Table 1
Performance of in silico systems

	Ames result	+	−	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	−	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	−	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	−	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	−	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	−	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	−	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

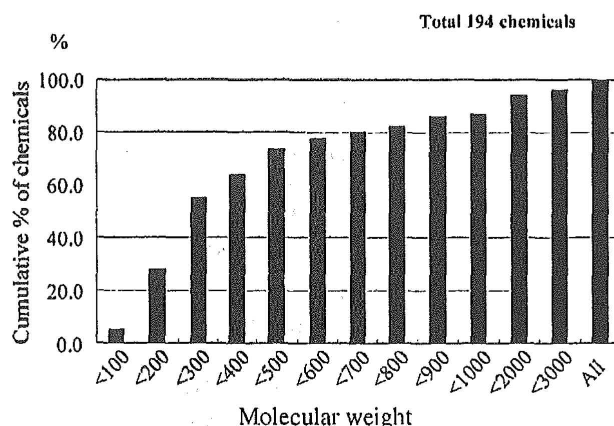

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

Ames \ In silico	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	279	40	319				
-	42	249	291	**87.8**	**85.6**	**86.7**	**86.8**
Total	321	289	610				
	+++	---					
+	166	1	167				
-	3	127	130	**99.4**	**97.7**	**98.7**	**42.2**
Total	168	129	297				

ECJ database

Ames \ In silico	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	19	7	26				
-	23	147	170	**73.1**	**86.5**	**84.7**	**95.1**
Total	42	154	196				
	+++	---					
+	13	2	15				
-	5	94	99	**86.7**	**94.9**	**93.9**	**55.3**
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Referen
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative a
Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Sourc
Bupropion	34911-55-2	+	−	−	−	1
Citalopram	59729-33-8	+	−	−	−	1
Naloxone	465-65-6	+	−	−	−	1
Oxcarbazepime	28721-07-5	+	−	−	−	1
Quetiapine	111976-69-7	+	−	−	−	1
Rabeprazole	117976-89-3	+	−	−	−	1
Zolmitriptan	139264-17-8	+	−	−	−	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	−	−	−	2
2-Chloropyridine	109-09-1	+	−	−	−	2
Pyrogallol	87-66-1	+	−	−	−	2
o-Phenylphenol	90-43-7	+	−	−	−	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	−	−	−	3
2-Vinylpyridine	100-69-6	+	−	−	−	3
Fosfomycin	23155-02-4	−	+	+	+	1
Toremifene	89778-26-7	−	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	−	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	−	+	+	+	3
Methidathion	950-37-8	−	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	−	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive An results but were negative in all three in silico syste (DEREK, MCase, TOPKAT/AWorks), and those tl were negative in the Ames test but gave positive evalı tions in all three systems, are summarized in Table 4. T table, which includes data from Synder et al. [11] ε White et al. [13] shows there are 19 exceptional cheı cals from both drug and non-drug families. Althougl would be unrealistic to expect zero exceptions using t approach, further improvement of the prediction syste is needed. We do not have good reasons to explain discordance, therefore we will verify the results fr both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decis tree (Fig. 2), in order to evaluate chemical inductior gene mutation. We may use the decision tree to pri(tize chemicals to be assayed by in vitro and/or in v tests. A final goal being that eventually, chemical mı genicity will be evaluated by in silico systems alone regulatory use. The decision tree consists of three stε namely to assess the molecular weight, the existence epoxy groups, and the in silico evaluation for genot icity. Based on the purpose of the in silico evaluati the tree might be altered by the different final call of in silico evaluation, i.e., regarding as positive (negat all three systems show positive (negative). The ch(of definition for final call applying to the decision should be based on the balance between accuracy of e

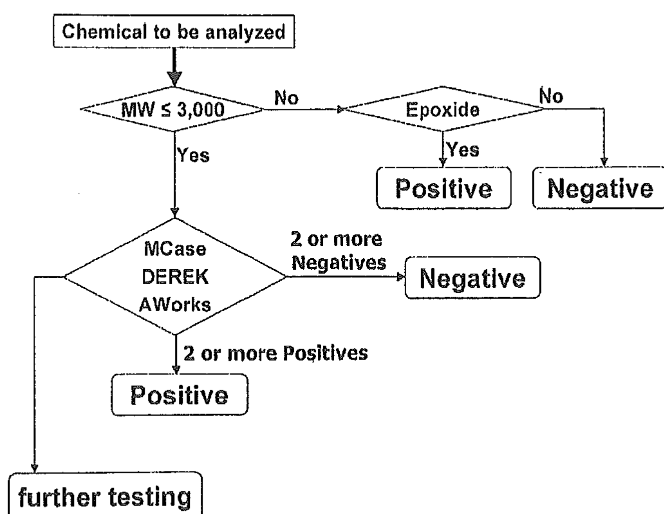

Fig. 2. Decision tree. In in silico evaluation, when two or more give positive then the final call is "positive" and two or more negative then call "negative".

uation and applicability, which are especially important for regulatory purpose. The decision should be made on a case-by-case basis depending upon the purpose of the decisions to be made.

Acknowledgements

The authors thank Dr. D. Kirkland, Covance, for his critical review and kind English edition and Mr. T. Ehara, MHLW, for his invaluable discussion. Authors also want to thank Ms. H. Akiyama (CTC laboratories, Japan), Akamatsu and Naitoh (Charles River Japan, Japan), and Kitajima, Suiroi, and Yuta (Fujitsu Kitakyushu, Japan) for their technical assistance. This work supported by the Health and Labour Sciences Research Grants (H15-Chemistry-003).

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Allergenicity evaluation of *p*-chloro-*m*-cresol and *p*-chloro-*m*-xylenol by non-radioactive murine local lymph-node assay and multiple-dose guinea pig maximization test

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Received 25 January 2003; accepted 3 April 2003

Abstract

p-Chloro-*m*-cresol (PCMC) and *p*-chloro-*m*-xylenol (PCMX) are known to cause allergic contact dermatitis. For risk assessment of skin sensitizers, information on dose–response profiles in the induction and elicitation phases and cross-reactivity with analogous chemicals are important. In the non-radioactive local lymph-node assay (LLNA) using 5-bromo-2′-deoxyuridine instead of ^{3}H-methyl thymidine, significant effect on lymph node cell proliferation was detected at 10% PCMC and 25% PCMX, while in the multiple-dose guinea pig maximization test (GPMT) at least one animal tested in the group was sensitized at a 5 ppm induction dose of either chemical. When mean skin reaction score in an animal group maximally sensitized with each allergen with the GPMT was plotted against log challenge concentration, linear regression lines with high correlations were obtained in both cases. The calculated elicitation threshold was lower for PCMC than PCMX. The area under the linear regression line between the threshold point and 1% of the elicitation concentration, another index of relative elicitation potency, was also greater for PCMC. Bidirectional cross-reactivity between PCMX and PCMC was detected in the GPMT. PCMC was thus identified in both LLNA and GPMT as a stronger sensitizer than PCMX in both the induction and elicitation phases. These results suggest that the non-radioactive LLNA is a simple and useful method for evaluating allergenicity in the induction phase, while the GPMT using a maximally sensitized animal group is more suitable for assessing the dose–response profile and cross-reactivity in the elicitation phase.
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Keywords: Contact dermatitis; Murine local lymph-node assay; Guinea pig maximization test; 5-Bromo-2′-deoxyuridine (BrdU); Risk assessment

1. Introduction

p-Chloro-*m*-cresol (PCMC) and *p*-chloro-*m*-xylenol (PCMX) are substituted phenols with antiseptic properties. Both chemicals are widely used as biocides and preservatives in cosmetics and medical products used in dermatology and general

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doi:10.1016/S0300-483X(03)00161-6

skin care, and in electrode paste. One of the toxicological concerns with the use of these chemicals is their ability to cause allergic contact dermatitis. Many cases of skin sensitization by PCMC and PCMX following prolonged use and direct contact with not only normal but also damaged skin have been reported (Adams, 1981; Andersen and Hamann, 1984; Libow et al., 1989). In the process of risk assessment of a chemical, hazard identification should be followed by dose–response evaluation. However, information on the evaluation of contact sensitizer potency is in general lacking.

The guinea pig maximization test (GPMT) is a standard method for the identification of contact sensitizers (Magnusson and Kligman, 1969). Its purpose is to establish whether a chemical is a skin sensitizer using a single dose equal to the tolerable maximum dose to perform induction and challenge. Accordingly, the result is qualitative and not suitable for quantitative assessment of allergenic potency. There have been attempts to modify the standard GPMT for the dose–response evaluation of allergenicity by using varied induction and challenge doses. (Nakamura et al., 1994; Andersen et al., 1995). Our group has also used the multiple-dose GPMT to quantitatively evaluate the allergenicity of several skin sensitizers (Yamano et al., 2001a,b).

The murine local lymph-node assay (LLNA) was proposed by Kimber et al. (1989) as an alternative to the GPMT, which, although sensitive, is time-consuming and includes potentially harmful elicitation protocols. The LLNA identifies skin-sensitizing chemicals as a function of events with the induction phase, reducing stress and harm to the experimental animals. It has been validated rigorously and accepted as a stand-alone method for the detection of contact allergens by the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (NIH, 1999). It does, however, have several disadvantages, such as occasional false negative results with weak sensitizers identified as positive in the GPMT, and the use of radioisotopes to quantify lymph-node cell proliferation. Thus, various improvements have been proposed.

Regarding the use of radioisotopes, Boussiquet-Leroux et al. (1995) used 5-bromo-2′-deoxyuridine (BrdU) instead of ^{3}H-thymidine and measured BrdU incorporation into lymph nodes immunohistochemically. Takeyoshi et al. (2001) developed a method closer to the original, with the injection of BrdU after topical application of chemical allergens and the use of ELISA to measure BrdU concentration in the auricular lymph nodes. In the present study, we further improved this latter method by incorporating an evaluation of the effects of tested chemicals on cell counts in the lymph nodes.

The GPMT has been adopted by various countries and organizations as a standard method and has given rise to a large-scale database on contact sensitizers. Comparison of dose–response profiles in the induction phase between the GPMT and the LLNA would provide valuable additional information for risk assessment. Moreover, dose–response profiles in the elicitation phase and cross-reactivity with analogous chemicals are essential, but the LLNA cannot evaluate these profiles. We therefore undertook a comparison of the allergenicity of PCMC and PCMX in the induction phase by the multiple-dose GPMT and the non-radioactive LLNA. Allergenic potencies for the elicitation phase and cross-reactivity were evaluated by the GPMT using a maximally sensitized group of animals.

2. Methods

2.1. Chemicals

PCMC and PCMX were obtained from Tokyo Kasei Co., Ltd, Tokyo, Japan, BrdU from Nakalai Tesque Inc., Kyoto, Japan, and 2,4-dinitrochlorobenzene (DNCB) from Katayama Chemical Inc., Osaka, Japan. All other reagents used were of superior grade. The Chemical structures of PCMC and PCMX are shown in Fig. 1.

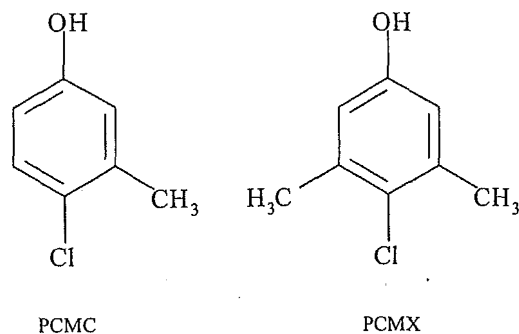

Fig. 1. Chemical structures of PCMC and PCMX.

2.2. Non-radioactive murine local lymph-node assay

Six- to eight-week-old female BALB/c mice from CLEA Japan (Tokyo, Japan) were used. The assay was performed according to the method of Takeyoshi et al. (2001) with some modifications. Briefly, groups of mice ($n = 4$) were exposed to 25 μl of various concentrations of the test chemicals in vehicle (acetone/olive oil, 4:1, AOO) or vehicle alone through application to the dorsum of both ears on three consecutive days (days 0–2). On day 4, BrdU (150 mg/kg/15 ml saline) was administered intraperitoneally to each mouse. The next day, a pair of auricular lymph nodes from each mouse was excised and weighed. Single-cell suspensions were prepared by gentle mechanical disaggregation in saline. After filtration through gauze and washing with centrifugation, cells were resuspended in 4 ml of saline. The total cell count in each suspension was measured using an automatic cell counter (CDA-500, Sysmex Corp., Kobe, Japan) and the suspensions were then diluted with saline to a concentration of 4×10^5 cells/ml and dispensed (50 μl) into the wells of a flat-bottom microplate. After drying at 60 °C for 2 h, 200 μl of Fix-Denat solution (Roche Molecular Biochemicals, Mannheim, Germany) was added to each well and left for 30 min at room temperature. After removal of the Fix-Denat solution, 100 μl of a working solution of peroxidase-conjugated anti-BrdU antibody (Roche Molecular Biochemicals) was added and the plate sealed and incubated for 90 min at room temperature. The plate was then washed with PBS and incubated for 15–30 min in the dark with 100 μl of *o*-phenylenediamine dihydrochloride solution for ELISA (Sigma-Aldrich Co., St. Louis, MO). The enzymatic reaction was stopped by addition of 50 μl of 4 N HCl and the plate read at 492 nm with a reference wavelength of 540 nm. In each experiment, lymph node cells from naïve mice (not treated with BrdU) were assayed concomitantly and the absorbance value obtained for the non-specific binding of BrdU antibody to lymph cells subtracted from all other values.

2.3. Guinea pig maximization test

Five- to six-week-old female Hartley guinea pigs from SLC (Shizuoka, Japan) were used. The GPMT was performed as described previously (Nakamura et al., 1994). Five or ten animals were used for each sensitization group. The induction profiles for PCMC and PCMX were evaluated by varying the intradermal induction concentrations in line with those for topical induction (Yamano et al., 2001b). Two weeks after topical induction, 0.1 ml aliquots of various concentrations of test chemicals in acetone were applied all at once to a shaved area of the flank for challenge. Forty-eight hours after the challenge, each site was scored for erythema (0–4) and edema (0–3) according to the criteria of Sato et al. (1981). Total group scores (erythema plus edema) with the same challenge concentration in one group were summed and divided by the number of animals of the group to give the mean response (MR) value, an index of skin reaction to challenge with a given concentration of the test chemical. For each challenge concentration group, the percentage of animals showing a positive reaction was taken as the sensitization rate (SR). In some cases, MR values were plotted against log challenge concentrations and a linear regression calculated. The area under the linear regression line between the x-intercept and 1% challenge concentration was defined as the relative elicitation potency index value of the allergen (elicitation AUL) (Yamano et al., 2001a).

2.4. Statistical analysis

In the LLNA, index values for each chemical-treated group and vehicle-treated control group were compared via Dunnett's or Steel's multiple comparison method using the StatLight computer package (Yukms Corp., Tokyo, Japan).

3. Results

Dose–response profiles for the allergenicity of PCMC and PCMX in the induction phase were evaluated by the non-radioactive LLNA (Table 1). Stimulation index (SI), which is obtained by multiplying the cellularity index by the BrdU index, takes account of the effect of the tested chemical on the total incorporation of BrdU into the auricular lymph nodes. DNCB dose-dependently increased both the cellularity of the lymph nodes and BrdU incorporation per well of cells, with a statistically significant increase in SI observed at doses of 0.1% and more. PCMC and PCMX were much less potent sensitizers in the LLNA, with the lowest concentrations inducing a significant effect on SI values calculated at 10 and 25%, respectively.

GPMT results for PCMC and PCMX are shown in Table 2. An intradermal induction concentration of 50 000 ppm for both chemicals and a challenge concentration of 50 000 ppm PCMC and 500 000 ppm PCMX were selected as the maximum tolerable doses for each step of the GPMT. For both PCMC and PCMX, the minimum dose needed to induce sensitization was 5 ppm, and animals were optimally sensitized at 5000 ppm. Dose–response profiles of PCMC and PCMX in the induction phase were compared between the LLNA and the GPMT (Fig. 2). When GPMT skin reaction scores (MR) at the maximum non-irritating challenge concentrations of PCMC (50 000 ppm) and PCMX (500 000 ppm) were plotted against log induction concentrations, a bell shape were observed for both chemicals, although values for PCMC were consistently higher than for PCMX. The dose–response curves of PCMC and PCMX showed a similar bell shape when the SI values in the LLNA were plotted against induction

Table 1
LLNA results with DNCB, PCMC and PCMX

Chemical	%	Cellularity index	BrdU incorporation index	SI
DNCB	0.000	1.0±0.1	1.0±0.3	1.0±0.3
	0.010	0.9±0.2	1.3±0.3	1.2±0.2
	0.025	0.9±0.4	1.1±0.3	1.0±0.2
	0.050	1.0±0.0	1.2±0.3	1.2±0.3
	0.100	3.2±1.2*	1.6±0.2	5.2±2.6*
	0.250	3.8±0.6*	1.9±0.5*	7.0±1.5*
	1.000	4.4±0.3*	2.8±0.9**	12.8±5.0*
PCMC	0	1.0±0.2	1.0±0.3	1.0±0.4
	5	1.3±0.2	1.1±0.3	1.5±0.6
	10	1.6±0.4*	1.4±0.4	2.1±0.6*
	25	2.4±0.4**	2.4±0.6**	6.1±2.5*
	50	2.8±0.5**	1.8±0.9	5.1±2.9*
PCMX	0	1.0±0.2	1.0±0.3	1.0±0.4
	5	1.2±0.3	1.0±0.3	1.2±0.6
	10	1.3±0.5	1.4±0.4	1.7±0.3
	25	1.6±0.5	2.1±0.8**	3.6±2.1*
	50	2.1±0.8**	1.7±0.4	3.7±2.1*

Values are given as means±S.D. Cellularity index = Total lymph node cell count in a chemical-treated animal/Mean lymph node cell count in control group. BrdU incorporation index = BrdU incorporation (OD) per well of cells from a chemical-treated animal/Mean BrdU incorporation (OD) per well of cells from control group. SI = Cellularity index × BrdU incorporation index. *, **Significantly different from control, $P < 0.05$ and 0.01, respectively.

Table 2
GPMT results with PCMC and PCMX

Challenge dose (ppm)	MR (SR)[a]					
	Induction dose[b]					
	0 ppm[c]	0.5 ppm	5 ppm	50 ppm	5000 ppm	50 000 ppm
PCMC						
160	0.0	0.0	nd	0.0	0.0	0.0
500	0.0	0.0	0.0	0.0	0.3(20)	0.0
1600	0.0	0.0	nd	0.0	1.2(70)	0.1(10)
5000	0.0	0.0	0.0	0.0	2.8(100)	0.3(20)
16 000	0.0	0.0	nd	0.0	3.9(100)	1.3(70)
50 000	0.0	0.0	0.2(20)	0.5(30)	5.4(100)	3.2(80)
PCMX						
1600	0.0	0.0	nd	0.0	0.0	0.0
5000	0.0	0.0	0.0	0.0	0.8(60)	0.3(30)
16 000	0.0	0.0	nd	0.0	1.7(80)	0.5(30)
50 000	0.0	0.0	0.0	0.0	2.3(80)	1.2(60)
160 000	0.0	0.0	nd	0.0	3.3(90)	1.8(70)
500 000	0.0	0.0	0.4(40)	0.3(30)	3.8(90)	2.8(90)

nd, not determined; MR, mean response; SR, sensitization rate (%).
[a] Skin reactions were evaluated 48 h after challenge.
[b] Each group of animals was sensitized using the same dose of PCMC or PCMX for both intradermal and topical induction procedures.
[c] Animals were treated with vehicle in both intradermal and topical induction procedures.

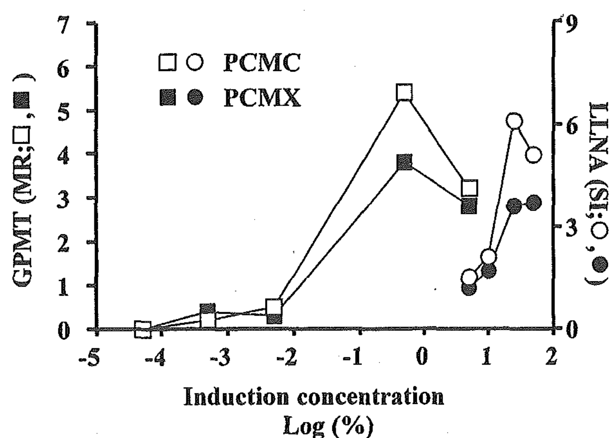

Fig. 2. Comparison of dose–response profiles for induction phase of PCMC and PCMX in GPMT and LLNA. For GPMT, each symbol represents the MR score of a group treated with the relevant induction concentration and measured 48 h after challenge with the maximum concentration of each compound (50 000 ppm for PCMC and 500 000 ppm for PCMX). For LLNA, each symbol represents dose-related SI values for PCMC and PCMX as per Table 1.

concentrations. Again, SI values for PCMC were consistently higher. The dose ranges required for inducing positive results in the LLNA appeared to be much higher than in the GPMT.

The dose–response profiles for the challenge phase were evaluated by the GPMT using maximally sensitized groups of animals. As shown in Fig. 3, when mean skin reaction values (MR) were plotted against log challenge concentrations, linear regression showed a good fit for both PCMC and PCMX. The evaluated elicitation threshold concentrations were 440 and 1370 ppm for PCMC and PCMX, respectively, indicating that PCMC is a stronger sensitizer than PCMX in eliciting skin reaction in maximally sensitized animals. The elicitation AUL, the area under the linear regression line between the threshold point and 1% of challenge concentration, is another index of the relative elicitation potency of an allergen and reflects the integrated degree of skin reaction that would emerge among a maximally sensitized population exposed to the allergen. The elicitation

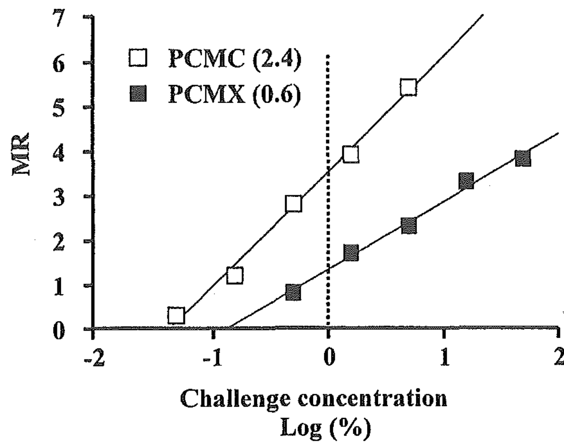

Fig. 3. Relationship between challenge concentration and skin-reaction score for PCMC and PCMX in GPMT. In order to evaluate the challenge profile, a group of animals maximally sensitized with each allergen (induction dose of 5000 ppm for both PCMC and PCMX) was used. Each symbol represents the MR score of the group (MR) 48 h after challenge with the relevant compound. Values in parenthesis indicate the AUL values of PCMC and PCMX, which are calculated as the area under the linear regression line between the x-intercept and a vertical dotted line at a concentration of 1%.

AUL of PCMC (2.4) was four times higher than that of PCMX (0.6).

As shown in Table 3, PCMC-sensitized animals cross-reacted to PCMX, and vice versa. To obtain nearly equipotent skin reaction, a concentration of PCMX two orders higher or a concentration of PCMC one order lower than for the corresponding parent chemical was required.

Table 3
Cross-reactivity between PCMC- and PCMX-sensitized animals in the GPMT

Challenge	MR	SR
PCMC-sensitized animals		
0.5% PCMC	1.6	90
50% PCMX	0.9	90
PCMX-sensitized animals		
5% PCMC	1.1	60
50% PCMX	1.8	100

Animals were sensitized with 5000 ppm PCMC or PCMX for both intradermal and topical induction procedures and were rechallenged 1 week after the challenge with PCMC or PCMX described in Table 2. Skin reactions were evaluated 48 h thereafter. MR, mean response; SR, sensitization rate (%).

4. Discussion

Although the LLNA is an accepted alternative method to the traditional guinea pig test, the need for special facilities to handle radioisotopes hinders its widespread use in routine screening for skin sensitizers. To circumvent the use of radioisotopes, several non-radioactive endpoints in the LLNA have been developed. Lymph cell IL-2 production corrected for the CD4+ T cell subset is a good index and can detect strong and moderate sensitizers (Hariya et al., 1999). BrdU, a non-radioactive alternative to ^{3}H-thymidine, has been used in various fields as it can be assayed using specific antibodies. Its use to replace radioisotopes in the LLNA has been approached from various angles. Flow cytometric analysis and immunohistochemical staining have been developed to count BrdU-positive cells in lymph nodes (Boussiquet-Leroux et al., 1995; Suda et al., 2002). Compared to the modified LLNA as mentioned above, the method of Takeyoshi et al. (2001) using ELISA to quantify BrdU in lymph nodes is closer to the original LLNA and easy to perform, but results for a number of allergens suggest an insufficient sensitivity, because the increase in lymph node cell counts is not taken into account when calculating SI values. The SI value presented in the present study, incorporating both the cellularity index and the BrdU index, more precisely reflects the total incorporation of BrdU into lymph nodes, i.e. lymph node proliferation by skin sensitizers. Moreover, using an individual SI value as a statistical unit gives more objective criteria to judge positive results than the EC_3, used in the standard LLNA. In the modified non-radioactive LLNA, DNCB gave positive results at doses of 0.1% and more, in the same range than previous reports by several laboratories using the standard radioactive LLNA method (Loveless et al., 1996).

Using the non-radioactive LLNA and multiple-dose GPMT, we compared dose–response profiles of PCMC and PCMX in the induction phases. Recently, a similar comparison of both methods

was made by Van Och et al. (2001) using three rubber accelerators. In their study, the rank order of allergenicity of benchmark concentrations differed to some extent in the GPMT and the LLNA. Such a discrepancy is likely to occur due to difference in the induction procedure among these methods. In the GPMT, in addition to the use of an adjuvant as a vehicle, the intradermal injection bypasses the skin penetration step of the test chemical, so that the sensitizing capability of chemicals with specific physiochemical properties is augmented. The results of the present study showed that PCMC is a stronger sensitizer than PCMX in the induction phase in both the LLNA and GPMT. In addition, the shapes of the dose–response curves for PCMC and PCMX were similar with both methods, except that concentrations two orders higher than in the GPMT were required to obtain positive results in the LLNA. Van Och et al. (2001) reported a discrepancy of similar magnitude in the sensitivity of the GPMT and LLNA. Since topical application is a more relevant route of exposure for practical situations, the LLNA is more suited to risk evaluation in the induction phase, but the apparent lower sensitivity of the LLNA compared to the GPMT presumes its possible inability to identify weak sensitizers. PCMC and PCMX, the test chemicals used in the present study, are not necessarily weak sensitizers, at least not as measured by the GPMT. PCMC and PCMX ranked third and the fourth among seven known allergens for induction potency evaluated using the same multiple-dose GPMT as in the present study (Yamano et al., 2001a). Whether the non-radioactive LLNA is able to detect weak sensitizers is under investigation.

From a practical point of view, risk in the elicitation phase is an important factor in the risk assessment of skin sensitizers. As risk is typically evaluated on the basis of the worst-case scenario, the GPMT using optimally induced animals to evaluate the elicitation profiles would seem to be ideal. Although a threshold concentration in the skin elicitation reaction appears to be the most useful index to compare the potency of allergens, the study of the dose–response profile is a better index. Recently, we proposed an index to compare the relative elicitation potencies of chemical allergens in the GPMT (Yamano et al., 2001a). The proposed index value, the area under the linear regression line between the threshold point and 1% of the elicitation concentration in Fig. 3, reflects the integrated degree of skin reaction that would occur when a maximally sensitized animal is exposed to the allergen. The elicitation AUL takes into account both the threshold and the dose–response profile from the slope of the regression line. This means that, in practical terms, when the slope of the regression line is steep, i.e. when the severity of the skin reaction increases sharply with a slight increase in challenge concentration, the elicitation AUL can reach a higher value than for an allergen with a lower threshold concentration, but a gentler slope. In the present study, PCMC was identified as a stronger sensitizer than PCMX as judged from both threshold concentrations and elicitation AUL values.

Cross-reactivity is another important factor in risk assessment for skin sensitizers. Allergic skin reaction are often caused by a chemical without a history of previous exposure. As PCMC and PCMX are closely related chemicals, cross-reactivity has been a matter of concern. Lewis and Emmett (1987) speculated from the results of patch testing in PCMC- and PCMX-sensitized patients that cross-reactivity occurs only after the initial sensitization to PCMX. However, it is difficult to know the exposure history of patients to chemical allergens. The use of strictly controlled experimental animals sensitized to a specific allergen is essential to predict the risk of cross-reactivity between closely related chemicals. As shown in Table 3, bidirectional cross-reactivity between PCMC and PCMX was detected in the GPMT indicating that T lymphocytes stimulated with PCMC could recognize PCMX and vice versa. Our results also suggest that skin reactions to PCMC in PCMX-sensitized animals are much more likely to occur than the reverse. This is consistent with the human care report by Lewis and Emmett (1987).

Of the various fields of toxicology, risk assessment in the area of contact sensitizers seems to lag far behind. Various factors, such as allergen concentration in and leaching from products, the

duration and frequency of exposure, and skin condition should be considered case by case in risk assessment. Above all, dose–response profiles for the induction and elicitation phases are prerequisite. The non-radioactive LLNA is a simple and useful method for evaluating the allergenicity of a chemical in the induction phase, while the GPMT using a maximally sensitized group of animals is more suitable for assessing the dose–response profile of an allergen in the elicitation phase. The GPMT is also effective in detecting cross-reactivity among related chemicals. Comparison of the two methods using a wide range of allergens and in light of human data is however still needed to validate the non-radioactive LLNA and to extrapolate animal data to humans.

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報　文

家庭用品に使用される化学物質の皮膚感作性試験（VI）

抗菌剤2-chloroacetamide、2-bromo-2-nitropropane-1,3-diol、zinc bis(2-pyridylthio-1-oxide) のモルモットにおける皮膚感作性

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Allergenicity Evaluation of Chemicals for Use in Household Products (VI)

Contact Allergenicity of Antimicrobial Agents 2-Chloroacetamide, 2-Bromo-2-nitropropane-1,3-diol and Zinc bis(2-pyridylthio-1-oxide) in Guinea Pigs

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Abstract

Three antimicrobial agents, 2-chloroacetamide (CAA), 2-bromo-2-nitropropane-1,3-diol (BNPD) and zinc bis(2-pyridylthio-1-oxide) (ZPT) were evaluated for their skin sensitization potency in a modified guinea pig maximization test (GPMT). These chemicals have low estimated octanol-water partition coefficients and are therefore presumed to have low skin penetration capability. CAA produced weak skin sensitization, BNPD seemed to cause very weak sensitization of guinea pig skin as only one of ten animals had a positive skin reaction to it, and ZPT failed to elicit a positive skin reaction even at maximal concentrations of intradermal injection (5,000 ppm) and elicitation (50,000 ppm). Guinea pigs sensitized with CAA cross-reacted to the structural analogue 2-chloro-N-(hydroxymethyl) acetamide, which is known as a contact sensitizer, but not to trichloroacetamide. BNPD-sensitized animals did not react to formaldehyde, which is a degradation product of BNPD. CAA and ZPT are thus defined as positive and negative, respectively, for their skin sensitization potency in the GPMT. BNPD, however, could not be assessed clearly as having a weak skin sensitization potency in the GPMT.

Keywords: 2-chloroacetamide, 2-bromo-2-nitropropane-1,3-diol, bronopol, zinc bis(2-pyridylthio-1-oxide), zinc pyrithione, skin sensitization, guinea pig maximization test

I．緒　言

家庭用品には抗菌剤、可塑剤、酸化防止剤など様々な用途に多くの化学物質が使用されており、特に抗菌剤は近年の清潔志向を反映してその使用が増加している。しかし、それらの安全性、特に皮膚感作性については未だ十分に検討されているとは言い難い。我々は現在まで、抗菌剤を中心に24種の化学物質（抗菌剤15種を含む）の皮膚感作性を試験し、その内抗菌剤13種を含む17種が皮膚感作性を有することを報告した［1-12］。今回は化学構造式から推定して極性が高いと考えられる3種類の抗菌剤2-chloroacetamide（CAA）、2-bromo-2-nitropropane-1,3-diol（BNPD）およびzinc bis(2-pyridylthio-1-oxide)（ZPT）についてモルモットを用いた皮膚感作性試験を実施した。

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2-chloroacetamideは接着剤、皮革、塗料、化粧品、切削油などに抗菌剤として使用されており、それらの使用に伴うCAA感作症例が報告されている［13, 14］。その他、壁用塗料から揮散したCAAに暴露された住人が接触性皮膚炎を発症した例もある［15, 16］。一方、実験動物においてはCAAに皮膚感作性は認められない［17］とされていたが、最近CAAに皮膚感作性があるとの報告［18］がなされ、結論が出るに至っていない。

BNPDは抗菌剤として化粧品、外用薬や切削油などに使用されている。BNPDの使用濃度としては化粧品や外用薬には0.01～0.1%、切削油などには0.01～0.2%が推奨されている。皮膚感作性に関してはBNPD含有化粧品、軟膏、潤滑油などの使用に伴ういくつかの症例報告がある［20-23］。しかし、イギリスを中心としたヨーロッパの病院での調査では、BNPDに対するアレルギーと判定されたヒトは0.21%と少数であった［24］。一方、モルモットを用いた皮膚感作性試験では、惹起方法によって結果が異なる［19, 25］ことから、BNPDの皮膚感作性試験にあたってはその皮膚透過性に留意する必要があると考えられる。

ZPTは薬用シャンプーやローション用の抗菌剤として使用されるほか船底塗料用防汚剤としても利用されている化学物質である。ZPTの皮膚刺激性には種差があり、ウサギやマウスでは皮膚刺激性があるが、モルモットでは皮膚刺激性は観察されていない［26］。皮膚感作性に関しては、ZPT含有薬用シャンプーで感作された患者の症例報告が数報ある［27-31］。しかし、大規模なZPTのパッチテスト調査ではZPTに対するアレルギー陽性と判定された割合はわずかに0.08%で、ヒトのZPTに対する感作率は非常に低い［32］。一方、実験動物を用いた皮膚感作性に関する報告はない。

今回、家庭用品に使用される化学物質の安全性評価の一環として、抗菌剤のCAA、BNPDおよびZPTについて、それらの皮膚感作性をモルモットmaximization法［33］のNakamuraらによる改良法（GPMT）［34］を用いて検討した。

Ⅱ．実験材料および方法

1．試験物質（Fig. 1）

1）2-chloroacetamide

（CAA：CAS No. 79-07-2、東京化成工業製）

純度：>97.0 %

2）2-bromo-2-nitropropane-1,3-diol

（BNPD：CAS No. 52-51-7、Myacide Pharma BP製）

別名：bronopol

純度：約100%

3）zinc bis(2-pyridylthio-1-oxide)

（ZPT：CAS No. 13463-41-7、東京化成工業製）

別名：zinc pyrithione、omadine zinc、zinc 1-hydroxy-2-pyridine-thione

純度：約100%

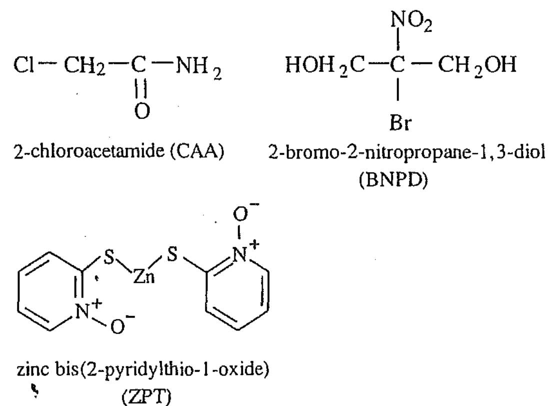

Fig. 1 Chemical structures of compounds tested

2．BNPD分解物

formaldehyde（formalin、米山薬品工業製）

純度：formaldehydeとして約37%

3．交差反応性検討物質（Fig. 2）

1）2-chloro-N-(hydroxymethyl)acetamide

（CHMAA：CAS No. 2832-19-1、Aldrich Chem. Co.）

別名：N-methylol-chloroacetamide

純度：98 %

2）trichloroacetamide

（TCAA：CAS No. 594-65-0、和光純薬製）

純度：> 95%

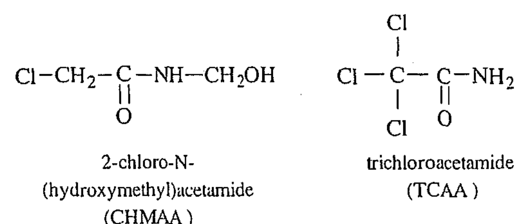

Fig. 2 Chemical structures of compounds tested for cross-reactivity to CAA.

4．陽性対照物質

2,4-dinitrochlorobenzene

（DNCB：CAS No. 97-00-7、片山化学工業製）

5．その他の試薬

フロインド完全アジュバンド（FCA：ヤトロン製）、白色ワセリン、ラウリル硫酸ナトリウム（SLS）、特級アセトン、局方オリブ油、注射用生理食塩液。

6．使用動物

4週齢のStd:Hartley系の雌性モルモット（クリーン）を日本SLC（株）から購入し、2週間の馴化飼育の後実験に供した。投与前日に一般状態および投与部位の皮膚に異常のみられない動物を選択し、各群に無作為に振り分けた。動物は馴化期間および実験期間ともに、ステンレス製2連ユニットケージ（W40cm×L26cm×H20cm）に個別に収容し、固型飼料（オリエンタル酵母（株）製RC-4）および水道水を自由に摂取させた。飼育は温度23±2℃、点燈7:00～19:00に調節された動物飼育室で行った。

7．皮膚感作性試験方法

各試験物質毎に皮膚刺激性試験（皮内投与、閉塞貼付、開放塗布）を実施し、その結果を基に各試験物質の皮膚感作性の有無を調べるとともに第2次感作処置の効果を検討した。

CAA、BNPDおよびZPTはいずれも適用できる最高投与量を第1次感作および第2次感作に用いる群（高用量群）、高用量群で用いた第1次感作濃度の1/10000を第1次および第2次感作に用いる群（低用量群）、第1次感作濃度を0％（溶媒投与）とし、第2次感作濃度を高用量群で用いた第2次感作濃度とする群（閉塞貼付群）、および対照群を設定した。使用動物数は各群5例とした。

以上の試験の結果、皮膚感作性が認められた化学物質については用量反応性を検討した。CAAは閉塞貼付群で感作が成立したので、第2次感作濃度を最高濃度で固定し、第1次感作濃度のみを変化させる従来の方法は採らず、第1次および第2次感作濃度を同一濃度として変化させた。BNPDは高用量群で感作が成立した可能性が示唆された。また、閉塞貼付群の1例にごく軽度の紅斑が見られたが、この結果から直ちにこの群で感作が成立したとは判断できなかった。そこで、今回は第2次感作濃度は適用可能な最高濃度である1％に固定して、第1次感作濃度だけを変化させる従来法を用いた。ZPTは皮膚感作性が認められなかったので、確認のために高用量群と同じ投与量で再度試験を実施した。

3種の試験物質の皮膚感作性試験ではそれぞれ溶媒を投与する非感作群および陽性対照群を前報［5］に準じて設けた。

各試験物質の調製方法、感作方法および惹起方法は前報［5］に準じて行った。なお、ZPTは皮膚刺激性が低いので、第2次感作誘導に際して10%SLSワセリン軟膏処置を実施した。CAAおよびBNPDには適度な皮膚刺激性があるので10%SLSワセリン軟膏処置は省略した。

3種の試験物質の皮膚感作性試験で用いた投与量、惹起濃度および使用動物数はTable 2, 4, 5に示した。皮膚反応の評価に関しては惹起暴露48および72時間後に前報［5］に従って判定した。

なお、BNPDおよびZPT感作群については、皮膚透過性が低いと考えられたので、惹起暴露1週間後に18G注射針で皮膚に井型のすり傷を付けて惹起する実験を行った。用いた群はBNPD感作群およびZPT感作群のいずれも5,000 ppm感作群および対照群とした。

CAA感作群については、惹起暴露1週間後にCAAと類似の構造を有する化合物CHMAAおよびTCAAで惹起暴露し、これらの化合物との交差反応性を検討した。用いた群は本実験で最も強く感作された5,000 ppm CAA感作群および対照群とした。各物質の惹起濃度はTable 3に示した。BNPD感作群については、BNPD分解物であるformaldehydeの感作誘導に対する影響を検討するために、無傷の皮膚での惹起に際して、20,000 ppm formalinのアセトン溶液を同時に塗布した。

Ⅲ．実験結果

1．皮膚感作性の有無および第2次感作処置の効果

CAA、BNPDおよびZPTについて皮膚感作性の有無、非感作濃度の推定および第2次感作処置の皮膚感作性に及ぼす効果について検討した。

Table 1に示すように、CAAでは高用量群（第1次感作濃度5%、第2次感作濃度25%）および閉塞貼付群に陽性反応が観察された。BNPDではデータには示さなかったが、惹起濃度5,000 ppmでは高用量群（第1次感作濃度5,000 ppm、第2次感作濃度1%）と閉塞貼付群の各1例に軽度の紅斑が認められた。ZPTではデータには示さ

Table 1　Skin sensitization test of CAA 48 and 72 hrs after challenge dose (dose-finding study)

Induction		N	Challenge	48 hrs		72 hrs	
id injection[1]	topical		topical ppm	SR[2]	MR[3]	SR	MR
			0	0	0.0	0	0.0
			50	0	0.0	0	0.0
0％	0％	5	500	0	0.0	0	0.0
			5,000	0	0.0	0	0.0
			50,000	0	0.0	0	0.0
			0	0	0.0	0	0.0
			50	0	0.0	0	0.0
5 ppm	5 ppm	5	500	0	0.0	0	0.0
			5,000	0	0.0	0	0.0
			50,000	0	0.0	0	0.0
			0	0	0.0	0	0.0
			50	0	0.0	0	0.0
5％	25％	5	500	0	0.0	0	0.0
			5,000	40	0.4	40	0.4
			50,000	100	1.2	100	1.2
			0	0	0.0	0	0.0
			50	0	0.0	0	0.0
0％	25％	5	500	0	0.0	0	0.0
			5,000	20	0.2	20	0.2
			50,000	20	0.2	20	0.2

1) Intradermal injection
2) Sensitization rate (%) = number of positive animals / total number of animals × 100
3) Mean response = summation of numerical scoring / total number of animals

なかったが、高用量群（第1次感作濃度5,000 ppm、第2次感作濃度25%）を含むいずれの群にも陽性反応は認められなかった。

2．CAA感作群の皮膚反応性

惹起暴露後の各観察時点における陽性反応率および平均評価点をTable 2に示した。対照群ではいずれの観察時間でも皮膚反応はみられず、用いた最高惹起濃度（50,000 ppm）でもCAAに皮膚刺激性はなかった。50 ppm感作群では陽性反応はみられなかったが、500 ppm以上のCAA感作群で陽性反応が認められた。最高惹起濃度である50,000 ppmで惹起した時の48時間後の陽性反応率と平均評価点は500、5,000および50,000 ppm感作群でそれぞれ60%と0.7、100%と2.4、70%と1.2であった。このように最も強く感作が成立したのは5,000 ppm感作群であり、この群で求められたCAAの最低惹起濃度は1,600 ppmであった。

なお、CAAを処置したいずれの動物にも観察終了時まで、一般状態に特記すべき異常は認められなかった。

3．CAAとCHMAAおよびTCAAとの交差反応性

惹起の1週間後に対照群および5,000 ppm CAA感作群をCAAと類似構造を有する化合物CHMAAおよびTCAAで惹起した時の各観察時点における陽性反応率および平均評価点をTable 3に示した。対照群ではCHMAAおよびTCAAのアセトン溶液を塗布しても紅斑は観察されず、皮膚刺激性は認められなかった。CAA感作群では10,000 ppm CHMAAに対して48時間の観察時点で60 %の動物に陽性反応が確認され、CAAとCHMAAの交差反応性が確認された。しかし、より低濃度の1,000 ppm CHMAAの惹起では陽性反応は観察されなかった。一方、TCAAは50,000 ppmで惹起しても陽性反応は観察されなかった。

4．BNPD感作群の皮膚反応性

BNPD皮膚感作性試験の無傷の皮膚およびすり傷を付けた皮膚での結果をTable 4に示した。無傷の皮膚では、惹起濃度50および500 ppmでは、対照群を含む全ての群に皮膚反応はみられなかった。惹起濃度5,000 ppmでは、50および500 ppm感作群に皮膚反応は認められなかったが、5,000 ppm感作群で惹起24時間以降に1例の動物に軽度の紅斑が認められた。この惹起濃度ではいずれの観察時点でも対照群に紅斑は認められず、皮膚刺激性はないものと考えられた。惹起濃度25,000 ppmでは、惹起24時間以降に対照群の動物に軽度の紅斑が認められ、皮膚刺激性があることが明らかになった。この惹起濃度における惹起48時間後の平均評価点は対照群0.2、50 ppm感作

Table 2 Skin sensitization test of CAA 48 and 72 hrs after challenge dose.

Induction id injection[1] ppm	Induction topical ppm	N	Challenge topical ppm	48 hrs SR[2]	48 hrs MR[3]	72 hrs SR	72 hrs MR
0	0	5	0	0	0	0	0
			500	0	0	0	0
			1600	0	0	0	0
			5,000	0	0	0	0
			16,000	0	0	0	0
			50,000	0	0	0	0
50	50	10	0	0	0	0	0
			500	0	0	0	0
			1600	0	0	0	0
			5,000	0	0	0	0
			16,000	0	0	0	0
			50,000	0	0	0	0
500	500	10	0	0	0	0	0
			500	0	0	0	0
			1600	0	0	0	0
			5,000	20	0.2	20	0.2
			16,000	60	0.7	60	0.6
			50,000	60	0.7	60	0.6
5,000	5,000	10	0	0	0	0	0
			500	0	0	0	0
			1600	30	0.3	20	0.2
			5,000	90	1.3	70	0.8
			16,000	90	1.8	90	1.3
			50,000	100	2.4	100	1.7
50,000	250,000	10	0	0	0	0	0
			500	0	0	0	0
			1600	10	0.1	0	0.0
			5,000	50	0.5	50	0.5
			16,000	50	0.7	50	0.6
			50,000	70	1.2	70	0.8

1) Intradermal injection
2) *Sensitization rate (%) = number of positive animals / total number of animals × 100*
3) Mean response = summation of numerical scoring / total number of animals

Table 3 Cross-reactivity to CHMAA and TCAA 48 and 72 hrs after challenge dose.

Induction id injection[1] ppm	Induction topical ppm	N	Challenge topical ppm	48 hrs SR[2]	48 hrs MR[3]	72 hrs SR	72 hrs MR
0	0	5	1,000 CHMAA[4]	0	0	0	0
			10,000 CHMAA	0	0	0	0
			50,000 TCAA[5]	0	0	0	0
5000	5000	10	1,000 CHMAA	0	0	0	0
			10,000 CHMAA	60	0.8	50	0.7
			50,000 TCAA	0	0	0	0

Animals sensitized to 5000 ppm CAA were treated with CHMAA or TCAA 1 week after challenge procedure.
1) Intradermal injection
2) Sensitization rate (%) = number of positive animals / total number of animals × 100
3) Mean response = summation of numerical scoring / total number of animals
4) 2-chloro-N-(hydroxymethyl)acetamide
5) trichloroacetamide

群0.4、500 ppm感作群0.3、5,000 ppm感作群0.8で、5,000 ppm感作群の平均評価点は対照群よりいくぶん高い傾向がみられたが、統計的な有意差はなかった。また、この惹起濃度でみられた紅斑の程度は5,000 ppm感作群の1例を除いて全て軽度の紅斑であった。この5,000 ppm感作群の1例では48時間後の観察で中等度の紅斑と軽度の浮腫が観察された。なお、このモルモットは惹起濃度5,000 ppmで軽度の紅斑が観察された動物である。

惹起1週間後に対照群と5,000 ppm感作群について皮膚にすり傷を付けてBNPD（500、5,000および25,000 ppm）を塗布した（Table 4）。その結果、無傷の皮膚で陽性反応の認められた5,000 ppm感作群の1例は、惹起濃度5,000 ppmで紅斑評価3と浮腫評価1（無傷の皮膚では紅斑評価：1、浮腫評価：0）が観察され、惹起濃度25,000 ppmでは、紅斑評価4と浮腫評価2（無傷の皮膚では紅斑評価：3、浮腫評価：1）が観察され、すり傷を付けた皮膚で紅斑および浮腫の程度が強くなった。なお、すり傷を付けた皮膚での皮膚反応の評価に際しては、斑点状の紅斑は陽性反応から除外した。すり傷を付けた皮膚でも惹起濃度5,000 ppmでは対照群に紅斑は認められず、刺激性はないものと考えられた。以上から、少なくとも1例のモルモットはBNPDに感作された可能性があると推察された。

BNPDを処置したいずれの動物にも観察終了時まで、一般状態に特記すべき異常は認められなかった。

5．BNPD感作性に対する分解物formaldehydeの寄与

BNPD分解物の一つであるformaldehydeがBNPD感作性に寄与しているかどうかを検討するために、7,400 ppm formaldehyde（formalinとして20,000 ppm）をBNPDの惹起と同時に塗布した（無傷の皮膚）。しかし、5,000 ppm感作群の1例を含む全ての動物で陽性反応は認められなかった（Table 4）。

6．ZPT感作群の皮膚反応性

ZPTの皮膚感作性試験の無傷の皮膚およびすり傷を付けた皮膚での結果をTable 5に示した。ZPTのいずれの惹起濃度（500、5,000、50,000 ppm）でもZPT感作群および対照群に紅斑はみられなかった。また、1週間後にすり傷を付けた皮膚にZPT（5,000および50,000 ppm）を塗布したが、陽性反応は認められなかった。

なお、ZPTを処置したいずれの動物にも観察終了時まで、一般状態に特記すべき異常は認められなかった。

Table 4 *Skin sensitization test of BNPD 48 and 72 hrs after challenge dose.*

Induction		N	Challenge	intact skin				abraded skin[1]			
id injection[2]	topical		topical	48 hrs		72 hrs		48 hrs		72 hrs	
ppm	%		ppm	SR[3]	MR[4]	SR	MR	SR	MR	SR	MR
0	0	5	0 BNPD	0	0	0	0	0	0	0	0
			50 BNPD	0	0	0	0	—[6]	—	—	—
			500 BNPD	0	0	0	0	0	0	0	0
			5,000 BNPD	0	0	0	0	0	0	0	0
			25,000 BNPD	20	0.2	20	0.2	40	0.4	20	0.2
			7,400 formaldehyde[5]	0	0	0	0	—	—	—	—
50	1	10	0 BNPD	0	0	0	0	—	—	—	—
			50 BNPD	0	0	0	0	—	—	—	—
			500 BNPD	0	0	0	0	—	—	—	—
			5,000 BNPD	0	0	0	0	—	—	—	—
			25,000 BNPD	40	0.4	20	0.2	—	—	—	—
			7,400 formaldehyde	0	0	0	0	—	—	—	—
								—	—	—	—
500	1	10	0 BNPD	0	0	0	0	—	—	—	—
			50 BNPD	0	0	0	0	—	—	—	—
			500 BNPD	0	0	0	0	—	—	—	—
			5,000 BNPD	0	0	0	0	—	—	—	—
			25,000 BNPD	30	0.3	20	0.2	—	—	—	—
			7,400 formaldehyde	0	0	0	0	—	—	—	—
5,000	1	10	0 BNPD	0	0	0	0	0	0	0	0
			50 BNPD	0	0	0	0	—	—	—	—
			500 BNPD	0	0	0	0	0	0	0	0
			5,000 BNPD	10	0.1	10	0.1	10	0.4	10	0.3
			25,000 BNPD	50	0.8	30	0.6	20	0.7	10	0.6
			7,400 formaldehyde	0	0	0	0	—	—	—	—

1) BNPD was applied on the abraded skin of animals sensitized to 5000 ppm BNPD 1 week after challenge procedure.
2) Intradermal injection
3) Sensitization rate (%) = number of positive animals / total number of animals × 100
4) Mean response = summation of numerical scoring / total number of animals
5) 20,000 ppm formalin
6) Not examined

Table 5 Skin sensitization test of ZPT 48 and 72 hrs after challenge dose.

Induction		N	Challenge	intact skin				abraded skin[1)]			
id injection[2)]	topical		topical	48 hrs		72 hrs		48 hrs		72 hrs	
ppm	%		ppm	SR[3)]	MR[4)]	SR	MR	SR	MR	SR	MR
0	0	5	0	0	0	0	0	0	0	0	0
			500	0	0	0	0	—[5)]	—	—	—
			5,000	0	0	0	0	0	0	0	0
			50,000	0	0	0	0	0	0	0	0
5,000	25	15	0	0	0	0	0	0	0	0	0
			500	0	0	0	0	—	—	—	—
			5,000	0	0	0	0	0	0	0	0
			50,000	0	0	0	0	0	0	0	0

1) ZPT was applied on the abraded skin of animals sensitized to 5,000 ppm ZPT 1 week after challenge procedure.
2) Intradermal injection
3) Sensitization rate (%) = number of positive animals / total number of animals × 100
4) Mean response = summation of numerical scoring / total number of animals
5) Not examined

7．DNCB感作群の皮膚反応性

BNPDおよびZPT感作性試験と平行して実施した。データには示していないが、惹起濃度100 ppmでは24時間以降、惹起濃度1,000 ppmでは3時間以降に陽性反応が観察された。惹起48時間後における平均評価点は惹起濃度100 ppmで2.0、1,000 ppmで4.6であった。惹起濃度10 ppmでは陽性反応は観察されなかった。また、CAA感作性試験と同時に実施したDNCB感作性試験でもほぼ同様の結果が得られた。

以上、今回用いたロットのモルモットは陽性対照物質に対し良好な皮膚感作性反応を示すことが確認された。なお、DNCBを処置したいずれの動物にも観察終了時まで、一般状態に特記すべき異常は認められなかった。

Ⅳ．考 察

化学構造式から推定したオクタノール-水分配係数（estimated log Pow）がマイナスの値を有する3種の抗菌剤CAA、BNPDおよびZPTについてモルモットを用いた皮膚感作性試験を実施した。

1．CAAの皮膚感作性

CAAを含有する各種の家庭用品の使用に伴う感作症例報告［13-16］があることから、CAAはヒトで皮膚感作性があると推察される。一方、実験動物におけるCAAの皮膚感作性に関して、GPMTにおいて9%CAAで感作誘導を行い、3%で惹起しても皮膚感作性は認められず、Buehler法においても0.21%CAAで感作誘導しても皮膚感作性がないと報告されている［17］。しかし最近、GPMTでCAAに皮膚感作性が認められたと報告されたが、その詳細は不明である［18］。このように実験動物におけるCAAの皮膚感作性の有無については明確ではなかったが、今回の試験によりCAAはGPMTにおいて皮膚感作性を有することが確認された。

CAAの感作濃度に関しては最高非感作濃度は50 ppmであり、5,000 ppmでは100 %の動物に陽性反応がみられ、最も強く感作が成立した。今回用いた最高感作濃度である50,000 ppm感作群では5,000 ppm感作群に比べてむしろ感作能は低下した。このことは高濃度のCAA皮内投与により免疫系への毒性作用が発現したためと考えられる。CAAのオクタノール-水分配係数（log Pow）は-0.53と比較的極性が高く、皮膚透過性に問題があると推察された。しかし最も強く感作された5,000 ppm感作群では惹起濃度と平均評価点の間にきれいな用量相関性が認められたことからCAAの場合には極性が高いことは特に問題にはならなかった。なお、最低惹起濃度は1,600 ppmであった。

5,000 ppm感作群における皮膚反応（MR）と惹起濃度との用量反応性から求められたCAAの相対的惹起力価［7］は0.8であった。これまでに本試験法により皮膚感作性を評価した抗菌剤は17種類であり、そのうち13種（相対的惹起力価：0.1～21.9）に陽性反応が認められた［1-12］。この中でCAAの相対的惹起力価は低いものから5番目と比較的低いものであった。なお、同時に実施した陽性対照のDNCBの相対的惹起力価は10.0と算定された。

Nakamuraら［34］は化学物質の皮膚感作性の強さを表すためにa値：最低感作濃度、およびb値：最高感作群で平均評価点約1.0を与える惹起濃度の2つの値を用いることを提案している。今回の試験におけるCAAのa値は500 ppmであった。また5,000 ppm感作群におけるb値