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A quantitative approach to assess the potency of skin sensitizers in the elicitation phase

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ABSTRACT

The concept that thresholds exist for the induction of allergic contact dermatitis by chemicals with skin sensitizing properties has been used for a quantitative risk assessment approach. In this approach the potency of skin sensitizers as determined in the Local Lymph Node Assay is used to calculate the threshold for induction of sensitization. These are then used to estimate safe exposure levels for consumers. Whether these exposure levels will protect subjects that are already sensitized is unknown. The elicitation of allergic contact dermatitis supposedly occurs above a certain threshold as well and this threshold is most likely lower than that for the induction. It is unclear if induction thresholds can be extrapolated to elicitation thresholds. The aim of this study was to assess the potency of sensitizers with different sensitizing potencies in the elicitation phase in a mouse model for elicitation. Mice were sensitized by topical application on days 0 and 7 using equipotent concentrations of oxazolone, 2,4-dinitrochlorobenzene (DNCB) and eugenol to ensure that the sensitization strength would not influence the elicitation potency. Mice were challenged on day 21 by topical application on the ears in a dose-dependent manner and dose–response data were used to calculate the elicitation potency. Unexpectedly, sensitizers with different sensitizing potencies induced not the same dose–response curves in sensitized mice. The most potent sensitizer in the elicitation phase was oxazolone, followed by DNCB and eugenol. Similar to the induction phase, under equipotent sensitization conditions strong sensitizers such as oxazolone and DNCB elicit allergic reactions at lower concentrations than weak sensitizers such as eugenol. Our results indicate that elicitation thresholds cannot be readily deduced from sensitization thresholds.

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1. Introduction

Allergic contact dermatitis is an important occupational and consumer health problem that results from exposure to chemicals with skin sensitizing properties (Kimber et al., 2002). In the EU, skin sensitizers are allowed to be used in consumer products, but they have to be classified and labeled according to the Globally Harmonized System (GHS) for classification and labeling of chemicals. Some strong potent sensitizers are not allowed to be used in certain product groups, whereas other sensitizers are restricted to a maximum allowed concentration. The guinea pig test methods (Buehler test and Guinea Pig Maximization Test) or the murine Local Lymph Node Assay (LLNA) are used for hazard identification and characterization (OECD, 1992, 2000; ICCVAM, 1999; ECVAM, 2008).

Like other toxicological effects, skin sensitization occurs only when the dose applied on the skin exceeds a certain threshold

(Basketter et al., 2002; Boukhman and Maibach, 2001; Kimber et al., 1999). The intrinsic potency of a chemical is one of the factors that determine this threshold dose. Potency can be defined as the relative ability of a chemical to induce sensitization and the LLNA offers the opportunity to estimate this potency based on dose–response data. Skin sensitizing potency is expressed as the EC3 value, which is the estimated concentration that induces a threefold stimulation of lymphocyte proliferation (EC3 value) (Basketter and Cadby, 2004; Ryan et al., 2007; Van Och et al., 2005). This EC3 value correlates relatively well with human potency assessed in clinical settings (Griem et al., 2003; Schneider and Akkan, 2004; Basketter et al., 2005).

The concept of thresholds for skin sensitization has been used as the starting point for quantitative risk assessment. In this approach EC3 values are used as a point-of-departure to calculate levels below which no sensitization will occur, called the NESIL (No Expected Sensitization Induction Level). Different safety factors are then applied to calculate safe exposure levels (Robinson et al., 2000; Api et al., 2008; Api and Vey, 2008; Basketter et al., 2008b). In the future, such quantitative approaches might be used to determine concentration limits that are safe for consumers, rather than the

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current concentration limits that are arbitrary chosen. In this way the risk of becoming sensitized would be reduced significantly. This quantitative approach focuses on the sensitization phase only and the estimated safe exposure levels will probably not prevent elicitation responses in subjects that are already sensitized. Similar to sensitization, there is most likely a dose level at which sensitized subjects will not develop a clinical skin reaction, hence, for elicitation thresholds may exist as well. These thresholds are likely to be lower than the threshold dose for the induction of sensitization (Allenby and Basketter, 1989, 1993; Andersen et al., 2001; Frosch et al., 1995; Johansen et al., 1996; McFadden et al., 1998; Menné, 1994).

It is unclear whether induction thresholds of sensitizers can be readily extrapolated to elicitation thresholds. The complexity is that elicitation thresholds not only depend on the chemical itself but also on the exposure dose that induced sensitization, and the strength of the sensitization. Studies in human volunteers have demonstrated that an inverse relationship exists between the strength of sensitization and the elicitation threshold (Boukman and Maibach, 2001; Friedmann, 2007; Friedmann et al., 1983). This means that at a higher sensitization dose, a lower dose is needed for elicitation responses (Scott et al., 2002). To investigate if it is possible to calculate the potency of sensitizers in the elicitation phase a mouse model for elicitation was developed. Studies in mice offer the opportunity to select *equipotent* concentrations for sensitization, to ensure that the degree of sensitization is similar for the different chemicals. Subsequently, dose–response data in the elicitation phase were used to calculate the elicitation potency.

2. Methods

2.1. Animals

Young adult (6–8 weeks of age) female inbred BALB/cOlaHsd mice were obtained from Harlan (Boxmeer, The Netherlands) and acclimatized for at least 7 days before the start of the study. The animals were bred under specified pathogen-free (SPF) conditions. During the experiments, the animals were housed barrier maintained under conventional conditions in light-, humidity-, and temperature controlled rooms. All animals were housed in macrolon cages. The mice were fed a standard pellet diet (RM3 [E] SQC, Special Diet Service, Witham, UK) and unfluoridated tap water *ad libitum*. All other husbandry conditions were maintained according to all applicable provisions of the following national laws: Experiments on Animals Decree, and Experiments on Animals Act. All animal experiments were performed according to all applicable national laws, and had permission from the Commission of Animal Welfare of the National Institute for Public Health and the Environment.

2.2. Chemicals

Chemicals were selected that vary in their potency as determined in the LLNA (Table 1). The following sensitizers were included: oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; purity at least 90%), 2,4-dinitrochlorobenzene (DNCB; purity at least 98%) and eugenol (purity at least 99%) all from Sigma, St. Louis, MO, USA. Other chemicals used were acetone (Biosolve, Valkenswaard, the Netherlands) and raffinated olive oil (Sigma).

2.3. Experimental design elicitation model

In the elicitation model, mice were sensitized on days 0 and 7 and challenged on day 21. All exposures were done by topical application of 25 μ l of the compounds dissolved in acetone:olive oil (4:1) solution (AOO) on the dorsum of both ears (50 μ l per animal). Control mice were treated with AOO alone. This site of exposure for sensitization was chosen because it offered the possibility to measure cell proliferation in the auricular lymph nodes at day 9 as an indicator for the strength of sensitization. The increase in cell proliferation in the auricular lymph node was used as a read-out to assess if the chemicals were dosed at equipotent concentrations. In all experiments a parallel group was included that was sacrificed at day 9. To assess the elicitation response, mice were sacrificed on day 23 and auricular lymph nodes were dissected and single cell suspensions were prepared to measure cell proliferation using *ex vivo* labeling of the proliferating lymph node cells with [^{3} H]-thymidine as described previously (De Jong et al., 2002; van Och et al., 2003). The [^{3} H]-thymidine incorporation is expressed per animal, being the measured counts per minute (cpm) times the cell number of the two LN and divided by the cell number in culture. The mean [^{3} H]-thymidine incorporation per experimental group $\pm$ SEM was calculated. Stimulation indices (SI) were calculated by dividing the [^{3} H]-thymidine

incorporation of the experimental group with the mean [^{3} H]-thymidine incorporation of the vehicle group.

2.4. Pilot experiments to determine the optimal sensitization dose of DNCB

Preliminary experiments with DNCB were performed to determine the sensitization dose that is required to measure an elicitation response in the auricular lymph nodes. Mice were sensitized with 0.2% or 0.4% DNCB ($n=4$). Control mice ($n=6$) were exposed to the vehicle, AOO, only. At day 21 mice were challenged on the ears with 0.045 or 0.09% DNCB or with the vehicle. At day 23, mice were sacrificed and auricular lymph nodes were dissected to measure cell proliferation.

2.5. Determination of equipotent sensitization concentrations for oxazolone and eugenol

Equipotent concentrations of oxazolone and eugenol were determined in mice sensitized to increasing concentrations of these compounds on day 0 and 7. For oxazolone, mice were exposed to 0.06%, 0.09%, 0.135%, 0.18% and 0.27%. For eugenol, the exposure concentrations were 5%, 10%, 25% and 50%. Cell proliferation was measured at day 9 and dose–response data were analyzed by nonlinear regression analysis, as described previously (van Och et al., 2000). This was done using PROAST software (Slob, 2002). The concentration that resulted in a 9-fold induction was retrieved from these dose–response curves. For oxazolone and eugenol, the concentration that induced a 9-fold increase in proliferation was 0.12% and 35%, respectively.

2.6. Elicitation responses and calculation of elicitation potency

The elicitation potency was determined in mice sensitized to DNCB, oxazolone and eugenol using equipotent concentrations. To assess dose–response relationships, mice were challenged with increasing concentrations on day 21 by topical application on the ears. The concentrations used were percentages of the sensitization dose selected for each chemical: 12.5, 25, 50, 75 and 100% of the sensitization concentration. Table 1 shows the concentrations used for each sensitizer. In each experiment, a control group was included that was sensitized and challenged with AOO. Proliferation in this group was used as a reference value to determine the potency in the elicitation phase. Finally, mice that were sensitized with the vehicle were challenged with the two highest elicitation concentrations to assess if a single application of the sensitizers did not induced cell proliferation by a non-specific (irritation) response.

In each experiment, parallel groups were included in which cell proliferation was measured on day 9 to assess if sensitization resulted in an equipotent immune response.

The relative potency for elicitation was estimated as the concentration that induced a 3-fold increase of proliferation at day 23. This EC_{3_{el}} value in the elicitation phase (EC_{3_{el}}) was determined by analyzing the dose–response data by nonlinear regression analysis using PROAST software (Slob, 2002). The EC_{3_{el}} is calculated by plotting the elicitation concentration expressed as a percentage of the sensitization dose and not by using the actual exposure concentration. In this way the EC_{3_{el}} of the three sensitizers reflects the amount of chemical in relation to the sensitization dose. The lower this value, the more potent the compound is.

2.7. Elicitation responses induced by eugenol

Additional experiments with eugenol were performed using the same experimental protocol for sensitization and elicitation. Mice were sensitized with 25 or 50% eugenol on days 0 and 7. On day 21 mice were challenged with concentrations that were related to the sensitization concentration (12.5, 25, 50, 75 and 100% of the sensitization concentration) and proliferation was assessed in the auricular lymph nodes at day 23. The proliferation was compared to the dose–response curve of mice sensitized with 35% eugenol.

2.8. Statistics

In the pilot experiments with DNCB, cell proliferation in the auricular lymph nodes in the different experimental groups was statistically analyzed after logarithmic transformation, using a one-way ANOVA, followed by a Bonferroni post hoc test. A p value of <0.05 was considered to be statistically significant. Outliers were detected using Grubb's test and rejected for further analysis.

3. Results

3.1. Pilot studies to determine the optimal sensitization dose

DNCB was used in pilot experiments to determine the optimal sensitization dose required to measure an elicitation response in the auricular lymph nodes. A significant increase in cell proliferation was induced after sensitization with 0.2% or 0.4% DNCB and challenge with 0.045 and 0.09% DNCB (Fig. 1). In mice sensitized

Table 1

Concentrations used for sensitization and elicitation.

Sensitizers	EC3 value LLNA ^a	Sensitization concentration	Elicitation concentration ^b
Oxazolone	0.002–0.013%	0.12%	0.015 (12.5%) 0.03 (25%) 0.06 (50%) 0.09 (75%) 0.12 (100%)
DNCB	0.05–0.09%	0.2%	0.025 (12.5%) 0.05 (25%) 0.1 (50%) 0.15 (75%) 0.2 (100%)
Eugenol	9.8–13%	35%	4.4 (12.5%) 8.8 (25%) 17.5 (50%) 26.3 (75%) 35 (100%)

Q5 ^a The EC3 value is the estimated concentration that induces a 3-fold induction of cell proliferation in the LLNA (Gerberick et al., 2005; Loveless et al., 1996; van Och et al., 2000);

^b The elicitation dose is expressed as the actual dose applied (w/v %) and the % of the sensitization dose in brackets.

with 0.4% DNCB, cell proliferation was still enhanced at day 23, as shown in mice that were challenged with the vehicle. At day 30, this increase in cell proliferation was back to control values (not shown). It was decided to continue with a dose of 0.2% DNCB, since at this dose the sensitization response was back to control values and it was sufficiently high to measure elicitation responses at day 23.

In parallel groups that were sacrificed on day 9 it was shown that 0.2% DNCB induced a stimulation index (SI) of 9.3 (± 1.63). Hence, the sensitization concentration of the other chemicals selected should induce an equipotent response, meaning that for the other sensitizers tested in this model a dose that increased cell proliferation with a fold change of 9 (± 2) should be used as well. Table 1 summarizes the concentrations used in the elicitation experiments.

3.2. Elicitation responses of oxazolone, DNCB and eugenol

A prerequisite in the elicitation model was that all sensitizers should be dosed at equipotent concentrations. The proliferation

Table 2

Proliferation responses in the auricular lymph nodes after sensitization.

Sensitizer	SI value ^a
Oxazolone	7.8 $\pm$ 1.8
DNCB	9.3 $\pm$ 3.2
Eugenol	7.1 $\pm$ 2.1

^a Parallel groups in the elicitation experiments were sensitized at day 0 and 7. At day 9, mice were sacrificed and proliferation in the auricular lymph nodes was measured. Proliferation is expressed as the mean SI value ($\pm$ SEM), which was calculated by dividing the mean [³H]-thymidine incorporation per group by the mean [³H]-thymidine of the control group.

response in the auricular lymph nodes at day 9 was used as a read-out for this. In parallel experiments it was shown that after sensitization the SI values for oxazolone, DNCB and eugenol were in the predefined range of SI values between 7 and 9 (Table 2).

The proliferation in the elicitation phase was measured at day 23 (Fig. 2). Although mice were sensitized with equipotent concentrations, the dose–response curves for the three sensitizers are clearly different. Oxazolone increased proliferation at the lowest elicitation dose tested (12.5% of the sensitization concentration). The dose–response curve for oxazolone reached a plateau at the three highest concentrations. DNCB induced a linear dose response curve from 25% of the sensitization dose onward. Eugenol increased cell proliferation only after challenge with the two highest elicitation concentration.

3.3. Elicitation potency

To compare the potency of these skin sensitizers, the EC_{3_{el}} value was calculated using the dose–response curves shown in Fig. 2. This EC_{3_{el}} value is used as a metric to compare the intrinsic potency of these three sensitizers. Oxazolone was the most potent sensitizer in the elicitation phase; a 3-fold induction of cell proliferation was induced when 3% of the sensitization concentration was applied. For DNCB, 14% of the initial sensitization dose was required, whereas for eugenol, the EC_{3_{el}} was 73% (Table 3).

3.4. Elicitation responses of eugenol, a weak sensitizer

To further investigate the relationship between degree of sensitization and elicitation threshold for the weak sensitizer eugenol mice were sensitized to 25 or 50% eugenol. The extent of sensitization was different compared to the 35% dose. The SI value in mice sensitized with 25% eugenol was 4.6 (± 0.39). Sensitization

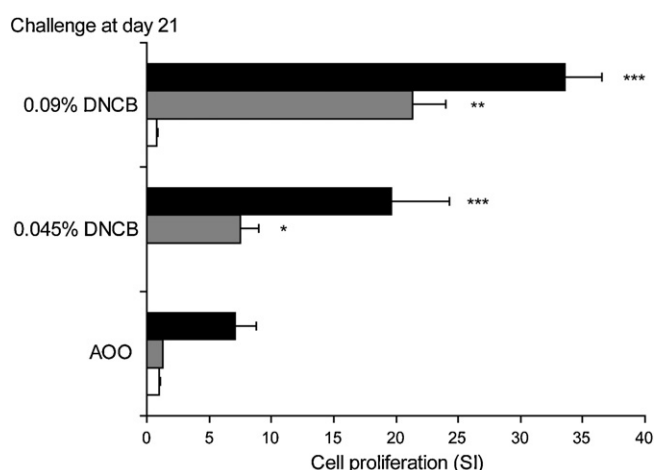


Fig. 1. Elicitation response induced by DNCB. Mice were sensitized at day 0 and 7 with 0.2% DNCB (grey bars), 0.4% DNCB (black bars) or the vehicle (white bars). At day 21, mice were challenged by topical application on the ears with 0.045% or 0.09% DNCB. Cell proliferation was measured at day 23 and is expressed as the stimulation index (SI). SIs were calculated by dividing the mean [³H]-thymidine incorporation per group by the mean [³H]-thymidine of the control group (sensitized and challenged with vehicle). Statistical analysis was performed after logarithmic transformation, using a one-way ANOVA followed by a Bonferroni post hoc test; **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

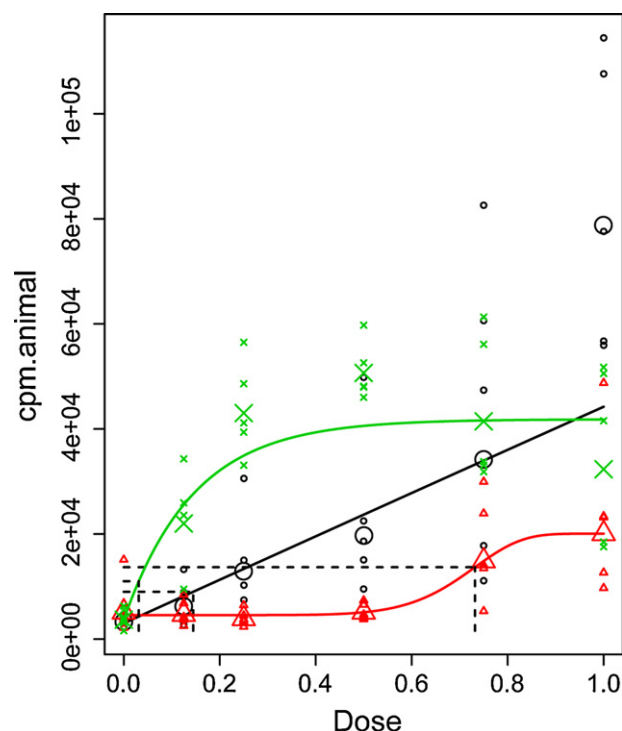


Fig. 2. Dose–response data and fitted models of the elicitation response. Proliferation in the auricular lymph nodes was assessed at day 23 by measuring [^{3} H]-thymidine uptake and is expressed in cpm. Dose–response curves were generated for oxazolone ($\times$), DNCB ($\circ$) and eugenol (Δ). The elicitation concentration is expressed relative to the dose used for sensitization (in %). The horizontal dashed line represent the 3-fold induction of lymph node proliferation; vertical dashed line is the associated dose, representing the $EC_{3_{el}}$.

Table 3
Potency of oxazolone, DNCB and eugenol in the elicitation phase.

Sensitizer	$EC_{3_{el}}^a$	90% confidence interval	Actual concentration ^b
Oxazolone	3%	1.7–5.1	0.0037%
DNCB	14%	8.5–25	0.028%
Eugenol	73%	52–84	26%

^a The $EC_{3_{el}}$ is interpolated from the dose–response curve and is the dose that induced a 3-fold increase in cell proliferation compared to the control group. The $EC_{3_{el}}$ is expressed as the percentage of the sensitization concentration.

^b The actual concentration is the concentration of each compound needed to induce an EC_3 in the elicitation phase.

with 50% eugenol resulted in a SI value of $26.8 (\pm 10.1)$ at day 9. The dose responses in the elicitation phase confirm the inverse relationship between sensitization strength and elicitation response (Fig. 3). Mice sensitized with 25% eugenol did not respond in the elicitation phase, demonstrating that these mice were not sensitized, although a moderate increase in proliferation was found on day 9. The sensitization concentration of 50% induced a strong response on day 9 that was more pronounced than in the mice sensitized with 35% eugenol. This indicates that weak sensitizers can induce a strong elicitation response when mice are sensitized with a dose that is higher than the equipotent dose selected.

4. Discussion

The aim of this study was to investigate if thresholds for the induction of skin sensitization can be extrapolated to thresholds for elicitation. For this reason an experimental elicitation model was used in which mice were sensitized to equipotent concentrations. In this way the strength of sensitization would supposedly

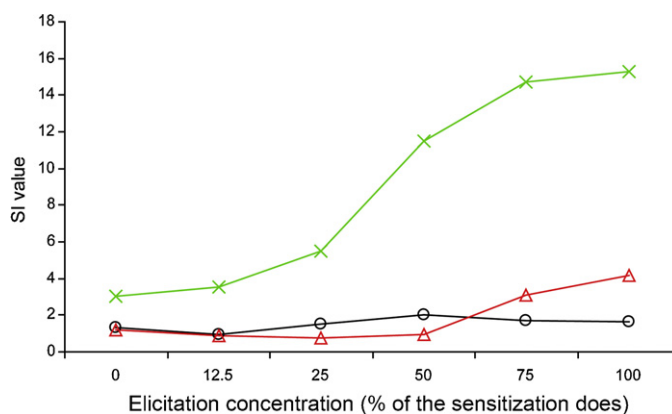


Fig. 3. Elicitation responses induced by different sensitization concentrations of eugenol. Mice were sensitized to 25% ($\circ$), 35% (Δ) or 50% eugenol ($\times$). Proliferation in the auricular lymph nodes was assessed at day 23 by measuring [^{3} H]-thymidine uptake and is expressed in cpm. The elicitation concentration is expressed as relative to the dose used for sensitization.

not influence the elicitation response induced by sensitizers with different sensitizing potencies. Remarkably, the dose–response curves in the elicitation phase were quite different, while it was expected that under these equipotent circumstances the dose–response curves in the elicitation phase would be similar. The dose response data were used to calculate the elicitation potency and oxazolone was the most potent sensitizer, followed by DNCB and eugenol. It is apparent that the ranking of these compounds is similar to the potency ranking in the sensitization phase. These results imply that under equipotent sensitization conditions strong sensitizers will elicit allergic symptoms at much lower concentrations than weak sensitizers. Hence, elicitation thresholds cannot easily be deduced from sensitization thresholds.

Apparently, strong potent sensitizers are able to trigger an elicitation response more efficiently. The immunological processes and cellular and molecular interactions that are involved in the induction and elicitation of skin sensitization are relatively well known and all skin sensitizers have to activate the complete cascade to induce sensitization (Kimber and Dearman, 2002; Vocanson et al., 2009). The magnitude of certain immunological responses appears to differ between strong and weak sensitizers. Innate signals produced by keratinocytes are essential in the elicitation response (Martin et al., 2011). Studies in a murine model for allergic contact dermatitis showed that in the elicitation phase the strong sensitizer trinitrochlorobenzene (TNCB) induced a more pronounced increase of the effector cytokine IFN- γ and the proinflammatory cytokines IL-1 β and IL-6 in the skin than the weak sensitizer Arnica. Remarkably, TNCB and Arnica induced similar levels of the anti-inflammatory cytokine IL-10. Furthermore, the degree of skin inflammation was related to the recruitment of T cells into the exposed skin that was more pronounced for strong sensitizers (Lass et al., 2010; Martin et al., 2011). This illustrates that strong sensitizers will probably more efficiently recruit T cells into the skin and in this way more easily elicit allergic contact dermatitis. It has been further hypothesized that at the strength of the T cell response differs also between strong and weak sensitizers. Furthermore, the quality and diversity of the T cell response might be different in the sense that strong sensitizers induce more effector T cells than regulatory cells and they probably induce a more diverse T cell repertoire compared to weak sensitizers (Kimber et al., 2012). These hypotheses might explain the differences between strong and weak sensitizers observed in this elicitation model, but should be substantiated with further research.

Besides the sensitizing potency of chemicals, the actual risk that humans become sensitized depends on other factors as well. The

most important are concentration, frequency and duration of exposure and condition of the exposed skin (Basketter et al., 2002, 2006). Thresholds for elicitation depend strongly on the degree of induction of skin sensitization making it difficult to determine a human threshold for elicitation, since in real life the induction of sensitization will most probably differ considerably. In subjects suffering from allergic contact dermatitis, it was shown that there is little variation in the dose that elicited a positive patch test in subjects sensitized to weak compared to strong sensitizers. Hence, in humans there was no clear relationship between sensitization potency derived from the LLNA and the threshold for elicitation in humans (Fischer et al., 2011). On the other hand, our results, although based on a limited number of chemicals, indicate that strong sensitizers should be treated with more caution than weak sensitizers. Not only will stronger sensitizers induce skin sensitization at a lower concentration, they also appear to have a lower threshold to elicit allergic responses. In this regard the situation for both induction and elicitation seems to be similar. Therefore, using quantitative approaches to establish safe exposure limits will be a step forward in terms of preventing the induction of allergic contact dermatitis. The initial focus should be on strong skin sensitizers since these will most likely have the biggest impact on the disease burden.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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