



Preventing false-negatives in the in vitro skin sensitization testing of acid anhydrides using interleukin-8 release assays



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ABSTRACT

In vitro safety tests may be used as replacements for animal tests owing to their accuracy and high-throughput performance. However, several in vitro skin sensitization tests produce false-negative results such as acid anhydride. Here, we investigated the relationship between false-negative results of acid anhydride and its hydrolysis by aqueous vehicle. Differences in the pattern of hydrolysis for phthalic anhydride (PAH) due to addition of 1 drop of stock solution of PAH in liquid paraffin (LP) dispersion medium and PAH in DMSO were analyzed in a cell-free system. The results showed that use of LP dispersion medium stabilized the concentration of PAH in water over 5 min by sustained-release, although almost all PAH converted to phthalic acid in water within 5 min using DMSO. Additionally, treatment of THP-1 cells with PAH and phthalic acid using LP dispersion medium for 5 min resulted in a 32-fold increase in IL-8 release for PAH as compared with that in the vehicle control. In contrast, for PAH using aqueous vehicle and phthalic acid using LP dispersion medium, there were no significant increases in IL-8 release. Similarly, using LP dispersion medium, trimellitic anhydride significantly increased IL-8 release was observed.

1. Introduction

Recently, animal tests have been affected by increasing ethical, social, and political concerns regarding animal welfare. Indeed, several countries have banned the testing of cosmetic products and their ingredients on animals (Adler et al., 2011). Accordingly, it is necessary to develop alternatives to animal experiments, particularly for in vitro safety testing. Several in vitro safety tests have been proposed. Currently, most researchers are focusing on the development of tests based on not only cytotoxicity but also complex toxic mechanisms, such as skin sensitization tests.

Skin sensitization is a phenomenon in which rash occurs on the skin, i.e., allergic contact dermatitis (ACD), owing to T-cell-mediated complicated immune responses. In the induction phase of skin sensitization, antigen-presenting cells, such as dendritic cells (DCs), and Langerhans cells are activated. Sensitizers cause morphological and functional changes in DCs, altering the expression of CD40, CD80, CD83, CD86, and HLA-DR (Aiba et al., 1997; Aiba, 1998; Arrighi et al., 2001; De Smedt et al., 2001) and the production of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-8/CXCL8, and IL-12p40 (Coutant et al., 1999; De Smedt et al., 2001; Aiba et al., 2003; Toebak et al., 2006).

Accordingly, various skin sensitization tests have been developed; in

particular, augmentation of IL-8 mRNA expression or protein synthesis/release in THP-1 cells has been suggested as a promising biomarker for skin sensitization (Miyazawa et al., 2008; Nukada et al., 2008; Mitjans et al., 2008, 2010; Takahashi et al., 2011; Kimura et al., 2015; Parise et al., 2015). IL-8 levels are significantly increased by poison ivy/oak in human patch-tests (Griffiths et al., 1991). Importantly, IL-8 is well established as a potent chemotactic peptide for neutrophils and T lymphocytes (Leonard et al., 1990). Thus, analysis of IL-8 in THP-1 cells, based on ACD mechanisms, may provide a promising alternative skin sensitization test.

However, similar to other in vitro skin sensitization tests, these tests still produced false-negative results (e.g., for water-insoluble chemicals, pro/pre-hapten, volatile chemicals, and chemicals which are categorized as weak sensitizers) compared with local lymph node assay (LLNA) and human clinical data. For example, Nukada et al. (2008) reported that three skin sensitizers (i.e., phthalic anhydride [PAH], ethylene diamine, and geraniol) out of 16 did not show the capacity to induce IL-8 production.

PAH is classified as a strong skin sensitizer (EC₃ value = 0.16% w/v) by LLNA (Dearman et al., 2000). Although the false-negative results observed for PAH are thought to be related to its water-insolubility, hydrolysis, and respiratory sensitization, no studies have described the

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cause of false-negative results for PAH using IL-8 analysis by THP-1 cells. More importantly, several studies have shown that almost all in vitro skin sensitization tests do not evaluate PAH correctly (e.g., the IL-8 Luc assay, h-CLAT, the Myeloid U937 Skin Sensitization Test, the test based on reactive oxygen species production in THP-1 cells, and the ARE-Nrf2 luciferase test) (Kimura et al., 2015; Ashikaga et al., 2010; Piroird et al., 2015; Saito et al., 2013; Emter et al., 2013). Therefore, we hypothesized that false-negative results for PAH may not be associated with cell lines, tissues, skin sensitization-related endpoints, or criteria for evaluation, but may instead be attributed to a common factor of in vitro tests, i.e., hydrolysis of aqueous vehicle. Indeed, further supporting this hypothesis, trimellitic anhydride (TAH), which has also been classified as a strong skin sensitizer (EC_{30} value = 0.60% w/v) by LLNA (Dearman et al., 2000), was shown to have false-negative results by IL-8 analysis in THP-1 cells (Mitjans et al., 2008) and the IL-8 Luc assay (Kimura et al., 2015).

In this study, we aimed to determine whether hydrolysis of acid anhydride was the underlying cause of the false-negative results from in vitro skin sensitization tests measuring IL-8 release in a cell-free system and in THP-1 cells. Furthermore, we investigated whether the preventing hydrolysis of acid anhydride could be prevented using liquid paraffin (LP) could be used to accurately evaluate these chemicals. LP has been used in cosmetics and for medical purposes for many years (Freund and McDermott, 1942), and LP exhibits chemical stability and water nonmiscibility. Therefore, we selected LP as a dispersion medium in this study. Our findings provide important insights into the applications of these alternative methods for toxicity evaluation.

2. Materials and methods

2.1. Cells

THP-1 cell, human monocytic leukemia cells, were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% heated-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 units/mL penicillin (Wako Pure Chemical Industries Ltd.), 100 µg/mL streptomycin (Wako Pure Chemical Industries Ltd.), and 0.05 mM 2-mercaptoethanol (Wako Pure Chemical Industries Ltd.) at 37 °C with 5% CO₂. THP-1 cells were routinely passaged and maintained at densities of $1-8 \times 10^5$ cells/mL. Cells were used before passage 30 for all experiments.

2.2. Chemicals

The test chemicals are listed in Table 1, along with their Chemical

Abstract Service (CAS) numbers, octanol-water partition coefficient ($\log K_{ow}$) values calculated with KOWWIN in EPI Suite (Environmental Protection Agency, Washington, DC, USA), skin sensitization potencies based on EC_{30} LLNA values, clinical case reports, patch tests, and human maximization test results. PAH, maleic anhydride (MAH), TAH, nickel sulfate hexahydrate (NiSO₄), and sodium lauryl sulfate (SLS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Phthalic acid, DMSO, and LP were purchased from Wako Pure Chemical Industries Ltd. and were of the highest available purity.

2.3. UV spectrophotometric analysis

The absorption measurements of PAH and phthalic acid were made in a SpectraMax M5e (Molecular Devices, LLC, Sunnyvale, CA, USA) at room temperature. The absorption spectra of PAH and phthalic acid were determined immediately and at 1 week after dissolving in distilled water (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) or dimethyl sulfoxide (DMSO; Japanese Industrial Standards Special Grade; Wako Pure Chemical Industries Ltd.). A quartz cell (optical path length = 1 cm) was then filled with 2 mL of these solutions, and the absorbance was measured at every 4 nm from 250 to 360 nm. The hydrolysis of PAH was determined by adding a stock solution of PAH in 10 µL DMSO and 200 µL liquid paraffin (LP; Wako Pure Chemical Industries Ltd.) to a 1-cm quartz cell containing 2 mL distilled water at room temperature. Optical density readings at 300 nm were taken every 1 min after mixing well.

2.4. Preparation of chemicals and chemicals exposure of THP-1 cells

Twenty-four-hour aqueous vehicle exposure tests were carried out as follows. Test chemicals were first solubilized in saline or DMSO and diluted. These solutions were diluted into culture medium. Fifty microliters of this diluted solution aqueous solution was then further mixed with 50 µL of 2×10^6 THP-1 cells/mL prepared in 96-well flat-bottomed plates. The exposure plates were then incubated for 24 h at 37 °C in an atmosphere containing 5% CO₂. The final concentrations of saline and DMSO were less than 0.2% and 0.8% (v/v), respectively.

For the 5-min LP dispersion medium exposure assays, test chemicals were first dispersed in LP and diluted. These suspensions (100 µL) were then mixed with 100 µL THP-1 cell suspension (1×10^7 cells/mL) prepared in 1.5 mL tubes for 5 min at room temperature. After 5 min of exposure, cells were washed twice with phosphate-buffered saline and resuspended in 1 mL culture medium. Next, 100 µL cell suspension was transferred to individual wells of a 96-well plate, and plates were then incubated for 24 h at 37 °C in an atmosphere containing 5% CO₂.

Table 1
List of chemicals tested.

Test chemicals	Abbreviation	CAS No.	Estimated $\log K_{ow}$ ^a	EC_{30} (%) and potency category ^{**}	Human data ^{***}
Phthalic anhydride	PAH	85-44-9	2.07	0.16 Strong ^a	Positive ^d
Maleic anhydride	MAH	108-31-6	1.62	0.16 Strong ^a	Positive ^e
Trimellitic anhydride	TAH	552-30-7	1.95	0.60 Strong ^a	Positive ^d
Nickel sulfate hexahydrate	NiSO ₄	10101-97-0	2.04	4.80 Moderate ^b	Positive ^f
Phthalic acid		88-99-3	1.07	nf ^{***}	nf ^{***}
Sodium lauryl sulfate	SLS	151-21-3	1.69	1.5–17.0 Moderate–weak ^c	Negative ^f

^a $\log K_{ow}$ value calculated with KOWWIN in EPI Suite.

^{**}The potency of skin sensitization was estimated using LLNA (^dDearman et al., 2000; ^bRyan et al., 2002; ^cLoveless et al., 1996).

^{***}nf, not found.

^{****}The clinical case report was from ^dMoffitt and Sansom (2002), patch test data were from ^eMotolese et al. (1993), and human maximization test data were from ^fKligman, 1966.

2.5. Analysis of cell viability

After incubation and exposure to test chemicals, cytotoxicity was assessed with water-soluble tetrazolium salt (WST-8) assays using a Cell Counting Kit-8 (Dojin Laboratory Co. Ltd., Kumamoto, Japan) in 96-well plates according to the manufacturer's instructions. The half-maximal inhibitory concentration (IC_{50}) values of PAH and phthalic acid were calculated by linear regression analysis of the data. Prior to analysis, the background absorbance values of the test chemicals were determined.

2.6. Analysis of IL-8 release

After incubation and exposure to test chemicals, cell-free culture supernatants were collected and centrifuged at $700 \times g$ for 10 min. IL-8 production was assessed using a specific sandwich enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Inc., Minneapolis, MN, USA). Procedures were conducted according to the manufacturer's instructions. Results are expressed in pg/mL.

2.7. Statistical analysis

Cell-based assays were performed at least three times, and representative results are shown. The data presented are expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance followed by Dunnett's post-hoc test was used to evaluate statistical significance using EZR (Kanda, 2013), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 3.3.0). For comparisons with data from vehicle-treated cells, differences with p values of less than 0.05 (asterisks) were considered statistically significant.

3. Results

3.1. Comparison between the absorption spectra of PAH and phthalic acid dissolved in vehicle

First, we confirmed the measurement wavelengths that could be used to distinguish between PAH and phthalic acid. Fig. 1 shows the molar extinction coefficient of PAH 1 week after dissolving in distilled water and that of phthalic acid immediately after dissolving in distilled water; these values were similar, ranging from 250 to 360 nm. In contrast, the molar extinction coefficient of PAH immediately after dissolving in DMSO was higher than that of phthalic acid immediately after dissolving in DMSO. In particular, at 300 nm, the molar extinction coefficient of PAH immediately after dissolving in DMSO ($\epsilon = 2586$) was 8.6 fold higher than that of phthalic acid immediately after dissolving in DMSO ($\epsilon = 302$). These results demonstrated that measuring absorbance at 300 nm could distinguish between PAH and phthalic acid, consistent with a previous study examining the time of PAH hydrolysis using methanol (Peters, 1959). In contrast, the molar extinction coefficient of PAH 1 week after dissolving in DMSO at 300 nm decreased by nearly 10% ($\epsilon = 273$) as compared with that immediately after dissolving in DMSO ($\epsilon = 2586$). This molar extinction coefficient value was similar to that of phthalic acid immediately after dissolving in DMSO ($\epsilon = 302$).

3.2. Hydrolysis of PAH by vehicle

To demonstrate whether PAH was hydrolyzed during the preparation of test solutions, we examined the hydrolysis of PAH following addition of 1 drop of a stock solution of PAH or phthalic acid in DMSO to a quartz cell containing distilled water by measuring changes in absorbance at 300 nm. The maximum absorbance (0.9) of distilled water solution contain PAH solute (final concentration of PAH in solution: 0.5 mM) was observed at 300 nm. Subsequently, this value

decreased each minute, rapidly reaching a minimum value. The absorbance of distilled water solution containing PAH solute was comparable to that of phthalic acid solution (final concentration of phthalic acid in solution: 0.5 mM) after 5 min (Fig. 2a). Moreover, changes in absorbance were not observed from 5 min onward (data not shown). To maintain the concentration of PAH in distilled water over a long period, we dropped an LP suspension of PAH into distilled water. Because LP and distilled water were not compatible, absorbance measurements were made at 300 nm using distilled water. For the LP dispersion-liquid containing PAH, the absorbance at 300 nm increased from 0 min (absorbance = 0.4) to 5 min, with maximum induction at 2–4 min (absorbance = 0.9; Fig. 2b). When LP dispersion-liquid containing PAH was mixed, the absorbance at 300 nm increased. Dropping only LP and LP dispersion-liquid containing phthalic acid did not cause this increase in absorbance at 300 nm.

3.3. Effects of 5-min exposure to PAH or phthalic acid in LP dispersion medium on IL-8 release

To examine whether PAH was hydrolyzed under cell culture conditions, we carried out cytotoxicity assays using THP-1 cells. THP-1 cells were exposed to PAH or phthalic acid with aqueous vehicle (culture medium containing 0.8% v/v DMSO) for 24 h. This vehicle did not affect cell viability relative to the DMSO-free vehicle (data not shown). From this analysis, the IC_{50} value for PAH was 1315 μ g/mL in aqueous vehicle after a 24-h exposure; that for phthalic acid was 1305 μ g/mL. However, in LP dispersion medium after a 5-min exposure, the IC_{50} value for PAH was 736 μ g/mL, which was only half that of phthalic acid (1421 μ g/mL).

Based on the cytotoxicity results observed using LP dispersion medium, we investigated the effects of LP dispersion medium exposure on IL-8 release in THP-1 cells. THP-1 cells were exposed to PAH or phthalic acid with the same aqueous vehicle for 24 h. Alternatively, THP-1 cells were exposed to PAH or phthalic acid suspended in LP dispersion medium for 5 min. The release of IL-8 was measured in supernatants 24 h after exposure and culture. Under our experimental conditions, the concentration of aqueous vehicle-induced IL-8 released from THP-1 cells was 9.4 ± 6.4 pg/mL ($n = 6$). In contrast, that observed for LP dispersion medium after a 5-min exposure was 25.6 ± 25.0 pg/mL ($n = 9$). With aqueous vehicle, PAH failed to stimulate significant IL-8 release (Fig. 3a). This observation was consistent with a previous analysis by Nukada et al. (2008). In contrast, with LP dispersion medium, PAH significantly induced IL-8 release (Fig. 3b; maximum IL-8: 821 pg/mL; cell viability: 26%; exposure concentration: 1250 μ g/mL). Finally, with aqueous vehicle, phthalic acid stimulated IL-8 release (Fig. 3c; maximum IL-8: 178 pg/mL; cell viability: 38%; exposure concentration: 1608 μ g/mL). However, with LP dispersion medium, phthalic acid did not significantly augment IL-8 release.

3.4. Effects of 5-min exposure to other acid anhydride skin sensitizers using LP dispersion medium on IL-8 release

To investigate whether LP dispersion medium contributes to IL-8 release in response to exposure to other acid anhydrides, we used the skin sensitizers MAH and TAH. After exposure to MAH with aqueous vehicle (culture medium containing 0.2% v/v DMSO) for 24 h, THP-1 cells exhibited baseline IL-8 release of 8.3 ± 8.6 pg/mL ($n = 12$). Because TAH is a water-insoluble chemical, we selected aqueous vehicle containing 0.8% v/v DMSO as culture medium.

With aqueous vehicle, MAH caused an increase in IL-8 release (Fig. 4a; maximum IL-8: 1506 pg/mL; cell viability: 41%; exposure concentration: 400 μ g/mL). Thus, MAH increased IL-8 release by over 180-fold compared with that of the vehicle control. Significant augmentation of IL-8 release was also induced within a wide cytotoxicity range, also affecting cell viability. Similarly, using LP dispersion medium, MAH also increased IL-8 release (Fig. 4b; maximum IL-8:

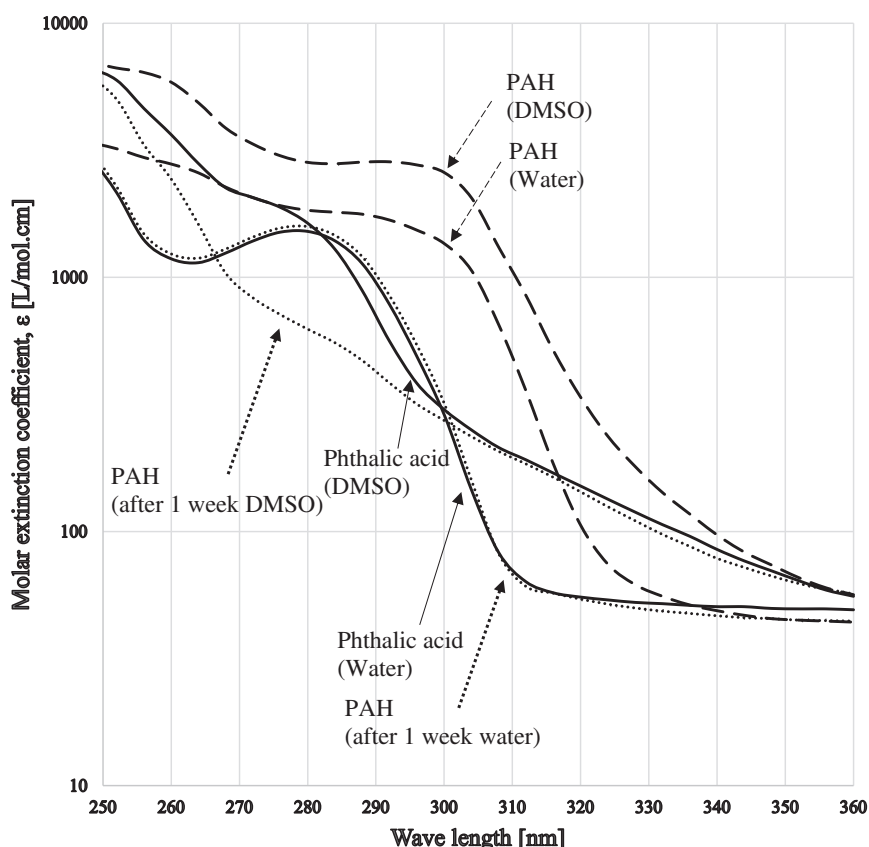


Fig. 1. Absorption spectra of 0.5 mM phthalic anhydride (PAH; broken line) and phthalic acid (solid line) determined immediately after dissolving in DMSO or distilled water. The spectra for 0.5 mM PAH (dotted line) dissolved in DMSO or distilled water after a lapse of 1 week are also shown.

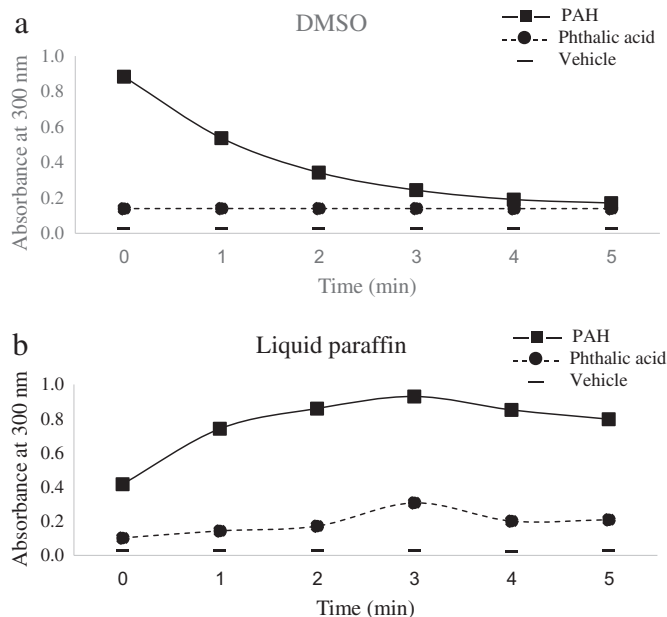


Fig. 2. The hydrolysis of phthalic anhydride (PAH; solid line) was measured following addition of 1 drop of stock solution of PAH or phthalic acid (broken line; a: DMSO vehicle, b: liquid paraffin vehicle) to a 1-cm quartz cell containing 2 mL distilled water. The absorbance of the distilled water layer was measured at 300 nm with a spectrometer every 1 min.

1191 pg/mL; cell viability: 32%; exposure concentration: 313 μ g/mL).

Notably, with aqueous vehicle, TAH failed to produce a significant increase in IL-8 release (Fig. 4c), consistent with a previous study by

Mitjans et al. (2008). In contrast, using LP dispersion medium, TAH significantly increased IL-8 release (Fig. 4d; maximum IL-8: 432 pg/mL; cell viability: 49%; exposure concentration: 1250 μ g/mL). Under these conditions, TAH increased IL-8 release by over 17-fold compared with that of the vehicle control.

3.5. Effects of 5-min exposure to other skin sensitizer and irritant using LP dispersion medium on IL-8 release

To investigate whether LP dispersion medium contributed to IL-8 release following exposure to skin sensitizers other than acid anhydrides, we used NiSO_4 and the skin irritant SLS. THP-1 cells were exposed to NiSO_4 or SLS with aqueous vehicle (culture medium containing 0.2% v/v saline) for 24 h. Under these conditions, baseline IL-8 release of 22.2 ± 13.9 pg/mL ($n = 6$) was observed.

With aqueous vehicle, NiSO_4 , a moderate skin sensitizer, significantly increased IL-8 release (Fig. 5a; maximum IL-8: 57,207 pg/mL; cell viability: 26%; exposure concentration: 142 μ g/mL) by more than 100-fold compared with that of the vehicle control, and significant augmentation of IL-8 release was induced in the range of cell viability from 19% to 95%. Similarly, with LP dispersion medium, NiSO_4 also increased IL-8 release (Fig. 5b; maximum IL-8: 3447 pg/mL; cell viability: 19%; exposure concentration: 5000 μ g/mL). However, this amount of IL-8 was only one-sixteenth that obtained from aqueous vehicle exposure for 24 h, and significant augmentation of IL-8 release was induced in the range of cell viability from 21% to 67%.

The skin irritant SLS, increases IL-8 release (Fig. 5c; maximum IL-8: 162 pg/mL; cell viability: 1.5%; exposure concentration: 71 μ g/mL), and augmentation of IL-8 release was induced in the range of cell viability from 1.5% to 10%. However, with LP dispersion medium, SLS did not significantly induce augmentation of IL-8 release (Fig. 5d).

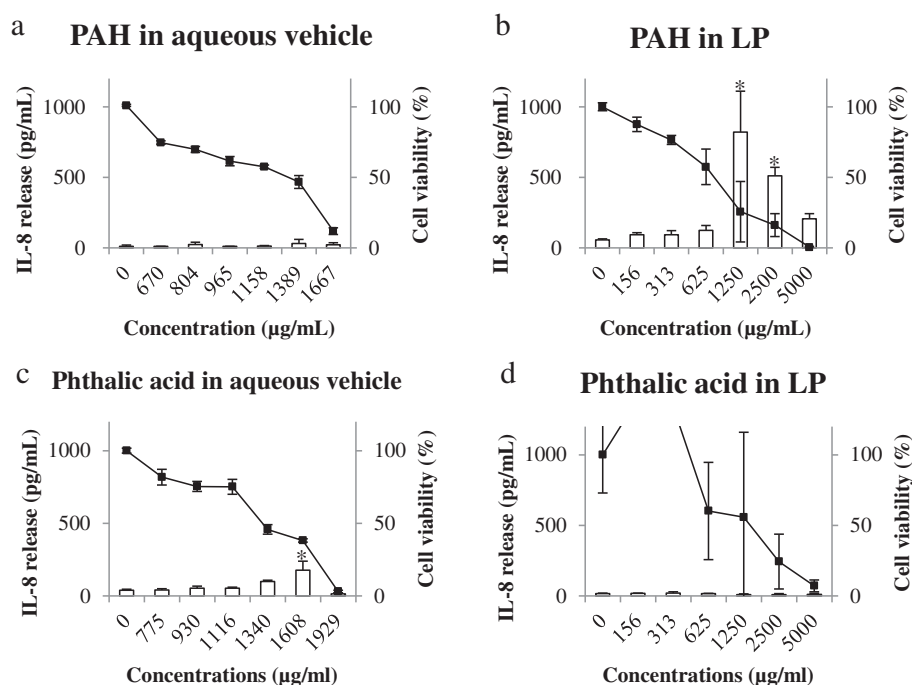


Fig. 3. THP-1 cells were treated with various concentrations of phthalic anhydride (PAH; a) or phthalic acid (c) in aqueous vehicle (culture medium with 0.8% v/v DMSO) for 24 h. Alternatively, THP-1 cells were cultured in medium for an additional 24 h after a 5-min exposure to various concentrations of PAH (b) and phthalic acid (d) in liquid paraffin (LP) dispersion medium. IL-8 secretion in culture medium supernatants was measured by ELISA (open bars) and expressed as pg/mL, while cell viability was measured by using WST-8 assays (black squares).

4. Discussion

In this study, we aimed to help prevent false-negative results in in vitro skin sensitization test using analysis of IL-8 release by THP-1 cells. In particular, we examined why acid anhydrides, which exhibit strong skin sensitizing potential, often show false-negative results in these

assays. PAH, MAH, and TAH are highly immunogenic in LLNA, exhibiting strong sensitization potential (Dearman et al., 2000). Therefore, we believe that developing improve methods for evaluation of acid anhydride should be a research priority.

In previous studies, PAH was evaluated as false negative in IL-8 release assays and several in vitro skin sensitization tests (Ashikaga

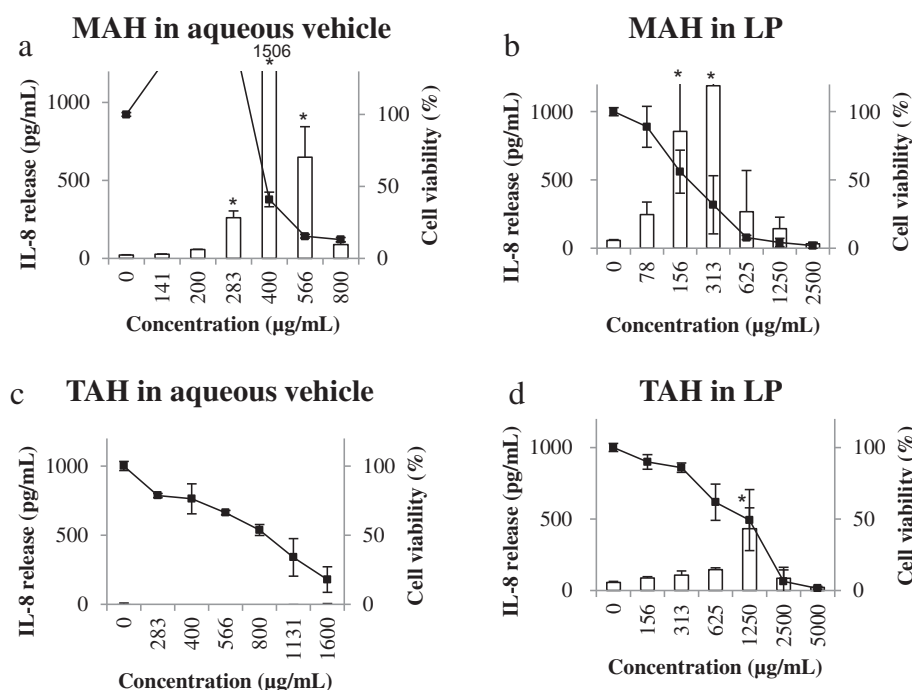


Fig. 4. THP-1 cells were treated for 24 h with various concentrations of maleic anhydride (MAH; a) and trimellitic anhydride (TAH; c) in aqueous vehicle (culture medium with 0.2% and 0.8% v/v DMSO). THP-1 cells were cultured in medium for an additional 24 h following a 5-min exposure to various concentrations of MAH (b) or TAH (d) in liquid paraffin (LP) dispersion medium. IL-8 secretion in culture medium supernatants was measured by ELISA (open bars) and expressed as pg/mL, while cell viability was measured using WST-8 assays (black squares).

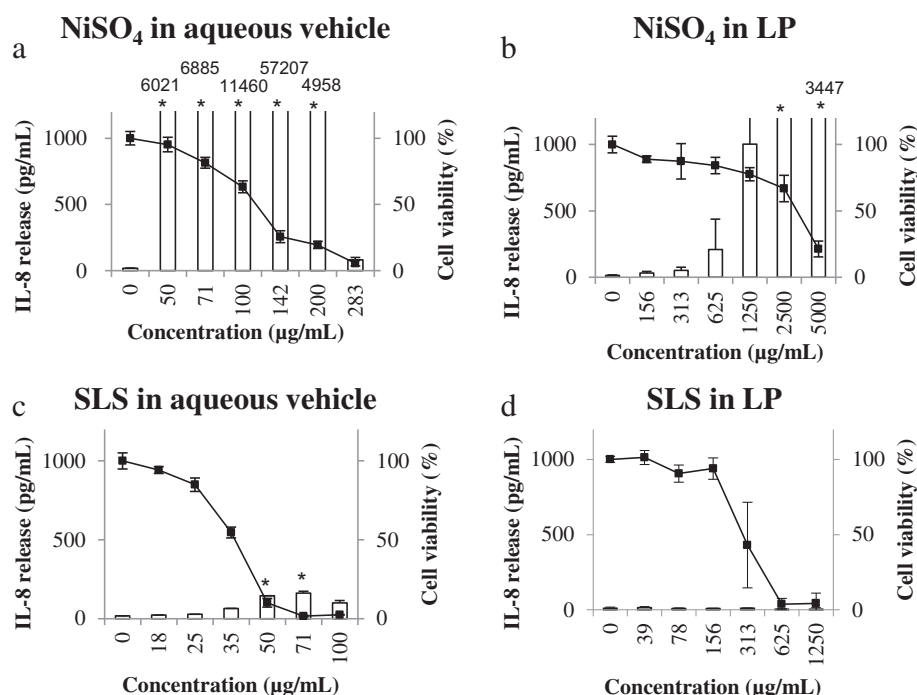


Fig. 5. THP-1 cells were treated for 24 h with various concentrations of NiSO₄ (a) or SLS (c) in aqueous vehicle (culture medium with 0.2% v/v saline). THP-1 cells were cultured in medium for an additional 24 h following a 5-min exposure to various concentrations of NiSO₄ (b) or SLS (d) in liquid paraffin (LP) dispersion medium. IL-8 secretion in culture medium supernatants was measured by ELISA (open bars) and expressed as pg/mL, while cell viability was measured using WST-8 assays (black squares).

et al., 2010; Emter et al., 2013; Saito et al., 2013; Takenouchi et al., 2013; Piroird et al., 2015; Kimura et al., 2015). In LLNA, PAH is dissolved in acetone/olive oil (4:1 v/v) solution (Dearman et al., 2000). However, in all of the above in vitro tests, PAH is dissolved in aqueous vehicle, i.e., culture medium containing DMSO. Generally, in vitro test results are more strongly affected by water than in vivo test results (e.g., water solubility, hydrolysis reactions, hydration reactions). PAH is known to form the corresponding phthalic acid quickly via hydrolysis. The rate constant for the forward reaction for PAH in aqueous solution at 25 °C is $7.9 \times 10^{-3} \text{ s}^{-1}$ (Hawkins, 1975). Therefore, we hypothesized that hydrolysis of PAH may be the underlying cause of the false-negative results.

In order to clarify these results and validate our hypothesis, we measured the hydrolysis of PAH in a cell-free system using ultraviolet (UV) spectrophotometry, which is relative inexpensive, highly sensitive, and quick. In addition, this method has been used to measure the hydrolysis of PAH in previous studies (Peters, 1959; Hawkins, 1975). However, quantitative analysis of unknown substances is difficult using UV spectroscopy. Our preliminary analysis revealed that the greatest difference between PAH and phthalic acid were observed at 300 nm; subsequent analyses at this wavelength revealed that PAH was hydrolyzed within approximately 5 min in water containing DMSO or within 1 week in DMSO. This could be explained by the hygroscopic properties and chemical reactivity of DMSO. For example, the amount of water taken up by DMSO into an open bottle at constant relative humidity of 98% at 22 °C is 40 g per 100 g DMSO (Lebel and Goring, 1962), and the product is obtained from the reaction of DMSO and acetic anhydride within 12–24 h (Albright and Goldman, 1965).

In this study, we used LP as a dispersion medium for PAH. By mixing an LP/water binary-phase solvent, we found that PAH was present for at least 5 min in the water phase because of the sustained release of PAH from LP. Therefore, we further analyzed the validity and effectiveness of our LP dispersion medium methods for in vitro skin sensitization tests using THP-1 cells of acid anhydrides. Notably, when dispersed in LP medium, PAH exhibited an IC₅₀ that was approximately half that of phthalic acid, despite having similar IC₅₀ values in aqueous vehicle, confirming that PAH was hydrolyzed by aqueous vehicle to

phthalic acid. Moreover, PAH in LP dispersion medium induced an increase in IL-8 release from THP-1 cells, further validating our hypothesis. Similar results were observed for MAH and TAH, supporting that the use of LP dispersion medium prevented the hydrolysis of acid anhydrides, facilitating THP-1 cell exposure.

In GARD assay, which are in vitro assays based on a genomic biomarker signature, both PAH and TAH scored as respiratory sensitizers despite the use of aqueous vehicle (Forreryd et al., 2015). The predictive genomic biomarker signature was identified on the assumption that PAH and TAH were the respiratory sensitizers, regardless of aqueous environment. In this procedure, whether PAH and TAH were hydrolyzed was not considered. Moreover, the Ensembl transcript ID, which was used as a gene identifier, did not include IL-8 as a predictor gene in the GARD Respiratory Prediction Signature. Therefore, it may not be possible to compare our data with the results of this assay. In accordance with the considerations above, we could not determine whether both PAH and TAH were scored as sensitizers, despite the use of aqueous vehicle.

Notably, MAH showed a preference for LP dispersion medium, although it also significantly induced augmentation of IL-8 release in aqueous vehicle. This phenomenon was considered in terms of the sensitization potential properties of hydrolyzed MAH. Most importantly, maleic acid classified clear positive results in LLNA (Kreiling et al., 2008). Therefore, it is likely that hydrolysis did not suppress IL-8 release induced by MAH. Furthermore, acid anhydrides bind covalently to free amino groups, particularly the amino group of lysine (Newman Taylor et al., 1987; Lindh and Jönsson, 1998; Lalko et al., 2012). However, Lalko et al. (2012) found that MAH also increased cysteine reactivity when compared with PAH and TAH in chemical analyses. Thus, we hypothesized that the skin sensitization mechanism of MAH may differ from those of PAH and TAH.

However, for MAH and NiSO₄, the maximum IL-8 release in LP dispersion medium was significantly decreased compared with that in aqueous vehicle, which may have been related to exposure time. Accordingly, additional studies are needed to develop a long-term exposure method using LP dispersion medium.

In our study, SLS augmented IL-8 release during a 24-h exposure

using aqueous vehicle. Toebak et al. (2006) reported similar results in monocyte-derived undifferentiated dendritic cells exposed to subtoxic concentrations of SLS. For this same reason, phthalic acid caused a slight augmentation of IL-8 release during a 24-h exposure using aqueous vehicle.

5. Conclusion

We found that PAH, MAH, maleic acid, and TAH induced IL-8 release in THP-1 cells, whereas phthalic acid and trimellitic acid did not. Moreover, the false-negative results of PAH and TAH were caused by hydrolysis by aqueous vehicle. Furthermore, 5-min exposure to LP dispersion medium may be a promising method for evaluation of skin sensitization by acid anhydrides. These findings may have applications in in vitro skin sensitization tests using THP-1 cells (e.g., h-CLAT). However, additional studies are needed to develop a long-term exposure method using LP dispersion medium.

Transparency document

The <http://dx.doi.org/10.1016/j.tiv.2017.04.008> associated with this article can be found, in online version.

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