

Sensitizing potential of chlorothalonil in the guinea pig and the mouse

ANDERS BOMAN¹, JOHAN MONTELIUS², RIITTA-LIISA RISSANEN³ AND CAROLA LIDÉN¹

¹Department of Medicine, Occupational and Environmental Dermatology, Karolinska Institutet, Stockholm, Sweden

²National Institute for Working Life, Toxicology and Risk Assessment, Stockholm, Sweden

³National Institute for Working Life, Occupational Dermatology, Stockholm, Sweden

The fungicide chlorothalonil is used extensively under several tradenames for the protection of various horticultural and fruit crops and bananas against fungal infections. It is also used as fungicide in wood preservation and as a preservative in paints. Clinical experience has shown chlorothalonil to be a contact allergen and several cases of allergic contact dermatitis attributed to chlorothalonil have been described. 2 previous guinea pig maximization test studies have shown the sensitizing potential of chlorothalonil to be high. The sensitizing property of chlorothalonil was studied by us with the predictive test methods the local lymph node assay and the cumulative contact enhancement test. In the local lymph node assay, chlorothalonil induced a dose-dependent increase in proliferation with a maximal stimulation index of 19.2 and 27.2. In the cumulative contact enhancement test, a statistically significant dose-dependent high sensitization rate was seen with a maximal sensitization rate of 100%. In conclusion, it is evident that chlorothalonil is an extremely potent contact allergen, inducing sensitization using only topical exposure on intact skin.

Key words: chlorothalonil; cumulative contact enhancement test; local lymph node assay; guinea pig; mouse; predictive testing; pesticide; fungicide. © Munksgaard, 2000.

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The fungicide chlorothalonil (Fig. 1) is a colourless crystalline compound with vapor pressure 0.01 mmHg at 40°C. It is almost insoluble in water (0.6 mg/kg) and has low solubility in organic solvents. Chlorothalonil is used extensively under several tradenames – Bravo, Dac, Daconil, Dacosoil, Exotherm, Forturf, Nopocide, Sweep, TCIN, m-TCPN, Termil, TPN – for the protection of various horticultural and fruit crops and bananas against fungal infections. It is also used as fungicide in wood preservation and as a preservative in paints (1–9, 10). Chlorothalonil has recently been classified as possibly carcinogenic to humans (Group 2B) (11).

Chlorothalonil has irritant properties in humans and experimental animals (12, 13). In addition, clinical experience has shown chlorothalonil to be a contact allergen and 17 cases of allergic contact dermatitis attributed to chlorothalonil have been described in the literature (2–7, 14). 1 of the cases was also photocontact allergic (14). Chlorothalonil has been described as a possible cause of erythema dyschromicum perstans (ashy dermatosis) in 39 exposed banana field workers, of whom 34 were patch test positive to the substance (10).

2 guinea pig maximization test studies have shown the sensitizing potential of chlorothalonil to be high, with an allergenicity rating of IV – V according to the Magnusson-Kligman classification (8, 9, 15).

Sensitization rate	Grade	Classification
0–8	I	weak
9–28	II	mild
29–64	III	moderate
65–80	IV	strong
81–100	V	extreme

In contrast, an unpublished study in guinea pigs, by the producer of chlorothalonil, using an occluded topical exposure method, revealed no sensitizing potential (16). The contact sensitizing property of chlorothalonil was questioned by the producer (17), and the skin reactions elicited were suggested to be attributed solely to its irritant properties, and to overestimation inherent in the predictive method used. This interpretation, however, itself has been challenged (18).

The aim of the present study was to investigate the sensitizing potential of chlorothalonil with

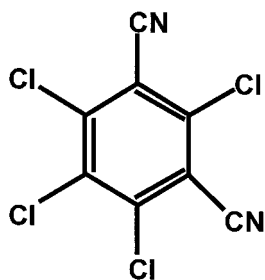


Fig. 1. Structural formula of chlorothalonil.

standardized predictive test methods including topical applications only – the local lymph node assay and the cumulative contact enhancement test.

Materials and Methods

Chemicals

Chlorothalonil (2,4,5,6-tetrachloro-1,3-isophthalonitril, 1,3-dicyano-2,4,5,6-tetrachlorobenzene) – CAS no [1897–45–6] (Mw 265.91) purity min. 99.0% was produced by the Institute of Organic Industrial Chemistry, Warsaw, Poland (purchased from Referensmaterial AB, Ulricehamn, Sweden) and used as delivered. [*methyl*- ^3H] Thymidine (specific activity 2.0 Ci/mmol) was purchased from Amersham International plc, Amersham, UK. Olive oil for pharmaceutical use (Apoteksbolaget AB, Stockholm), dimethyl formamide and methyl ethylketone p.a. (Merck, Darmstadt, Germany) were all used as delivered.

Local lymph node assay in mice

The local lymph node assay (LLNA) was carried out as recommended by Kimber and Basketter (19). Briefly, mice, in groups of 4, received 25 μl of chlorothalonil in the concentrations indicated (0.5–0.003% w/v), dissolved in dimethylformamide (DMF), on the dorsum of both ears on 3 consecutive days. Control mice were treated identically, but with an equal volume of DMF alone. 5 days after the 1st treatment, all the mice were injected intravenously through the tail vein with 20 μCi [^3H]thymidine in 250 μl of phosphate buffered saline (PBS). After 5 h, the mice were sacrificed and the draining auricular lymph nodes were excised, pooled for each group and the average lymph node weight was determined. A single-cell suspension of lymph node cells was prepared. After washing in PBS and precipitation with trichloroacetic acid, the incorporated thymidine was determined by β -scintillation counting, as previously described (20). Results are expressed as mean dpm (disintegrations/minute)/lymph node for each experimental

group and a stimulation index (SI), i.e., test group value/control group value, was calculated for each concentration tested. A chemical is classified as a sensitizer if 2 criteria are fulfilled (19): 1. at least 1 concentration of the test chemical induces a stimulation index of a $3\times$ or greater value than that of the vehicle control; 2. the result must not be incompatible with a biological dose response.

Cumulative contact enhancement test in guinea pigs

Animals

Outbred Dunkin-Hartley guinea pigs (Møllegaards Breeding and Research Centre, Ll. Skensved, Denmark and AB Sahlins Försöksdjursfarm, Malmö, Sweden) with a mean weight of 300 g were used for sensitization. The animals were allowed 1 week of acclimatization prior to exposure. They were fed C-vitamin-enriched guinea pig feed (SDS pellets from AB Sahlins Försöksdjursfarm, Malmö, Sweden) and given water *ad lib*. To minimize the risk of cross-contamination of animals with chlorothalonil, exposed animals and control animals were kept in separate rooms and kept separated during handling throughout the experiment, except for the final reading of test reactions.

Pre tests. Extensive preliminary testing was carried out for range-finding and establishing induction and challenge concentrations. The concentration found suitable for induction in Series I was 3% in petrolatum. The test compound was dissolved in acetone which was subsequently dissolved in petrolatum and later evaporated under vacuum.

Induction

Series I. The animals were exposed according to the cumulative contact enhancement test (CCET) method (21, 22). 4 occluded exposures of 24 h each were performed during 2 weeks with the compound dispersed on filter paper (Whatman, UK) on Blenderm[®] (3M, St. Paul, MN, USA) and fixed with acrylic bandage (Beiersdorf, Germany).

Series II. The animals were exposed according to the CCET method as in the previous series, but induction followed a multiple dose-response protocol (22–24). The injection of Freund's complete adjuvant on day 9 was omitted to avoid nonspecific irritation. The animals were divided into 6 groups, with 8 or 9 animals in each, and were treated with the test chemical in the concentrations 3.0, 1.0, 0.3, 0.1, 0.03, and 0% (control group) in pet. All the animals were given 4 occluded induction treatments.

Table 1. Cell proliferation induced by chlorothalonil in the local lymph node assay

Treatment	Concentration (w/v%)	Lymph node weight (mg/node)	[³ H]Thymidine incorporation (dpm/node)	Stimulation index
<i>experiment 1:</i>				
control	—	2.7	161	—
chlorothalonil	0.02	5.2	1783	11.1
	0.1	6.4	2454	15.3
	0.2	9.4	3080	19.2
	0.5	8.9	2759	17.2
<i>experiment 2:</i>				
control	—	2.7	121	—
chlorothalonil	0.003	2.5	248	2.1
	0.01	3.6	1137	9.4
	0.03	5.7	1668	13.8
	0.1	6.8	2222	18.4
	0.3	9.2	3289	27.2

Groups of mice ($n=4$) received 25 μ l of chlorothalonil dissolved in vehicle (DMF) in the concentrations indicated, on the dorsum of both ears daily for 3 consecutive days. Control animals were treated in the same way with the vehicle alone. Proliferation was assessed according to the description in *Materials and Methods*.

Table 2. Sensitization and challenge of guinea pigs with chlorothalonil according to the CCET method. Induction concentration: 3% chlorothalonil in pet.

	Reading time (h)	Number of positive animals							
		Challenge concentration (%)							
		0.03	0.01	0.003	0.001	0.0003	0.0001	0.00003	Vehicle
exposed	48	11	12	7	6	3	0	0	0
($n=15$)	72	14	14	12	9	3	1	0	0
control	48	3	2	0	0	1	0	1	0
($n=15$)	72	1	0	0	0	0	0	0	0
Fisher's exact test									
p	48	<0.01	<0.001	<0.01	<0.05	NS	NS	NS	NS
p	72	<0.001	<0.001	<0.001	<0.01	NS	NS	NS	NS

Challenge

After 1 week of rest, the animals were challenged with closed patch test for 24 h using Finn Chambers® (Epitest Ltd Oy, Tuusula, Finland) on Scanpor® tape (Alpharma AS, Norway) fixed to the body with acrylastic bandage (Acrylastic, Beiersdorf, Germany) (23). The animals in Series I and II were challenged with chlorothalonil in the concentrations 0.03, 0.01, 0.003, 0.001, 0.0003, 0.0001, 0.00003, and 0%, using methyl ethylketone/olive oil in the proportions 5:6 (MEK/OO) as vehicle. The test preparations were prepared by dissolving the compound in MEK and then adding olive oil.

The reactions were assessed 48 h after the application of the patches according to a 4-grade scale (15): no visual reaction (0), slight or spotted erythema (+), moderate and confluent erythema (++), erythema and edema (+++). To reduce the influence of possible false-positive reactions, all + reactions were treated in the data analysis as negative (Table 2), thus minimizing the risk of over-

estimation of sensitizing potential of the test chemical (24).

Data analysis

The results from the CCET test series were analyzed using Fisher's exact test (Instat, GraphPad Software, San Diego, CA, USA) and for dose-response relationship using a logistic dose response analysis program (25, 26).

Results

LLNA

The sensitizing potential of chlorothalonil was tested in the LLNA on 2 different occasions (experiments 1 and 2) (Table 1). Chlorothalonil induced a dose-dependent increase in proliferation, with a maximal stimulation index of 19.2 (experiment 1) at 0.2% and 27.2 (experiment 2) at 0.3%. Chlorothalonil should thus be classified as a sensitizer according to the LLNA (Table 1, Fig. 2).

Table 3. Sensitization and challenge of guinea pigs with chlorothalonil according to a multi dose-response protocol in the CCET method. 72-h reading

Induction conc. (%)	n	Number of positive animals							
		Challenge concentration (%)							
		0.03	0.01	0.003	0.001	0.0003	0.0001	0.00003	Vehicle
3	(9)	8	8	8	4	0	1	0	1
1	(9)	9	9	6	6	3	0	0	0
0.3	(8)	7	7	4	4	1	1	0	0
0.1	(8)	7	6	6	5	0	0	0	0
0.03	(8)	6	6	5	2	1	0	0	0
control pet.	(8)	0	0	0	0	0	0	0	0

CCET

The preliminary range-finding tests for the CCET sensitizing experiment showed that chlorothalonil was highly irritant and subsequently on induction the concentration of 3% chlorothalonil in petrolatum was chosen, with the highest challenge concentration as 0.03% chlorothalonil in MEK/OO (5/6).

The results from the CCET series studies are summarized in Tables 2 and 3. Induction with 3% according to the CCET method (Series I) resulted in a highest sensitization rate of 73% (11 positive animals of 15 exposed) at the highest challenge concentration at the 48-h reading and 93% (14 positive animals of 15 exposed) at the 72-h reading (Table 2). The challenge results were analyzed for a dose-response relationship and the fitted non-monotone curves are shown in Fig. 3. Applying logistic regression analysis to the challenge results showed that there was a statistically significant dose response. The EC_{50} for challenge at the 48-h reading was $3.1 \cdot 10^{-3}\%$ and at the 72-h reading $9.4 \cdot 10^{-4}\%$.

Induction according to the CCET method using a dose-response protocol (Series II) resulted in clear sensitization with highest sensitization rate 100% in the 1% sensitization group at 0.03 and 0.01% challenge concentrations. The sensitization and challenge results were analyzed for dose-response relationships. A dose-response relationship for sensitization was statistically significant at the 48-h reading for the 0.01% challenge concentration (Fig. 4). The challenge results gave dose response in all concentrations tested, and an example of a fitted non-monotone curve is shown in Fig. 5. Logistic regression analysis applied to the challenge results showed that there was a statistically significant dose response. The EC_{50} for sensitization at 0.03% challenge concentration at the 48-h reading was $2.4 \cdot 10^{-3}\%$ (0.09 mM) and at the 72-h reading $7.1 \cdot 10^{-4}\%$ (0.03mM).

Discussion

The present study, like previous ones, shows clearly that chlorothalonil is a potent contact sensitizer in mice and guinea pigs. Based on theoretical reason-

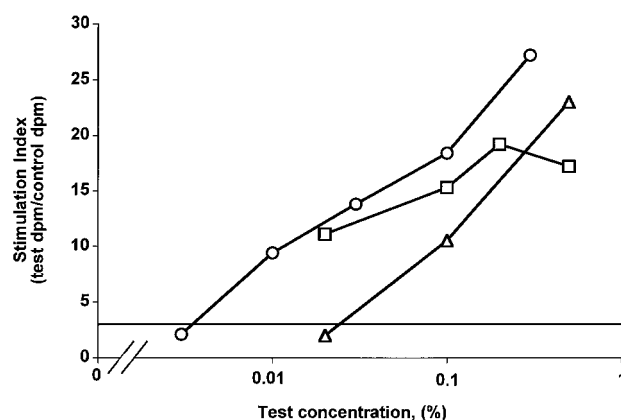


Fig. 2. Proliferation induced by chlorothalonil and DNCB in mice using the LLNA. Chlorothalonil in DMF Series I ($\square$), Series II ($\circ$) and DNCB in acetone/olive oil ($\triangle$) (from 19).

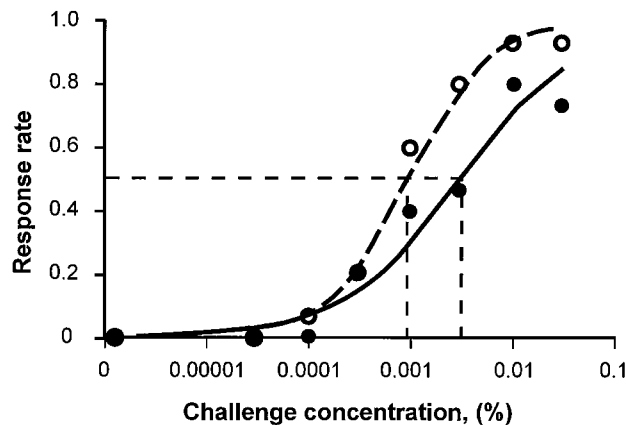


Fig. 3. Dose-response curve obtained for challenge with chlorothalonil at constant induction concentration (3%) Series I. 48-h ($\bullet$) and 72-h ($\circ$) reading.

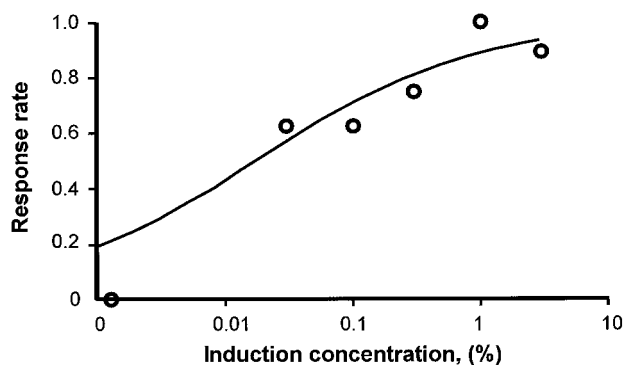


Fig. 4. Dose-response curve obtained for induction with chlorothalonil at constant challenge concentration (0.01 %) Series II. 48-h reading.

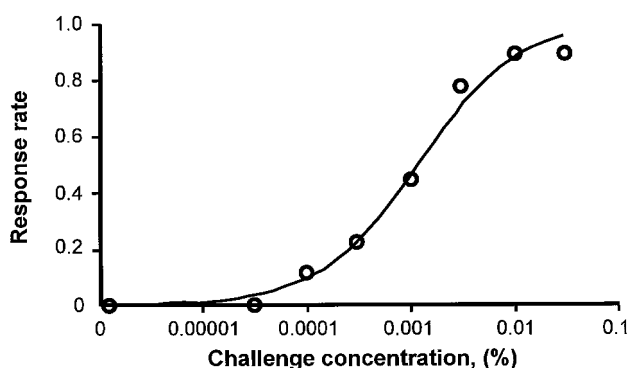


Fig. 5. Dose-response curve obtained for challenge with chlorothalonil at constant induction concentration (3 %) Series II. 72-h reading.

ing and known physicochemical data of various halobenzenes, chlorothalonil has been predicted to be a contact sensitizer, and a nucleophilic aromatic substitution was suggested as the hapten-protein reaction mechanism (27). The sensitizing property of chlorothalonil is well known from clinical experience and has also been documented by experimental studies using the guinea pig maximization test (8, 9). We chose the predictive test methods LLNA and CCET for this study since they both comprise, or are suitable for, dose-response exposure at induction and have only 1 exposure route, i.e., topical.

According to our results, chlorothalonil fulfils the current criteria for a chemical to be classified as a sensitizer in the LLNA, i.e., a dose-dependent increase in proliferation and at least 1 test concentration inducing a $3\times$ or greater increase (SI-value ≥ 3) in isotope incorporation compared with vehicle-treated controls (19). The increase in lymph node weight roughly follows the increase in stimulation index, but is a less sensitive indicator of lymph node activation.

It has been proposed that the lowest concen-

tration required to induce a stimulation index of 3 or greater in the LLNA, expressed as a $EC_{3\%}$ -value, may be used to rank the skin-sensitizing potential of chemicals (19, 28–32). The $EC_{3\%}$ -value for chlorothalonil was determined to be 0.002% (0.075 mM) in experiment 1 and to be 0.035% (0.13 mM) in experiment 2, using methods described recently (32). In Fig. 2 the sensitizing potential of chlorothalonil is compared with the extreme sensitizer DNCB. About 1 order of magnitude lower concentration of chlorothalonil is needed to induce a $3\times$ or greater increase in proliferation compared to DNCB (Fig. 2) (data from 20) and the dose-response curve coincides with those obtained with DNFB and oxazolone (20). The $EC_{3\%}$ -value for DNCB was determined to be 0.03% (1.4 mM) for DNFB, and for oxazolone to be 0.01% (0.6 mM). Chlorothalonil would thus, according to the LLNA, possess a skin-sensitizing potential comparable to those of the extreme contact sensitizers DNCB, DNFB, and oxazolone.

The ability of the LLNA to discriminate between allergens and irritants has been questioned by some investigators (20, 33–35). This inability has so far been restricted to weak-to-moderate allergens and not to strong-to-extreme allergens such as chlorothalonil.

The results from the present CCET study show that chlorothalonil is a potent sensitizer when delivered topically. The occlusive dressing applied in the CCET technique represents a slight departure from the normal route – open exposure. However, this imitates exposure under occupational conditions when contaminated gloves or boots are worn. Occlusion also prevents the animals from ingesting the compound during the animals natural fur-grooming behaviour (Lidén et al. unpublished observations), which may influence the sensitization process. The absence of adjuvant injection ensures that there is no nonspecific augmentation of the immune system in the animals, giving false-positive reactions at challenge.

The dose-response of the challenge and the sensitization results, including concentration gradients, was analysed according to Andersen et al. (24, 25). In Series I, a non-monotone dose-response relationship was seen for the challenge concentrations used at both readings (Fig. 3). This proved statistically acceptable and the EC_{50} for eliciting challenge reactions were 0.003% and 0.001% at the 48- and 72-h readings, respectively.

In Series II, a dose-dependent increase in sensitization rate was seen, however not statistically significant, ranging from 50–100% sensitization for the 3 highest challenge concentrations. The lack of statistical significance was due to the high sensitization rate in the groups tested. The EC_{50} for

sensitization was $7.1 \cdot 10^{-4}\%$ at the 72-h reading at the highest challenge concentration (0.03%). There was a clear, statistically significant dose-response relationship for challenge at all the sensitization concentrations used.

In conclusion, it is evident that chlorothalonil is an extremely potent contact allergen, inducing sensitization in 2 different species following 2 methods using only topical exposure on intact skin and no adjuvant on exposure. There was a strong, clearly dose-dependent proliferation of lymphocytes in the LLNA test, with a stimulation index well above the $3 \times$ control limit. The concentration range was comparable to our previous results with DNCB and oxazolone. Strong dose-dependent sensitization and elicitation reactions were also shown in the CCET.

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Address:

Anders Boman
Department of Occupational and Environmental Dermatology
Stockholm County Council
Norrbäcka
SE-171 76 Stockholm
Sweden