

Suitability of macrophage inflammatory protein-1 β production by THP-1 cells in differentiating skin sensitizers from irritant chemicals

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Background: Worldwide restrictions in animal use for research have driven efforts to develop alternative methods.

Objective: The study aimed to test the efficacy of the macrophage inflammatory protein-1 β (MIP-1 β) assay for testing chemicals' skin-sensitizing capacity.

Methods: The assay was performed using 9 chemicals judged to be sensitizing and 7 non-sensitizing by the standard *in vivo* assays. THP-1 cells were cultured in the presence or absence of 4 doses, 0.01x, 0.1x, 0.5x, or 1x IC₅₀ (50% inhibitory concentration for THP-1 cell proliferation) of these chemicals for 24 hr, and the MIP-1 β level in the supernatants was determined. Skin sensitization by the test chemicals was determined by MIP-1 β production rates. The MIP-1 β production rate was expressed as the relative increase in MIP-1 β production in response to chemical treatment compared with vehicle treatment.

Results and Conclusion: When the threshold MIP-1 β production rate used was 100% or 105% of dimethyl sulfoxide, all the sensitizing chemicals tested (dinitrochlorobenzene, hexyl cinnamic aldehyde, eugenol, hydroquinone, dinitrofluorobenzene, benzocaine, nickel, chromium, and 5-chloro-2-methyl-4-isothiazolin-3-one) were positive, and all the non-sensitizing chemicals (methyl salicylate, benzalkonium chloride, lactic acid, isopropanol, and salicylic acid), with the exception of sodium lauryl sulfate, were negative for MIP-1 β production. These results indicate that MIP-1 β could be a biomarker for classification of chemicals as sensitizers or non-sensitizers.

Key words: contact dermatitis; MIP-1 β ; skin-sensitizing chemicals; THP-1 cell line. © Blackwell Munksgaard, 2008.

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The Council of the European Union has banned marketing cosmetic ingredients tested on animals after March 2009 (1). Because of this regulation, cosmetic industries are developing alternatives to animal experimentation to test the efficacy or toxicity of cosmetic ingredients and other products. The Organisation for Economic Co-operation and Development (OECD) recently adopted a local lymph node assay (LLNA) using mice as an alternative model for testing epidermal sensitization to chemicals, where guinea-pigs had been tradition-

ally used (2). Because the LLNA still uses an experimental animal, development of *in vitro* methods for screening skin sensitizers has been undertaken using human or animal cell lines (3–5). The human cell line activation test (h-CLAT), which was recently developed by Japanese research groups, has been examined to determine whether the assay can be internationally accepted as a new *in vitro* skin sensitization test (6, 7). The assay determines the skin-sensitizing potential of a chemical, based on the chemically mediated

enhancement of surface CD54 or CD86 expression, or production of macrophage inflammatory protein-1 β (MIP-1 β), from the THP-1 human monocytic cell line (3, 6, 7). In addition to the establishment of standard protocols and the inter-laboratory validations conducted thus far, the h-CLAT should be internationally evaluated using as many chemicals as possible to verify its suitability as an animal testing alternative. Because a very limited number of known chemical sensitizers or irritants have been tested using the h-CLAT (3, 6, 7), we examined the use of the h-CLAT as an alternative *in vitro* skin sensitization test by evaluating changes in MIP-1 β production in response to 16 chemicals, including 7 sensitizers and 3 non-sensitizers, that have not been previously tested in this system.

Materials and Methods

Chemicals

The 9 sensitizers and 7 non-sensitizers used in this study were chosen based on the reported LLNA results (8, 9). The sensitizing chemicals were dinitrochlorobenzene (DNCB; CAS no. 97-00-7), hexyl cinnamic aldehyde (HCA; CAS no. 101-86-0), hydroquinone (HQ; CAS no. 123-31-9), dinitrofluorobenzene (DNFB; CAS no. 70-34-8), benzocaine (CAS no. 94-09-7), eugenol (CAS no. 97-53-0), nickel sulfate hexahydrate (nickel; CAS no. 10101-97-0), potassium dichromate (chromium; CAS no. 7778-50-9; Sigma-Aldrich, St Louis, MO, USA), and 5-chloro-2-methyl-4-isothiazolin-3-one (MCI; CAS no. 26172-55-4; Fluka, St Louis, MO, USA). Non-sensitizing chemicals were sodium lauryl sulfate (SLS; CAS no. 151-21-3), isopropanol (CAS no. 67-63-0; Bio Basic, Markham Ontario, Canada), methyl salicylate (MS; CAS no. 119-36-8), benzalkonium chloride (BC; CAS no. 8001-54-5), lactic acid (CAS no. 50-21-5), salicylic acid (SA; CAS no. 69-72-7), and dimethyl sulfoxide (DMSO; CAS no. 68-68-5; Sigma-Aldrich). DMSO was used to prepare stock solutions of DNCB, HCA, eugenol, HQ, DNFB, benzocaine, MS, and SA. The final DMSO concentration in the culture medium was less than 0.1%. Stock solutions for the other chemicals were made in physiological saline solution. The stock solutions were diluted in culture medium prior to addition to culture wells.

Cells and culture media

The THP-1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were seeded at 1×10^5 cells/ml in complete RPMI-1640 medium supplemented

with sodium pyruvate (1 mM), heat-inactivated fetal bovine serum (10%), 2-mercaptoethanol (50 μ M), glucose (4.5 g/l), and HEPES (10 mM) in a humidified 37 celsius, 5% CO₂ incubator. Cells were passaged every 72 hr.

Determination of the IC₅₀ for THP-1 cell proliferation

A colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich), known as the MTT assay, was conducted as described elsewhere (7, 10) to determine the IC₅₀ (50% inhibitory concentration) for THP-1 cell proliferation. Briefly, THP-1 cells (10^5 cells/well) were cultured in 0.1 ml of culture medium containing various concentrations of chemicals in 96-well culture plates for 24 hr followed by a 4-hr incubation after adding 10 μ l MTT stock solution (5 mg/ml). Thereafter, 0.04 N HCl in isopropanol (100 μ l/well) was added to each well, and the wells were vigorously mixed to dissolve the MTT. The plates were read using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek CERES UV 900C, Winooski, VT, USA) at 570 nm with 630 nm as the reference wavelength. The IC₅₀ values were determined using 4-parameter kinetic curves automatically generated by the plate reader software.

Evaluation of MIP-1 β production from THP-1 cells treated with chemicals

The THP-1 cells (10^6 cells/well) were plated in 1 ml of culture medium in 24-well culture plates. After a 4-hr stabilization period, chemicals were added and the cells were incubated for an additional 24 hr. 4 concentrations (1x, 0.5x, 0.1x, and 0.01x of the IC₅₀ value) were chosen as test doses (6, 7). Levels of MIP-1 β in the culture supernatants were assayed by a sandwich ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The lower limit of detection for MIP-1 β was 20 pg/ml.

Statistical analysis

Data were initially evaluated for normal distributions. Statistically significant differences among groups were tested using SIGMASTAT (Systat, Richmond, CA, USA) by single-factor ANOVA and Dunnett's *t*-test or by Kruskal–Wallis ANOVA and Dunn's test, depending on the normality of the data. The significant differences were further confirmed by Student's *t*-test or Mann–Whitney *U*-test. Differences with $P < 0.05$ were considered significant.

Results

Determination of IC₅₀

The MTT assay to determine IC₅₀ values was performed at least 3 times for each test chemical. The mean ($\pm$ SD) IC₅₀ values (μ g/ml) for the sensitizer chemicals are 100.2 ± 53.5 for DNCB, 154.1 ± 93.1 for HCA, 216.8 ± 38.8 for eugenol, 43.5 ± 7.6 for HQ, 6.9 ± 0.1 for DNFB, 579.5 ± 53.0 for benzocaine, 137.9 ± 9.1 for nickel, 3.0 ± 1.1 for chromium, and 478.6 ± 254.4 for MCI (data not shown). The IC₅₀ values (μ g/ml) of the non-sensitizing chemicals tested are 28.5 ± 3.1 for SLS, $18\,683.1 \pm 433.3$ for isopropanol, 825.0 ± 526.1 for MS, 3.2 ± 0.2 for BC, 2257.9 ± 601.0 for lactic acid, and 4386.3 ± 3737.9 for SA. Because DMSO did not show any cytotoxicity at 5000 μ g/ml, the maximum concentration tested, the IC₅₀ value of DMSO was set to 5000 μ g/ml.

Level of MIP-1 β production following application of chemicals

We investigated the effects of the chemicals on production of MIP-1 β by THP-1 cells. The sensitizing chemicals, in general, enhanced production of MIP-1 β with statistically significant increases at some concentrations (Table 1). Compared with MIP-1 β levels in the vehicle control cells, the highest MIP-1 β production was obtained from THP-1 cells treated with a 1x IC₅₀ concentration

of nickel (1132%) followed by chromium 0.01x IC₅₀ (314%), DNCB 1x (246%), MCI 1x (187%), HQ 0.5x (171%), HCA 0.1x (167%), benzocaine 0.5x (165%), DNFB 1x (145%), and eugenol 0.1x (136%). In contrast, no significant upregulation of MIP-1 β production was observed in response to the non-sensitizing chemicals. Although no significant increases were detected, a substantial increase in MIP-1 β production was observed with SLS, in that a 152% increase in response to treatment with the 1x IC₅₀ concentration was obtained, compared with the vehicle control. However, significant decreases in MIP-1 β production were observed with higher concentrations of BC (0.5x and 1x IC₅₀), lactic acid (0.5x and 1x IC₅₀), and SA (1x IC₅₀). This suppression was not considered to be attributable to cytotoxicity of the chemicals because the cell viability assayed by propidium iodide uptake was $97.4 \pm 2.3\%$ for 0.5x IC₅₀ BC, $96.9 \pm 1.6\%$ for 1x IC₅₀ BC, $96.5 \pm 2.6\%$ for 0.5x IC₅₀ lactic acid, $96.7 \pm 1.1\%$ for 1x IC₅₀ lactic acid, and $97.7 \pm 0.3\%$ for 1x IC₅₀ SA (data not shown).

Classification of the tested chemicals as sensitizers or non-sensitizers based on the level of MIP-1 β production

Next, we examined whether enhancement of MIP-1 β production can be used as a biomarker for skin sensitization capacity. To classify the

Table 1. Level of MIP-1 β production by THP-1 cells at 24 hr after initiation of chemical exposure

Chemicals	MIP-1 β production rate (means $\pm$ SEM) ^a			
	0.01x IC ₅₀	0.1x IC ₅₀	0.5x IC ₅₀	1x IC ₅₀
Sensitizers				
DNCB	120.6 $\pm$ 14.0	140.4 $\pm$ 8.4 ^b	184.6 $\pm$ 12.8 ^b	246.0 $\pm$ 68.5
HCA	137.3 $\pm$ 9.2 ^b	167.0 $\pm$ 3.7 ^b	105.6 $\pm$ 41.5	55.8 $\pm$ 26.8
Eugenol	131.0 $\pm$ 22.5	136.2 $\pm$ 11.9 ^b	125.8 $\pm$ 17.7	74.2 $\pm$ 18.3
HQ	117.5 $\pm$ 2.7 ^b	125.3 $\pm$ 4.0 ^b	170.8 $\pm$ 6.7 ^b	145.1 $\pm$ 3.6 ^b
DNFB	115.7 $\pm$ 3.3 ^b	125.7 $\pm$ 4.2 ^b	121.0 $\pm$ 3.2 ^b	144.7 $\pm$ 20.4 ^b
Benzocaine	144.3 $\pm$ 9.0 ^b	162.3 $\pm$ 23.5 ^b	165.3 $\pm$ 26.9	135.7 $\pm$ 8.2 ^b
MCI	96.7 $\pm$ 5.2	75.0 $\pm$ 2.3 ^b	104.3 $\pm$ 4.5	186.7 $\pm$ 24.2 ^b
Nickel	84.3 $\pm$ 23.9	123.3 $\pm$ 14.0	525.0 $\pm$ 123.8 ^b	1132.3 $\pm$ 41.9 ^b
Chromium	313.7 $\pm$ 142.5	143.7 $\pm$ 8.5 ^b	147.0 $\pm$ 26.2	151.0 $\pm$ 19.3 ^b
Irritants				
SLS	97.9 $\pm$ 9.0	86.2 $\pm$ 16.4	123.2 $\pm$ 14.3	152.4 $\pm$ 27.5
MS	81.5 $\pm$ 3.7 ^b	62.4 $\pm$ 21.1	62.8 $\pm$ 14.6	63.2 $\pm$ 21.0
BC	101.0 $\pm$ 4.7	68.8 $\pm$ 17.6	39.1 $\pm$ 6.8 ^b	33.2 $\pm$ 9.5 ^b
Lactic acid	95.3 $\pm$ 10.8	78.3 $\pm$ 15.8	42.3 $\pm$ 12.3 ^b	42.0 $\pm$ 5.5 ^b
Isopropanol	80.7 $\pm$ 16.6	76.7 $\pm$ 14.3	84.0 $\pm$ 40.0	80.3 $\pm$ 17.5
SA	97.3 $\pm$ 25.4	80.0 $\pm$ 16.7	61.3 $\pm$ 22.9	72.0 $\pm$ 8.5 ^b
DMSO	82.0 $\pm$ 12.6	91.6 $\pm$ 3.3	84.5 $\pm$ 17.4	105.2 $\pm$ 16.0

BC, benzalkonium chloride; DMSO, dimethyl sulfoxide; DNCB, dinitrochlorobenzene; DNFB, dinitrofluorobenzene; HCA, hexyl cinnamic aldehyde; HQ, hydroquinone; IC₅₀, 50% inhibitory concentration; MCI, 5-chloro-2-methyl-4-isothiazolin-3-one; MIP-1 β , macrophage inflammatory protein-1 β ; MS, methyl salicylate; SA, salicylic acid; SLS, sodium lauryl sulfate.

^aThe MIP-1 β production rate, percentage relative to control cells, was determined by dividing the amount of MIP-1 β (pg/ml) produced by chemically treated cells by the amount of MIP-1 β produced by vehicle control-treated cells and multiplying by 100 for relative percent expression. Data are expressed as the means $\pm$ SEM of at least 3 separate experiments.

^bSignificantly ($P < 0.05$) different from the vehicle control.

tested chemicals as sensitizers based on the MIP-1 β production rate, we set a threshold MIP-1 β production rate value. The MIP-1 β production rate is defined as the relative increase (%) in MIP-1 β production by chemically treated THP-1 cells compared with vehicle control-treated cells. Our classifications were compared with the existing classifications judged by LLNA or standard guinea-pig/human test methods (8, 9, 11, 12). Using a threshold value of 100% to identify chemicals with a significantly higher MIP-1 β production level than that of the vehicle control, DNCB, HCA, eugenol, HQ, DNFB, MCI, and chromium were defined as sensitizers (Fig. 1). Nickel currently has no data available for its skin-sensitizing capacity, but we classify it as a strong sensitizer because it elicited an 1132% increase in MIP-1 β production compared with that of the vehicle control. Among the chemicals considered to be non-sensitizers by the existing classification methods, all the chemicals we tested, with the exception of SLS, were classified as non-sensitizers because they did not elicit a significant difference in

MIP-1 β production between the chemical-treated and the vehicle control-treated THP-1 cells. At the 1x IC₅₀ concentration, SLS produced a 152% enhancement in MIP-1 β production, although this level was not statistically significant. SLS, which is considered an irritant when tested using the guinea-pig/human methods, also causes a positive response in LLNA (8). If our threshold value is set at 152%, the highest MIP-1 β production rate among the non-sensitizers tested, DNCB, HCA, hydroquinone, benzocaine, nickel, and chromium are positive for enhancement of MIP-1 β production, resulting in classification as sensitizers, while eugenol and DNFB are negative. All the non-sensitizing chemicals tested were negative.

Discussion

The present study was undertaken as part of the Korea Food & Drug Administration's plan for establishing alternatives to animal experimentation for the evaluation of skin-sensitizing chemicals. Any new alternative assay should be

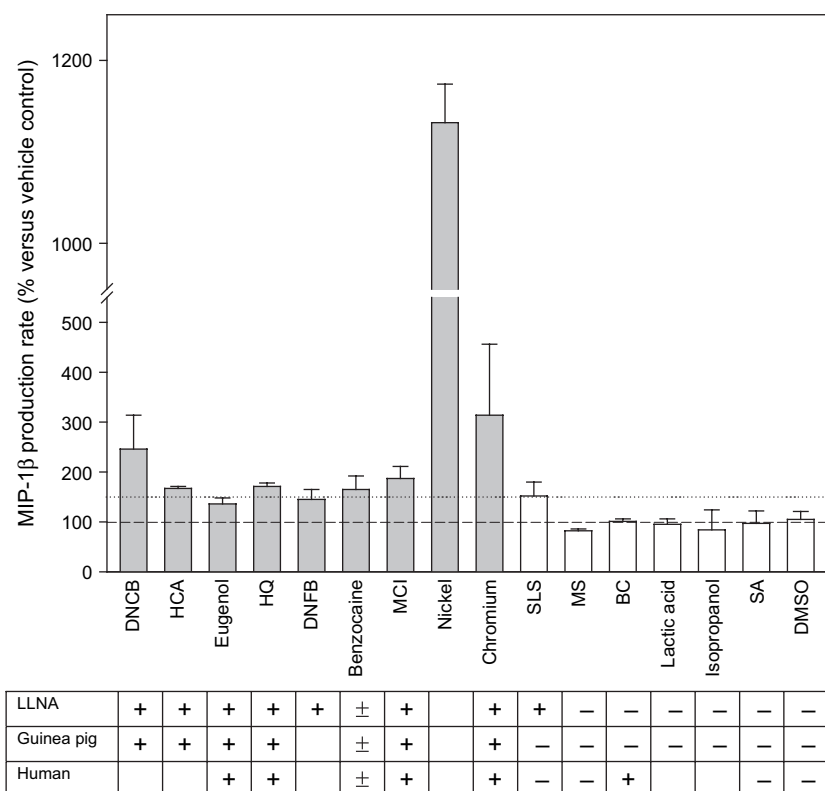


Fig. 1. Comparison of the MIP-1 β production rates calculated in Table 1. The highest MIP-1 β production rate for each chemical is presented. Positive (+) or negative (-) skin-sensitizing activity, presented below the histogram, refers to previously published data (8, 9, 11, 12) obtained using the guinea-pig maximization test, the Buehler test, or the human maximization test. The short dash line indicates the level of MIP-1 β production from vehicle control THP-1 cells (100%). The dotted line indicates the highest MIP-1 β production rate (152% from 1x IC₅₀ of SLS) among the non-sensitizers. BC, benzalkonium chloride; DMSO, dimethyl sulfoxide; DNCB, dinitrochlorobenzene; DNFB, dinitrofluorobenzene; HCA, hexyl cinnamic aldehyde; HQ, hydroquinone; IC₅₀, 50% inhibitory concentration; MCI, 5-chloro-2-methyl-4-isothiazolin-3-one; MIP-1 β , macrophage inflammatory protein-1 β ; MS, methyl salicylate; SA, salicylic acid; SLS, sodium lauryl sulfate.

standardized to achieve a high degree of reproducibility and provide a reliable and highly sensitive and specific biomarker. Furthermore, the assay would be most useful if laboratories from many different countries validate the assay. Because the initial suggestion that MIP-1 β production by the THP-1 human monocytic cell line could provide biomarker for skin sensitization by chemicals (3), inter-laboratory validation has been carried out by only a few laboratories with a limited number of chemicals (6). Therein, our present study, which included 10 previously untested chemicals, 7 sensitizers and 3 non-sensitizers, further contributes to international validation of the MIP-1 β assay as an alternative *in vitro* test method for skin sensitization. The chemicals newly tested here are HCA, eugenol, HQ, DNFB, benzocaine, MCI, chromium, lactic acid, isopropanol, and SA.

MIP-1 β is a chemokine produced by a broad range of immune cells including monocytes, macrophages, and T lymphocytes (13). MIP-1 β plays an important *in vivo* role in recruiting T lymphocytes into local lymph nodes during DNFB-mediated contact hypersensitivity (14), which indicates that MIP-1 β production is relevant for identifying skin allergenic chemicals (15). In comparison to the guinea-pig maximization test, the Buehler test, or the human maximization test, which has been internationally adopted as an *in vivo* method for evaluation of skin sensitizers, the h-CLAT assay, including the assessment of MIP-1 β production, may have several advantages, including the non-animal-based system, the human origin of the THP-1 cell line, the avoidance of subjective visual assessment as performed in the *in vivo* assays mentioned above, and the commercial availability of an MIP-1 β ELISA kit.

The most important issue in validating an alternative method may be how accurately sensitizing chemicals can be differentiated from non-sensitizing chemicals. The OECD uses a stimulation index value of 3 as a threshold value for classification of chemicals as sensitizers by LLNA (2). Similarly, we tried to establish an effective threshold rate value for MIP-1 β production. The maximum MIP-1 β production rate in response to 4 different doses of each chemical (0.01x, 0.1x, 0.5x, and 1x IC₅₀) was calculated and considered for classification. A threshold MIP-1 β production rate value of 100% showed very good concordance between our MIP-1 β assay results and the *in vivo* skin sensitization test results published previously (8, 9, 11, 12) (Fig. 1). Furthermore, benzocaine, a chemical whose skin-sensitizing activity has been questioned (15), was also classified as a sensitizer because a significant increase (165%) in MIP-1 β production was observed.

When the highest MIP-1 β production rate (152% by SLS) among the non-sensitizers was used as the threshold value, two sensitizers (eugenol and DNFB) were judged to be non-sensitizers. Hirota and Moro (3) also reported approximately 150% MIP-1 β production in response to SLS, which was the highest among the non-sensitizing chemicals they tested (DMSO, Triton-X-100, MS, and BC). Eugenol has been persistently classified as a sensitizer by traditional *in vivo* assays (8, 9, 11, 12). DNFB has been reported as a positive skin sensitizer by LLNA, but no data using the guinea-pig or human *in vivo* test methods are available. Although SLS has been reported to induce a positive LLNA response, SLS is still classified as a non-sensitizing irritant because the SLS-mediated LLNA positive response was speculated to occur through an unknown, but probably non-immune-mediated, mechanism (8). Thus, instead of the 152% rate induced by SLS, the rate of 105% in response to DMSO, the second highest MIP-1 β production rate among the non-sensitizing chemicals tested, may be the preferred threshold MIP-1 β production rate value. When this threshold value is applied, all the sensitizing chemicals we tested are identified as sensitizers and all the non-sensitizing chemicals we tested, with the exception of SLS, are classified as non-sensitizers.

Nickel sulfate has been classified as a modest skin sensitizer by the *in vivo* assays using mouse or guinea-pig (8, 12). Whereas, our results showed the highest MIP-1 β production from THP-1 cells treated with nickel among the 9 sensitizing chemicals, resulting in classification of the nickel compound as a strong sensitizer. The discrepancy in classification between the existing *in vivo* results and our results could be explained by several possibilities. Most nickel salts are hydrophilic compounds, which are difficult to penetrate the skin. Because skin sensitization potential has been found to depend on chemicals' capacity of percutaneous absorption (16), nickel sulfate with lesser skin permeability may not be effectively presented to antigen-presenting cells at the site of nickel sulfate exposure, which could lead to the lesser cell proliferation in lymph nodes draining the site of nickel exposure. Meanwhile, nickel can directly contact with THP-1 cells and bind to the class II major histocompatibility complex (MHC) on the cells (17). Direct association of nickel with MHC molecules can cause an immediate activation of the cells and as a consequence stimulate production of MIP-1 β . Hirota and Moro (3) also reported the highest MIP-1 β production in response to nickel among the sensitizing chemicals they tested (DNCB, p-phenylenediamine, cobalt, 2-mercaptobenzothiazole, formaldehyde,

hydroxycitronellal, citral, mercuric chloride, and ammonium tetrachloroplatinate). Thus, the skin-sensitizing potential of nickel might be influenced by its availability to immune component cells such as antigen-presenting cells.

The sensitizing chemicals tested except DNCB and nickel did not show a dose–response relationship between the concentration of chemical and the level of MIP-1 β production (Table 1). This observation implies that the concentration is not necessarily related with the chemical's potency to enhance MIP-1 β production. MCI was in fact only positive at the highest concentration (1x IC₅₀) where cell viability was significantly lower ($67.2 \pm 19.1\%$) than the vehicle control ($95.5 \pm 2.1\%$). Although we do not have a definite clue for this phenomenon at the present moment, one possibility could be considered. Miyazawa et al. (18) and Hulette et al. (19) reported that upregulation of CD54 or CD86 expression from THP-1 cells or dendritic cells, an activation marker of the cells, was observed at slightly cytotoxic doses of chemical allergens, causing 70–95% (18) or 85–90% (19) cell viability. The association of cell activation with cytotoxicity needs to be further investigated.

In conclusion, MIP-1 β appears to be a good candidate for a biomarker for classification of chemical sensitizers. Additional studies should be performed at various laboratories using a broad range of chemicals to further validate the assay.

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