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IL-1 α and IL-1 β as alternative biomarkers for risk assessment and the prediction of skin sensitization potency

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ABSTRACT

Potential biomarkers of skin sensitization in RAW264.7 mouse macrophages were investigated as alternatives to animal experiments and risk assessment. The concentrations that resulted in a cell viability of 90% (CV90) and 75% (CV75) were calculated by using a water-soluble tetrazolium salt (WST)-1 assay and used to analyze the skin sensitization potency of 23 experimental materials under equivalent treatment conditions. In addition, the expression of interleukin (IL)-1 α , IL-1 β , IL-31, tumor necrosis factor (TNF)- α , inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), and cyclooxygenase-2 (COX-2) was analyzed utilizing Western blotting. In the cell viability analysis, skin sensitizers were generally more cytotoxic and exhibited increased skin sensitization potency. However, nonsensitizers did not show any marked cytotoxic tendency. Biomarker analysis demonstrated that IL-1 α , IL-1 β , and the combination of IL-1 α and IL-1 β (IL-1 α + IL-1 β) predicted reliably skin sensitization potential (1) sensitivities of 94.4%, 83.3%, and 83.3%, specificities of 100%, 100%, and 100%, and (2) accuracies of 95.7%, 87%, and 87%, respectively. These observations correlated most reliably as indicators for skin sensitization potency. Data suggest that IL-1 α and IL-1 β may serve as potential biomarkers for skin sensitization and provide an alternative method to animal experiments for prediction of skin sensitization potency and risk assessment.

KEYWORDS

IL-1 α ; IL-1 β ; skin sensitization; biomarker; risk assessment

Introduction

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity reaction induced by low-molecular-weight allergens termed haptens. The sources of haptens include various substances such as metal ions and cosmetic raw materials (Castañeda et al. 2017). These reactions are commonly encountered worldwide and are deemed a major public health problem (Jo et al. 2018). Therefore, the risk assessment of various components, including cosmetic ingredients, is performed for safety management (Choi et al. 2018; Kim et al. 2017; Lim et al. 2018a, 2018b). Previously, skin sensitization risk assessments were conducted using a stimulation index (SI) value of 3, derived from local lymph node assay (LLNA) in mice, or the no expected sensitization induction level (NESIL), derived from human repeat insult patch testing (HRIPT) (Gerberick et al. 2001). However, animal and human experiments are limited owing to their cost and ethical implications. In particular, the testing of cosmetic

products and cosmetic ingredients on animals has been banned in Europe since March 2013 (EC (European Commission) 2013), which has necessitated the development of alternative methods and risk assessment (Casey 2016; Lee and Kacew 2018). For risk assessment, the EC3 (%), defined as the amount of a chemical that is required to elicit a three-fold increase in lymph node cell proliferative activity (SI $\geq$ 3), is considered a critical value derived from animal experiments.

At present, alternative methods for skin sensitization include *in silico* and cell-based methods (Nepal et al. 2018). Of these, cell-based experiments were performed employing keratinocytes (Andreas et al. 2011; Corsini et al. 2013, 2009; Dos Santos et al. 2011; Emter, Ellis, and Natsch 2010; Natsch et al. 2013), dendritic cells (DC) and DC-like cells (Ade et al. 2006; Ashikaga et al. 2010, 2006; Johansson et al. 2013, 2011; Lambrechts et al. 2010), and T cells (Vocanson et al. 2008).

Keratinocytes are known to secrete cytokines including tumor necrosis factor alpha (TNF- α), interleukin (IL)-18, and interferon (IFN)- γ , in response to hapten sensitization and to stimulate DC and T cells (Roggen 2014). Dendritic cells play an important role in the process of skin sensitization through the capture of haptens and release of prostaglandin E₂ (PGE₂) and reactive oxygen species (ROS), both of which act as Toll-like receptor (TLR) ligands (Christensen and Haase 2012; Erkes and Selvan 2014). These ligands bind to TLR2 and TLR4 and activate the nuclear factor kappa beta (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, which induce the expression of costimulatory molecules such as cluster of differentiation (CD)54, CD80, and CD86 on the cell surface, cytokines IL-1 α , IL-1 β , and TNF- α , and chemokines (Corsini et al. 2013; Kim et al. 2018; Lee and Kim 2018; Novak et al. 1999; Toebak et al. 2009). Therefore, DC function as antigen-presenting cells (APC) and induce T cell activity. Activated T cells induce ACD at hapten-exposed sites through proliferative and inflammatory responses (Banchereau and Steinman 1998); that is to say, skin sensitization is the consequence of immune responses by T cells. However, the use of DC obtained from the spleen and blood cells for testing of materials is limited owing to their low acquisition rates and high cost (Van Helden, Van Leeuwen, and Figdor 2008). In addition, a long time (typically 6–9 days) is required to induce the differentiation of DC precursors to DC (Van Helden, Van Leeuwen, and Figdor 2008). Further, experiments utilizing these primary cells demonstrated variations in expression of cytokines, cell surface molecules, and T cell activation (Van Helden, Van Leeuwen, and Figdor 2008). In contrast, RAW264.7 cells are known to play a role similar to that of DC in processing external antigens and are easy to culture (Van Helden, Van Leeuwen, and Figdor 2008). To circumvent the problems associated with DC, RAW264.7 cells were selected for our investigations.

In this study, 23 experimental materials were tested in RAW264.7 cells as a replacement for animal experiments for prediction of EC3 (%). To determine the most appropriate biomarkers to predict skin sensitization potential in these cells,

quantitative analysis was performed on skin sensitization-related biomarkers including IL-1 α , IL-1 β , IL-31, TNF- α , inducible nitric oxide synthase (iNOS), PGE₂, and cyclooxygenase-2 (COX-2) (Christensen and Haase 2012; Erkes and Selvan 2014; Jin et al. 2009; Laouini et al. 2005; Novak et al. 1999; Toebak et al. 2009). In addition, the association between these biomarkers and indicators of skin sensitization potency was determined to predict the most reliable indicator for skin sensitization potency and risk assessment.

Materials and methods

Tested chemicals

The following 18 skin sensitizers were selected: p-benzoquinone (BQ), 1-benzoylacetone (BA), 2,4-dinitrochlorobenzene (DNCB), potassium dichromate (PD), cyanuric chloride (CC), lauryl gallate (LG), methylisothiazolinone (MI), 2-aminophenol (2-AP), isoeugenol (IE), 3-aminophenol (3-AP), nickel sulfate (NS), citral (CI), hexyl cinnamaldehyde (HCA), eugenol (EU), imidazolidinyl urea (IU), linalool (LL), limonene (LI), and pyridine (PY). The following five nonsensitizers were selected: benzalkonium chloride (BKC), glycerol (GL), isopropanol (IP), methyl salicylate (MS), and sodium lauryl sulfate (SLS). The EC3 (%) values of all materials were based upon published data from LLNA experiments (Basketter, Gerberick, and Kimber 1998; Basketter et al. 1996, 2003; Basketter and Kimber 2006; OECD (Organisation for Economic Co-operation and Development) 2010; Ryan et al. 2002; Sakaguchi et al. 2009) and classified by skin sensitization potency (ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) 2003a; 2003b) (Table 1). All materials were purchased from Sigma-Aldrich (St. Louis, MO, USA). Experimental materials PD, MI, NS, IU, PY, BKC, GL, IP, and SLS were dissolved in saline, CC was dissolved in ethanol, and remaining materials (BQ, BA, DNCB, LG, 2-AP, IE, 3-AP, CI, HCA, EU, LL, LI, and MS) were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of ethanol or DMSO in culture medium were less than 1% for ethanol and less than 0.2% for DMSO.

Table 1. Determination of CV90 and CV75 for 23 experimental materials.

Chemicals	CAS number	CV90 ^a (µg/ml)	CV75 ^a (µg/ml)	LLNA		Ref.
				EC3 (%)	Potency ^d	
p-Benzoquinone (BQ)	106-51-4	0.306	1.154	0.0099	Extreme	Sakaguchi et al. 2009
1-Benzoylacetone (BA)	93-91-4	29.702	37.863	0.04	Extreme	Basketter and Kimber 2006
2,4-Dinitrochloro Benzene (DNCB)	97-00-7	0.101	0.124	0.049	Extreme	OECD (Organisation for Economic Co-operation and Development) 2010
Potassium dichromate (PD)	7778-50-9	0.101	0.209	0.08	Extreme	Basketter and Kimber 2006
Cyanuric chloride (CC)	108-77-0	8.881	21.137	0.09	Extreme	Basketter and Kimber 2006
Lauryl gallate (LG)	1166-52-5	0.081	0.271	0.30	Strong	Basketter and Kimber 2006
Methyl isothiazolinone (MI)	2682-20-4	2.496	3.226	0.4	Strong	Basketter et al. 2003
2-Aminophenol (2-AP)	95-55-6	1.014	1.979	0.50	Strong	Basketter and Kimber 2006
Isoeugenol (IE)	97-54-1	11.652	16.322	1.3	Moderate	Basketter and Kimber 2006
3-Aminophenol (3-AP)	591-27-5	3.121	4.956	3.2	Moderate	Basketter and Kimber 2006
Nickel sulfate (NS)	7786-81-4	9.005	12.970	4.8	Moderate	Ryan et al. 2002
Citral (CI)	5392-40-5	3.389	10.571	9.2	Moderate	OECD (Organisation for Economic Co-operation and Development) 2010
Hexyl cinnamaldehyde (HCA)	101-86-0	2.218	4.038	9.7	Moderate	OECD (Organisation for Economic Co-operation and Development) 2010
Eugenol (EU)	97-53-0	10.772	29.054	10.1	Weak	OECD (Organisation for Economic Co-operation and Development) 2010
Imidazolidinyl urea (IU)	39236-46-9	5.218	8.967	24	Weak	OECD (Organisation for Economic Co-operation and Development) 2010
Linalool (LL)	78-70-6	53.124	86.864	30	Weak	Basketter and Kimber 2006
Limonene (LI)	138-86-3	229.532	308.525	69	Weak	Basketter and Kimber 2006
Pyridine (PY)	110-86-1	221.495	419.973	72	Weak	Sakaguchi et al. 2009
Benzalkonium chloride (BKC)	8001-54-5	0.187	0.521	-	None	Basketter, Gerberick, and Kimber 1998
Glycerol (GL)	56-81-5	2887.124	4559.192	-	None	Basketter et al. 1996
Isopropanol (IP)	67-63-0	1000 ^b	5000 ^c	-	None	Basketter and Kimber 2006
Methyl salicylate (MS)	119-36-8	372.686	470.171	-	None	Basketter and Kimber 2006
Sodium lauryl sulfate (SLS)	151-21-3	7.487	8.932	-	False positive	Basketter et al. 1996

^a The results of the CV90 and CV75 are experimental data.

^{b,c} As cytotoxicity was not observed at the highest treatment concentration of 10,000 µg/ml, (b) and (c) were calculated as 10,000 µg/ml × 0.1 and 10,000 µg/ml × 0.5, respectively.

^d Extreme: EC3 (%) < 0.1, strong: 0.1 ≤ EC3 (%) < 1, moderate: 1 ≤ EC3 (%) < 10, weak: 10 ≤ EC3 (%) < 100 (ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) 2003a 2003b); EC3 (%): the amount of a chemical that is required to elicit a three-fold increase in lymph node cell proliferative activity (stimulation index, SI ≥ 3)

Cells and medium

RAW264.7 mouse macrophage cells were purchased from the American Type Cell Culture (ATCC) (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Corning, Tewksbury, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Corning) and 1% penicillin-streptomycin (P/S) (Corning). In all experiments, RAW264.7 cells were subcultured after reaching 80% confluence and grown at 37°C in a humidified atmosphere of 5% CO₂.

Cytotoxicity assay

The cytotoxicity of experimental materials was measured by using a water-soluble tetrazolium salt (WST)-1 assay. RAW264.7 cells were seeded at 2×10^4 cells/well on a 96-well plate. After 24 hr, medium containing 1% FBS and various concentrations up to a maximum of 10,000 µg/ml of test substance was added and cells were cultured for 48 hr. Subsequently, 20 µl WST-1 reagent (EZ-Cytox; DoGenBio, Seoul, Korea) was added to each well and the cell plate was incubated at 37.5°C for 1 hr. Cell viability was calculated through measurement of

absorbance of the cell plate at 450 nm by employing a spectrophotometer (VERSA Max Microplate Reader) (Molecular Devices Corp., CA, USA).

Determination of treatment concentrations for experimental materials

To determine equivalent treatment conditions for experimental materials, the concentrations that resulted in a cell viability of 90% (CV90) and 75% (CV75) were calculated by log-linear interpolation from the cytotoxicity results of the experimental materials from the following equations:

$$\log CV90 = \frac{(90 - C) \times \log(B) - (90 - A) \times \log(D)}{A - C}$$

where A is the minimum value of cell viability over 90% in testing groups, C is the maximum value of cell viability below 90% in testing groups, and B and D are the concentrations corresponding to A and C, respectively.

$$\log CV75 = \frac{(75 - C) \times \log(B) - (75 - A) \times \log(D)}{A - C}$$

where the minimum value of cell viability over 75% in testing groups, C is the maximum value of cell viability below 75% in testing groups, and B and D are the concentrations corresponding to A and C, respectively.

Chemical treatment of RAW264.7

RAW264.7 cells were seeded in a 60-mm cell culture dish at 1×10^5 cells/ml. After 24 hr, cells were treated with medium containing 1% FBS and each substance at the CV90 or CV75 for 48 hr. At least three independent experiments were performed.

Western blotting

After treatment, cells were harvested, washed with cold PBS, and then lysed with protein extraction solution (PRO-PREP) (iNtRON Biotechnology, Seongnam, Korea) at 4°C for 20 min, and centrifuged for 5 min at 10,500g. Protein quantitation was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, Ill., USA) of the supernatant from lysed cells. Cell samples for western blotting, prepared to contain

20 µg of protein per 10 µl, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked for 1 hr in a Tris-buffered saline with Tween 20 (TBST) containing 5% nonfat dry milk or 5% bovine serum albumin (BSA), and then incubated overnight at 4°C with an appropriate primary antibody [β -actin, 1:1,000; IL-1 β , 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); IL-1 α , 1:200; IL-31, 1:500 (Abcam, Cambridge, MA, USA); TNF- α , 1:500; COX-2, 1:500 (Cell Signaling Technology, Danvers, MA, USA); iNOS, 1:500 (Novus Biologicals, Littleton, CO, USA); PGE2, 1:500 (Biorbyt, Cambridge, UK)]. The membranes were washed with TBST and incubated with an appropriate secondary antibody [anti-mouse, 1:10,000 (Novus Biologicals, Littleton, CO, USA) or anti-rabbit, 1:5000 (GeneTex, Irvine, CA, USA)] for 1 hr at room temperature 25°C $\pm$ 2°C. The membranes were washed with TBST, reacted with ECL solution (Luminata Forte western HRP substrate) (Merck Millipore, Darmstadt, Germany), and developed by using a Davinch Chemi Imager (Davinch-K, Seoul, Korea). The quantification of band intensities was computed densitometrically by using ImageJ software (NIH, Bethesda, MD, USA). The band intensities were normalized to expression of B-actin, with the band intensities of the control group set as 1.

Data analysis and statistics

The relative band intensity (RBI) value, used as an indicator of the biomarker expression level, was calculated from the following equation:

RBI value = the band intensity of chemical-treated cells/the band intensity of control cells $\times$ 100.

The RBI value and standard error of mean (SEM) value were calculated for each biomarker and concentration; the RBI value was employed to set the cut-off value as a reference value for classification of a compound as a skin sensitizer or nonsensitizer. A substance was classified as a skin sensitizer when at least one of the RBI values was above the cut-off value. In contrast, a substance

was classified as a nonsensitizer when all the RBI values were below the cut-off value.

Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were analyzed through the comparison of our classification results for the test substances with findings from LLNA. In addition, the minimum concentration that corresponded to the cut-off value of the substance classified as a skin sensitizer was defined as the effective concentration (EC). The EC value was calculated by linear interpolation of the RBI value and from cell viability data for each material. The combination EC was calculated as the mean amount of EC values for two or more biomarkers. The correlation coefficient (R) was calculated to determine the correlation of cell viability and EC3 (%), and EC value and EC3 (%). Pearson's test was used for statistical analysis, with *P* values of < 0.05 considered statistically significant.

Results

Determination of CV90 and CV75 for experimental materials

The cell viability of the 18 skin sensitizers and five nonsensitizers was confirmed by WST-1 assay using pulmonary macrophage RAW264.7 cells. Log-linear interpolation was used to calculate the CV90 and CV75 for the experimental materials. Cell viability analysis revealed that skin sensitizers were more cytotoxic, with a lower EC3 (%), and that cytotoxicity was lower when the EC3 (%) was higher (Table 1). However, this trend was not observed in nonsensitizers. The non-sensitizers BKC and SLS exerted relatively potent cytotoxicity, whereas IP was not cytotoxic at the maximum treatment concentration of 10,000 µg/ml (Table 1).

Correlation between cell viability and EC3 (%)

The correlation between CV90, CV75, and EC3 (%) was evaluated for the 18 skin sensitizers. EC3 (%) was placed on the horizontal axis of the graph and CV90 and CV75 of each material were placed on the vertical axis of the graph (Figure 1). The correlation coefficient of was significant for CV90 and EC3 (%) at 0.6967 and correlation coefficient of CV75 and EC3 (%) significant at 0.7021.

Effects of experimental substances on biomarkers related to skin sensitization

The expression of biomarkers related to skin sensitization in response to exposure to skin sensitizers and non-sensitizers was measured by utilizing western blotting. The results for each substance are shown in Figure 2 (CV90) and Figure 3 (CV75). The RBI values of IL-1α were ≤ 120 for all skin sensitizers, except IE (Table 2 and Figure 4). For extreme and potent substances, the RBI values were higher at CV90, whereas those of moderate and weak substances were higher at CV75. The RBI values of non-sensitizers did not exceed 100. Further, the RBI values of IL-1β were ≤ 150 for all skin sensitizers except BQ, BA, and IE, but did not exceed 100 for all non-sensitizers. Most RBI values were higher at CV90. The RBI values of IL-31 were ≤ 150 for all skin sensitizers, except BQ, BA, PD, CC, LG, MI, and 2-AP, and values were significantly higher in the moderate and weak groups at CV90. For nonsensitizers, the RBI values were all below 100. The RBI values of TNF-α were ≤ 200 for skin sensitizers, except BQ, BA, IE, EU, LL, LI, and PY, and ≥ 200 for nonsensitizers, except IP. Data demonstrated that RBI values for skin sensitizers were higher at CV90, whereas those for

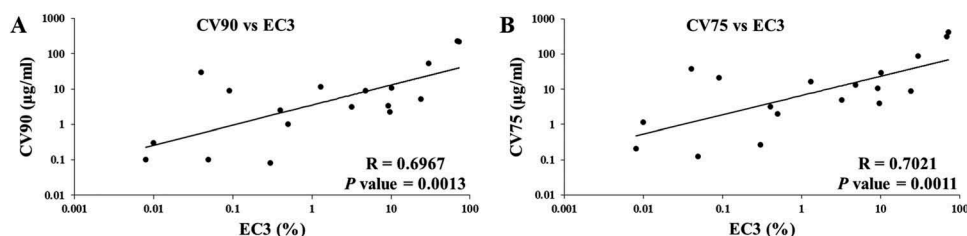


Figure 1. Correlation between cell viability and EC3 (%) for 18 skin sensitizers. Cell viability was determined by the WST-1 assay. CV90 and CV75 were calculated by log-linear interpolation. The EC3 (%) was selected for the horizontal axis of the graph and the CV90 and CV75 for each material was selected for the vertical axis. The correlation coefficient (R) was calculated to determine the correlation of the cell viability and the EC3 (%). Pearson's test was used for statistical analysis; *P* values of < 0.05 were considered statistically significant. (a) The correlation between CV90 and EC3 (%) and (b) the correlation between CV75 and EC3 (%).

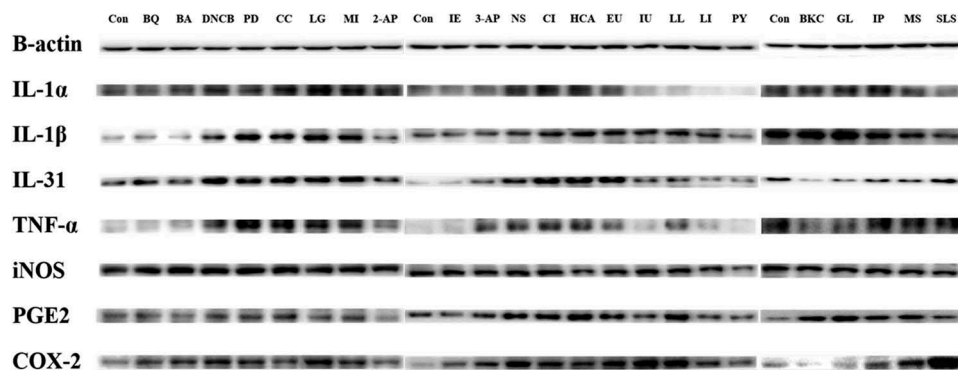


Figure 2. The expression of biomarkers related to skin sensitization in response to exposure of RAW264.7 cells to skin sensitizers and nonsensitizers (CV90) was measured by using western blotting.

(Abbreviations: Con, control; BQ, p-benzoquinone; BA, 1-benzoylacetone; DNCB, 2,4-dinitrochlorobenzene; PD, potassium dichromate; CC, cyanuric chloride; LG, lauryl gallate; MI, methylisothiazolinone; 2-AP, 2-aminophenol; IE, isoeugenol; 3-AP, 3-aminophenol; NS, nickel sulfate; CI, citral; HCA, hexyl cinnamaldehyde; EU, eugenol; IU, imidazolidinyl urea; LL, linalool; LI, limonene; PY, pyridine; BKC, benzalkonium chloride; GL, glycerol; IP, isopropanol; MS, methyl salicylate; SLS, sodium lauryl sulfate)

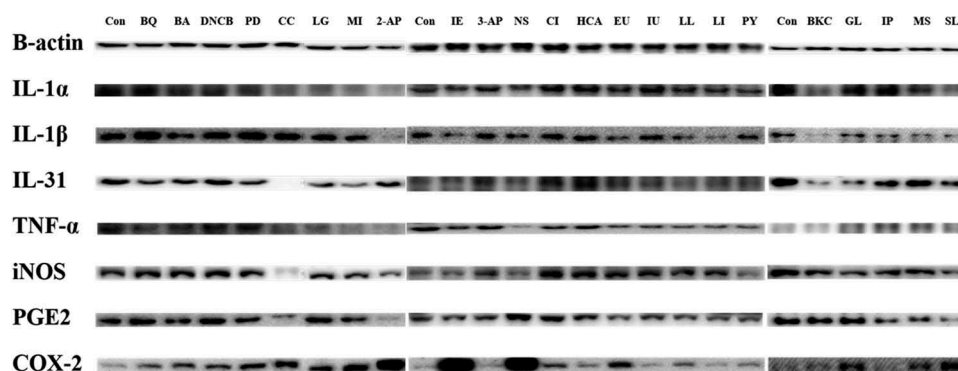


Figure 3. The expression of biomarkers related to skin sensitization in response to the exposure of RAW264.7 cells to skin sensitizers and non-sensitizers (CV75) was measured by using western blotting.

(Abbreviations: Con, control; BQ, p-benzoquinone; BA, 1-benzoylacetone; DNCB, 2,4-dinitrochlorobenzene; PD, potassium dichromate; CC, cyanuric chloride; LG, lauryl gallate; MI, methylisothiazolinone; 2-AP, 2-aminophenol; IE, isoeugenol; 3-AP, 3-aminophenol; NS, nickel sulfate; CI, citral; HCA, hexyl cinnamaldehyde; EU, eugenol; IU, imidazolidinyl urea; LL, linalool; LI, limonene; PY, pyridine; BKC, benzalkonium chloride; GL, glycerol; IP, isopropanol; MS, methyl salicylate; SLS, sodium lauryl sulfate)

nonsensitizers were greater at CV75. The RBI values of iNOS were ≥ 120 for skin sensitizers, except CC, 2-AP, IE, NS, and PY; however, the values were not above 100 for non-sensitizers, except for MS. For skin sensitizers, RBI values were 100 or below at CV90, but changed at CV75. The RBI values for PGE₂ of ≥ 120 with BQ, LG, MI, 3-AP, NS, CI, HCA, EU, IU, LL, LI, and PY, and ≥ 120 for the remaining skin sensitizers. The RBI values were more than 120 for non-sensitizers and these values were higher at CV90. For COX-2, RBI values were ≥ 150 for all skin sensitizers; however, the RBI values for nonsensitizers, with the exception of GL, MS, and SLS, did not exceed 150.

Classification value and accuracy

Based upon the RBI values of biomarkers related to skin sensitization, studies were undertaken to differentiate the cut-off value (reference value) between skin sensitizers and nonsensitizers (Table 3). From the results provided in Table 3, the accuracy of the cut-off values was determined (Table 4). The sensitivity, specificity, and accuracy were 94.4% (17/18), 100% (5/5), and 95.7% (22/23), respectively, for IL-1 α , based upon the cut-off value of 120; 83.3% (15/18), 100% (5/5), and 87% (20/23) for IL-1 β , based upon the cut-off value of 150; 61.1% (11/18), 100% (5/5), and 69.6% (16/23) for IL-31, based upon the cut-off value of 150; 61.1% (11/18), 80% (4/5), and

Table 2. RBI (relative band intensity) values of biomarkers related to skin sensitization in response to the exposure of RAW264.7 cells to skin sensitizers and non-sensitizers.

LLNA potency ^a	Chemicals	RBI values						COX-2							
		IL-1 α		IL-1 β		IL-31		TNF- α		iNOS		PGE2			
		CV90	CV75	CV90	CV75	CV90	CV75	CV90	CV75	CV90	CV75	CV90	CV75	CV90	CV75
Extreme	BQ	123.0 $\pm$ 20.6	94.4 $\pm$ 8.3	117.8 $\pm$ 10.8	142.3 $\pm$ 13.6	115.9 $\pm$ 19.4	92.1 $\pm$ 2.0	169.7 $\pm$ 39.8	108.5 $\pm$ 0.5	83.0 $\pm$ 22.3	151.5 $\pm$ 2.3	106.0 $\pm$ 0.9	125.4 $\pm$ 19.4	163.1 $\pm$ 14.2	238.3 $\pm$ 21.9
	BA	151.4 $\pm$ 52.1	81.3 $\pm$ 0.8	117.4 $\pm$ 16.1	90.4 $\pm$ 0.9	88.5 $\pm$ 17.3	83.5 $\pm$ 1.8	145.1 $\pm$ 28.4	105.1 $\pm$ 0.1	88.5 $\pm$ 31.3	173.1 $\pm$ 29.3	107.9 $\pm$ 23.4	96.5 $\pm$ 9.4	191.2 $\pm$ 22.8	248.8 $\pm$ 6.7
	DNCB	275.7 $\pm$ 129.0	98.8 $\pm$ 9.0	225.1 $\pm$ 59.1	110.7 $\pm$ 3.0	159.2 $\pm$ 54.6	112.4 $\pm$ 3.2	295.0 $\pm$ 54.4	112.9 $\pm$ 3.9	87.9 $\pm$ 33.0	177.9 $\pm$ 38.0	93.5 $\pm$ 5.3	116.3 $\pm$ 5.9	259.1 $\pm$ 34.2	261.8 $\pm$ 43.6
	PD	273.3 $\pm$ 138.0	65.1 $\pm$ 3.7	265.9 $\pm$ 126.7	113.1 $\pm$ 5.4	146.1 $\pm$ 43.2	89.1 $\pm$ 12.5	409.6 $\pm$ 78.6	106.3 $\pm$ 6.2	79.1 $\pm$ 39.6	128.0 $\pm$ 26.2	107.2 $\pm$ 4.4	96.3 $\pm$ 6.7	270.2 $\pm$ 67.2	293.3 $\pm$ 62.0
Strong	CC	238.5 $\pm$ 105.9	44.0 $\pm$ 7.6	275.0 $\pm$ 94.6	82.2 $\pm$ 23.9	146.9 $\pm$ 53.2	34.2 $\pm$ 19.2	437.3 $\pm$ 60.8	76.0 $\pm$ 1.6	83.3 $\pm$ 41.4	56.9 $\pm$ 20.9	107.0 $\pm$ 6.2	57.1 $\pm$ 8.4	212.3 $\pm$ 49.4	383.7 $\pm$ 4.2
	LG	234.8 $\pm$ 82.4	58.5 $\pm$ 7.1	313.7 $\pm$ 106.1	96.9 $\pm$ 8.4	125.3 $\pm$ 47.4	79.6 $\pm$ 4.1	515.4 $\pm$ 107.7	74.2 $\pm$ 6.0	69.0 $\pm$ 30.0	138.9 $\pm$ 28.9	75.9 $\pm$ 2.2	123.4 $\pm$ 10.4	244.2 $\pm$ 69.1	205.2 $\pm$ 1.5
	MI	204.8 $\pm$ 53.0	67.6 $\pm$ 18.9	238.5 $\pm$ 111.0	84.4 $\pm$ 0.7	115.5 $\pm$ 54.3	72.0 $\pm$ 6.5	411.2 $\pm$ 33.6	61.8 $\pm$ 10.1	73.5 $\pm$ 29.4	120.8 $\pm$ 19.2	80.6 $\pm$ 9.4	132.1 $\pm$ 10.4	201.3 $\pm$ 51.4	368.4 $\pm$ 19.7
	2-AP	153.4 $\pm$ 23.3	34.8 $\pm$ 7.9	201.1 $\pm$ 49.5	40.9 $\pm$ 14.3	71.3 $\pm$ 34.6	104.6 $\pm$ 16.4	271.5 $\pm$ 17.1	41.2 $\pm$ 7.0	78.8 $\pm$ 18.2	92.3 $\pm$ 18.8	58.7 $\pm$ 4.0	48.4 $\pm$ 15.0	143.3 $\pm$ 40.6	642.5 $\pm$ 44.9
Moderate	IE	92.9 $\pm$ 0.9	103.2 $\pm$ 20.9	145.4 $\pm$ 22.0	64.7 $\pm$ 0.04	179.1 $\pm$ 23.0	84.0 $\pm$ 9.1	107.0 $\pm$ 28.3	72.2 $\pm$ 7.3	60.7 $\pm$ 10.3	75.0 $\pm$ 4.3	83.4 $\pm$ 1.1	82.0 $\pm$ 1.0	401.6 $\pm$ 43.5	931.3 $\pm$ 304.7
	3-AP	85.3 $\pm$ 2.5	240.9 $\pm$ 86.5	217.0 $\pm$ 58.2	101.6 $\pm$ 12.6	454.3 $\pm$ 54.8	126.5 $\pm$ 14.8	216.4 $\pm$ 54.8	122.5 $\pm$ 19.6	54.9 $\pm$ 10.7	127.0 $\pm$ 33.5	131.2 $\pm$ 11.5	105.9 $\pm$ 5.3	475.0 $\pm$ 19.6	221.8 $\pm$ 26.8
	NS	98.2 $\pm$ 18.3	206.2 $\pm$ 82.9	232.3 $\pm$ 53.7	63.6 $\pm$ 20.8	670.2 $\pm$ 133.3	89.1 $\pm$ 2.4	225.9 $\pm$ 61.0	58.3 $\pm$ 28.0	47.6 $\pm$ 12.8	78.2 $\pm$ 18.3	178.8 $\pm$ 12.6	145.3 $\pm$ 4.7	628.8 $\pm$ 14.4	790.4 $\pm$ 220.7
	CI	101.0 $\pm$ 10.7	380.2 $\pm$ 153.9	314.9 $\pm$ 115.5	85.8 $\pm$ 25.0	901.3 $\pm$ 210.1	166.7 $\pm$ 5.6	234.3 $\pm$ 41.5	131.5 $\pm$ 43.5	71.3 $\pm$ 3.5	177.0 $\pm$ 28.9	179.4 $\pm$ 19.9	154.6 $\pm$ 15.3	562.0 $\pm$ 2.4	367.7 $\pm$ 49.2
Weak	HCA	118.3 $\pm$ 1.8	297.8 $\pm$ 86.8	355.0 $\pm$ 119.5	100.1 $\pm$ 28.6	1008.9 $\pm$ 254.9	224.0 $\pm$ 9.7	230.6 $\pm$ 49.3	139.2 $\pm$ 34.5	73.9 $\pm$ 4.3	172.9 $\pm$ 34.1	229.0 $\pm$ 28.4	140.9 $\pm$ 14.0	529.5 $\pm$ 22.1	219.0 $\pm$ 8.4
	EU	68.9 $\pm$ 10.8	250.3 $\pm$ 73.4	393.2 $\pm$ 152.6	97.7 $\pm$ 2.1	981.9 $\pm$ 198.6	180.2 $\pm$ 2.5	149.3 $\pm$ 38.9	109.3 $\pm$ 40.0	48.2 $\pm$ 4.8	168.1 $\pm$ 15.7	167.9 $\pm$ 5.7	136.7 $\pm$ 34.4	585.4 $\pm$ 34.7	578.1 $\pm$ 124.8
	IU	39.2 $\pm$ 0.9	315.9 $\pm$ 93.2	383.0 $\pm$ 156.5	108.1 $\pm$ 21.4	719.6 $\pm$ 133.3	194.8 $\pm$ 28.1	85.2 $\pm$ 7.9	237.3 $\pm$ 110.0	53.9 $\pm$ 5.2	204.8 $\pm$ 22.9	130.1 $\pm$ 12.9	264.5 $\pm$ 90.8	753.2 $\pm$ 26.0	223.5 $\pm$ 53.7
	LL	27.5 $\pm$ 3.0	175.2 $\pm$ 31.9	287.1 $\pm$ 95.0	66.0 $\pm$ 13.5	655.8 $\pm$ 70.6	131.6 $\pm$ 15.0	181.6 $\pm$ 25.6	138.5 $\pm$ 58.1	40.5 $\pm$ 8.1	164.1 $\pm$ 27.0	185.3 $\pm$ 13.3	178.7 $\pm$ 51.9	692.4 $\pm$ 9.4	282.4 $\pm$ 11.6
None	LI	8.2 $\pm$ 0.2	151.2 $\pm$ 27.6	297.1 $\pm$ 79.9	43.0 $\pm$ 3.3	558.4 $\pm$ 27.5	175.4 $\pm$ 18.3	86.4 $\pm$ 11.2	101.8 $\pm$ 36.4	47.4 $\pm$ 12.2	135.7 $\pm$ 17.5	126.7 $\pm$ 15.0	203.6 $\pm$ 56.1	746.7 $\pm$ 179.6	149.1 $\pm$ 25.9
	PY	10.3 $\pm$ 0.2	146.0 $\pm$ 24.2	219.5 $\pm$ 90.5	87.5 $\pm$ 17.8	509.1 $\pm$ 40.8	219.3 $\pm$ 43.0	59.3 $\pm$ 7.7	67.1 $\pm$ 16.4	29.3 $\pm$ 0.03	104.9 $\pm$ 3.0	114.7 $\pm$ 22.6	217.6 $\pm$ 51.6	350.5 $\pm$ 7.6	277.3 $\pm$ 24.9
	BKC	65.1 $\pm$ 17.7	58.6 $\pm$ 16.8	62.3 $\pm$ 12.8	52.5 $\pm$ 19.3	29.3 $\pm$ 6.3	27.6 $\pm$ 6.3	58.1 $\pm$ 10.5	68.1 $\pm$ 7.9	72.3 $\pm$ 29.8	57.7 $\pm$ 3.2	249.8 $\pm$ 79.1	59.2 $\pm$ 8.0	55.4 $\pm$ 14.6	91.5 $\pm$ 5.9
	GL	62.0 $\pm$ 18.1	62.5 $\pm$ 1.2	61.7 $\pm$ 14.9	84.6 $\pm$ 10.4	56.0 $\pm$ 8.2	30.3 $\pm$ 2.8	59.9 $\pm$ 7.6	168.1 $\pm$ 19.4	85.0 $\pm$ 28.6	42.6 $\pm$ 6.9	375.4 $\pm$ 191.6	57.9 $\pm$ 10.7	118.8 $\pm$ 10.7	214.9 $\pm$ 35.1
	IP	56.0 $\pm$ 21.5	56.3 $\pm$ 3.3	58.8 $\pm$ 5.3	78.3 $\pm$ 12.8	86.4 $\pm$ 27.2	39.6 $\pm$ 5.2	91.7 $\pm$ 31.6	203.3 $\pm$ 23.5	89.0 $\pm$ 28.3	34.6 $\pm$ 3.5	316.3 $\pm$ 80.6	45.0 $\pm$ 4.2	126.3 $\pm$ 33.1	79.0 $\pm$ 17.0
	MS	36.4 $\pm$ 10.6	34.4 $\pm$ 4.2	64.4 $\pm$ 12.1	70.0 $\pm$ 10.9	77.1 $\pm$ 17.3	51.5 $\pm$ 3.9	103.1 $\pm$ 37.1	197.3 $\pm$ 22.8	101.2 $\pm$ 30.5	54.3 $\pm$ 3.6	456.3 $\pm$ 149.3	51.5 $\pm$ 2.1	213.5 $\pm$ 7.4	147.8 $\pm$ 32.0
	SLS	16.8 $\pm$ 4.9	17.3 $\pm$ 1.9	49.5 $\pm$ 15.6	78.9 $\pm$ 20.6	116.1 $\pm$ 28.6	38.9 $\pm$ 0.7	70.4 $\pm$ 19.3	194.0 $\pm$ 22.4	99.6 $\pm$ 35.3	24.4 $\pm$ 0.2	401.4 $\pm$ 137.3	41.5 $\pm$ 8.2	300.9 $\pm$ 8.4	270.5 $\pm$ 29.8

The expression of biomarkers in response to each substance were quantified by ImageJ software. The RBI values of the control group were set to 100. The data are expressed as the mean RBI $\pm$ SEM of a minimum of three experiments.

^a Extreme: EC3 (%) < 0.1, strong: 0.1 $\leq$ EC3 (%) < 1, moderate: 1 $\leq$ EC3 (%) < 10, weak: 10 $\leq$ EC3 (%) < 100 (ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) 2003a; 2003b) (Abbreviations: BQ, p-benzoquinone; BA, 1-benzoylacetone; DNCB, 2,4-dinitrochlorobenzene; PD, potassium dichromate; CC, cyanuric chloride; LG, lauryl gallate; MI, methylisothiazolone; 2-AP, 2-aminophenol; IE, isoeugenol; 3-AP, 3-aminophenol; NS, nickel sulfate; CI, citral; HCA, hexyl cinnamaldehyde; EU, eugenol; IU, imidazolidinyl urea; LL, linalool; LI, limonene; PY, pyridine; BKC, benzalkonium chloride; GL, glycerol; IP, isopropanol; MS, methyl salicylate; SLS, sodium lauryl sulfate)

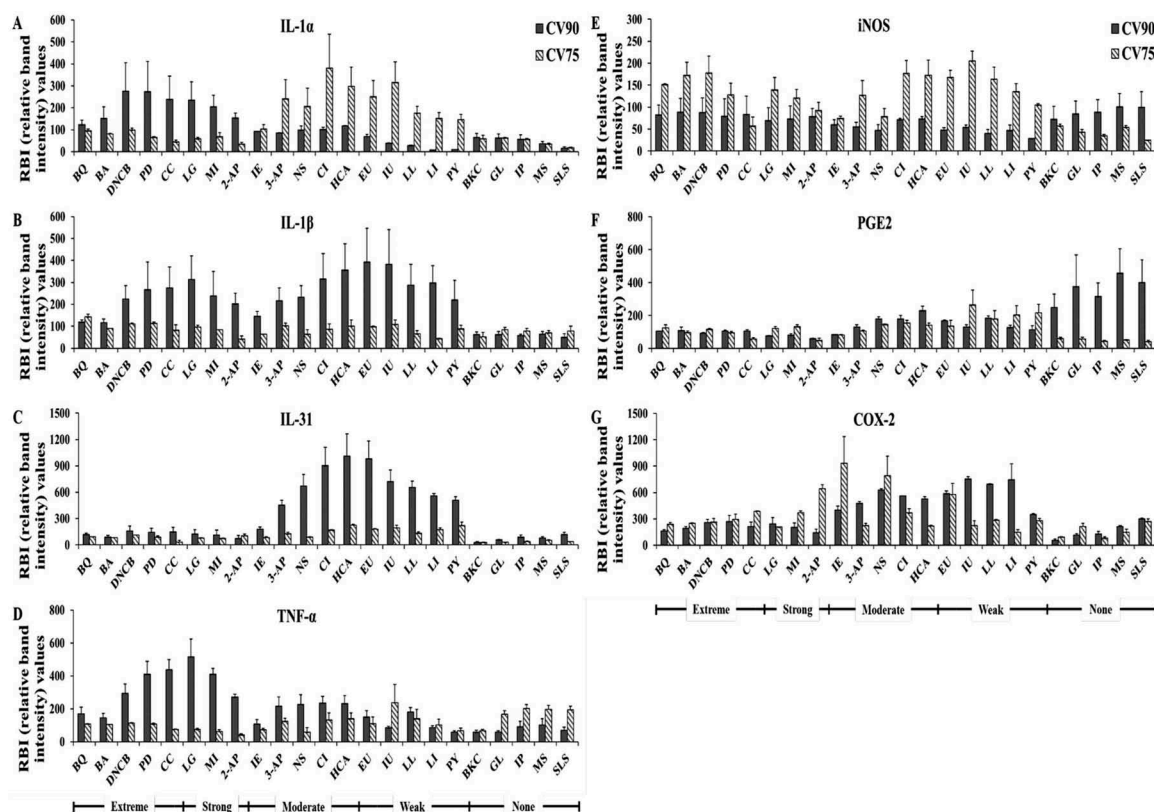


Figure 4. Histogram of RBI values of biomarkers related to skin sensitization in response to the exposure of RAW264.7 cells to skin sensitizers and nonsensitizers. The expression of biomarkers in response to each substance was quantified by ImageJ software. The RBI values of the control group were set to 100. The data were expressed as the mean RBI $\pm$ SEM for a minimum of three experiments. IL-1 α (a), IL-1 β (b), IL-31 (c), TNF- α (d), iNOS (e), PGE2 (f), and COX-2 (g).

(Abbreviation: BQ, p-benzoquinone; BA, 1-benzoylacetone; DNCB, 2,4-dinitrochlorobenzene; PD, potassium dichromate; CC, cyanuric chloride; LG, lauryl gallate; MI, methylisothiazolinone; 2-AP, 2-aminophenol; IE, isoeugenol; 3-AP, 3-aminophenol; NS, nickel sulfate; CI, citral; HCA, hexyl cinnamaldehyde; EU, eugenol; IU, imidazolidinyl urea; LL, linalool; LI, limonene; PY, pyridine; BKC, benzalkonium chloride; GL, glycerol; IP, isopropanol; MS, methyl salicylate; SLS, sodium lauryl sulfate)

65.2% (15/23), respectively, for TNF- α , respectively, based upon the cut-off value of 200; 72.2% (13/18), 100% (5/5), and 78.3% (18/23), respectively, for iNOS, based on the cut-off value of 120; 66.7% (12/18), 0% (0/5), and 52.2% (12/23), respectively, for PGE₂, based upon the cut-off value of 120; 100% (18/18), 40% (2/5), and 87% (20/23), respectively, for COX-2, based upon the cut-off value of 150; 83.3% (15/18), 100% (5/5), and 87% (20/23) for IL-1 α + IL-1 β , based upon the cut-off values of 120 (IL-1 α) and 150 (IL-1 β), respectively. The biomarkers with over 85% accuracy included IL-1 α , IL-1 β , COX-2, and IL-1 α + IL-1 β , with a maximum accuracy of 95.7% for IL-1 α . The specificity for PGE₂ and COX-2 was relatively low (0% and 40%, respectively).

Determination of the relationship between EC value and EC3 (%) of skin sensitizers

Based upon the cut-off values, the minimum concentration corresponding to the cut-off value (EC) for skin sensitizers was calculated for each biomarker (Table 5). In addition, the correlation between EC and EC3 (%) for each substance was determined and whether this value was significant identified (Figure 5; Table 6). The horizontal axis of the graph contained the EC3 (%) and vertical axis displayed the EC value of each material (Figure 5). The correlation coefficients for EC (120) and EC3 (%) for IL-1 α were found to be significant at 0.7528 and that for EC (200) and EC3 (%) for TNF- α 0.7319. The correlation coefficients for EC (150) and EC3

Table 3. Discrimination results according to the cut-off values of the biomarkers for test substances.

LLNA potency ^a	Chemicals	Cut-off value							IL-1α + IL-1β IL-1α ≥ 120 & IL-1β ≥ 150
		IL-1α ≥ 120	IL-1β ≥ 150	IL-31 ≥ 150	TNF-α ≥ 200	iNOS ≥ 120	PGE2 ≥ 120	COX-2 ≥ 150	
Extreme	BQ	+	-	-	-	+	+	+	-
	BA	+	-	-	-	+	-	+	-
	DNCB	+	+	+	+	+	-	+	+
	PD	+	+	-	+	+	-	+	+
	CC	+	+	-	+	-	-	+	+
Strong	LG	+	+	-	+	+	+	+	+
	MI	+	+	-	+	+	+	+	+
	2-AP	+	+	-	+	-	-	+	+
Moderate	IE	-	-	+	-	-	-	+	-
	3-AP	+	+	+	+	+	+	+	+
	NS	+	+	+	+	-	+	+	+
	CI	+	+	+	+	+	+	+	+
	HCA	+	+	+	+	+	+	+	+
Weak	EU	+	+	+	-	+	+	+	+
	IU	+	+	+	+	+	+	+	+
	LL	+	+	+	-	+	+	+	+
	LI	+	+	+	-	+	+	+	+
	PY	+	+	+	-	-	+	+	+
None	BKC	-	-	-	-	-	+	-	-
	GL	-	-	-	-	-	+	+	-
	IP	-	-	-	+	-	+	-	-
	MS	-	-	-	-	-	+	+	-
	SLS	-	-	-	-	-	+	+	-

The discrimination results are shown according to the cut-off values for test substances.

A positive sign (+) indicates that at least one of the RBI values of CV90 and CV75 was greater than or equal to the cut-off value.

A negative sign (-) indicates that all the RBI values of CV90 and CV75 were below the cut-off value.

^a Extreme: EC3 (%) < 0.1, strong: 0.1 ≤ EC3 (%) < 1, moderate: 1 ≤ EC3 (%) < 10, weak: 10 ≤ EC3 (%) < 100 (ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) 2003a; 2003b)

(Abbreviations: BQ, p-benzoquinone; BA, 1-benzoylacetone; DNCB, 2,4-dinitrochlorobenzene; PD, potassium dichromate; CC, cyanuric chloride; LG, lauryl gallate; MI, methylisothiazolinone; 2-AP, 2-aminophenol; IE, isoeugenol; 3-AP, 3-aminophenol; NS, nickel sulfate; CI, citral; HCA, hexyl cinnamaldehyde; EU, eugenol; IU, imidazolidinyl urea; LL, linalool; LI, limonene; PY, pyridine; BKC, benzalkonium chloride; GL, glycerol; IP, isopropanol; MS, methyl salicylate; SLS, sodium lauryl sulfate)

Table 4. Determination of accuracy according to the cut-off values of the biomarkers.

Biomarkers	Cut-off value	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
IL-1α	≥ 120	94.4 (17/18)	100 (5/5)	100 (17/17)	83.3 (5/6)	95.7 (22/23)
IL-1β	≥ 150	83.3 (15/18)	100 (5/5)	100 (15/15)	62.5 (5/8)	87 (20/23)
IL-31	≥ 150	61.1 (11/18)	100 (5/5)	100 (11/11)	41.7 (5/12)	69.6 (16/23)
TNF-α	≥ 200	61.1 (11/18)	80 (4/5)	91.7 (11/12)	36.4 (4/11)	65.2 (15/23)
iNOS	≥ 120	72.2 (13/18)	100 (5/5)	100 (13/13)	50 (5/10)	78.3 (18/23)
PGE ₂	≥ 120	66.7 (12/18)	0 (0/5)	70.6 (12/17)	0 (0/6)	52.2 (12/23)
COX-2	≥ 150	100 (18/18)	40 (2/5)	85.7 (18/21)	100 (2/2)	87 (20/23)
IL-1α + IL-1β	IL-1α ≥ 120 and IL-1β ≥ 150	83.3 (15/18)	100 (5/5)	100 (15/15)	62.5 (5/8)	87 (20/23)

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated through the comparison of our classification results for the test substances with the LLNA results. IL-1α was the most accurate, at an accuracy of 95.7%.

(%) were significant for IL-1β, IL-31, and COX-2 at 0.7637, 0.5771, and 0.4263, respectively, whereas the coefficients for iNOS and PGE₂ were significant at 0.6786 and 0.6117, respectively. The correlation coefficient for the combination EC and EC3 (%) for IL-1α + IL-1β was marked at as 0.8633. Therefore, the biomarker with the highest correlation between EC value and EC3 (%) was the combination of IL-1α + IL-1β.

Discussion

In this study, the analysis of cell viability and expression of biomarkers related to skin sensitization was conducted in response to the application of 18 skin sensitizers and 5 nonsensitizers by using mouse macrophages. First, CV90 and CV75 were set to compare skin sensitization potency after treatment with skin sensitizers and non-sensitizers under the same conditions. CV90 and CV75 were

Table 5. EC (effective concentration) values of the biomarkers for the test substances classified as skin sensitizers.

LLNA potency ^a	Chemicals	EC values (µg/ml)							
		IL-1α	IL-1β	IL-31	TNF-α	iNOS	PGE2	COX-2	IL-1α + IL-1β
		EC (120)	EC (150)	EC (150)	EC (200)	EC (120)	EC (120)	EC (150)	Combination EC ^b
Extreme	BQ	0.267	-	-	-	0.764	0.918	0.242	-
	BA	11.553	-	-	-	32.741	-	16.291	-
	DNCB	0.011	0.040	0.085	0.052	0.109	-	0.032	0.026
	PD	0.012	0.030	-	0.033	0.191	-	0.030	0.021
	CC	1.283	2.537	-	2.633	-	-	3.954	1.910
Strong	LG	0.012	0.019	-	0.020	0.220	0.257	0.028	0.015
	MI	0.477	0.901	-	0.802	3.213	3.055	1.232	0.689
	2-AP	0.380	0.501	-	0.591	-	-	1.027	0.441
	IE	-	-	7.362	-	-	-	1.932	-
Moderate	3-AP	3.531	1.334	0.440	2.681	4.777	2.003	0.416	2.432
	NS	9.806	3.403	0.790	7.151	-	2.285	0.851	6.604
	CI	3.878	0.788	0.211	2.524	6.699	0.854	0.367	2.333
	HCA	2.236	0.435	0.122	1.698	3.065	0.344	0.258	1.335
	EU	15.924	1.837	0.611	-	21.722	3.174	1.110	8.881
Weak	IU	6.313	0.922	0.421	8.048	6.860	3.468	0.399	3.617
	LL	74.250	14.194	4.779	-	74.818	12.455	4.484	44.222
	LI	291.280	58.216	25.038	-	294.511	171.754	17.747	174.748
	PY	381.967	92.682	27.071	-	-	231.670	44.214	237.324

Calculation of the EC values according to the cut-off values of the biomarkers for the test substances classified as skin sensitizers. The EC value was calculated by linear interpolation using the RBI value and from the cell viability data for each material.

A negative sign (-) indicates that the test substances were classified as non-sensitizers by our classification results.

^a Extreme: EC₃ (%) < 0.1, strong: 0.1 ≤ EC₃ (%) < 1, moderate: 1 ≤ EC₃ (%) < 10, weak: 10 ≤ EC₃ (%) < 100 (ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) 2003a; 2003b)

^b The combination EC was the mean value of the EC (cut-off 120) for IL-1α and the EC (cut-off 150) for IL-1β.

defined as concentrations that exerted stimulatory effects on cells, but did not exhibit significant cytotoxicity. Potent cytotoxic actions may result in the rupture of the cell membrane and diffusion of the cytoplasmic structure (Becker et al. 1994; Sakaguchi et al. 2009), and lead to unspecified reactions (Python, Goebel, and Aeby 2007). Therefore, experiments at the CV90 and CV75 were conducted.

The cell viability analysis revealed that skin sensitizers were more cytotoxic with a lower EC₃ (%) and that cytotoxicity was less frequent when the EC₃ (%) was higher (Table 1). However, this trend was not observed after treatment with non-sensitizers. The nonsensitizers BKC and SLS displayed relatively potent cytotoxic actions, whereas IP did not cytotoxicity at the maximum applied treatment concentration of 10,000 µg/ml (Table 1); thus, there was a limit to the prediction of skin sensitization potency based upon cytotoxicity. Hence, skin sensitization potency through the quantitative evaluation of biomarkers was determined.

The test substances were found to produce various effects on different biomarkers (Figures 2 and

3). To classify skin sensitizers using biomarkers, it is important to select appropriate biomarkers and evaluate their expression. Such biomarkers need to exhibit higher expression than that in the reference conditions as a result of their overexpression in response to skin sensitization. Expression below that in the reference conditions is suggestive of absence of sensitization of cells by the test agent. Of the biomarkers evaluated, IL-1α, IL-1β, COX-2, and IL-1α + IL-1β were deemed appropriate, as indicated by an accuracy of ≥ 85% obtained from their respective cut-off values (Table 4). Previously Jung et al. (2016) used IL-1α to classify skin sensitizers in human keratinocytes treated with 0.01 × CV75, 0.1 × CV75, 0.5 × CV75, and CV75 and the expression level of IL-1α measured by employing an enzyme-linked immunosorbent assay (ELISA) kit. In contrast with our classification criteria, the criteria for classification of substances in that experiment were based upon a threefold increase in cytokine expression in the test group as compared to that in control with an accuracy of 68% achieved (Jung et al. 2016). Alternatively, IL-1β was utilized as a biomarker for classification of skin sensitizers in U937 cells, which are DC-like

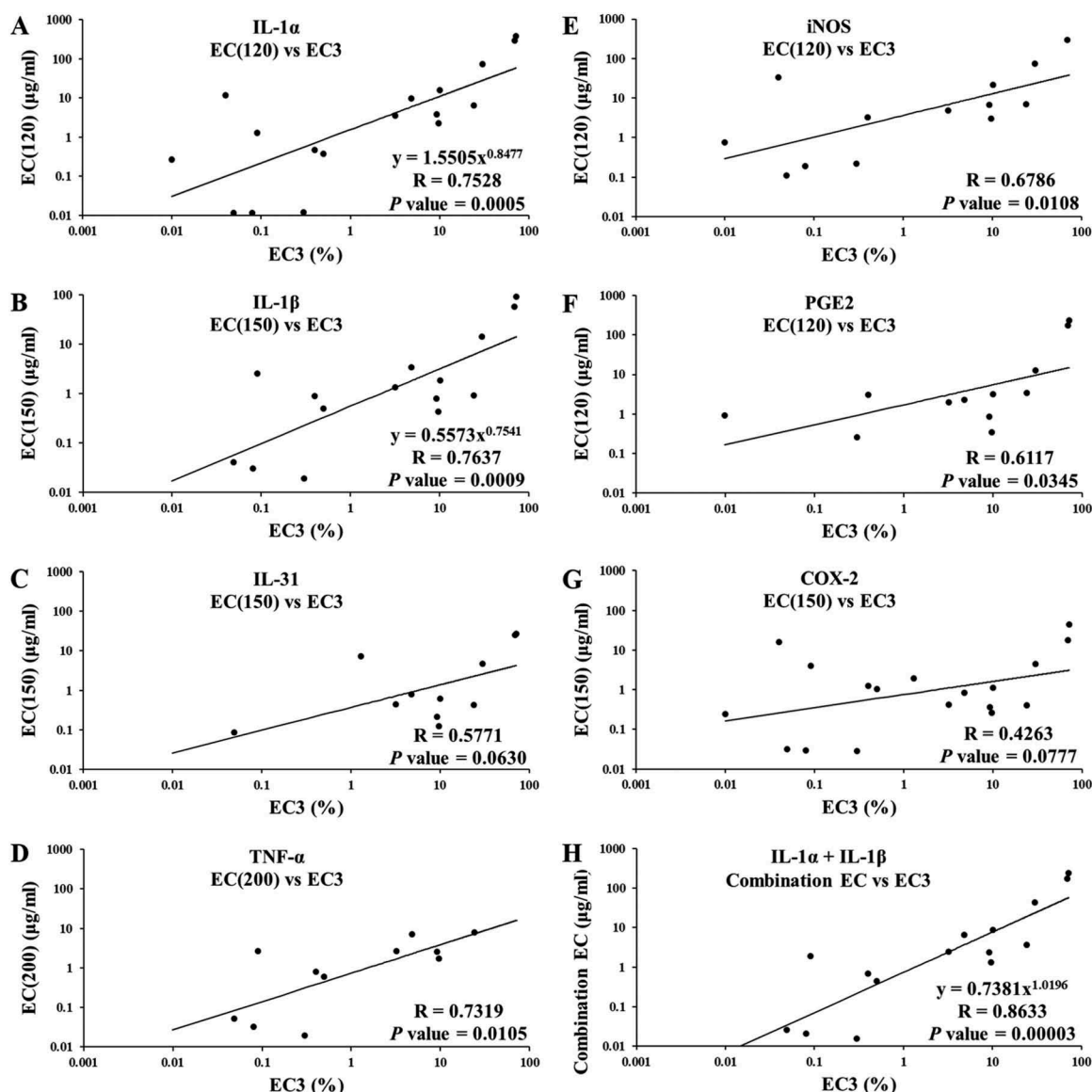


Figure 5. Correlation between the EC value and the EC3 (%) of each biomarker. The EC value was calculated by linear interpolation of the RBI value and from cell viability data for each material. The combination EC was calculated as the mean value of the EC (cut-off 120) for IL-1 α and EC (cut-off 150) for IL-1 β . The EC3 (%) was plotted on the horizontal axis of the graph and EC value of each biomarker was placed on the vertical axis. The correlation coefficient (R) was evaluated to determine the correlation between EC and EC3 (%). IL-1 α (a), IL-1 β (b), IL-31 (c), TNF- α (d), iNOS (e), PGE2 (f), COX-2 (g), and IL-1 α + IL-1 β (h).

*EC3 (%): the amount of a chemical that is required to elicit a three-fold increase in lymph node cell proliferative activity (stimulation index, SI ≥ 3)

cells (Python, Goebel, and Aeby 2007). Similar to our findings, Python, Goebel, and Aeby (2007) observed specific changes in IL-1 β expression in response to skin sensitizer treatments, but no relevant changes detected in cells treated with non-sensitizers. In our analysis using IL-1 β , non-sensitizers did not markedly alter IL-1 β levels compared to those in control (Figure 2, 3, and 4b; Table 2). In our experiments, the RBI values of COX-2 were ≥ 150 for all skin sensitizers, but it

was not possible to accurately discriminate RBI values of ≥ 150 for nonsensitizers GL, MS, and SLS (Table 2). SLS is a typical irritant and produces false-positive results in animal experiments and cell-based studies (An et al. 2009; Python, Goebel, and Aeby 2007). In our analysis using COX-2, SLS produced a false-positive result (Figure 2, 3, and 4a; Table 2).

To predict skin sensitization potency using cell-based experiments, the EC values were calculated

Table 6. Determination and statistical significance of the correlation between the EC value and the EC3 (%) for each biomarker.

Biomarkers	Cut-off value	Number of substances ^a	Correlation coefficient (R)	P value ^b
IL-1 α	≥ 120	17	0.7528	0.0005
IL-1 β	≥ 150	15	0.7637	0.0009
IL-31	≥ 150	11	0.5771	0.0630
TNF- α	≥ 200	11	0.7319	0.0105
iNOS	≥ 120	13	0.6786	0.0108
PGE ₂	≥ 120	12	0.6117	0.0345
COX-2	≥ 150	18	0.4263	0.0777
IL-1 α + IL-1 β	IL-1 α ≥ 120 & IL-1 β ≥ 150	15	0.8633	0.00003

The correlation coefficient (R) was calculated to determine the correlation of EC value and EC3 (%). Pearson's test was used for statistical analysis; P values of < 0.05 were considered statistically significant.

^a The number of substances classified as skin sensitizers by cut-off value.

^b Statistical significance of the correlation (*P value < 0.05)

for all substances classified as skin sensitizers by the biomarker analysis and their correlation with EC3 (%) was determined (Tables 5 and 6; Figure 5). The correlation coefficients between the EC value and the EC3 (%) were highest for IL-1 α , IL-1 β , and IL-1 α + IL-1 β . A significant correlation coefficient of ≤ 0.7 was exhibited in the case of TNF- α . However, TNF- α was excluded from predictable biomarkers for EC3 (%) because the accuracy was calculated to be as low as 65%. In conclusion, the most appropriate biomarkers for the prediction of skin sensitization potency in mouse macrophages were IL-1 α and IL-1 β .

These results suggest alternative biomarkers to animal experimental methods that may enable prediction of skin sensitization potency and risk assessment by using RAW264.7 mouse macrophages. This alternative to animal testing is expected to reduce the costs and overcome the limitations of animal and human experiments. Further studies need to be undertaken to confirm the relevance of the selected biomarkers for various substances and examine new biomarkers to improve the efficiency of the methodologies involved in skin sensitization testing.

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