Allergenicity Evaluation of *N*-(1-Methylheptyl)-*N*′-Phenyl-*p*-Phenylenediamine and 2-(Thiocyanomethylthio) Benzothiazole by the Guinea Pig Maximization Test

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In order to regulate contact sensitizers in commercial products, not only is identification of chemical allergens essential but also quantitative evaluation of their allergenicity. We have so far reported threshold values of chemical allergens for both induction and challenge phases in the modified guinea pig maximization test (GPMT). In this study, we examined *N*-(1-methylheptyl)-*N*′-phenyl-*p*-phenylenediamine (MHPPD), an antioxidant for rubber products, and 2-(thiocyanomethylthio) benzothiazole (TCMTB), a biocide for plastic and wood products. MHPPD and TCMTB exhibited distinct profiles of contact allergenicity with the GPMT; that is, both could sensitize guinea pigs by the second induction procedure of topical application alone, even without the first induction procedure of intradermal injection. The calculated challenge threshold values for MHPPD and TCMTB were 0.9 and 0.8 ppm, respectively, which were at least one tenth lower than those of chemical allergens previously examined by us. Octanol-water partition coefficients (log *p*) used as an index of skin permeability were not necessarily higher for MHPPD and TCMTB than those of other allergens, thus, the skin permeability of these compounds did not seem to be related to their ability to cause sensitization by topical application alone or their low challenge threshold values. The results suggest that the sensitizing potential of both compounds is due to their molecular basis. Cross-reactivities among homologues of both allergens were examined to evaluate their antigenic determinants.

Key words —— contact dermatitis, guinea pig maximization test, antioxidant, biocide, cross-reactivity

INTRODUCTION

N-(1-Methylheptyl)-*N*′-phenyl-*p*-phenylenediamine (MHPPD), an antioxidant for rubber products, and 2-(thiocyanomethylthio) benzothiazole (TCMTB), a biocide for plastic and wood products, are commercially available, respectively. No data on the allergenicity of MHPPD and TCMTB in human or experimental animals have been reported so far. Three *p*-phenylenediamine (PPD)-type antioxidants, *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD), *N*-isopropyl-*N*′-phenyl-*p*-phenylenediamine (IPPD) and *N*-cyclohexyl-*N*′-phenyl-*p*-phenylenediamine (CPPD), have long been used in the rubber industry, and all three are human sensitizers. In particular, IPPD has been reported often as the cause of contact dermatitis in various countries.[1–4] Another PPD-type antioxidant, *N*-1,3-dimethylbutyl-*N*′-phenyl-*p*-phenylenediamine (DMBPPD) was identified to be a causative compound in contact dermatitis from rubber boots.[5] Among MHPPD, DPPD, IPPD, CPPD and DMBPPD, there is a difference in *N*-alkyl substituents. Thus, it is important to collect information on MHPPD in terms of its relative potential allergenicity and cross-reactivity with homologue PPD-type antioxidants. On the other hand, TCMTB may produce 2-mercaptobenzothiazole (MBT), a rubber vulcanizing agent and a typical rubber allergen,[6,7] as its major metabolite.[8]

In the process of risk assessment of a chemical, hazard identification should be followed by dose–response evaluation. In this study, we evaluated the dose–response profiles of MHPPD- and TCMTB-allergenicity by varying both induction and challenge doses in the GPMT. Furthermore, cross-reactivities among their homologues were examined with animals sensitized to MHPPD and TCMTB to evaluate their antigenic determinants.

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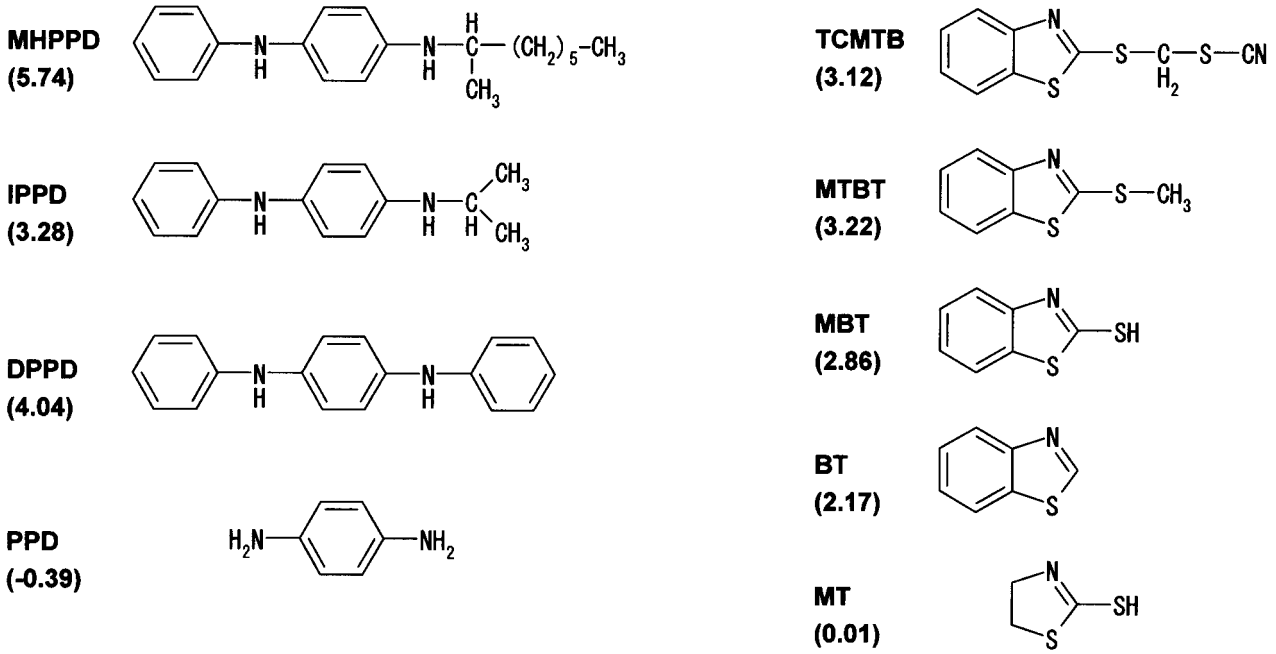


Fig. 1. Chemical Structures of MHPPD, TCMTB and Their Analogous Compounds
Values in the parentheses are the octanol-water coefficients (log *p*) of the chemicals.

MATERIALS AND METHODS

Chemicals —— MHPPD was obtained from Seiko Kagaku Co., Ltd., Tokyo, Japan; PPD, 2-(methylthio) benzothiazole (MTBT), MBT, benzothiazole (BT), and 2-mercaptothiazoline (MT) were from Wako Pure Chemical Industries, Osaka, Japan; DPPD and IPPD were from Tokyo Kasei Co., Ltd., Tokyo, Japan; and TCMTB was from Buckman Laboratories, Tennessee, U.S.A. The purity of TCMTB was 60%, containing 40% diethylene glycol monomethyl ether, according to the manufacturer. All other chemicals were of analytical grade. The octanol-water partition coefficients (log *p*) of the chemicals were calculated using SRC's LOGKOW/KOWWIN program. The structures and log *p* values of the chemicals used in the GPMT are shown in Fig. 1.

Guinea Pig Maximization Test —— Five- to six-week-old female Hartley guinea pigs from SLC (Shizuoka, Japan) were used. Essentially, the procedure described by Nakamura *et al.*[9] was followed. Four to 10 animals were used for each sensitization group.

For the first (intradermal) induction with MHPPT or TCMTB, olive oil and Freund's complete adjuvant (FCA) emulsified with an equal volume of distilled water were used as vehicles. Two injections (0.05 ml each) of test chemicals were administered using both vehicles, in addition to two injections of emulsified FCA without test chemicals. The shoulder region was the induction site. Seven days after the first induction, the second induction procedure involved the test compounds in 200 mg white petrolatum were applied occlusively for 48 hr over the injection site. Two weeks after the second induction, 0.1 ml aliquots of various concentrations of test compound in acetone were applied all at once to the shaved area of the flank for challenge. Three to five concentrations were set in a logarithmic scale. Forty-eight hrs after the challenge, each site was scored for erythema (0 to 4) and edema (0 to 3) according to the criteria of Sato *et al.*[10] Total scores (erythema plus edema) with the same challenge concentration in a group were summed and divided by the number of animals in the group to give the mean response (MR) value, an index for skin reaction to a given concentration of test compound as the challenge. The percentage of animals showing a positive reaction for each challenge concentration in the group was used as the sensitization rate (SR). The relative challenge potency index values of the allergens were calculated as reported in our previous paper.[11]

To evaluate cross reactivity among corresponding homologues of MHPPD and TCMTB, MHPPD- or TCMTB-sensitized animals were prepared. Fifty ppm MHPPD-sensitized animals were selected and challenged with 5000 ppm PPD, DPPD and IPPD

in 0.1 ml acetone all at once, one week after the challenge with MHPPD. Each challenge site was scored as described above. In the same manner, 100 ppm TCMTB-sensitized animals were challenged and evaluated with 10000 ppm MTBT, MBT, BT and MT.

Five vehicle control animals and five animals sensitized with 50 ppm MHPPD were newly prepared to evaluate cross-reactivity by means of intradermal injection. Two weeks after the second induction, these animals were intradermally injected with olive oil, 500 ppm and 5000 ppm MHPPD, PPD, DPPD and IPPD in olive oil all at once to the shaved area of the flank for the challenge. The injection volume was 0.02 ml each. Forty-eight hrs after the challenge, the diameter of the redness at each site was measured as an index of skin reaction. Likewise, control and 100 ppm TCMTB-sensitized animals were prepared and intradermally injected with olive oil, 100 ppm and 1000 ppm TCMTB, 1000 ppm and 10000 ppm MTBT, MBT, BT, and MT in olive oil and evaluated.

RESULTS

Contributions of the Topical Induction Step of MHPPD and TCMTB in the GPMT

The intradermal induction concentrations of 5000 ppm MHPPD and 1000 ppm TCMTB, the topical induction concentrations of 100% MHPPD and 5% TCMTB, and the challenge concentration of 5000 ppm MHPPD and 100 ppm TCMTB were selected as maximum tolerable doses for each step of the GPMT, as recommended in the original method by Magnusson and Kligman.[12] MHPPD and TCMTB sensitized all ten guinea pigs with maximum induction and challenge doses. Notably, these chemicals could sensitize animals by the topical induction procedure alone, although the mean skin reaction scores of these groups were lower than those of the maximally sensitized groups (Table 1).

Dose–Response Profiles of MHPPD and TCMTB for Both the Induction and Challenge Phases in the GPMT

The skin sensitization test results of MHPPD and TCMTB are shown in Table 2. Skin reaction scores (MR and SR) with maximum challenge concentrations for MHPPD (5000 ppm) and TCMTB (100 ppm) were used to evaluate the dose–response profiles for the induction phases. When MR and SR were plotted against log induction concentrations of MHPPD or TCMTB, skin reaction saturation was observed with both compounds at higher induction concentrations (Fig. 2). Optimal induction concentrations for MHPPD and TCMTB were 50 ppm and 100 ppm, respectively. These maximally sensitized groups of animals were used in the subsequent evaluation of the dose–response profiles for the challenge phases for MHPPD and TCMTB. MR scores were plotted against log challenge concentrations, and linear regression lines were well fitted both to MHPPD and TCMTB throughout the challenge dose ranges employed (Fig. 3). The calculated threshold values for challenge, x-intercepts of regression lines, were 0.9 ppm and 0.8 ppm for MHPPD and TCMTB, respectively. The calculated relative challenge potency index values; the area of a triangle enclosed by the x-axis, a linear regression line and a vertical line at 1% of challenge concentration for each allergen in Fig. 3, for MHPPD and TCMTB, were 13.7 and 21.9, respectively.

Table 1. Results of GPMT for MHPPD and TCMTB (Preliminary Study)

Chemical	Induction		Challenge[a)]	MR (SR)	n
	1st (ppm)	2nd (%)	(ppm)		
MHPPD	0[b)]	0[c)]	5000	0.0	5
	0[b)]	5	5000	1.8 (60)	10
	0[b)]	100	5000	2.5 (75)	4
	50000	100	5000	2.9 (100)	10
TCMTB	0[b)]	0[c)]	100	0.0	5
	0[b)]	5	100	1.2 (100)	10
	0.1	5	100	2.4 (100)	10
	1000	5	100	3.6 (100)	10

a) Animals were challenged 2 weeks after topical induction, and skin reactions were evaluated 48 hr thereafter. *b*) Animals were treated with vehicles (olive oil and emulsified complete adjuvant). *c*) Animals were treated with vehicle (white petrolatum). MR: mean response. SR: sensitization rate (%).

Cross-Reactivities among Homologues of MHPPD and TCMTB Applied Either Topically or Intradermally in MHPPD- or TCMTB-Sensitized Animals

The results of the topical challenge of test compounds are shown in Table 3. MHPPD-sensitized animals exhibited cross-reactivity to IPPD and DPPD but not to PPD, although the degrees of skin reactions by IPPD or DPPD were far weaker than by MHPPD itself. Likewise, TCMTB-sensitized animals exhibited cross-reactivity to MTBT and

Table 2. Results of GPMT for MHPPD and TCMTB

Challenge dose	MR (SR)[a] Induction dose[b]					
MHPPD	0 ppm[c]	0.5 ppm	5 ppm	50 ppm	500 ppm	5000 ppm
0 ppm	0.0	0.0	0.0	0.0	0.0	0.0
0.5 ppm	0.0	0.0	0.0	0.0	0.0	0.0
5 ppm	0.0	0.0	0.0	0.0	0.0	0.0
50 ppm	0.0	0.0	0.1 (10)	2.8 (100)	2.4 (100)	0.6 (100)
500 ppm	0.0	0.0	0.7 (40)	4.8 (100)	4.5 (100)	4.0 (100)
5000 ppm	0.0	0.0	1.1 (40)	6.1 (100)	6.1 (100)	5.5 (100)
TCMTB	0 ppm[c]	1 ppm	10 ppm	100 ppm	1000 ppm	
0 ppm	0.0	0.0	0.0	0.0	0.0	
0.1 ppm	0.0	0.0	0.0	0.0	0.0	
1 ppm	0.0	0.0	0.0	0.3 (30)	0.9 (60)	
10 ppm	0.0	0.0	0.2 (20)	2.8 (100)	3.2 (90)	
100 ppm	0.0	0.0	2.1 (60)	5.5 (100)	5.3 (100)	

a) Skin reactions were evaluated 48 hr after challenge. *b*) A group of animlas were sensitized with the same dose of MHPPD or TCMTB for both intradermal and topical induction procedures. *c*) Animals were treated with vehicles for both intradermal and topical induction procedures. MR: mean response. SR: sensitization rate (%).

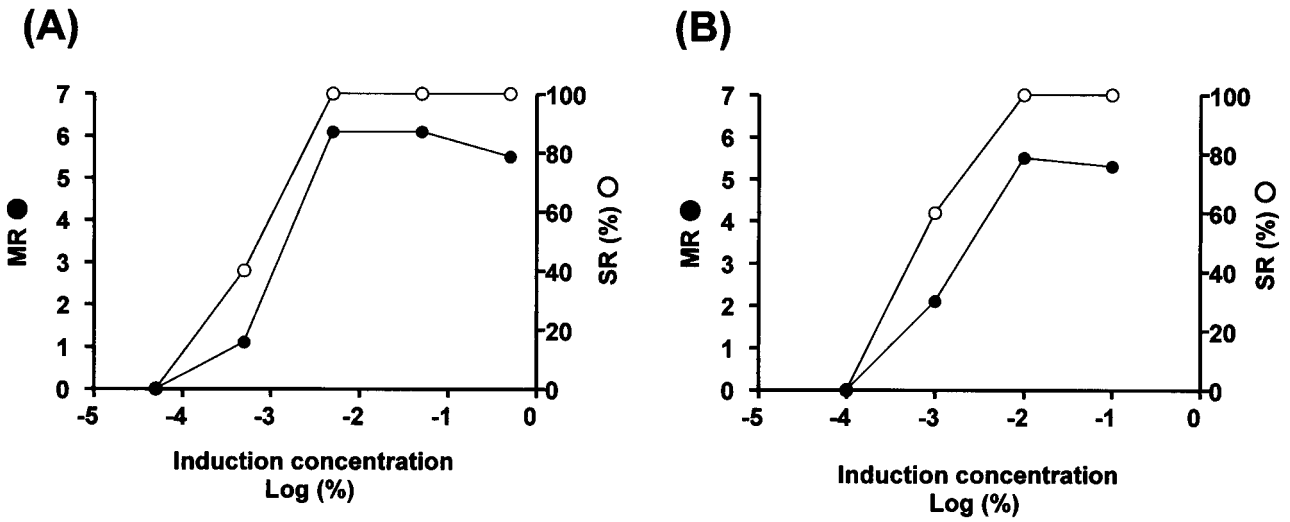


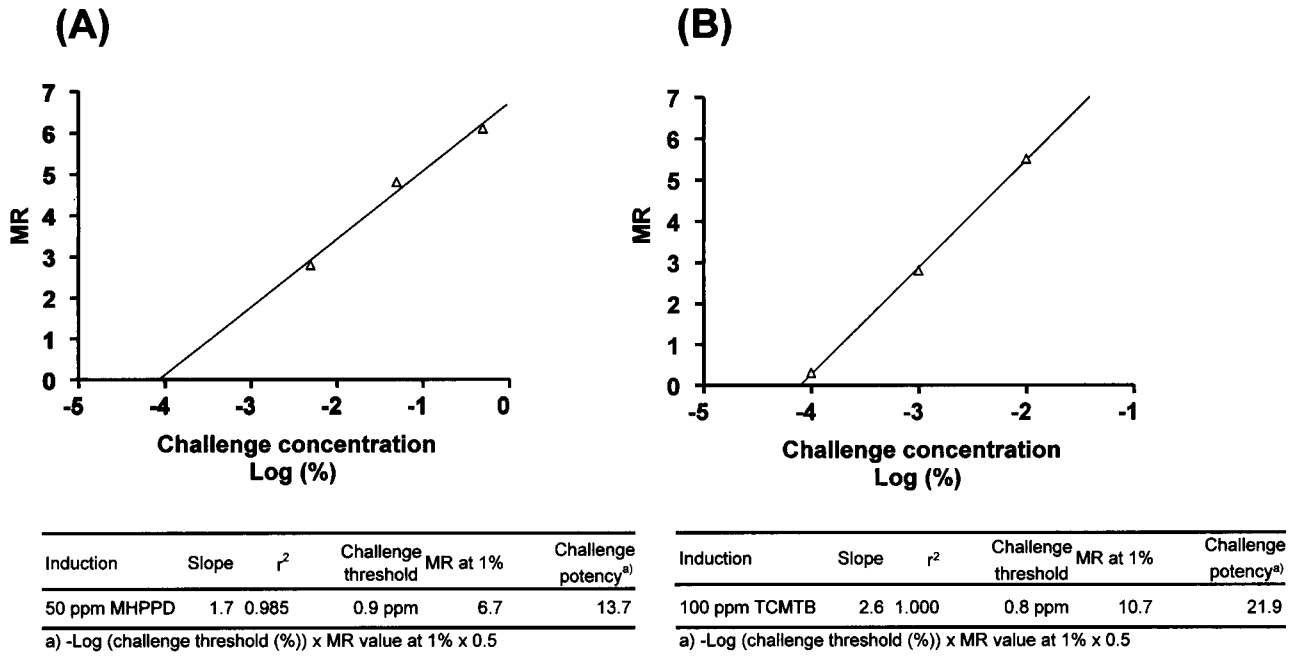
Fig. 2. Relationship between Induction Concentration and Maximum Skin Reaction Score for MHPPD and TCMTB

Dose–related skin reaction for the induction concentration was evaluated with MHPPD (A) and TCMTB (B). Each symbol represents the mean response score (closed circle) and sensitization rate (open circle) of the group 48 hr after challenge with a maximum concentration of each compound (5000 ppm for MHPPD, 100 ppm for TCMTB).

MBT. In this case, too, the skin reaction by MTBT and MBT was at least one-hundredth weaker than by TCMTB itself. As shown in Fig. 4, when MHPPD- or TCMTB-sensitized animals were intradermally challenged, a dose–related increase in the diameter of redness at the injection site was observed by MHPPD or TCMTB, and not by any other homologues.

DISCUSSION

In previous studies, we have evaluated the dose–response profiles of allergens for the induction phase by varying the intradermal induction concentration under the fixed maximum topical induction concentration, since the topical induction procedure alone had failed to sensitize animals, even at the maxi-



Induction	Slope	r^2	Challenge threshold	MR at 1%	Challenge potency[a)]
50 ppm MHPPD	1.7	0.985	0.9 ppm	6.7	13.7

a) -Log (challenge threshold (%)) x MR value at 1% x 0.5

Induction	Slope	r^2	Challenge threshold	MR at 1%	Challenge potency[a)]
100 ppm TCMTB	2.6	1.000	0.8 ppm	10.7	21.9

a) -Log (challenge threshold (%)) x MR value at 1% x 0.5

Fig. 3. Relationship between the Challenge Concentration and Skin Reaction Score for MHPPD and TCMTB

The dose–related skin reaction to the challenge concentration was evaluated with MHPPD (A) and TCMTB (B). In order to evaluate the challenge profile, the maximally sensitized group of animals with each allergen (50 ppm for MHPPD, 100 ppm for TCMTB) was used. Each symbol represents the mean response score of the group 48 hr after challenge with each compound. Variables of linear regression lines for each allergen are summarized in the tables.

Table 3. Cross-Reactivity of MHPPD- or TCMTB-Sensitized Animals to Related Compounds after Topical Application

Challenge	MR	SR
MHPPD-sensitized animals		
5000 ppm MHPPD[a)]	6.1	100
5000 ppm IPPD[b)]	1.7	70
5000 ppm DPPD[b)]	0.9	70
5000 ppm PPD[b)]	0.0	0
TCMTB-sensitized animals		
100 ppm TCMTB[a)]	5.5	100
10000 ppm MTBT[b)]	0.8	60
10000 ppm MBT[b)]	0.9	60
10000 ppm BT[b)]	0.0	0
10000 ppm MT[b)]	0.0	0

Animals were sensitized with 50 ppm MHPPD or 100 ppm TCMTB for both intradermal and topical induction procedures. *a*) Animals were challenged 2 weeks after the 2nd induction, and skin reactions were evaluated 48 hr thereafter. *b*) Animals were rechallenged 1 week after the challenge with MHPPD or TCMTB, and skin reactions were evaluated 48 hr thereafter. MR: mean response. SR: sensitization rate (%).

mum tolerable dose. The peculiar characteristic displayed by MHPPD and TCMTB as contact sensitizers is that they can sensitize guinea pigs by the topical induction procedure alone in the GPMT. Accordingly, the induction profiles for MHPPD and TCMTB were evaluated by varying the topical induction concentrations together with those of the intradermal induction.

In the Buehler test, with a repeated topical induction procedure, allergens with high log p values can sensitize guinea pigs with relatively low induction concentrations,[13] because of their high skin penetration abilities. The log p values ranged from 0.64 to 5.73 for seven contact allergens which had failed to sensitize animals with the topical induction procedure alone in our previous study.[11] In this context, log p values of MHPPD (5.74) and TCMTB (3.12) were within the range. Thus, the skin penetration rates do not seem to be related to their sensitizing abilities by the topical induction procedure.

Besides skin penetration ability, the reactivity of a chemical would play an important role in sensitization, since chemical allergens are haptens and it is a prerequisite for them to be associated with macromolecules to obtain immunogenicity. The threshold values of the challenge phase for MHPPD and TCMTB were 0.9 and 0.8 ppm, respectively, which are at least one-tenth lower than those for the seven chemical allergens mentioned above, ranging from 15 to 3500 ppm.[11] Recently, we proposed an index to compare the relative challenge potencies of chemi-

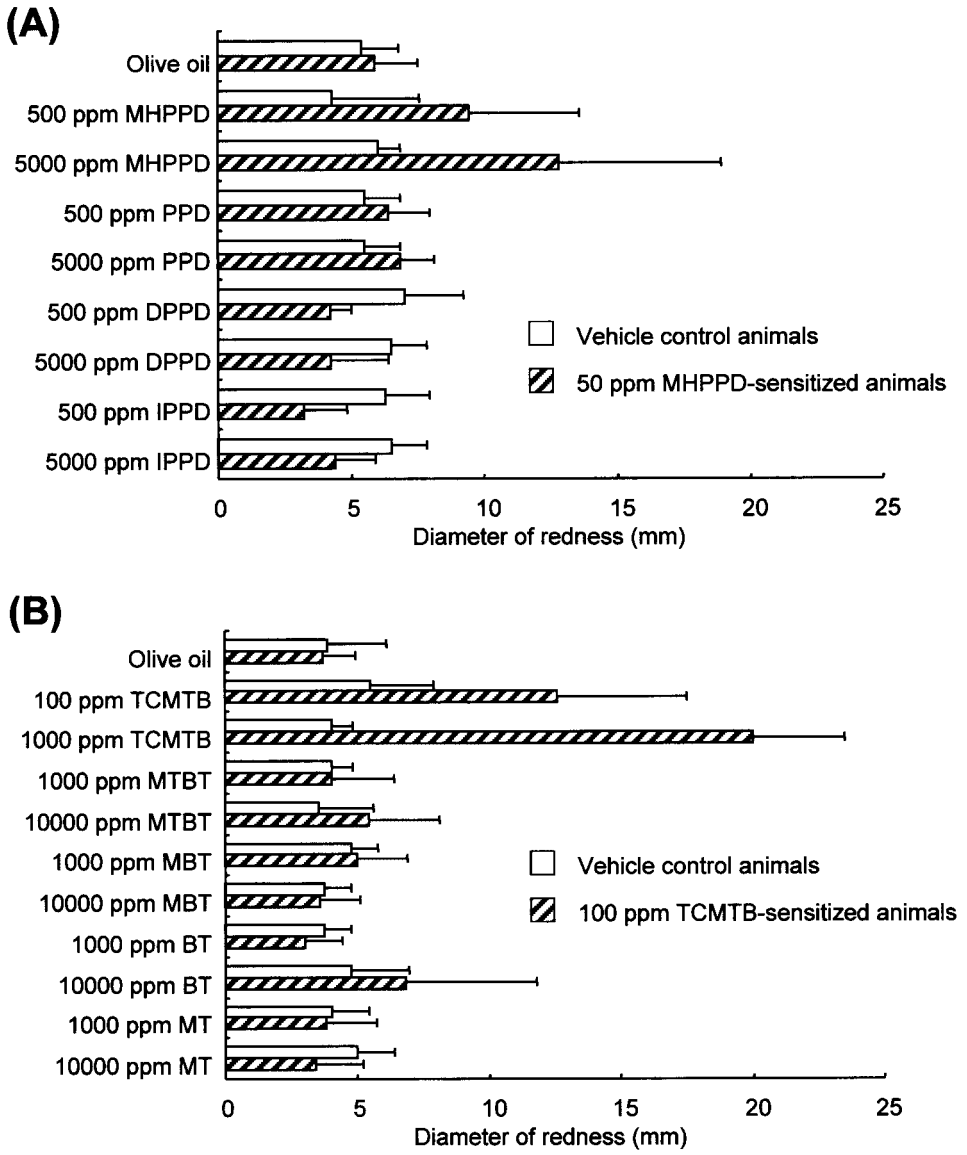


Fig. 4. Cross-Reactivity of MHPPD- or TCMTB-Sensitized Animals to Their Homologues after Intradermal Injection

Animals were sensitized with 50 ppm MHPPD (A) or 100 ppm TCMTB (B) for both the 1st (intradermal) and 2nd (topical) induction procedures. Control animals were treated with vehicles only. Two weeks after the topical induction, animals were intradermally injected with tested compounds in 0.02 ml olive oil at one time. Each value represents the mean ± S.D. of the diameter of redness at each site 48 hr after injection.

cal allergens in the GPMT.[11] The proposed index value reflects the integrated degree of skin reaction that would occur when a maximally sensitized animal is exposed to the allergen. This value includes both the threshold and the dose–response profile, a slope of regression line, in its mathematical expression. The calculated relative challenge potency index values for MHPPD (13.7) and TCMTB (21.9) are higher than those for the seven allergens.[11] Taken together, these results suggest that with regard to MHPPD and TCMTB, sensitizing and challenging potentials per molecule are higher than previously evaluated allergens.

Cross-reactivity between the chemicals indicates that those chemicals have a common antigenic determinant in their structures. IPPD and DPPD exhibited cross-reactivity in animals sensitized to MHPPD after topical application, suggesting that a minimum antigenic structure for MHPPD is that of *N*-phenyl-*p*-phenylenediamine. Nakamura *et al.*[9] investigated dose–response profiles of the allergenicity of IPPD for both the induction and challenge phases in the GPMT. In that case, even in the maximally sensitized group (1000 ppm and 12.5% for intradermal and topical induction, respectively), a challenge with 100 ppm IPPD resulted in a nega-

tive skin reaction, and the MR score at 1000 ppm IPPD was 2.6. In our study with the maximally sensitized group (50 ppm for both 1st and 2nd induction), the challenge with 50 ppm MHPPD resulted in a positive skin reaction, and the calculated MR score at 1000 ppm from the linear regression line was 5.1. Accordingly, MHPPD seems a more potent sensitizer than IPPD in the GPMT. Momma *et al*.[14] reported that the allergenicity of DMBPPD, another *N*-phenyl-*p*-phenylenediamine antioxidant, in guinea pigs is more potent than IPPD. The numbers of carbon atoms in the alkyl side chains in IPPD, DMBPPD and MHPPD are three, six and eight, respectively. It is likely that *N*-phenyl-*p*-phenylenediamine antioxidants with long alkyl side chains have potent sensitizing ability.

As for TCMTB, its minimum antigenic determinant seems to be the structure of 2-thiobenzothiazole. Although MTBT and MBT exhibited cross-reactivity to TCMTB-sensitized animals after topical application, the degree of skin reactions caused by these compounds was far weaker compared to that by TCMTB, despite the use of a 100-fold concentration in the topical application. Although MBT is a major metabolite of TCMTB in rats,[8] the animals seemed to be sensitized by TCMTB itself and not by the metabolites.

One possible explanation for the weak or non-cross-reactivity of the test chemicals may be the difference in their ability to penetrate the skin across the stratum corneum compared to the corresponding parent compound, which is inferred from the wide range of log *p* values of the test chemicals. To avoid the influence of the difference in the skin penetration step, we evaluated cross-reactivity by means of intradermal injections. The cross-reactivity of IPPD and DPPD to MHPPD-sensitized animals, which was observed after topical application, was not detected after intradermal injection. A similar discrepancy in the results between topical and intradermal applications in the cross-reactivity study was found with TCMTB-sensitized animals. It seems that intradermal injection as a challenge procedure would be suitable for detecting allergens with a low skin penetration rate but not for those with weak reactivity, because the slight reaction would be masked by the basal skin reaction caused by the vehicle treatment.

In conclusion, MHPPD and TCMTB are to be said risky compounds as allergens, from the respect that they are able to sensitize animals with the topical induction procedure alone and have low challenge thresholds and high challenge potency index values in the GPMT. It goes without saying that compounds such as these should be treated carefully.

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