLAT), an *in vitro* skin sensitization test, is based on the augmentation of CD86 and CD54 expression in THP-1 cells following exposure to chemicals. The h-CLAT was found to be capable of determining the hazard of skin sensitization. In contrast, the local lymph node assay (LLNA), widely used as a stand-alone method in Europe and US, identifies the same hazard, but also classifies the potency by using the estimated concentration of SI = 3 (EC3). In this study, several values calculated from the h-CLAT data were evaluated for its correlation to the LLNA EC3 determination. A statistically significant correlation was observed between h-CLAT concentration providing a cell viability of 75% (CV75), h-CLAT estimated concentration of RFI = 150 for CD86 (EC150), and for CD54 (EC200) with LLNA's EC3. From EC150 and EC200, a minimum induction threshold (MIT) was determined as the smaller of either EC150 or EC200. MIT showed a correlation with EC3 ($R = 0.638$). Also, MIT had an approximate 80% accuracy for sub-categories of the globally harmonized system (GHS) when a tentative threshold of 13 $\mu\text{g/mL}$ was used. From these data, the h-CLAT values may be one of the useful tools to predict the allergic potency of chemicals.

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Introduction

Some chemicals, haptens with low molecular weights, might cause contact allergy via skin sensitization. For cosmetics, hazard identification of the ingredients and risk assessment of the final product for skin sensitization are very important because some cosmetic ingredients are potential sensitizers in sensitive individuals (e.g., some preservatives, hair dyes, or fragrance components). The skin sensitization potential of chemicals or cosmetic ingredients had been evaluated primarily by using guinea pig tests, such as the maximization test (Organisation for Economic Co-operation,

Abbreviations: h-CLAT, human Cell Line Activation Test (h-CLAT); EC3, estimated concentration that produces a stimulation index of 3 in the murine local lymph node assay; LLNA, murine local lymph node assay; CV75, tested concentration providing a cell viability of 75%; EC200, estimated concentration of RFI = 200 for CD54; EC150, estimated concentration of RFI = 150 for CD86; RFI, relative fluorescence intensity; MIT, minimum induction threshold; OECD, organization for economic cooperation and development; GHS, globally harmonized system of classification and labeling of chemicals; DPRA, direct peptide binding assay; KC, keratinocyte; DC, dendritic cell; COLIPA, European cosmetics toiletry and perfumery association; MFI, geometric mean fluorescence intensity; ECETOC, European centre for ecotoxicology and toxicology of chemicals; NOEL, no effect level; HRIPT, human repeated insult patch test; HMT, human maximization test; REACH, registration, evaluation, authorization and restriction of chemicals.

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1992; Botham et al., 1991). In the guinea pig test, the sensitization potential of test materials was determined by evaluating eczema and edema outcomes in the elicitation phase. The guinea pig tests have been used for a long time to discriminate between a skin sensitizer and non-sensitizer for chemicals and cosmetic ingredients. However, the guinea pig test could not provide a quantitative assessment (Kimber et al., 2001). To this, the murine local lymph node assay (LLNA) was developed to replace these guinea pig tests, where antigen-specific T cell proliferation is used to evaluate skin sensitization potential. The assay has since been adapted by the Organization for Economic Cooperation and Development (OECD) as a stand-alone test for skin sensitization: OECD Test guideline no. 429 (Organisation for Economic Co-operation, 2002). Currently, LLNA is used throughout the world. In Europe, LLNA is preferentially chosen to assess the skin sensitization potential of chemicals due to animal welfare concerns in the application of the "Registration, Evaluation, Authorization and Restriction of Chemicals" (REACH) regulations (Grindon et al., 2008; Angers-Loustau et al., 2011). The LLNA is able to provide a quantitative assessment of potency (EC3) and is used to assess the potency of chemicals based upon quantitative data. These data can be used for the risk assessment of final product (Gerberick et al., 2001a; Griem et al., 2003; Api and Vey, 2008). In addition, globally harmonized system of classification and labelling of chemicals (GHS) had been started. The purpose of this system is to classify and label all chemicals using

an internationally harmonized approach. In the 3rd edition of the GHS, the sub-categorization can be conducted based on the human evidence or the animal data. For this, EC3 is used to discriminate between a stronger sensitizer (GHS Category 1A) and a weaker sensitizer (GHS Category 1B) by setting the EC3 threshold at 2% (United Nations, 2009).

In terms of non-animal testing, several tests for skin sensitization have been developed in a response to a testing ban and marketing ban declared in 7th amendment of European Cosmetic Directive (Directive 2003/15/EC, 2003). Some of these *in vitro* methods currently being developed are focused on the major steps of the induction phase of skin sensitization (i.e. hapten binding to the protein, keratinocyte (KC) activation, dendritic cells (DC) activation, and T cell proliferation). For example, Cosmetics Europe (formerly known as the European Cosmetics Toiletry and Perfumery Association (COLIPA)) described such a project (Aeby et al., 2010). In terms of protein binding, Gerberick et al. developed the direct peptide reactivity assay (DPRA) (Gerberick et al., 2004, 2007). In this test, several model peptides, which consist of cysteine, lysine or other amino acids, are used to evaluate the peptide binding capacity of haptens (Gerberick et al., 2004). For DC activation, the cell-based *in vitro* assay, “human cell line activation test” (h-CLAT), was developed to predict the skin sensitizing potential of chemicals (Ashikaga et al., 2006, 2010; Sakaguchi et al., 2006). In the h-CLAT, human monocytic leukemia cell line THP-1 cells, which exhibit up-regulation of CD86 or CD54 expression following treatment with various sensitizers, but not non-sensitizers, were used. The h-CLAT showed that the overall accuracy among the approximately 100 chemicals evaluated was 84% against LLNA and 83% against human data (Ashikaga et al., 2010; Nukada et al., 2011). Furthermore, h-CLAT can be easily transferable to other laboratories, and the inter- and intra-laboratory reproducibility was relatively high after two ring trials conducted in Japan, Europe and/or the United States (Ashikaga et al., 2008; Sakaguchi et al., 2010). More importantly, several investigators revealed that three major ingredient categories, preservatives, hair dyes, and fragrances, could be identified by h-CLAT correctly against *in vivo* data (Kosaka et al., 2010; Okamoto et al., 2010; Sono et al., 2010). Based on these observations, h-CLAT could be used for hazard identification of various chemicals besides cosmetic ingredients.

In addition to the hazard identification, the risk assessment of skin sensitization is also important for chemicals. In situations where animal testing will be completely prohibited, potency categorization without animal tests will be needed. This means that any *in vitro* testing would need to not only provide the hazard identification but also the potency classification in the future. Previously, Sakaguchi et al. (2009) has reported that the EC150 and EC200, which means the estimated concentration of RFI = 150 for CD86 and RFI = 200 for CD54 in the h-CLAT, correlated to the EC3 of the LLNA with a limited number of the chemicals. In this study, we investigated whether h-CLAT can do the hazard identification and potency classification by analyzing the correlation between LLNA's EC3 and several candidate values from h-CLAT for a large number of chemicals.

Materials and methods

Laboratories

The h-CLAT was conducted independently by two laboratories, Kao Corporation and Shiseido Co., Ltd.

Chemicals

A total of 106 test chemicals, which had already been evaluated by LLNA (Basketter et al., 1999a; Warbrick et al., 1999; Dearman

et al., 2000; Ryan et al., 2000, 2002; Siebert, 2004; Gerberick et al., 2005; Basketter and Kimber, 2006; Aeby et al., 2009; Kern et al., 2010) were selected. This collection contained 75 sensitizers and 31 non-sensitizers, which are listed in Table 1. The majority of the test chemicals were purchased from the Sigma–Aldrich (St. Louis, MO, USA). Those chemicals which were not purchased from Sigma–Aldrich were the following chemicals: 2-aminophenol, lillial, and 2-hydroxypropyl methacrylate from the Wako Pure Chemical Industries (Osaka, Japan). Methylaldibromoglutaronitrile was purchased from the Alfa Aesar (Ward Hill, MA, USA). Penicillin G was purchased from the Duchefa Biochemie B.V. (Haarlem, Netherlands). Tween 80 was purchased from the Tokyo Chemical Industry (Tokyo, Japan). Final concentrations of the vehicle in h-CLAT were set at 1% for saline and 0.2% for DMSO.

human Cell Line Activation Test (h-CLAT).

Cells and medium

THP-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Irvine, CA, USA), 0.05 mM 2-mercaptoethanol and 1% of antibiotic–antimycotic (Invitrogen Corp.).

Cytotoxicity test and determination of the test doses

THP-1 cells were cultured in 24-well plates (1.0×10^6 cells/1 mL/well) with various concentrations of each chemical for 24 h. Chemical-treated cells were washed twice with phosphate-buffered saline (PBS) solution containing 0.1% (w/v) bovine serum albumin (FACS buffer). Then the cells were stained with propidium iodide (PI, 0.625 µg/mL). In this study, the cells were analyzed using flow cytometry. Both laboratories used the FACS calibur machine (Becton Dickinson, San Jose, CA, USA) and the CellQuest software was used to do the analyses. In the measurement of cell viability, PI negative (PI–) cells are defined as the living cells. The living-cell gate was set in PI negative area, and then 10,000 PI– cells were counted as living cell population. The ratio of the PI– cells to total-acquired cells was defined as the cell viability. The test concentration providing a cell viability of 75% (CV75) is derived from the dose response curve, and was calculated by log-linear interpolation. A total of 8 doses were applied and were based on the CV75: $1.2 \times CV75$, $1 \times CV75$, $1/1.2 \times CV75$, $1/1.2^2 \times CV75$, $1/1.2^3 \times CV75$, $1/1.2^4 \times CV75$, $1/1.2^5 \times CV75$ and $1/1.2^6 \times CV75$.

Chemical treatment procedure

THP-1 cells (1.0×10^6 cells/mL) were exposed to each chemical in 8 doses as described in the previous section. After a 24-h treatment, chemical-treated cells were washed twice with FACS buffer. After washing, Fc receptor blocking procedure was conducted: THP-1 cells were incubated with 0.01% of Globlins Cohn fraction II, III (Sigma–Aldrich) for 10 min on 4 °C. Next, cells were stained for 30 min at 4 °C with the following FITC-conjugated monoclonal antibodies (mAbs): anti-human CD54 (clone; 6.5B5) from DAKO (Glostrup, Denmark), anti-human CD86 (clone; FUN-1) from BD-PharMingen (San Diego, CA, USA) and FITC labeled-mouse IgG1 (clone; DAK-G01) from DAKO. Using the manufacturer's recommended dilutions, cells were incubated with the above mAbs at $6 \mu\text{L}/3 \times 10^5$ cells/50 µL for the anti-human CD86 mAb and $3 \mu\text{L}/3 \times 10^5$ cells/50 µL for the anti-human CD54 mAb. Also, FITC labeled-mouse IgG1 was used as an isotype control at a dilution of $3 \mu\text{L}/3 \times 10^5$ cells/50 µL. After washing and re-suspending with FACS buffer, the fluorescence intensities for a total of 10,000 PI–

Table 1
Test substances used (106 chemicals).

Chemical name	CAS	LLNA						h-CLAT				
		Potency category	EC3(%)	Result	CD86	CD54	CV75 (µg/mL)	Max RFI (CD86)	Max RFI (CD54)	EC150 (CD86)	EC200 (CD54)	MIT
Diphenylcyclopropenone	886-38-4	Extreme	0.003	P	–	+	6.0	121	349	–	3.92	3.92
Oxazolone	15646-46-5		0.003	P	+	–	166.6	249	183	2.71	–	2.71
MCI/MI (act. 1.5%)	Mixture/26172-55-4		0.005	P	+	–	3.2	156	127	2.21	–	2.21
Bandrowski's base	20048-27-5		0.008	P	+	+	2.3	215	5072	3.85	2.6	2.6
p-Benzoquinone	106-51-4		0.0099	P	+	+	4.3	170	140	2.68	2.25	2.25
1-Benzoylacetone	93-91-4		0.04	P	–	+	92.8	118	801	–	39.6	39.6
2,4-Dinitrochlorobenzene:DNCB	97-00-7		0.05	P	+	+	5.0	311	499	2.3	2.66	2.3
4-Nitrobenzyl bromide	100-11-8		0.05	P	+	+	3.6	504	468	0.95	0.91	0.91
Potassium dichromate	7778-50-9		0.08	P	+	+	3.2	161	300	2.09	1.06	1.06
Glutaraldehyde (act. 50%)	111-30-8	Strong	0.1	P	+	+	5.3	169	313	2.78	2.7	2.7
1,4-Dihydroquinone	123-31-9		0.11	P	+	–	5.0	208	145	2.13	–	2.13
1,4-Phenylenediamine	106-50-3		0.16	P	+	–	36.7	250	167	2.09	–	2.09
Maleic anhydride	108-31-6		0.16	P	–	+	658.0	56	217	–	298.4	298.4
Phthalic anhydride	85-44-9		0.16	N	–	–	400.0*	115	160	–	–	–
Benzyl bromide	100-39-0		0.2	P	+	+	7.5	230	624	3.2	2.86	2.86
Benzoyl peroxide	94-36-0		0.30	N	–	–	41.0	119	126	–	–	–
Lauryl gallate	1166-52-5		0.30	P	+	+	8.2	276	1042	2.02	2.91	2.02
Propyl gallate	121-79-9		0.32	P	–	+	125.0	110	1464	–	32.5	32.5
Cobalt chloride	7646-79-9		0.38	P	–	+	208.3	154	725	–	35.5	35.5
2-aminophenol	95-55-6	Moderate	0.4	P	+	–	6.0	350	162	0.89	–	0.89
Chloramine T	127-65-1		0.4	P	+	+	314.7	169	233	259.2	291	259.2
2-Nitro-1,4-phenylenediamine	5307-14-2		0.5	P	–	+	490.7	122	204	–	276.7	276.7
Formaldehyde (act. 37%)	50-00-0		0.61	P	+	+	5.8	298	282	4.3	5.14	4.3
Iodopropynyl butylcarbamate	55406-53-6		0.87	P	–	+	12.8	126	226	–	8.15	8.15
Methyldibromo glutaronitrile	35691-65-7		0.9	P	+	+	9.9	147	402	10.8	9.42	9.42
Isoeugenol	97-54-1		1.2	N	–	–	112.5	84	132	–	–	–
1-Naphthol	90-15-3		1.3	P	+	+	57.2	252	211	18.3	12.7	12.7
1-Phenyl-1,2-propanedione	579-07-7		1.3	P	+	+	123.1	203	633	81.5	56.7	56.7
Glyoxal (act. 40%)	107-22-2		1.4	P	–	+	396.0	72	242	–	286.7	286.7
2-Hydroxyethyl acrylate	818-61-1	Weak	1.4	P	+	+	24.4	218	208	10.8	19.49	10.8
2-Mercaptobenzothiazole	149-30-4		1.7	P	–	+	169.0	127	995	–	57.5	57.5
Methylisothiazolinone (act.9.7%)	2682-20-4		1.9	P	+	+	24.7	345	743	9.23	7.89	7.89
3-Dimethylaminopropylamine	109-55-7		2.2	P	+	+	276.7	189	433	131	165.8	131
Ethylene diamine	107-15-3		2.2	P	+	–	271.7	146	95	265.7	–	265.7
1,2-Benzisothiazolin-3-one	2634-33-5		2.3	P	–	+	1.83	138	1331	–	0.54	0.54
Methyl-2-nonynoate	111-80-8		2.5	P	+	–	191.7	206	94	91.8	–	91.8
Cinnamic aldehyde	104-55-2		3.0	P	+	+	28.0	212	422	10.2	12.3	10.2
Phenylacetaldehyde	122-78-1		3.0	P	+	+	27.0	212	546	11.5	13.4	11.5
3-Aminophenol	591-27-5		3.2	P	–	+	243.2	132	308	–	117.1	117.1
Diethyl sulfate	64-67-5		3.3	P	+	–	373.0	256	253	240.4	–	240.4
Benzylideneacetone	122-57-6		3.7	P	+	+	35.3	312	296	25.8	34.6	25.8
3-Propylenephthalide	17369-59-4		3.7	P	+	+	113.0	181	216	31.2	55.6	31.2
a-Methyl cinnamic aldehyde	101-39-3		4.5	P	+	+	139.0	215	301	40.3	70.16	40.3
Nickel sulfate	10101-97-0(7786-81-4)		4.8	P	+	+	150.0	322	4146	42.2	45.3	42.2
Tetramethylthiuramdisulfide	137-26-8		5.2	P	+	+	10.0	160	526	9.11	2.46	2.46
3,4-Dihydrocoumarin	119-84-6		5.6	P	–	+	810.0	98	617	–	531.7	531.7
Resorcinol	108-46-3		5.7	P	–	+	613.4	102	579	–	200.9	200.9
Diethylenetriamine	111-40-0		5.8	N	–	–	1221.6	88	95	–	–	–
Diethyl maleate	141-05-9		5.8	P	+	–	120.0	180	133	64	–	64
2-Methoxy-4-methyl-phenol	93-51-6		5.8	P	+	+	280.0	234	197	30.8	207.4	30.8
4-Chloroaniline	106-47-8		6.5	P	+	–	200.0	206	183	87.7	–	87.7
Trimellitic anhydride	552-30-7		9.2	P	+	–	250.0*	172	129	81.2	–	81.2
1-Bromohexane	111-25-1		10	N	–	–	163.3	131	117	–	–	–
Amyl cinnamic aldehyde	122-40-7		11	P	–	+	24.2	97	238	–	25.94	25.94

Hexyl cinnamic aldehyde	101-86-0		11	N	–	–	37.0	106	116	–	–	–
2,3-Butanedion	431-03-8		11	P	+	+	94.0	185	212	24.4	46.9	24.4
Citral	5392-40-5		13	P	+	+	24.0	176	278	8.41	15	8.41
Eugenol	97-53-0		13	P	+	+	143.2	309	214	64.4	137.2	64.4
Abietic acid	514-10-3		15	N	–	–	89.6	83	127	–	–	–
Oxalic acid	144-62-7		15	P	+	–	1000.0*	144	194	310.33	–	310.33
4-Allylanisole	140-67-0		18	P	–	+	186.0	106	213	–	207.6	207.6
Lillial	80-54-6		19	P	–	+	42.2	65	258	–	44.6	44.6
Phenyl benzoate	93-99-2		20	P	+	+	500.0*	236	415	166.2	236.4	166.2
Cinnamic alcohol	104-54-1		21	P	+	+	350.0	245	278	147	196.6	147
Benzocaine	60-09-3		22	P	+	–	545.5	205	168	74.2	–	74.2
Cyclamen aldehyde	103-95-7		22	N	–	–	49.8	78	96	–	–	–
Imidazolidinyl urea	39236-46-9		24	P	+	+	40.1	186	800	42.3	39.3	39.3
Geraniol	106-24-1		26	P	+	–	140.0	198	122	123.3	–	123.3
Ethyleneglycol dimethacrylate	97-90-5		28	P	–	+	563.6	174	207	–	535.43	535.43
Linalool	78-70-6		30	P	–	+	290.0	88	262	–	68.3	68.3
Penicillin G	61-33-6		30	P	–	+	5000.0*	125	285	–	3754.3	3754.3
Butyl glycidyl ether	2426-08-6		31	N	–	–	185.3	105	97	–	–	–
Hydroxycitronellal	107-75-5		33	P	+	+	700.0	313	312	35.4	26.8	26.8
Pyridine	110-86-1		72	P	+	–	4166.7	170	157	2370	–	2370
Aniline	62-53-3		89	P	+	+	930.0	168	416	927.9	550.8	550.8
Acetanisole	100-06-1	Non-sensitizer	NC	N	–	–	426.0	Not determined				
Benzalkonium chloride	8001-54-5		NC	N	–	–	3.0					
Benzoic acid	65-85-0		NC	N	–	–	1000.0*					
1-Bromobutane	109-65-9		NC	P	+	+	500.0					
1-Butanol	71-36-3		NC	N	–	–	1000.0*					
Chlorobenzene	108-90-7		NC	P	+	–	597.2					
Dextran	9004-54-0		NC	N	–	–	5000.0*					
Diethyl phthalate	84-66-2		NC	P	–	+	600.0					
Dimethyl formamide	68-12-2		NC	N	–	–	5000.0*					
Ethyl benzoylacetate	94-02-0		NC	N	–	–	571.1					
Ethyl vanillin	121-32-4		NC	N	–	–	569.5					
Furil	492-94-4		NC	N	–	–	300.0*					
Glycerol	56-81-5		NC	N	–	–	5000.0*					
4-Hydroxybenzoic acid	99-96-7		NC	N	–	–	1000.0*					
2-Hydroxypropyl methacrylate	923-26-2		NC	N	–	–	1000.0*					
Iodohehexane	638-45-9		NC	P	+	–	80.0					
Isopropanol	67-63-0		NC	N	–	–	5000.0*					
Kanamycin	59-01-8		NC	N	–	–	1000.0*					
Lactic acid	50-21-5		NC	N	–	–	2800					
6-Methyl coumarin	92-48-8		NC	N	–	–	276.9					
Methyl salicylate	119-36-8		NC	N	–	–	542.4					
Octanoic acid	124-07-2		NC	P	–	+	359					
Propylene glycol	57-55-6		NC	N	–	–	1000.0*					
Propyl paraben	94-13-3		NC	P	+	+	106.7					
Saccharin	81-07-2		NC	N	–	–	1000.0*					
Salicylic acid	69-72-7		NC	P	–	+	1000.0*					
Streptomycin sulfate	3810-74-0		NC	N	–	–	1000.0*					
Tween 80	9005-65-6		NC	N	–	–	5000.0*					
Vanillin	121-33-5		NC	N	–	–	650.0					
Zinc sulphate	7733-02-0		NC	P	–	+	670.8					
Sodium lauryl sulfate	151-21-3	FP	NC	N	–	–	60.0					

Table lists LLNA data for tested substances used in the study (adapted from Basketter et al., 1999a; Warbrick et al., 1999; Dearman et al., 2000; Ryan et al., 2000; Ryan et al., 2002; Siebert, 2004; Gerberick et al., 2005; Basketter and Kimber, 2006; Aeby et al., 2009; Kern et al., 2010). Results of h-CLAT including judgement, protein expression pattern, CV75, MaxRFI, EC150, EC200, and MIT are also provided. MCI/MI, Methylchloroisothiazolinone/methylisothiazolinone; CAS, Chemical Abstract Service number; P, positive; N, negative; NC, not calculated; FP, false positive; *highest tested dose were shown as an alternative to CV75.

living cells were analyzed by flow cytometry. For the calculation of the cell viability, the cells were analyzed as described in the previous section. For the calculation of protein expression, geometric mean of the FITC intensity was used.

Data analysis

The geometric mean as raw data was used to calculate the relative fluorescence intensity (RFI). An RFI value, which was calculated using the following formula, was used as an indicator of CD86 and CD54 relative expression. In the following formula, MFI indicates the “geometric mean fluorescence intensity”.

RFI (%) = 100

$$\times \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated Isotype}}{\text{MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells}}$$

When the cell viability was less than 50%, the RFI was not calculated. The reason is that the strewn labelling cytoplasmic structures that occur due to cell membrane destruction will interfere with fluorescent measurements (Becker et al., 1994).

Prediction model

All chemicals were tested in three independent experiments. If two of three independent experiments at any dose exceeded 150% of RFI for CD86, or exceeded 200% of RFI for CD54, the chemicals could be identified as a sensitizer. Otherwise it is identified as a non-sensitizer. The prediction model was based on the historical data and several previous studies (Ashikaga et al., 2008, 2010; Sakaguchi et al., 2009).

Calculation of key h-CLAT values

CV75 value was defined as the concentration where exposure to the chemical resulted in 75% cell viability; calculated using the formula previously shown. Maximum RFI (MaxRFI) was defined as the highest values of RFI through the eight doses in three independent experiments for each chemical. Estimated concentration required to produce a RFI of 150 or 200 for CD86 or CD54 (EC150 or EC200), respectively, was calculated based on the calculation rule of LLNA EC3 (Basketter et al., 1999b) by using a simple linear interpolation. In the case of the RFI value at the lowest dose above the positive criteria, EC150 (CD86) or EC200 (CD54) was calculated using by log-linear extrapolation following the formula. Because there is some variability between h-CLAT data from three independent assays, the median value of three experiments, not the average, was defined as EC150 (CD86) or EC200 (CD54) of the test chemical and was used as a threshold derived from the h-CLAT data. When the RFI exceeded the positive criteria in two of three experiments, the higher EC150 (CD86) or EC200 (CD54) was defined as a threshold of the chemicals.

Data analysis for the comparison between EC3 in LLNA and the key h-CLAT values

The correlation analysis was conducted to investigate the correlation between LLNA EC3 values and the values from the h-CLAT (CV75, MaxRFI, EC150, and EC200). The Spearman's rank correlation coefficient was used for the correlation analysis.

Results

The predictive performance of h-CLAT

A total of 106 chemicals were evaluated by h-CLAT as shown in Table 1. Among the 106 chemicals, the predictive performance of h-CLAT was calculated by comparing to the published LLNA data.

In Table 2, the accuracy of the h-CLAT against LLNA was 84% (Table 2). This value is similar to previous values calculated from the data of 100 chemicals (Ashikaga et al., 2010).

Four key h-CLAT values: CV75, maximum RFI (MaxRFI), EC150 and EC200

To investigate whether the h-CLAT can be used for potency categorization, several h-CLAT values were derived from 66 chemicals identified as sensitizers in the h-CLAT. Of the 75 tested, nine chemicals were not predicted as sensitizers and thus were not incorporated in the derivation of the four key values. In the h-CLAT, the test chemicals, which induce 1.5 times higher protein expression for CD86 or 2 times higher protein expression for CD54 against the vehicle control, are considered sensitizers. Therefore, we first tried to calculate the four values for 66 true positive chemicals in the h-CLAT. In this study, we focused on the CV75, MaxRFI, EC150 and EC200 obtained from the h-CLAT. All values are shown in Table 1.

CV75 is the indicator of cytotoxicity for each test material. As shown in Table 1, CV75 values were acquired for almost all test materials. However, CV75 for some test materials, which are insoluble in medium, could not be calculated due to the limitation of solubility. For example, oxalic acid and penicillin G have shown no cytotoxicity at the highest concentrations of the h-CLAT (i.e., 5000 µg/mL in saline and 1000 µg/mL in DMSO). In other cases, trimellitic anhydride and phenyl benzoate were not tested at the appropriate doses due to the limited solubility. These two chemicals had not shown any cytotoxicity at all. For these four test chemicals, highest tested doses were shown as an alternative to CV75 in Table 1.

MaxRFI can be defined as the strength to induce the higher protein expression. MaxRFI was determined for all test chemicals and are shown in Table 1. The highest RFI from the three independent assays was defined as the MaxRFI for each chemical.

EC150 and EC200 values were calculated with the appropriate formula based on the calculation rule for LLNA EC3. These values indicated the amount of test chemical needed to induce enough protein expression to be deemed a positive result. Most of the 66 true positive chemicals have both EC150 and EC200 values, though some chemicals only have one value. Thirty-two test chemicals of the 66 chemicals induced the augmentation of both CD86 and CD54 expression. In contrast, 15 test chemicals, such as oxazolone, 1,4-dihydroquinone, or ethylene diamine, had only EC150 value and 19 test chemicals, such as maleic anhydride, glyoxal, or lilial, have only EC200.

Correlation between the four key h-CLAT values and LLNA EC3

The correlation between for h-CLAT values and LLNA EC3 was examined. Comparison between the four h-CLAT values to LLNA

Table 2
Correspondence between LLNA and h-CLAT.

			h-CLAT	
			Sensitizer	Non-sensitizer
			74	32
LLNA	Sensitizer	75 ^a	66	9
	Non-sensitizer	31	8	23
Predictivity ^b			89%	59%
Overall accuracy ^c			82%	

^a Numbers in the table represented by “75” are numbers of corresponding chemicals. For example, the value 75 means that 75 chemicals were assigned as sensitizer in LLNA. The value 66 means that 66 chemicals were assigned as sensitizers in both LLNA and h-CLAT.

^b The percentage of the chemicals correctly assigned to as sensitizer or non-sensitizer in h-CLAT compared to LLNA, respectively.

^c The percentage of the chemicals that were correctly identified in h-CLAT compared to LLNA.

EC3 is shown in Fig. 1 and Table 3. As shown in Fig. 1, CV75, EC150, and EC200 were positively correlated with LLNA EC3. In contrast, there is a poor correlation between MaxRFI of CD86 and CD54 to EC3. The correlation coefficients for the comparison between CV75, EC150 and EC200 values to EC3 were 0.617, 0.761, and 0.586, respectively (Table 3). Among these values, EC150 show the highest correlation coefficient. (See Figs. 2 and 3).

Next, the correlations between the EC150 and EC200 from h-CLAT to LLNA EC3 were analyzed for chemicals grouped based on protein expression pattern in the h-CLAT. In the h-CLAT, the alteration of CD86 and CD54 expression was measured and those chemicals with positive results were separated into three groups as follows: CD86 and CD54 enhancement (CD86+CD54+), CD86 enhancement only (CD86+CD54–), and CD54 enhancement only (CD86–CD54+). Both EC150 and EC200 showed a statistical significance correlation against EC3 for all three chemical groups (Table 3). The EC values for chemical groups with a CD86 positive result (e.g. CD86+CD54– or CD86+CD54+) correlated against LLNA EC3. Interestingly, the EC150 and EC200 for the CD86+CD54+ group were very similar with an r value larger than 0.95, meaning that there was a high correlation.

h-CLAT induction threshold

As shown in the previous section, CV75, EC150 and EC200 correlated with LLNA EC3. Among these three values, EC150 and EC200 were calculated based on the same rule for the calculation of LLNA EC3. Thus, EC150 and EC200 may be able to function as the LLNA EC3 for the h-CLAT. In terms of the EC150 and EC200,

protein expression pattern that occurs following chemical treatment was different between the substances and remains a major issue for trying to determine h-CLAT threshold using the same rule for all chemicals. In other words, common rules are required, regardless of the difference in expression patterns, for establishing h-CLAT EC values and to predict the LLNA EC3. Therefore, a rule was established for EC150 and EC200: The smaller value of EC150 and EC200 was defined as a minimal induction threshold (MIT). This made a prediction of LLNA EC3 possible by using one defined value for all positive outcomes in the h-CLAT. After comparing LLNA EC3 and MIT, MIT was found to be positively correlated with LLNA EC3 (correlation index was 0.638) in the same manner as CV75, EC150, and EC200 (Fig. 1 and Table 3).

Usage of MIT for the potency categorization for strong and weak sensitizer

Currently, LLNA EC3 is used to make a risk assessment and obtain labeling by rank classifying cosmetics and chemicals tested in the LLNA (Gerberick et al., 2001b; Api and Vey, 2008; United Nations, 2009). European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) described previously a category classification in which a threshold separating strong sensitizers (extreme and strong in LLNA) and weaker sensitizers (moderate and weak in LLNA) (ECETOC, 2003; Loveless et al., 2010). This threshold was 1% of the LLNA EC3. Therefore, we investigated whether the MIT value could distinguish between strong sensitizers and weak sensitizers like the LLNA EC3. As shown in Fig. 1 and Table 4 and 17 out of 23 strong sensitizers in LLNA had a MIT below 10 µg/mL.

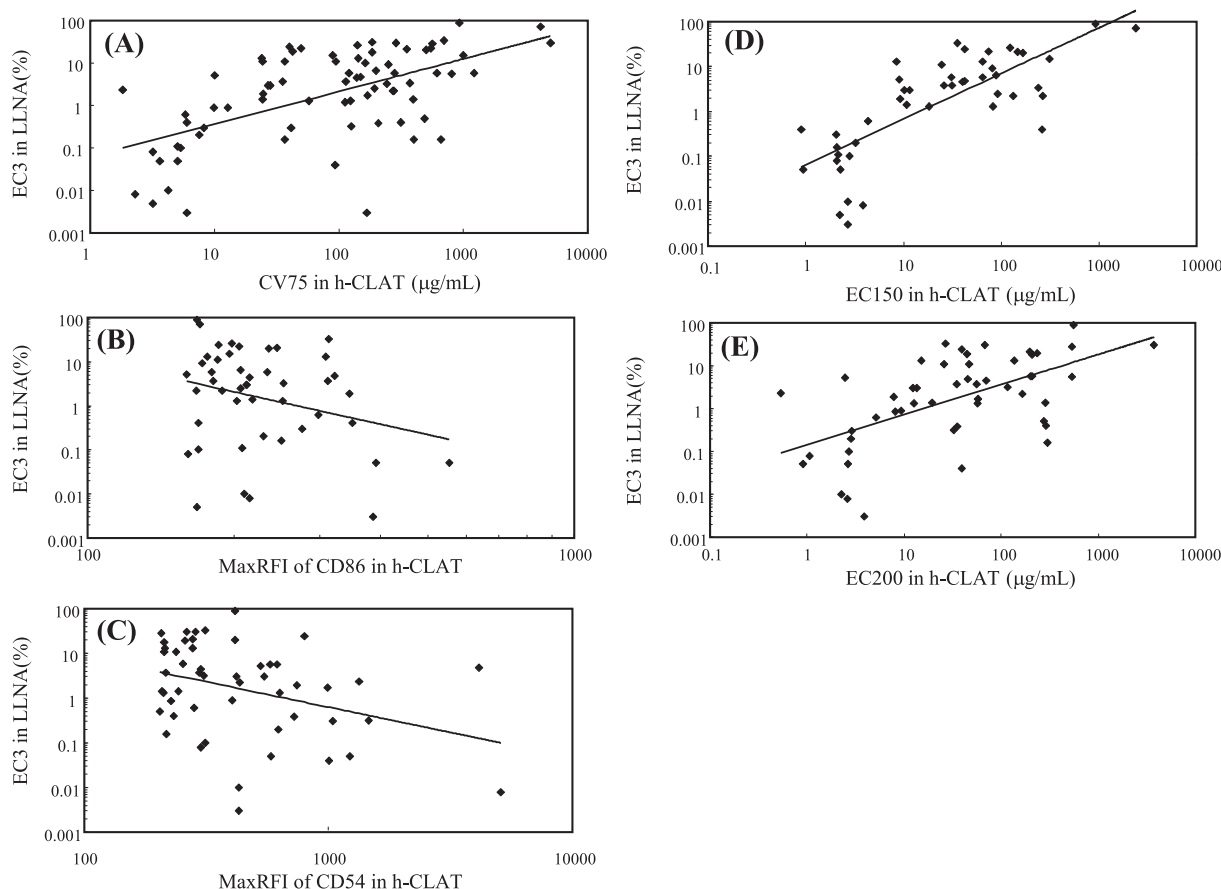


Fig. 1. The correlation between EC3 in LLNA and several h-CLAT values. The LLNA EC3 value and h-CLAT values were plotted in order to determine the correlation between the LLNA EC3 value and the h-CLAT values. (A) Correlation between CV75 in h-CLAT, (B) Maximum RFI of CD86, (C) Maximum RFI of CD54, (D) EC150, (E) EC200 and EC3 in LLNA.

Table 3
Results of the statistical analysis for the comparison between h-CLAT values and LLNA EC3.

	h-CLAT value ^a	vs ^b	groups ^c	Numbers of chemical ^d	r^2	r	Statistical significance ^e
1	CV75	EC3	both h-CLAT and LLNA positive	66	0.380	0.617	**
2	Max RFI (CD86)	EC3	both h-CLAT and LLNA positive	47	0.060	0.245	
3	Max RFI (CD54)	EC3	both h-CLAT and LLNA positive	51	0.123	0.351	
4	EC150	EC3	both h-CLAT and LLNA positive	47	0.578	0.761	**
5	EC200	EC3	both h-CLAT and LLNA positive	51	0.343	0.586	**
6	EC150	EC3	CD86+CD54+	32	0.5363	0.732	**
7	EC200	EC3	CD86+CD54+	32	0.5432	0.737	**
8	EC150	EC3	CD86+CD54–	15	0.6895	0.830	**
9	EC200	EC3	CD86–CD54+	19	0.1834	0.428	*
10	EC150	EC200	CD86+CD54+	32	0.9095	0.954	**
11	MIT	EC3	h-CLAT positive	66	0.4069	0.638	**

The correlation index was calculated for certain values.
 r^2 , determination coefficient; r , correlation coefficient.
^a Selected h-CLAT values (CV75, Max RFI, EC150, EC200, and MIT) used to determine the correlation index against EC3 or other values.
^b EC3 was compared to selected values as described before. In some cases, EC200 was compared to EC150.
^c Chemical group from which selected values were obtained for analysis.
^d The number of chemicals in the chemical group.
^e Statistical significance obtained for correlation. * $P < 0.05$, ** $P < 0.01$.

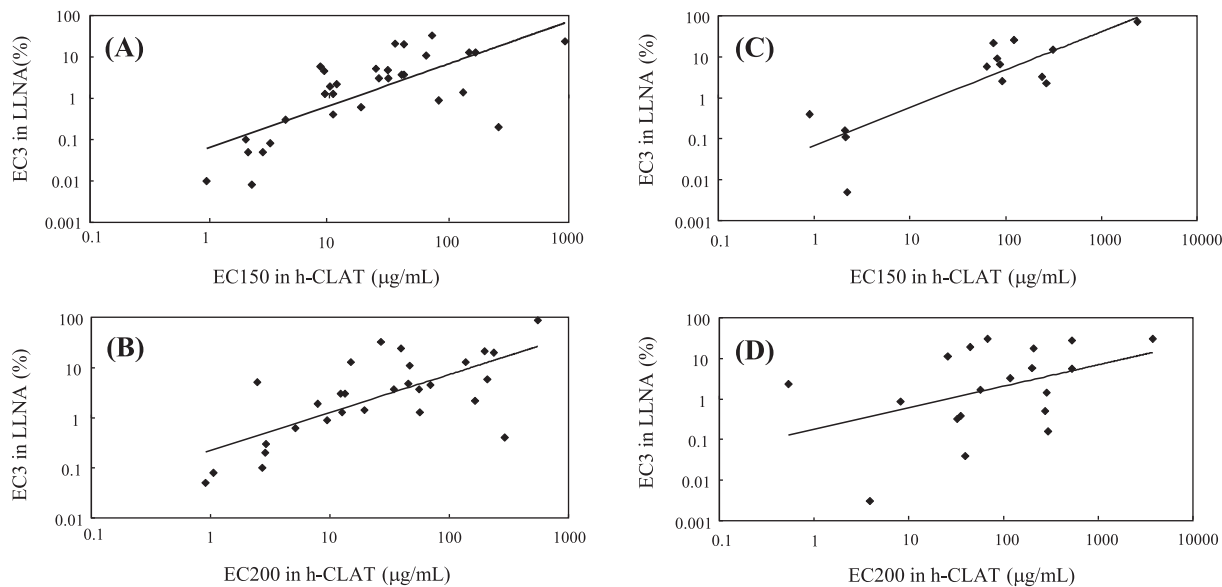


Fig. 2. The correlation between EC3 in LLNA and h-CLAT values based on protein expression pattern. The LLNA EC3 value and h-CLAT values were plotted in order to determine the correlation between three protein expression groups (A and B; CD86+CD54+group, C; CD86+CD54– group, D; CD86–CD54+group).

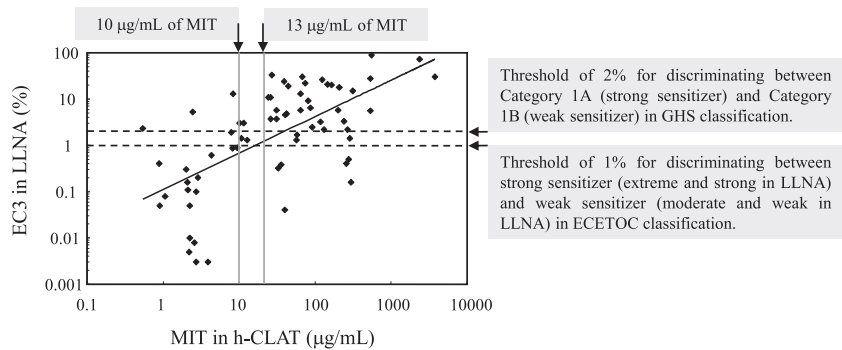


Fig. 3. Correlation between MIT in h-CLAT and EC3 in LLNA. The correlation between MIT and LLNA EC3 is described in the figure. Two solid lines indicate the threshold for differentiating a strong sensitizer from a weak sensitizer in ECETOC or GHS as shown in the figure. Two grey lines indicate the tentative threshold of MIT in h-CLAT for the prediction of potency categorization.

On the other hand, 39 out of 43 weak sensitizers had a MIT higher than 10 µg/mL. If the 10 µg/mL was set as the threshold for the separation of strong sensitizers and weak sensitizers in h-CLAT, the predictivity for the strong sensitizer was 81% and for the weak sensitizer was 87%.

Similarly, GHS has a sub-categorization for skin sensitization. For the sub-classification, EC3 is one of the criterion and other criteria include the induction threshold in a human repeated insult patch test (HRIPT) or human maximization test (HMT) and the reactivity in a guinea pig test (United Nations, 2009). In the 3rd revised edition of GHS, 2% of EC3 is used as a threshold to discriminate category 1A (strong sensitizer) and category 1B (weak sensitizer). When the 2% of EC3 threshold was used, 20 out of 29 strong sensitizers in GHS showed a lower MIT than 13 µg/mL. On the other hand, 34 out of 37 weak sensitizers in GHS had a higher MIT than 13 µg/mL. If the threshold was to be set at 13 µg/mL, the predictivity for discriminating strong and weak sensitizer using GHS criteria was 82%. Therefore, the MIT value may be used to rank classify a sensitizer.

Usage of MIT for the inter- or intra-laboratory reproducibility

MIT can be used to determine rank classification but MIT could also be used to check the inter- or intra-laboratory reproducibility. Because each flow-cytometer uses different algorithms for the detection of fluorescence intensity, the difference in the magnitude of raw data might not be able to be compared between the different facilities. In contrast, the MIT would not be affected by a difference of the flow-cytometer. In Table 5, the variability of MIT for DNCB acquired in the one laboratory is shown in upper table. As shown in Table 5, the results from four independent assays with DNCB and the corresponding MITs are listed. The variability of four MIT values was very narrow, average $\pm$ SD is 2.02 ± 0.62 µg/mL

Table 4

Accuracy of MIT for the potency categorization for strong and weak sensitizer.

Rank classification based on the ECETOC				h-CLAT MIT	
				<10 µg/mL Strong sensitizer 21	≥10 µg/mL Weak sensitizer 45
LLNA	<1%	Strong sensitizer	23	17	6
EC3	≥1%	Weak sensitizer	43	4	39
Predictivity				81%	87%
Overall accuracy				85%	
Rank classification based on the GHS				h-CLAT MIT	
				<13 µg/mL Strong sensitizer 25	≥13 µg/mL Weak sensitizer 45
LLNA	<2%	Strong sensitizer	29	20	9
EC3	>2%	Weak sensitizer	37	3	34
Predictivity				80%	76%
Overall accuracy				82%	

Upper table provides the predictive performance of h-CLAT MIT for discriminating between a strong and a weak sensitizer based on the ECETOC. According to the ECETOC, a strong sensitizer and a weak sensitizer can be identified using an EC3 threshold of 1%. The lower table provides the predictive performance of the h-CLAT MIT for distinguishing between a strong and a weak sensitizer based on the GHS sub-categorization. According to the GHS classification, a strong sensitizer and a weak sensitizer can be determined using an EC3 threshold of 2%. Numbers in the table are numbers of corresponding chemicals. The predictivity is the percentage of the chemicals that were correctly identified as sensitizer or non-sensitizer in h-CLAT compared to LLNA. The overall accuracy is the percentage of the chemicals that were correctly identified in h-CLAT compared to LLNA.

Table 5

Intra- or Inter-laboratory reproducibility measured by MIT.

Experiment	Judgment	CD86	CD54	MIT (µg/mL)
1	P	+	+	1.75
2	P	+	+	2.95
3	P	+	+	1.78
4	P	+	+	1.6
			Average	2.02
			SD	0.62
			CV (%)	31
Lab	Judgment	CD86	CD54	MIT (µg/mL)
A	P	+	+	1.90
B	P	+	+	0.71
C	P	+	+	1.98
D	P	+	+	1.65
E	P	+	+	1.92
			Average	1.63
			SD	0.53
			CV (%)	33

Upper table lists mean and SD values for MIT using four repeated experiments that were conducted in one laboratory. Variability of MIT values derived from four experiments where used as an indicator of intra-laboratory reproducibility.

Lower table provides mean and SD values for MIT based on 5 experiments obtained from five laboratories (lab. A–E), where each contributed one experiment. Inter-laboratory reproducibility was assessed using MIT variability.

P: positive in h-CLAT.

(CV: 31%). These data were then compared to the results obtained for DNCB in five other laboratories as referred to in Sakaguchi et al. (2009). From these data, MIT could be calculated as 1.90, 0.71, 1.98, 1.65, and 1.92 µg/mL for Lab A–E, respectively (Table 5). Four out of five MIT values were within the mean $\pm$ 2SD obtained by the current study. As a result, the h-CLAT has a good inter-laboratory reproducibility, and that MIT value can be used for the measurement of transferability.

Discussion

The h-CLAT has been found to be useful for identifying the hazard of skin sensitization by using CD86 and CD54 expression in THP-1 cells (Ashikaga et al., 2011). The predictive performance of h-CLAT for discriminating between a sensitizer and non sensitizer was shown in Table 2. The aim of this study was to investigate whether the h-CLAT could provide not only a hazard identification but also establish a potency categorization. Four key values derived from the h-CLAT (i.e., CV75, MaxRFI, EC150 and EC200) were considered as possible indicators for establishing the potency categorization. The consideration of the h-CLAT also provided us with an opportunity to better understand the usefulness of the h-CLAT for predicting the skin sensitizing potential of chemicals.

Generally, both the hazard (e.g., sensitization) and the strength of skin sensitization are important in understanding the risk associated with the chemical and to be able to do a risk assessment of cosmetic ingredients. Usually, the threshold of skin sensitization can be used for the risk assessment such as the no effect level (NOEL) from an HRIPT or HMT (Api and Vey, 2008). By contrast, the relative potency categorization based on the EC3 from LLNA has proven to be of value and provides the basis for current approaches to skin sensitization risk assessments (Gerberick et al., 2001a; Griem et al., 2003; Api and Vey, 2008). Earlier studies revealed that the LLNA EC3 highly correlated to the induction threshold of an HRIPT or HMT (Basketter et al., 2000; Gerberick et al., 2001a; Griem et al., 2003). The conservative threshold was defined based on the actual LLNA EC3 or threshold of HRIPT or HMT, and then the risk assessment was

done with conventional methods (Gerberick et al., 2001a). Therefore, the LLNA was expected to be the preferred approach for risk assessment of cosmetic ingredients, especially fragrances. However, all animal tests for safety assessment of cosmetics are prohibited in EU by the 7th amendment of the EU Cosmetic Directive (Directive 2003/15/EC, 2003). Therefore, alternates, *in vitro* methods, for the LLNA are required, so the possibility of the potency categorization by h-CLAT was examined.

EC3 value was derived from linear interpolation of LLNA dose response. The EC3 would be expected to be an indicator of risk because the EC3 highly correlates to the threshold of sensitization induction in humans (Gerberick et al., 2001b; Schneider and Akkan, 2004; Basketter et al., 2005). Therefore, the comparison between the values derived from the h-CLAT to the LLNA EC3 may not only provide a method for predicting hazard but also for predicting potency.

In current study, the relationship between the potency categorization value of the LLNA (EC3) and the CV75, Max RFI for CD86 and CD54, and EC150 and EC200 values of the h-CLAT were investigated in order to establish a potency categorization value for h-CLAT. Regarding the CV75, there was a correlation between CV75 and LLNA's EC3. CV75 is the index for the cytotoxicity of the chemical in the h-CLAT. Basketter et al. (2007) had reported that skin irritation potency correlated to the LLNA EC3. The elicitation concentration of the Guinea pig maximization test, which is the highest non-irritating concentration, is a measure of cytotoxicity and this measure correlated to the EC3 in LLNA (Basketter et al., 2007). Both the CV75 value and the skin irritation potency are measures of cytotoxicity, which may be the reason for a correlation between CV75 value and EC3 value.

Regarding the Max RFI, there was no statistically significant correlation between the Max RFI and EC3 in LLNA. There is no report indicating that the strength of the augmentation of the CD86 or CD54 protein expression in langerhans cells was proportional to the strength of skin sensitizing potency in certain chemicals. In fact, several researchers have investigated the response of contact allergens in several cell types. Python et al. has reported that the U937 was activated by contact allergens in association with augmentation of CD86 expression (Python et al., 2007). In the report, a difference between the magnitude of protein expression was not observed in U937 cells following exposure of a strong sensitizer (DNCB) and a weak sensitizer (citral). Moreover, Azam et al. (2006) obtained similar responses as Python et al. (2007) with MUTZ-3 cells. In cells other than dendritic cells, a similar phenomenon was observed in the keratinocyte response when treated with contact allergens. Emter et al. (2010) developed a keratinocyte-based reporter gene assay, which is based on the stress response of cells following contact allergen exposure. In these studies, differences the magnitude of protein/gene expression between the chemicals were observed but not between potency of skin sensitization.

By contrast to MaxRFI, the EC150 and EC200 had a correlation with EC3 from the LLNA. This means that the higher potency of skin sensitization would have a higher potential to induce protein expression in THP-1 cells. Meanwhile, the binding between the SH residues of protein and hapten is thought to be the critical step in the activation of langerhans cells (Bruchhausen et al., 2003). If this is correct, the strength of protein binding capacity might correspond to the strength of the augmentation of protein expression. To evaluate whether this hypothesis was correct or not, the protein binding capacity was investigated by Gerberick et al. (2007). Gerberick et al. (2007) have developed an *in vitro* skin sensitization test based on the chemical reactivity to model peptides. In this binding assay, the reducing ratio of model peptide was used for predicting the potency of the sensitizers. If the peptide reducing ratio is high, the binding capacity is low. In the

Gerberick et al. (2007) study, the mean reducing ratio for model peptide for 17 extreme and strong sensitizers in LLNA was $73.6 \pm 23.7\%$. On the other hand, the mean reactivity for 15 weak sensitizers tested in LLNA was $28.8 \pm 33.5\%$. A lower reactivity for model peptide was observed for weak sensitizers than strong sensitizers. This indicates that binding capacity to the peptide or protein also correlates to the potency of sensitization like the MIT. Moreover, the ability to induce protein expression might be correlated to the binding capacity of the chemicals to the peptide or protein.

In comparing EC150 or EC200 to EC3, EC200 seemed to have a lower correlation than the EC150 to EC3. Several studies have already showed a difference in protein expression pattern for CD86 and CD54 due to sensitizer exposure (Sakaguchi et al., 2009; Ashikaga et al., 2010). Because a similar difference was observed in another *in vitro* systems (dos Santos et al., 2009), the mechanisms of protein expression induction would appear to vary according to the sensitizer. The difference in protein expression was also observed for chemicals that enhanced CD54 protein expression only (CD86-CD54+) in the h-CLAT (24 h exposure). Although only CD54 protein expression was enhanced at 24 h, the same chemicals have induced CD86 protein expression up to 1.5 times at other time periods (data not shown), which indicate that the contact sensitizer can potentially induce the expression of both proteins. Though the reason of the lower correlation between EC200 and EC3 compared to EC150 is not clear, some speculations are available. One possibility is a self-fluorescence. If the chemical has a self-fluorescence, it might affect the flow cytometry analysis, causing the miscalculation of the RFI values. Therefore, it might affect to the calculation of EC values.

The MIT was defined for EC150 and EC200 as a universal and conservative value. A correlation with MIT and LLNA EC3 was observed, which would allow discriminating between strong and weak sensitizers. Recently, the third revised edition of GHS of classification and labelling of chemicals was published (United Nations, 2009). In latest edition, the sub-categorization of skin sensitization was added to distinguish strong sensitizers (sub-category 1A) from weak or other sensitizers (1B). EC3 of LLNA as well as human threshold are one way of determining the sub-category. Our results also suggest that the MIT might be able to differentiate between the GHS sub-categories by using the tentative threshold of $13 \mu\text{g/mL}$, which had approximately 80% accuracy (Table 4).

The utility of MIT as an indicator of reproducibility was also investigated. Needless to say, demonstration of long-term reproducibility will be necessary to support regulatory use of an *in vitro* model. Regarding the LLNA, the reproducibility between laboratories or within laboratory has been investigated before and after the acceptance of method. For example, hexylcinnamaldehyde (HCA) has been evaluated by LLNA a total of ten times in three laboratories (Dearman et al., 2001). Dearman et al. (2001) derived an average EC3 for HCA of 10.34 ± 0.59 . Similarly, isoeugenol has been evaluated in the LLNA in the same laboratory over 30 times. As reported by the authors, the average isoeugenol EC3 was calculated as $1.24 \pm 0.6\%$ (Basketter and Cadby, 2004). These results suggest that an indication of reproducibility can be obtained using the EC3 and since the h-CLAT MIT correlates with the EC3 of the LLNA, MIT can be used to evaluate the reproducibility of h-CLAT.

In conclusion, the results from this study suggest that MIT as well as CV75 might be useful markers to determine the potency of skin sensitizers. Moreover, MIT can also be an indicator of reproducibility of h-CLAT. To achieve the complete replacement of LLNA for potency categorization, MIT should be combined with other indicators obtained from other *in vitro* assays. The results in this

study would be helpful to construct a novel skin sensitization system without any animal tests.

Conflict of interest

The authors declare that we have no conflict of interest in connection with this paper.

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