Sensitization and cross-reactivity patterns of contact allergy to diisocyanates and corresponding amines: investigation of diphenylmethane-4,4′-diisocyanate, diphenylmethane-4,4′-diamine, dicyclohexylmethane-4,4′-diisocyanate, and dicylohexylmethane-4,4′-diamine

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Summary

Background. Isocyanates are used in polyurethane production. Dermal exposure to isocyanates can induce contact allergy. The most common isocyanate is diphenylmethane diisocyanate used for industrial purposes. The isomer diphenylmethane-4,4′-diisocyanate (4,4′-MDI) is used in patch testing. Diphenylmethane-4,4′-diamine (4,4′-MDA) is its corresponding amine. Concurrent reactions to 4,4′-MDI and 4,4′-MDA have been reported, as have concurrent reactions to 4,4′-MDI and dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI).

Objectives. To investigate the sensitization capacities and the cross-reactivity of 4,4′-MDI, 4,4′-MDA, 4,4′-DMDI, and dicyclohexylmethane-4,4′-diamine (4,4′-DMDA).

Methods. The guinea-pig maximization test (GPMT) was used.

Results. The GPMT showed sensitizing capacities for all investigated substances: 4,4′-MDI, 4,4′-MDA, 4,4′-DMDI, and 4,4′-DMDA (all $p < 0.001$). 4,4′-MDI-sensitized animals showed cross-reactivity to 4,4′-MDA ($p < 0.001$) and 4,4′-DMDI (all $p < 0.05$). 4,4′-MDA-sensitized animals showed cross-reactivity to 4,4′-DMDA ($p = 0.008$).

Conclusion. All of the investigated substances were shown to be strong sensitizers. Animals sensitized to 4,4′-MDI showed cross-reactivity to 4,4′-MDA and 4,4′-DMDI, supporting previous findings in the literature. The aromatic amine 4,4′-MDA showed cross-reactivity to the aliphatic amine 4,4′-DMDA.

Key words: allergic contact dermatitis; amines; cross-reactions; 4,4′-DMDA; 4,4′-DMDI; guinea-pig maximization test; isocyanates; 4,4′-MDA; 4,4′-MDI; occupational.

In modern life, plastic products are largely replacing traditional materials such as wood and metal, and new applications for plastics are constantly being introduced. One of the most common and versatile plastic materials is polyurethane (PUR). PUR products can appear in an astonishing variety of forms, for example flexible foams used in mattresses and car seats, rigid foams used in, for instance, thermal insulation and composite wood products, elastomers used in shoe soles, textiles, and sports equipment, and binders used in paint and lacquers.

PUR products are produced by reacting isocyanates with multifunctional alcohols (polyols). Isocyanate

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handling is a well-known occupational health hazard, mainly because of the adverse effects on the respiratory tract (1, 2). Strict rules on monitoring of air exposure limits apply to isocyanate work. Although skin exposure has been suggested to be an important route to diisocyanate asthma (3, 4), dermal exposure and the risk of developing contact allergy has not gained as much attention as the risks associated with airway exposure.

In spite of the large numbers of workers exposed and the fact that isocyanates are, theoretically, potent contact allergens, relatively few reports on contact allergy to isocyanates are found in the literature. Previous studies have shown that, concerning the most commonly used isocyanate in industry, diphenylmethane-4,4′-diisocyanate (4,4′-MDI), one reason could be inadequate patch test diagnostics, as test preparations of 4,4′-MDI are not stable (5, 6). The reliability of patch testing with 4,4′-MDI has thus been questioned, and it has been suggested that its corresponding amine, the more stable diphenylmethane-4,4′-diamine (4,4′-MDA), could serve as a marker for contact allergy to 4,4′-MDI (7–10). Concomitant reactions to 4,4′-MDI and 4,4′-MDA have been explained by the following possibilities: (i) both 4,4′-MDI and 4,4′-MDA are primary sensitizers (11), (ii) they show cross-reactivity to each other (12–14), or (iii) 4,4′-MDI is transformed to 4,4′-MDA upon reaction with water encountered in or on the skin (12, 15, 16). However, the last of these explanations is contradicted by biochemical *in vivo* data obtained by skin exposure to [^{14}C]4,4′-MDI, which suggest that there is almost no formation of 4,4′-MDA from 4,4′-MDI on exposed skin (17). Furthermore, several publications have described concurrent reactions between dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI) and 4,4′-MDA (11, 18–20), and it has been suggested that the concurrent reactions could be attributable to a similar immunological reaction as has been proposed for 4,4′-MDI and 4,4′-MDA (19). 4,4′-DMDI has been reported to be a potent skin sensitizer (20–22). In the early 2000s, the Department of Occupational and Environmental Dermatology investigated several workers at a company handling 4,4′-DMDI after an outbreak of contact dermatitis among the personnel, and concurrent reactions between 4,4′-DMDI, 4,4′-MDA and dicylohexanemethane-4,4′-diamine (4,4′-DMDA) were observed (20).

This prompted an investigation of the pattern of cross-reactivity between isocyanates and their corresponding amines. In this context, the term cross-reactivity refers to when an individual initially sensitized to one chemically defined substance (A) reacts to a second chemically defined substance (B) that he or she has not been in previous contact with. The first compound is the primary sensitizer, and the other is the secondary sensitizer (23). Cross-reactivity can occur because A and B are structurally similar, or because A is metabolized to a compound that is similar to B and vice versa, or because A and B are both metabolized into similar compounds (24). Cross-reactivity does not need to go in both directions; that is, if A is a primary sensitizer giving rise to a reaction to the secondary sensitizer B, it does not automatically imply that primary sensitization to B will also cause a reaction to A.

The aim of this study was to investigate the sensitizing potencies of 4,4′-MDI, 4,4′-MDA, 4,4′-DMDI, and 4,4′-DMDA, as well as the cross-reactivity patterns among them, by using the guinea-pig maximization test (GMPT). All investigated substances are specified in Table 1.

Materials and Methods

The study was approved by the Lund Ethical Committee on Animal Experiments, Lund, Sweden, and conducted in accordance with ethical standards (approval number M 340-12).

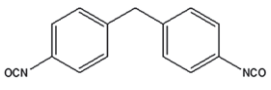
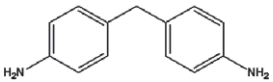
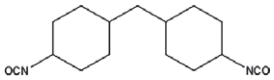
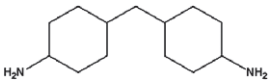
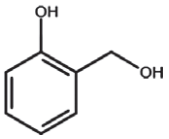
Chemicals

The investigated allergens were 4,4′-MDI, 4,4′-DMDI, and 4,4′-DMDA, which were obtained from Sigma-Aldrich Chemie (Steinheim, Germany), and 4,4′-MDA, which was obtained from TCI Europe (Zwijrdecht, Belgium). The vehicles were acetone of analytical grade from Scharlau Chemie (Sentemenat, Spain), ethanol from Kemetyl (Haninge, Sweden), liquid paraffin from Apoteksbolaget (Stockholm, Sweden), and propylene glycol from VWR International (Fontenay-sous-Bois, France). Sodium lauryl sulfate (SLS) and *N*,*N*-dimethylacetamide 99% were from Sigma-Aldrich (St Louis, MO, USA), 2-methylol phenol (2-MP) 97% was from Acros Organics (Geel, Belgium), and Imject® Freund's complete adjuvant (FCA) was from Thermo Scientific (Rockford, IL, USA).

Materials

The materials used for the assays were as follows: Comprilan® 6-cm elastic compression band (BSN Medical, Hamburg, Germany), Al test® (Imeco, Södertälje, Sweden), filter papers number 3 (Munktell Filter, Grycksbo, Sweden), and 1-ml syringes with 0.4 × 20-mm injection needles (Codan Triplus, Kungsbacka, Sweden). Adhesive bandages were from Durapore® 3M Health Care (St Paul, MN, USA), and impermeable plastic adhesive tape was from Acrylastic® (BSN Medical).

Table 1. All of the investigated substances, including the positive control, are listed with some common synonyms, their CAS numbers, their classification according to the CLP regulation[a], their log $P_{o/w}$, and their purity as stated by the manufacturers

				Harmonized classification[a]				
Name[b]	Synonyms	CAS no.	Structure	Class and category code	Hazards statement code	Specific concentration limits	Log $P_{o/W}$	Purity[c] (%)
Diphenylmethane-4, 4′-diisocyanate	4,4′-MDI; 4,4′-diisocyanatodi phenylmethane; 4,4′-methylenebis (phenyl isocyanate); 4,4′-methylenediphenyl diisocyanate	101-68-8		Skin sensitizer 1 Carcinogen 2 Respiratory sensitizer 1	H317 H351 H334	Respiratory sensitizer 1; H334: ≥ 0.1%	5.22	98
Diphenylmethane-4,4′-diamine	4,4′-MDA; 4,4′-methylene dianiline; 4,4′-dimethylenediamine; 4,4′-diaminodiphenyl methane	101-77-9		Carcinogen 1B Mutagen 2 Skin sensitizer 1	H350 H341 H317		1.59	>98
Dicyclohexylmethane-4, 4′-diisocyanate	4,4′-DMDI; 4,4′-HMDI; methylene bis(4-cyclohexy lisocyanate); 4,4′-methylenedicyclohexyl diisocyanate; hydrogenated MDI	5124-30-1		Respiratory sensitizer 1 Skin sensitizer 1	H334 H317	Respiratory sensitizer 1; H334: ≥ 0.5% Skin sensitizer 1; H317: ≥ 0.5%	6.11	91
Dicyclohexylmethane-4, 4′-diamine	4,4′-DMDA; 4,4′-HMDA; 4,4′-diaminodicyclo hexylmethane; 4,4′-methylenebis (cyclohexylamine)	1761-71-3		Classification not harmonized but notified classification as below[d] Skin sensitizer 1	H317		3.26	95
2-Methylol phenol	2-MP; 2-hydroxybenzyl alcohol	90-01-7		Classification neither harmonized nor notified			0.73	97

[a]Classification as found in Annex VI of Regulation (EC) No. 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP regulation).
[b]Name as used in this article.
[c]As stated on the package, other isomers of the substances can occur.
[d]Most commonly notified self-classification in ECHA's database (https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/notification-details/66995/968242; last accessed 19 December 2016).

Guinea-pig maximization test

The GPMT was essentially performed according to the original description (25–27), which is also the method described in OECD test guideline 406 that can be used to classify skin sensitizers according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (28). However, in order to standardize the test and objectify the evaluation of the patch test reactions, some modifications were made regarding, for example, the statistical calculations used to evaluate potency, blind readings, induction concentrations, and the introduction of a positive control group (29–32). The background for the introduction of these modifications can be found in Appendix S1.

Animals

Female albino guinea-pigs weighing 400 g (± 25 g) of the Hartley-Dunkin strain (HP Lidköpings Kaninfarm, Lidköping, Sweden) were used.

Topical irritancy

Before sensitization and cross-reactivity patterns can be assessed, the topical irritancy thresholds must be determined, in order to ensure that the chosen test concentrations do not give rise to irritant reactions. This was performed by applying different concentrations of each of the investigated substances intended for induction as a closed patch test for 2 days on both the neck and the flank of one side of 4 animals. All animals were pretreated with FCA. In order to maximize the number of test concentrations that could be evaluated, the animals were tested first on one side of the body and then on the other side (Fig. 1). Concentrations that did not cause irritation were chosen for topical induction and elicitation (Table 2).

Concentrations

Equimolar concentrations were used for all substances used in the study, with the exception of sensitization series A and C (Table 2). In series A, as a precautionary measure when testing with 4,4′-DMDI, because it was suspected of causing irritant reactions in the animals, it was tested at a non-equimolar concentration in relation to the test substance in challenge I. This also determined the concentration for the rest of the substances in challenge II. 4,4′-DMDI was later tested at two different concentrations in challenge II in series B, one of which was equimolar. However, as a precaution, the non-equimolar concentration for challenge I was used in series C. The concentrations used for induction and challenge are shown in Table 2. The use of equimolar concentrations constitutes a modification to OECD test guideline 406, in which it is stated that the concentration used for topical induction should be the highest that causes mild-to-moderate skin irritation, and that the concentration in the challenge should be the highest non-irritant concentration. The use of equimolar concentrations enables better comparisons in cross-reactivity studies, but may result in an underestimation of the sensitizing potential (for more information regarding the modification of test concentrations, see Appendix S1).

Induction

Twenty-four test animals (12 control animals and 6 positive control animals) were used for induction in each of the six sensitization series (Table 3) according to the following scheme, which is also described in Fig. 2.

Day 0. All animals were shaved on the neck, and three intradermal injections in a row on each side of the shoulder were then given, resulting in a total of six injections. For the test animals, the following injections were made in duplicate: (i) 0.1 ml of 40% FCA in water (wt/vol); (ii) 0.1 ml of the test substance (wt/vol) in propylene glycol or liquid paraffin; and (iii) 0.1 ml of a mixture of the test substance and FCA in propylene glycol or liquid paraffin in which the concentration of the test substance was the same as in (ii), and the concentration of FCA was the same as in (i). For (ii) and (iii), the vehicle varied according to whether the sensitizing substance was an isocyanate or an amine, as isocyanates can react with propylene glycol, which is normally the vehicle of choice. For sensitization series A, C, E, and F, liquid paraffin was used, and for sensitization series B and D, propylene glycol was used. For the control animals, the following injections were made in duplicate: (i) 0.1 ml of 40% FCA in water (wt/vol); (ii) 0.1 ml of propylene glycol; and (iii) 0.1 ml of 40% FCA in propylene glycol (wt/vol). For the positive control animals, the following injections were made in duplicate: (i) 0.1 ml of 40% FCA in water (wt/vol); (ii) 0.1 ml of 25% 2-MP in propylene glycol (wt/vol); and (iii) 0.1 ml of 25% 2-MP and 40% FCA in propylene glycol (wt/vol).

Day 6. All animals were shaved on the neck, and then subjected to pretreatment of the 2 × 4-cm area intended for topical induction, in order to induce irritancy. The area was treated with 0.2 ml of a preparation consisting of 10% SLS (vol/wt) in dimethyl acetamide/acetone/99.5% ethanol (DAE) 4:3:3 (vol/vol/vol).

Day 7. All animals were shaved on the neck, and epidermal induction was then performed in the test animals and the positive control animals by applying 0.2 ml of the sensitizing substance in acetone or ethanol, depending upon

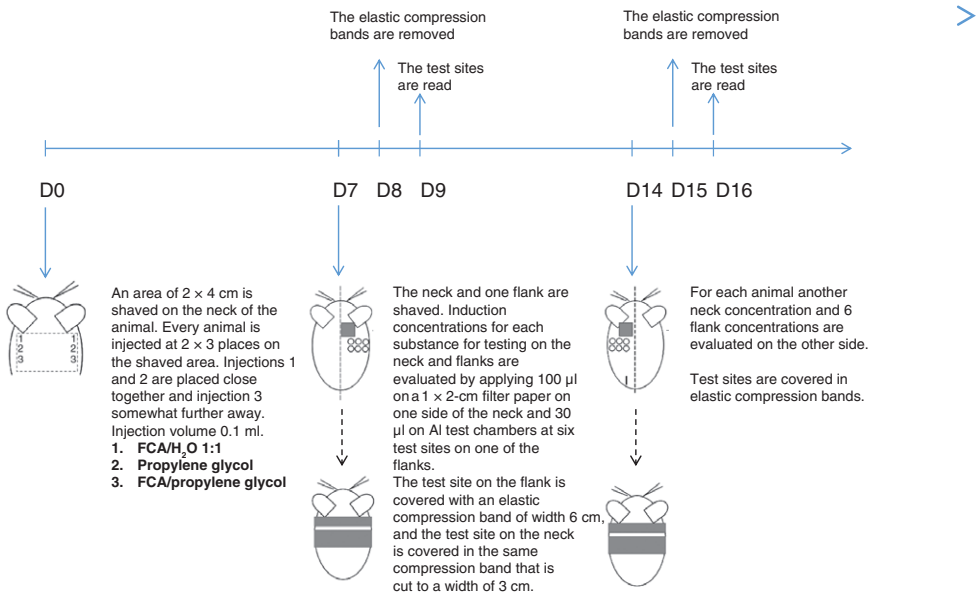


Fig. 1. Schematic figure of the evaluation of topical irritancy in order to find the optimal test concentrations to be used in the investigation of sensitization and cross-reactivity in the guinea-pig maximization test. For each evaluated test concentration, 4 animals were used. FCA, Freund's complete adjuvant.

Table 2. Concentrations for sensitization to, and challenge with, diphenylmethane-4,4′-diisocyanate (4,4′-MDI) and dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI), and their corresponding amines diphenylmethane-4,4′-diamine (4,4′-MDA) and dicyclohexylmethane-4,4′-diamine (4,4′-DMDA), as well as the positive control 2-methylol phenol (2-MP)

	Concentrations and vehicles (wt / vol)[a]					
	Sensitization			Challenge		
					Challenge II	
	Intradermal	SLS	Epidermal	Challenge I	Sensitization series A	Sensitization series B–F
4,4′-MDI	1.0% p.o. (40 mM)	10% DAE	1.0% ac (40 mM)	1.0% ac (40 mM)	0.6% ac (24 mM)	1.0% ac (40 mM)
4,4′-MDA	0.79% p.g. (40 mM)	10% DAE	0.79% EtOH (40 mM)	0.79% EtOH (40 mM)	0.48% EtOH (24 mM)	0.79% EtOH (40 mM)
4,4′-DMDI	1.0 p.o. (40 mM)	10% DAE	1.0% ac (40 mM)	0.63% ac[a] (24 mM)	0.63% ac (24 mM)	1.0% ac (40 mM)
4,4′-DMDA	0.84% p.g. (40 mM)	10% DAE	0.84% EtOH (40 mM)	0.84% EtOH (40 mM)	0.50% EtOH (24 mM)	0.84% EtOH (40 mM)
2-MP	5.0% p.g.	10% DAE	25.0% EtOH	15% EtOH	Not performed	

ac, acetone; DAE, dimethyl acetamide/acetone/99.5% ethanol 4:3:3 (vol/vol/vol); EtOH, ethanol; p.g., propylene glycol; p.o., liquid paraffin. Six different sensitization series, A–F, were performed. 4,4′-MDI was used as the induction substance in sensitization series A, E, and F. In sensitization series A, the test concentration in challenge II was lower because of uncertainties regarding the irritant capacity of 4,4′-DMDI.
[a]Non-equimolar concentrations of 4,4′-DMDI were used in sensitization series C because of a suspected risk of irritant reactions.

the nature of the sensitizing substance, on a 2 × 4-cm piece of filter paper placed on adhesive bandages. The patches were covered with impermeable plastic adhesive tape, and held in place with adhesive bandages. The patches were left in place for 48 h. The control animals were patch tested with the vehicle alone in the same manner as the test animals and the positive controls.

Challenge

The challenge procedure consisted of two parts: challenge I, in which the sensitization rate of the test substance used in the induction was assessed; and challenge II, in which cross-reactivity to other substances was assessed. Challenges I and II were performed at the same time but on different flanks of the animal; challenge I was performed on the left flank and challenge II on the right flank, according to the scheme shown in Fig. 2.

Day 21. All animals were shaved on their left flank, and the test animals and control animals were also shaved on their right flank. Challenge I (left flank, two patches) was performed by challenging 12 test animals with the induction

Table 3. Test reactions after sensitization to, and challenge with, diphenylmethane-4,4′-diisocyanate (4,4′-MDI) and dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI), and their corresponding amines diphenylmethane-4,4′-diamine (4,4′-MDA) and dicyclohexylmethane-4,4′-diamine (4,4′-DMDA), as well as the positive control 2-methylol phenol (2-MP)

		Number of positive animals				Evaluation of sensitizing capacity	
Sensitization substance	Sensitization series	C n = 12	T N = 24	V n = 12	2-MP n = 6	Fisher's exact test[a]	GHS and CLP regulation[b]
4,4′-MDI	A	1	18	0	4	Strong ($p < 0.001$)	1A
	E	3	2	0	6	$p = 0.19$	
	F	0	8	1	4	Weak ($p = 0.024$)	1B
4,4′-MDA	B	0	22	3	4[c]	Strong ($p < 0.001$)	1A
4,4′-DMDI	C	0	21	0	5	Strong ($p < 0.001$)	1A
4,4′-DMDA	D	1	18	3	5	Strong ($p < 0.001$)	1A
2-MP	A–F	–	–	–	28	NA[d]	1A

C, control animals; T, test animals receiving the suspected sensitizer; V, test animals receiving the vehicle; 2-MP, positive control animals tested with 2-MP; *n*, number of tested animals in each of the four groups.
[a]The proportion of positive animals in the test group was compared with the proportion of positive animals in the control group (see text).
[b]According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), substances are categorized as 1A if ≥ 60% of the test animals respond, and as 1B if ≥ 30% of the test animals respond when the intradermal induction dose is > 0.1% to ≤ 1%.
[c]Only 5 positive control animals were tested.
[d]NA, not applicable, because no control animals were tested with 2-MP.

substance in acetone or ethanol, depending on whether it was an isocyanate or an amine, on both the cranial and caudal patch. Six + 6 test animals were challenged with the induction substance on either the cranial or the caudal patch, and the vehicle (acetone or ethanol) alone on the other patch. Six of the control animals were tested with the induction substance on both patches, and 3 + 3 animals were patch tested with the induction substance on either the cranial or the caudal patch, and the vehicle alone on the other patch. Two of the positive control animals were tested with 2-MP on both the patches, and 2 + 2 animals were patch tested with 2-MP on either the cranial or the caudal patch, and the vehicle alone on the other patch. Al test® on a Durapore® adhesive band was used for patch testing. Thirty microlitres of test solution was applied. The patches were covered with impermeable plastic adhesive tape, and held in place with adhesive bandages.

Challenge II (right flank, six patches) was performed on 24 test animals and 12 control animals by patch testing with putatively cross-reacting substances. The distribution of the positions of the test substances was based on a Latin square table. In this article, the results of sensitization with 4,4′-MDI, 4,4′-MDA, 4,4′-DMDI and 4,4′-DMDA and their cross-reactivity patterns are described. Cross-reactivity to substances tested on the remaining two patches in challenge II are described elsewhere (Hamada et al., manuscript in preparation 2017).

Evaluation

Day 23. The minimum criterion for a positive reaction was confluent erythema covering the test area. All tests were evaluated blindly 24 h after the patch tests had been removed, that is, 48 h after test application. First, all of the left flanks of all the animals were read, and then, still blindly and without knowledge of the test outcome of the left side, the right flanks were read on the test animals and the control animals (Fig. 2).

Statistics

The proportion of positive animals in the test group was compared with the proportion of positive animals in the control group. Among the animals challenged with the induction substance on both the cranial and caudal patches (12 test animals and 6 negative control animals; Fig. 2), only one of the patches, chosen in advance, was included.

Statistical significance for the sensitizing capacity and cross-reactivity was calculated with a one-sided Fisher's exact test. When significant values ($p < 0.05$) were obtained, the compound was considered to be a sensitizer or to show cross-reactivity to other compounds on the basis of set criteria ($p < 0.001$, strong; $p < 0.01$, moderate; $p < 0.05$, weak).

Results

Six different sensitization series were performed on different occasions with the same method during the period

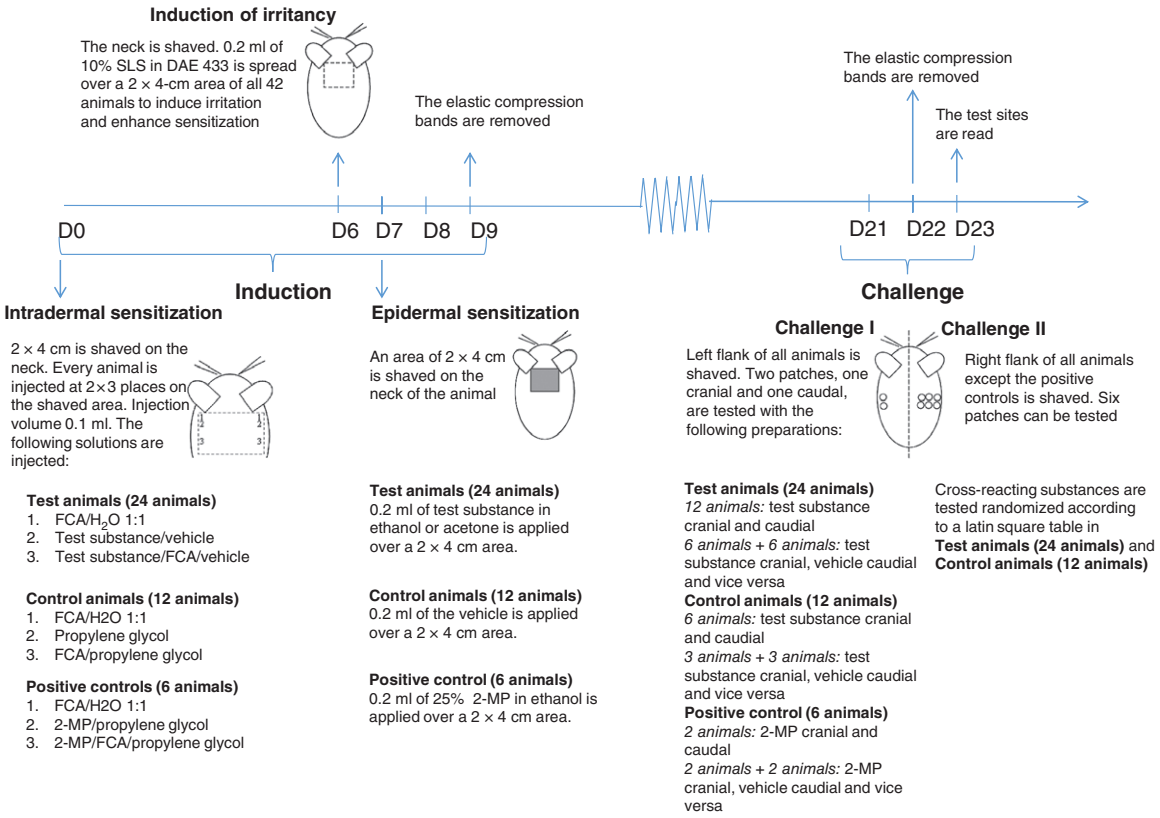


Fig. 2. Schematic figure of the performance of a sensitization series in the guinea-pig maximization test, in which one substance is evaluated in terms of its sensitizing capacity and cross-reactivity to another six investigated substances. DAE 433, *N*,*N*-dimethylacetamide; FCA, Freund's complete adjuvant; SLS, sodium lauryl sulfate; 2-MP, 2-methylol phenol.

May 2013 to September 2015. The results for each of these series are given in Tables 3 and 4. In Fig. 3, the cross-reactivity patterns for sensitization series B, C, D and F are compiled. In all sensitization series, at least 4 of the positive control animals showed positive reactions, indicating good performance of the method without negative influences resulting from, for example, sick animals or adjuvant with impaired effectiveness (Table 3).

Sensitizing capacity

4,4′-MDI was used as an induction substance on three different occasions. On the first and last occasions, it was found to be a sensitizer ($p < 0.001$ and $p = 0.024$, respectively), but on the second occasion the induction failed ($p = 0.19$) (Table 3). 4,4′-MDA, 4,4′-DMDI and 4,4′-DMDA were shown to be potent sensitizers (all $p < 0.001$).

Cross-reactivity

The pattern of cross-reactions between the investigated substances when tested in equimolar concentrations are shown in Fig. 3. Animals primarily sensitized to 4,4′-MDI showed cross-reactivity to the secondary sensitizers 4,4′-DMDI and 4,4′-MDA ($p = 0.016$ and $p < 0.001$, respectively), and animals primarily sensitized to 4,4′-MDA showed cross-reactivity to 4,4′-DMDA ($p = 0.008$).

Discussion

The GPMT is a well-recognized method used to detect contact sensitizers and their cross-reactivity patterns. The method was first developed by Magnusson and Kligman in 1969, and has been described in several articles (25, 26, 27, 33). It is also one of two guinea-pig tests described in OECD test guideline 406 (the other being the non-adjuvant Buehler test) that can be used in order to classify skin sensitizers according to the GHS (28). The GHS has been implemented in the EU by Regulation (EC) No. 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP regulation) (33), and thus results from GMPT studies can affect the classification of chemicals and mixtures within the EU.

Table 4. Test reactions after re-challenge with an equimolar concentration (40 mM) of diphenylmethane-4,4′-diisocyanate (4,4′-MDI) and dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI), and their corresponding amines diphenylmethane-4,4′-diamine (4,4′-MDA) and dicyclohexylmethane-4,4′-diamine (4,4′-DMDA)

		Number of positive animals after re-challenge with:			
Sensitization substance	Number of animals	4,4′-MDI	4,4′-MDA	4,4′-DMDI	4,4′-DMDA
4,4′-MDI[a]					
T	24	9	21	20	3
C	12	0	2	5	2
Cross-reactivity	–	–	Yes	Yes	No
4,4′-MDA					
T	24	3	No re-challenge	12	13
C	12	3		3	1
Cross-reactivity	–	No		No	Yes
4,4′-DMDI					
T	24	1	6	23	2
C	12	0	1	2	1
Cross-reactivity	–	No	No	–	No
4,4′-DMDA					
T	24	3	13	11	16
C	12	0	3	3	2
Cross-reactivity	–	No	No	No	–

C, control animals; T, test animals receiving the suspected sensitizer.
Test results were considered to show cross-reactivity when the p-value was < 0.001 according to a one-sided Fisher's exact test.
[a]Based on the results from sensitization series F.

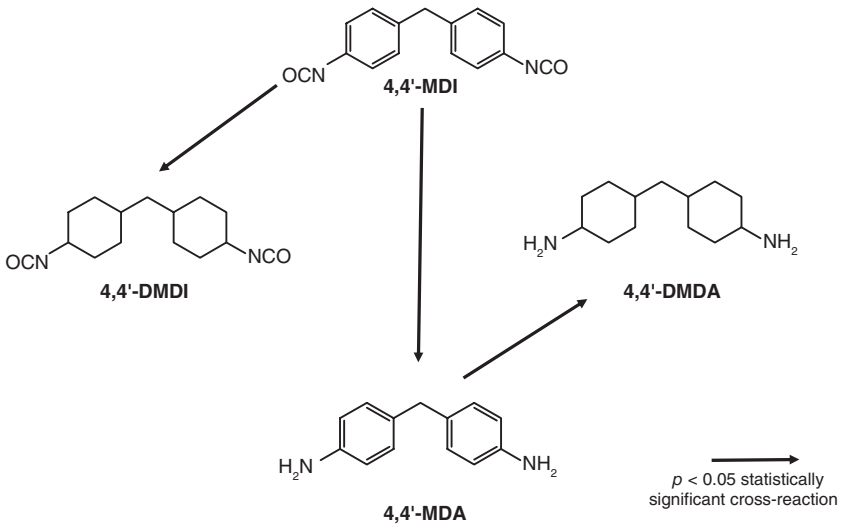


Fig. 3. Cross-reactivity pattern of the investigated substances in the guinea-pig maximization test. All of the depicted substances were used separately for induction and challenge. The arrows point from the induction substance towards the substance investigated for cross-reactivity. Arrows in bold denotes a significant number of reactions. 4,4′-DMDA, dicylohexylmethane-4,4′-diamine; 4,4′-DMDI, dicyclohexylmethane-4,4′-diisocyanate; 4,4′-MDA, diphenylmethane-4,4′-diamine; 4,4′-MDI, diphenylmethane-4,4′-diisocyanate.

This study was not performed for regulatory purposes, but rather for diagnostic and clinical reasons, and some changes from the original method as suggested by Bruze (29, 34) were made in order to standardize the test and make the evaluation of the patch test reactions objective. In Appendix S1, all changes from the original method and the rationale for making these are described.

Sensitizing capacity

In order to elicit allergic contact dermatitis, a chemical must have physicochemical characteristics suitable for penetration of the stratum corneum. Once in the viable epidermis, it must be able to form reaction products with proteins for the elicitation of an immune response. Thus, contact allergens are either protein-reactive in themselves or are metabolized in the skin into protein-reactive species (35). Isocyanates are theoretically potent contact allergens, because they possess electrophilic carbons that can be readily attacked by nucleophilic atoms present on macromolecules in the skin. However, it has been proposed that their reactivity is so high that they might polymerize before they penetrate the skin (36). Amines are

lipophilic and penetrate the skin quite readily. However, in order to react with proteins in the skin, the amines need to be metabolized.

In the literature, there are some animal studies investigating the sensitizing capacity of 4,4′-MDI. In 1976, Duprat et al. used the GPMT to study the sensitizing capacity of 4,4′-MDI, and concluded that the proportion of test animals that reacted to 4,4′-MDI 10% pet. showed it to be a strong allergen. In general, Duprat et al. used higher concentrations than in the present study, with intradermal injections of 5.0% 4,4′-MDI in olive oil and epicutanous sensitization with 25.0% 4,4′-MDI in pet. In the study presented here, there were apparent difficulties in sensitizing with 4,4′-MDI. It was used as an induction substance on three different occasions. On the first occasion, it was found to be a strong sensitizer, with 18 of 24 test animals reacting to 1% in acetone ($p < 0.001$). However, in this first sensitization series, there was a suspicion that 4,4′-DMDI might cause irritant reactions if patch tested equimolar to 1% 4,4′-MDI. Therefore, the concentrations of 4,4′-MDI in challenge I and challenge II were not the same. The concentration in challenge II was lower because we were able to patch test equimolar to a 'safe' concentration of 4,4′-DMDI. Expectedly, a lower proportion of test animals were positive in challenge II, in which they were patch tested with a lower concentration of 4,4′-MDI than in challenge I (18 of 24 positive animals in challenge I versus 7 of 24 animals in challenge II). In the second sensitization series, two concentrations of 4,4′-DMDI were investigated, and it was concluded that 1% did not cause irritant reactions. Hence, a new series was performed to induce with 4,4′-MDI and perform challenge II with equimolar concentrations to those in challenge I. On this occasion, the induction failed, and only 2 of 24 test animals were sensitized. As the positive controls reacted, there were no obvious reasons for the failure. 4,4′-MDI was used as an induction substance for a third time. On this occasion, 8 of 24 test animals ($p < 0.05$) reacted, making it as a weak allergen according to the set criteria.

In order to explain the different results, all steps in the study procedure were carefully revised. The only factor found that could have varied on the three occasions was, possibly, the concentration of 4,4′-MDI in the preparation when it was mixed with liquid paraffin and FCA to be used for the intradermal injections. Chemical analysis, presented elsewhere (Hamada et al., manuscript in preparation 2017), showed that 4,4′-MDI readily reacts with constituents in FCA, and that the injected concentration can vary according to the mixing procedure, the duration between preparation and injection, and the storage temperature.

4,4′-MDA, 4,4′-DMDI and 4,4′-DMDA were shown to be potent sensitizers. This is in accordance with clinical observations (18–20, 22). In fact, 4,4′-MDA is known to sensitize patients when tested at 0.5% pet. (37, 38).

Notably, all of the investigated substances fulfil the criteria for classification as subcategory 1A skin sensitizers according to the GHS and the CLP regulation, as ≥ 60% of the test animals responded at an intradermal induction dose of > 0.1% to ≤ 1%. Admittedly, 4,4′-MDI failed to induce sensitization in sensitization series E, and would only have been classified as a subcategory 1B skin sensitizer on the basis of the results from series F, as only 33% of the test animals responded at an intradermal induction dose of 0.1% to ≤1%. However, as suggested by Basketter et al. the higher-potency category should apply when multiple animal datasets lead to different categorization of the same substance (39).

Cross-reactivity

The results obtained in this study correspond to the clinical observations made in other studies, namely that 4,4′-MDA is a marker for 4,4′-MDI allergy, as animals primarily sensitized to 4,4′-MDI also react to the amine. However, in the clinical situation it is doubtful whether 4,4′-MDA is a good screening substance for 4,4′-MDI. In 2012, Engfeldt et al. published the results of consecutive patch testing in Belgium and Sweden with 4,4′-MDA and 4,4′-MDI (37). They concluded that positive reactions to 4,4′-MDA seem to be associated with contact allergy to *p*-phenylenediamine (PPD). As PPD is one of the most common contact allergens in the baseline series, this possible cross-reactivity might make 4,4′-MDA too blunt a tool to single out contact allergy to 4,4′-MDI; a positive reaction might say more about the patients' hair dyeing habits than his or her exposure to isocyanates. In order to give advice on the use of 4,4′-MDA as a marker for 4,4′-MDI in a patch test series, further exploration of the relationship between 4,4′-MDA and PPD is needed.

However, for an individual who is primarily sensitized to 4,4′-MDI, the fact that cross-reactivity to 4,4′-MDA can occur can be of clinical relevance. 4,4′-MDA is used as a hardener in PUR production, so a contact allergy to 4,4′-MDI in a worker at a PUR plant might lead to multiple exposure sources if 4,4′-MDA is used as a curing agent. Furthermore, 4,4′-MDA is also used as hardener in other plastic applications, such as epoxy, and possible exposure to 4,4′-MDA needs to be taken into consideration before a worker is reassigned other tasks because of a confirmed contact allergy to 4,4′-MDI.

4,4′-MDA is also a known rubber additive, and it is possible that an individual who has acquired contact allergy to 4,4′-MDI at work might react to rubber items later in life.

In the present study, animals primarily sensitized to 4,4′-MDI showed cross-reactivity to the secondary allergen 4,4′-DMDI. However, when 4,4′-DMDI was the primary sensitizer, no cross-reactivity to 4,4′-MDI was shown. There are, to our knowledge, no reports in the literature describing concurrent reactions between the two isocyanates. Instead, concurrent reactions between 4,4′-MDA and 4,4′-DMDI have been described (18–20). Possibly, the lack of concurrent reactions between the two isocyanates stems from the fact that commercially available patch test preparations of 4,4′-MDI have a high risk of false-negative reactions (5). The reason for the suggested cross-reactivity might seem evident when the two-dimensional structures of the isocyanates are considered. However, the spatial orientation of cyclohexane is quite different from that of the aromatic ring. Finally, it was shown that animals sensitized to 4,4′-MDA also showed cross-reactivity to 4,4′-DMDA. Concurrent reactions between 4,4′-MDA, 4,4′-DMDA and 4,4′-DMDI have been described in 2 patients working at a medical company where a lacquer based on 4,4′-DMDI was used (20). As with the isocyanates, the spatial orientation of the cyclohexane ring versus the benzene ring differs between the two amines.

Conclusions

All investigated substances were shown to be sensitizers. Regarding the evaluation of cross-reactivity, the previously noted clinical observation that 4,4′-MDA is a marker for 4,4′-MDI was verified, as animals sensitized to the isocyanate also reacted to the amine. Furthermore, animals sensitized to 4,4′-MDI cross-reacted to 4,4′-DMDI, and animals sensitized to 4,4′-MDA cross-reacted to 4,4′-DMDA.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Background to the modifications of the original method of GPMT.

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