

Validity of Methods to Predict the Respiratory Sensitizing Potential of Chemicals: A Study with a Piperidinyl Chlorotriazine Derivative That Caused an Outbreak of Occupational Asthma

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Received May 2, 2003; accepted August 28, 2003

A piperidinyl chlorotriazine (PCT) derivative, used as a plastic UV-stabilizer, caused an outbreak of occupational asthma. We verified, in BALB/c mice, the sensitizing potential of PCT in comparison to a known respiratory sensitizer (toluene diisocyanate [TDI]) and a known dermal sensitizer (oxazolone), using three different methods in order to evaluate the validity of current models of sensitization. These included the local lymph node assay (LLNA) and the mouse IgE test. In addition, respiratory hyper-reactivity was assessed following a novel protocol involving dermal sensitization (20 μ l of a 3% solution on each ear for three days) and intranasal challenge (0.1% or 1%, 10 μ l per nostril on day 10), followed, after 24 h, by a methacholine challenge (using whole-body plethysmography), bronchoalveolar lavage, and histology. PCT was also used for structure-activity relationship (SAR) models for (respiratory) sensitization. High concentrations of PCT (10 and 20%) resulted in significant responses in the local lymph node assay (LLNA; stimulation indices (SI) of 2.7 ± 0.9 and 3.2 ± 0.6 , respectively). The mouse IgE test was positive with 20% PCT only. Methacholine responsiveness was increased only in previously sensitized mice receiving a challenge with TDI or PCT. However, there was no evidence for pulmonary inflammation. The SAR studies indicated that PCT could be a respiratory sensitizer. Based on an approved test protocol such as the LLNA and the mouse IgE test, PCT proved to be a weak sensitizer when compared to TDI and oxazolone. However, in a protocol involving an intranasal challenge, PCT appeared to be a respiratory sensitizer of similar potency to TDI.

Key Words: local lymph node assay (LLNA); chemical sensitization; asthma; piperidinyl chlorotriazine (PCT); toluene diisocyanate (TDI).

The present study originated from an outbreak of occupational asthma in a plastic factory, where 18 subjects out of approximately 200 workers became affected in the course of

less than two years (Breysens *et al.*, 1996). The causal agent was found to be a piperidinyl chlorotriazine derivative (PCT) that had been recently introduced to stabilize colors in polymers. The exact route and levels of exposure have not been established. However, circumstantial evidence indicates that exposure mainly occurred via inhalation and that concentrations of PCT in air must have been very low, since the material was supplied as a granular, encapsulated master batch (30% PCT dispersed in polyamide 6) that was present in very low quantities in the final product (<1%). No evidence for contact dermatitis was found in the work force. PCT was detected as the probable causal agent by skin-prick testing (without the chemical being conjugated to a protein), and it was confirmed as the cause of occupational asthma by specific bronchial provocation challenges. These gave immediate or late bronchospastic reactions after, sometimes, very low durations of exposure (<10 s) to low amounts of the chemical (<10 mg/100 g lactose) (Breysens *et al.*, 1996). The prior hazard assessment of the chemical, performed according to prevailing OECD guidelines, had indicated that it was at most a weak (skin) sensitizer, on the basis of a guinea pig maximization test (Magnusson and Kligman, 1969) in which only one animal out of 20 had a positive response (unpublished data, provided by the manufacturer of PCT).

The aim of the present study was to evaluate the validity of various methods for predicting the respiratory-sensitizing potential when applied to an unknown chemical. We first set out to evaluate the response of PCT in the local lymph node assay (LLNA). This test assesses, in mice, the potential of chemicals to cause a primary T-lymphocyte proliferative response after their topical application on the skin (Kimber and Basketter, 1992). The LLNA has been validated (Kimber *et al.*, 1995; Kimber and Weisenberger, 1989) and accepted as a reliable method for the identification of chemical allergens (OECD guideline 429, 2002). Because the LLNA involves dermal application, it is, strictly speaking, only appropriate to assess the risk of allergic contact dermatitis. However, to the extent that the response in the LLNA depends on a chemical's intrinsic immunogenic potential, it can be assumed—and it has

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indeed been proposed (Briatico-Vangosa *et al.*, 1994)—that this test detects the “generic” ability of chemicals to cause sensitization. If this is the case, then the LLNA could be relevant not only for predicting the risk of allergic dermatitis but also for the risk of occupational asthma caused by inhaled chemical sensitizers. Obviously, the risk of respiratory sensitization must also depend on physical-chemical properties of the sensitizer, which determine its potential for being inhaled, its penetration through the airway mucosa, and its interaction with specific molecules in the respiratory tract. Although, the LLNA has not been thoroughly validated with regard to the risk of respiratory sensitization, it appears prudent to consider that any chemical that emerges as a sensitizer, via skin application, might also be a respiratory allergen if it can be inhaled. This possibility is supported by recent publications (Pauluhn *et al.*, 2002). However, the converse has not been established; i.e., it is not known whether a chemical that is negative in procedures involving skin sensitization could still be a respiratory sensitizer.

We also verified the response of PCT in the mouse IgE test. This test has been proposed, though not entirely validated, as allowing discrimination between dermal and respiratory sensitizers (Dearman *et al.*, 1992; Hilton *et al.*, 1995). We then tried to test whether PCT would also elicit a respiratory response when dermally sensitized animals were challenged via the respiratory tract. In all these tests, we compared PCT with a model respiratory sensitizer, toluene diisocyanate (TDI), and a model skin sensitizer, oxazolone. Additionally, PCT was subjected to a number of structure-activity relationship (SAR) models for (respiratory and dermal) sensitization.

MATERIALS AND METHODS

Chemicals. The piperidinyl chlorotriazine derivative, 1, 3, 5-triazine-2, 4-diamine, 6-chloro-N, N'-bis(2, 2, 6, 6-tetramethyl-4-piperidinyl) (CAS # 52185-43-0) (Fig. 1), was provided, as a powder, by the factory involved in the outbreak of occupational asthma (Breysens *et al.*, 1996). Industrial experience with PCT has indicated that it must be a potent sensitizer. The material has been withdrawn from industrial use.

Dimethylformamide (DMF) and acetone were obtained from VWR (Leuven, Belgium). 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) was obtained from Sigma (Antwerp, Belgium). Toluene diisocyanate (TDI) (98% 2,4 isomer) was obtained from Aldrich (Antwerp, Belgium). Olive oil was obtained from a local store. For the first set of experiments (LLNA and mouse IgE test), oxazolone and TDI were dissolved in acetone:olive oil (AOO; 4:1); PCT was dissolved in DMF. For the experiments with the intranasal challenge, oxazolone, TDI, and PCT were dissolved in acetone:olive oil (2:3). This ratio induced little or no nonspecific reactions after intranasal challenge. Concentrations of oxazolone and PCT are given as percent (w/v) as are concentrations of TDI, given as percent (v/v). The concentrations used for TDI and oxazolone were based on literature data (Hilton *et al.*, 1995).

Animals. For the LLNA and the mouse IgE test, female BALB/c mice (6–8 weeks) obtained from Charles-River (Germany) were used. For the respiratory challenge experiments, we used male BALB/c mice (± 20 g, 6–8 weeks) obtained from Harlan (Netherlands). The mice were housed in a conventional animal house with 12-h dark/light cycles. They received lightly acidified water and pelleted food (Trouw Nutrition, Gent, Belgium) *ad libitum*. All experimental procedures were approved by the local Ethical Committee of Animal Experiments.

In vivo local lymph node assay. The assay was performed as described by Kimber *et al.* (1989) with minor modifications. Briefly, groups of mice ($n = 3-5$) were exposed topically (days 1, 2, and 3) on the dorsum of both ears, to 25 μl of the test material (TDI [3%]; oxazolone [4%]; PCT [0.05–20%]) or vehicle. On day 5, all mice were injected intravenously with 20 μCi of methyl (^3H)-thymidine [$^3\text{HTdR}$], 2 Ci mmol^{-1} from ICN Pharmaceuticals (Asse, Belgium) in 250 μl of phosphate-buffered saline (PBS, pH 7.2). Five h later, the mice were killed by an overdose of pentobarbital (90 mg/kg) and the draining auricular lymph nodes were excised, pooled for each experimental group, and weighed. A single-cell suspension of lymph node cells (LNC) was prepared, and the LNC were washed twice. $^3\text{HTdR}$ incorporation was measured by β -scintillation counting (Beckman LS 5000CE) and expressed as disintegrations per min (dpm). The stimulation index (SI) was calculated as the ratio of $^3\text{HTdR}$ incorporation by lymphocytes from treated animals relative to that from concurrent vehicle-treated controls.

Mouse IgE test. The assay was performed as described by Hilton *et al.* (1995) with modifications. Briefly, on day 1, mice were exposed topically on both shaved flanks (12-h occlusion) to 50 μl of the test material (TDI [3%], oxazolone [4%]; PCT [10–20%]) or vehicle [AOO or DMF]. On day 8, the mice were treated on the dorsum of both ears with 25 μl of the material at half the application concentration used previously. On day 9, ear thickness was measured using a digimatic caliper, taking an average of two measurements per ear. On day 15, mice received an overdose of pentobarbital, and they were exsanguinated. A blood smear was prepared to count eosinophils in whole blood. Serum was separated by centrifugation and stored at -20°C until analysis. Serum IgE was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). For the coating, we used rat monoclonal anti-mouse IgE antibody (Biogenesis, Poole, England). In preliminary experiments, a standard curve was obtained using a mouse IgE standard (Monoclonal Anti-Dinitrophenyl, Sigma Catalog #D8406). However, when other batches of this mouse IgE standard were used in the actual experiments, they proved inadequate. Therefore, total serum IgE was expressed as changes in optical density (OD) in the final step of the ELISA, and values in treated mice were compared with those obtained in control mice (with all serum dilutions being similar and after subtraction of blank values).

Single respiratory challenge test

Treatment protocol. BALB/c mice received 20 μl of the test material (TDI, 3%; oxazolone, 3%; PCT, 3%) or vehicle on each ear for three consecutive days (days 1, 2, and 3). On day 10, they received an intranasal challenge with the same chemical (10 μl per nostril; TDI, 0.1%; oxazolone, 0.1%; PCT, 1%) or vehicle, under light anesthesia with diethyl ether. After the intranasal challenge, mice were placed, unrestrained, in a whole-body plethysmograph (EMKA Technologies, Paris, France) and their ventilation was followed for 6 h.

Test and control groups. Each experiment comprised four groups. In the 1/1 group, mice received both dermal application and intranasal challenge with the test chemical. Three control groups were included: 1/0 (dermal application of chemical; intranasal challenge with test vehicle), 0/1 (dermal application of vehicle; intranasal challenge with test chemical), and 0/0 (both dermal application and intranasal challenge with vehicle).

Measurement of airway responsiveness. Twenty-four h after intranasal challenge (day 11), reactivity to methacholine was assessed in the whole-body plethysmograph, following the procedure of Hamelmann *et al.* (1997). Briefly, the enhanced pause (Penh) was calculated for each mouse under resting conditions (baseline) and after nebulizing incremental doses of aerosolized methacholine (0, 10, 25, 50, or 100 mg/ml) for 1 min. An average Penh over 30 s was calculated during 3 min (6 measurements) and the mean of these 6 values was used for each condition.

Autopsy—broncho-alveolar lavage (BAL)—histology. After the methacholine challenge (day 11), mice were anesthetized with pentobarbital (90 mg/kg), ip, and sacrificed. A blood sample was drawn from the inferior vena cava. The left lung was removed from the thorax, weighed (wet weight), dried (24 h at $75-85^\circ\text{C}$), and weighed again (dry weight). The right lung was

laved three times with 0.4 ml sterile saline (0.9% NaCl) at room temperature. For differential cell counts, 250 μ l (40,000 cells/ml) of the resuspended cells were spun (1400 \times g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) upon microscope slides, air-dried and stained with the Diff-Quik® method. From each slide, 3 \times 100 cells were differentiated into macrophages, eosinophils, neutrophils, or lymphocytes.

After the lavage, the right lung was instilled with 10% formaldehyde until full inflation of all lobes was seen. Hematoxylin & eosin-stained slices (5 μ m sections) from all lung lobes were examined by an experienced pathologist, who evaluated lung injury without knowledge of the animal's treatment.

Mouse total IgE ELISA. Total IgE in serum (diluted 1/80) was measured using the OptEIA™ Mouse IgE Set from Pharmingen (San Diego, CA), according to the manufacturer's instructions, with the use of anti-mouse IgE, biotinylated anti-mouse IgE, and horseradish peroxidase conjugate.

Data analysis. All results are presented as means with standard deviation. Statistical analysis was performed using GraphPad Prism 3.01 (1999, Graph-Pad Software, Inc., San Diego CA) and consisted of one-way analysis of variance followed by Dunnett's test for comparing treated with control data. A level of $p < 0.05$ was considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Structure-activity relationship (SAR) of PCT. For the computational SAR modeling, we used the MCASE expert system (Klopman, 1984, 1992; Klopman and Rosenkranz, 1984, 1994). Application of this method yields molecular descriptors associated with biological activity (e.g., chemical sensitization). These are derived from learning sets composed of active and inactive molecules. The descriptors are readily recognizable: single continuous structural fragments that are embedded in the complete molecule (Fig. 1). The descriptors consist of either activating fragments (biophores) or inactivating fragments (biophobes). Each of these fragments is associated with a confidence level and a probability of activity that is derived from the distribution of these biophores and biophobes among active and inactive molecules in the learning sets. The biophores and biophobes can be used to facilitate mechanistic understanding of particular biological phenomena or as predictors of activity of unknown compounds.

For this study we used previously described MCASE SAR models. Three allergic contact-dermatitis (ACD) models were used: one developed solely from human data, one from data obtained for Guinea pigs (GP), and one model based on data from both species (human + GP) (Gealy *et al.*, 1996; Graham *et al.*, 1996). The human ACD model consisted of 767 compounds, 355 of which are allergens, and is approximately 76% predictive for compounds external to the model. The GP ACD model consisted of 217 compounds, 134 of which induced allergic responses, and is approximately 66% predictive. The human + GP ACD model consisted of 1033 compounds, 316 of which are allergens, and is approximately 88% predictive. The human respiratory hypersensitivity model has also been described previously (Graham *et al.*, 1997). It consisted of 80 compounds, 40 of which caused respiratory hypersensitization, and is approximately 77% predictive for compounds external to the model.

RESULTS

Local Lymph Node Assay

As expected from the literature (Hilton *et al.*, 1995; Kimber *et al.*, 1989), the LLNA was strongly positive after exposure to 3% TDI and 4% oxazolone. The lymph-node weight (LNW) increased 6-fold for TDI and 7-fold for oxazolone (Fig. 2a), and the stimulation indices reached mean values of around 30 (TDI) and 20 (oxazolone) (Fig. 2b). The LLNA, using 0.05%, 0.5% and 2% PCT, gave entirely negative results. However, when higher concentrations of PCT (10 and 20%) were tested later, positive responses were obtained, although the increases in LNW and values of SI (2.7 ± 0.9 and 3.2 ± 0.6 , respectively) were much less than for TDI or oxazolone.

Mouse IgE Test

On day 9, ear thickness was clearly increased after dermal challenge with TDI (0.39 ± 0.05 mm) and oxazolone (0.55 ± 0.04 mm) compared with the AOO control (0.26 ± 0.02 mm). PCT 20% (0.30 ± 0.07 mm) did not induce any significant swelling compared to the DMF control (0.28 ± 0.04 mm).

Figure 3 shows that the IgE concentration (day 15) in serum was significantly increased in mice treated with TDI, but not with oxazolone. In mice treated with PCT, only those who received the highest concentrations of PCT (20%) showed increased serum IgE concentrations.

Blood eosinophils (in percent of white blood cells) were increased, compared with the AOO control ($1.9 \pm 0.1\%$), in mice treated with TDI ($4.0 \pm 2.5\%$), but not with oxazolone ($1.5 \pm 1.9\%$). Eosinophils were not changed in mice treated with 10% PCT ($1.5 \pm 1.9\%$) and 20% PCT ($1.4 \pm 1.7\%$), compared with DMF ($2.0 \pm 1.5\%$).

Single Respiratory Challenge Test

Ventilatory function measurements during the first 6 h after intranasal challenge with TDI, oxazolone, and PCT did not show changes in the breathing pattern, and no early bronchoconstriction could be detected.

Figure 4 (left panel) shows the methacholine-response

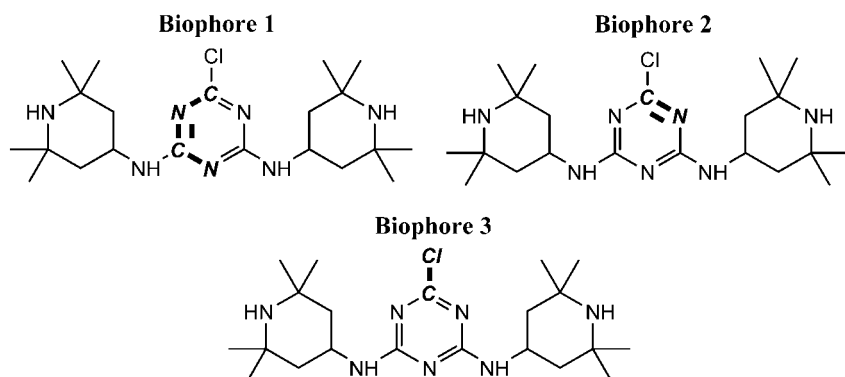
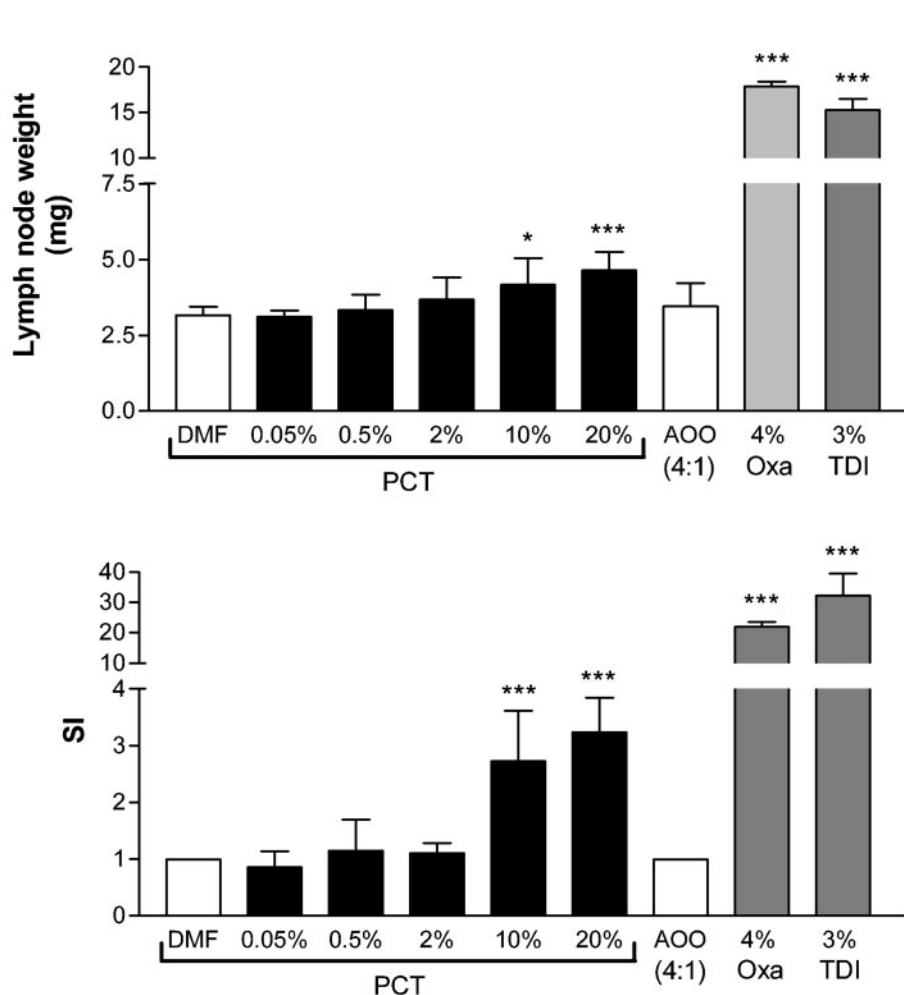


FIG. 1. Chemical structure of piperidinyl chlorotriazine derivative (PCT). MCASE biophore 1, 2, and 3 associated with chemical sensitization in human and guinea pigs and biophore 3 associated with respiratory hypersensitivity in humans are indicated in boldface type.

FIG. 2. Local lymph node assay (LLNA): (a) Auricular lymph node weight (LNW), (b) stimulation index (SI). On days 1, 2, and 3, mice were dermally sensitized on both ears. On day 5, they were intravenously injected with 20- μ Ci [methyl- 3 H]-thymidine (3 HTdR), and 5 h later the auricular lymph nodes were removed, pooled, and weighed (LNW). The SI was calculated as the ratio of 3 HTdR incorporation by lymphocytes from treated animals relative to that from concurrent vehicle-treated controls. Toluene diisocyanate (TDI) and oxazolone (OXA) were dissolved in acetone olive oil (AOO); piperidinyl chlorotriazine derivative (PCT) was dissolved in dimethylformamide (DMF). In each experiment, the lymph nodes of 3 to 5 mice were pooled. Bars represent means (\pm SD) of 3 to 7 experimental groups, except for TDI, where there were two groups (values of these two experiments are indicated by horizontal lines); * p < 0.05, *** p < 0.001, compared to controls.



curves obtained 24 h after the intranasal challenge. TDI-sensitized and -challenged mice (1/1) showed significantly higher methacholine reactivity than all control groups (1/0, 0/1, and

0/0) (Fig. 4a, left). Oxazolone-sensitized and -challenged mice (1/1) showed no significant difference in methacholine reactivity compared to the control groups (1/0, 0/1, 0/0) (Fig. 4b, left). PCT-sensitized and -challenged mice (1/1) showed a higher methacholine reactivity, compared with the control groups (1/0, 0/1, and 0/0) (Fig. 4c, left).

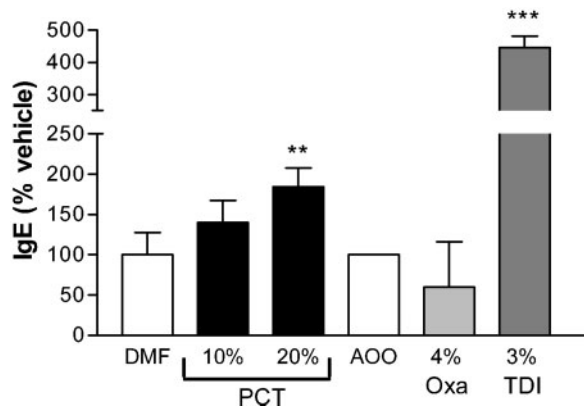
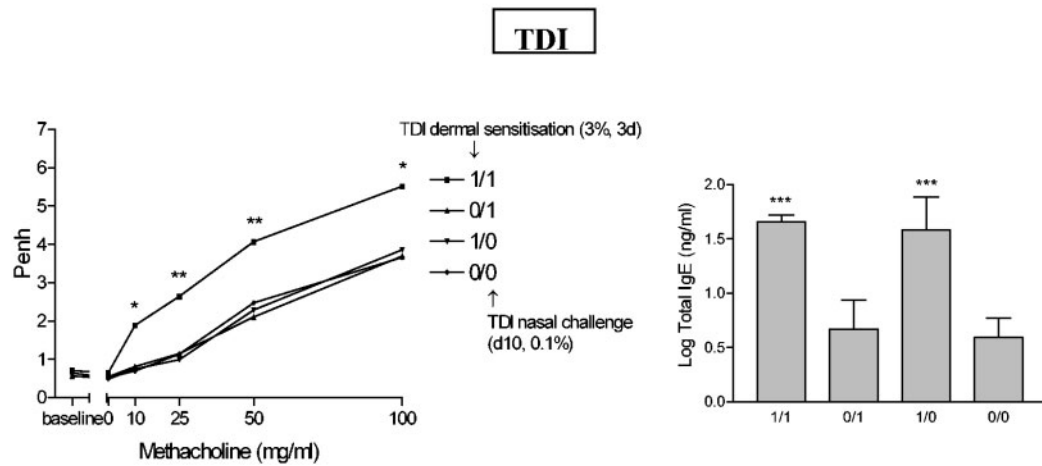


FIG. 3. Mouse IgE test: Total serum IgE (day 15) in mice dermally sensitized (day 1, on abdomen) and dermally challenged (day 8, on dorsum of both ears). Total serum IgE was measured by ELISA and expressed as changes in optical density (OD). Abbreviations as in Figure 2. Bars are means \pm SD; n = 8–14 mice; ** p < 0.01, and *** p < 0.001, compared to control.

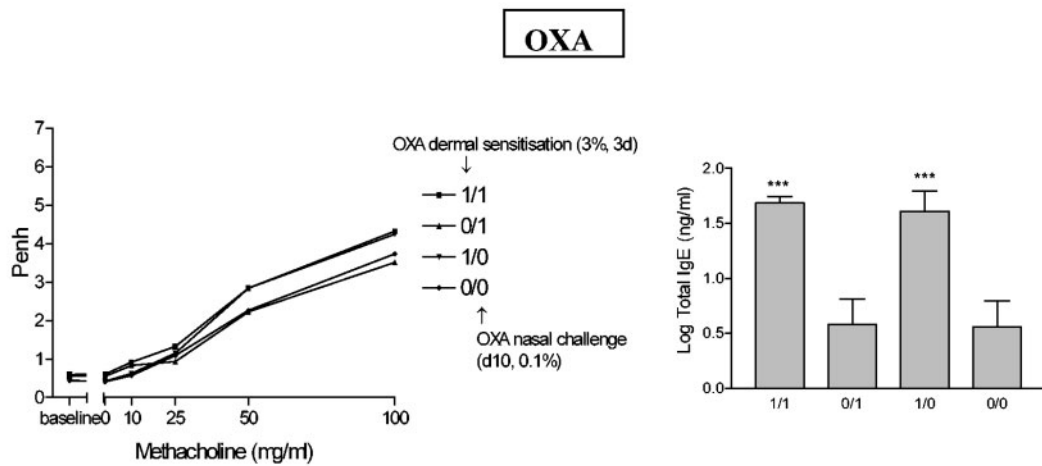
Figure 4 (right panel) shows total IgE in the serum of these mice at day 11. Mice sensitized with TDI (Fig. 4a, right) or oxazolone (Fig. 4b, right) (1/1 and 1/0) showed an increased total IgE, compared to mice sensitized with AOO (0/1 and 0/0). Mice treated with PCT (sensitization and/or challenge) (Fig. 4c, right) had slight, nonsignificant increases in serum total IgE.

Bronchoalveolar lavage and lung histology showed no signs of pulmonary inflammation (data not shown) in any of the groups or treatments. BALF of all mice contained only macrophages (99–100%) and no eosinophils, neutrophils, or lymphocytes could be detected. Dry and wet left-lung weights did not differ between treatment groups. Histology also did not reveal any inflammation in the mice treated with TDI, oxazolone, or PCT. There were no infiltrates of eosinophils,

a



b



c

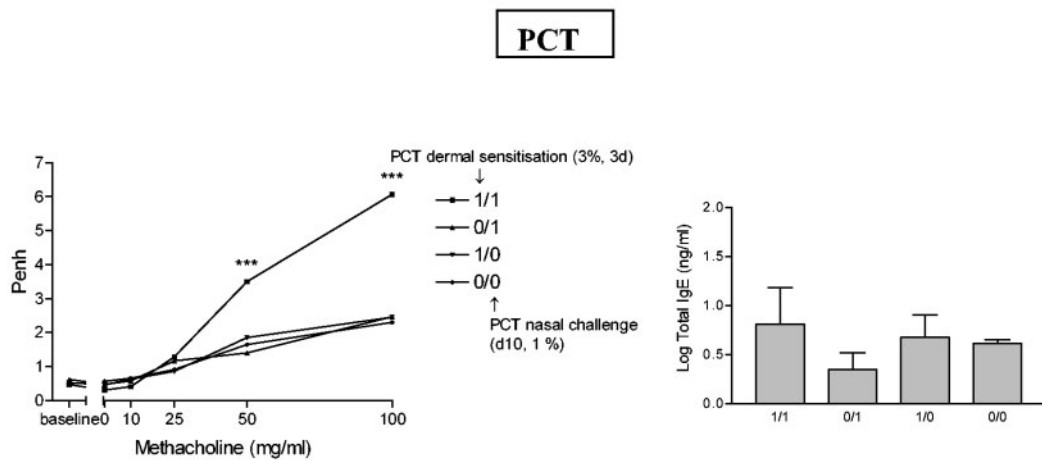


FIG. 4. Single respiratory challenge test: Methacholine responsiveness (left) and total serum IgE (right). On days 1, 2, and 3, mice were dermally sensitized with the test chemical or the vehicle. On day 10, they received an intranasal challenge with the same test chemical or the vehicle. On day 11, airway reactivity (Penh) to increasing concentrations of methacholine was assessed by whole-body plethysmography. Afterwards blood was drawn from the vena cava for analysis of serum IgE concentrations. (a) Toluene diisocyanate (TDI), $n = 8$ mice; (b) Oxazolone (OXA), $n = 8$ mice; (c) piperidinyl chlorotriazine derivative (PCT), $n = 10$ mice; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to 0/0 control.

neutrophils, or lymphocytes around blood vessels or the bronchi.

Structure-Activity Relationship (SAR) of PCT

PCT was predicted to be a contact allergen by both the GP and human + GP allergic contact dermatitis models. These predictions were based on the substituted triazine portion of the molecule (Biophores 1 and 2, Fig. 1). Three GP sensitizers contained biophore 1, while no nonsensitizers in this model possessed it. For the combined human + GP model, five sensitizers possessed biophore 2 and no nonsensitizers contained it. Moreover, for the human + GP model, 31 compounds contained biophore 3, which is a chloro-substituted triazine moiety; 27 of these were sensitizers and four were not. Likewise, PCT was also predicted to be a human respiratory sensitizer. This prediction was based on the same chloro-substituted triazine biophore as described for the human + GP model. In the instance of the respiratory sensitization model, five compounds possessed the biophore, all of which were respiratory sensitizers.

DISCUSSION

This study was initiated following the discovery that PCT, a novel UV stabilizer in polyester, had caused a major outbreak of occupational asthma. Clinical evidence, including positive responses to skin-prick testing with (unconjugated) PCT and positive bronchial challenge tests with low amounts, suggested that PCT was a (potent) respiratory sensitizer (Breysens *et al.*, 1996). On the basis of a standard hazard assessment, i.e. a guinea pig maximization test (Magnusson and Kligman, 1969), PCT had been considered to have only a weak sensitization potential, if any. The Magnusson-Kligman test was (and still is) considered a sensitive method for assessing the sensitization potential of chemicals (OECD guideline 406, 1992), although

its validity for predicting respiratory sensitization has never been established. The purpose of our study was to further investigate the sensitization potential of PCT, using more recent testing protocols such as the LLNA and the mouse IgE test, and to verify the validity of these procedures for identifying a potent respiratory sensitizer. Table 1 gives an overview of the results from the three treatment protocols.

Our initial experiments indicated that PCT was negative in the LLNA: three concentrations (0.05%, 0.5% and 2%) in a suitable solvent (DMF) gave consistent negative responses, although two concurrently tested control substances (TDI and oxazolone) yielded the expected responses (Hilton *et al.*, 1995). Having been advised to test higher concentrations, we obtained significantly positive responses with 10 and 20% PCT. However, the magnitude of the increases in LNW and the SI (2.7 and 3.2 for 10% and 20% PCT, respectively) were much lower than with TDI and oxazolone. Kimber *et al.* (1989) have proposed an SI of 3 or more as biologically significant. Chemicals having an EC₃ value (defined as the amount of chemical required to induce an SI of 3) above 10% are considered as having very low sensitizing potency (Kimber *et al.*, 2002). The positivity of PCT in the LLNA is reassuring with regard to the ability of the LLNA to identify sensitizers, including respiratory sensitizers, but the weak response suggests that the magnitude of the response should not be regarded as indicative of the risk of asthma (Kimber *et al.*, 2001). No evidence of contact dermatitis was found in the asthma outbreak caused by PCT (Breysens *et al.*, 1996).

Having established that PCT was capable of causing primary sensitization, we set out to verify whether this sensitization would result in a "respiratory type" of response in the mouse IgE test (Dearman *et al.*, 1992). As expected, the mouse IgE test was negative with oxazolone (a dermal sensitizer) and strongly positive with TDI (a respiratory sensitizer). Again, there was a weakly positive response to PCT, i.e., a nonsig-

TABLE 1
Overview of the Results of the Three Different Test Protocols

	Conc. (%)	LLNA		Mouse-IgE-test		Single respiratory challenge test	
		LNW	SI	Ear thickness	Total serum IgE	AHR	Total serum IgE
TDI	3	+++	+++	++	+++	++	+++
Oxazolone	4	+++	+++	+++	-		
Oxazolone	3					-	+++
PCT	0.05	-	-				
PCT	0.5	-	-				
PCT	2	-	-				
PCT	3					+++	±
PCT	10	+	+		±		
PCT	20	+	+	-	+		

Note. LLNA, local lymph node assay; LNW, lymph node weight; SI, stimulation index; AHR, airway hyperresponsiveness assessed by methacholine test. Conc., concentration of the test compound applied on the skin (three daily applications for the LLNA and the single respiratory challenge test, one application in the mouse IgE test). Empty cells indicate that the compound was not tested at this concentration: (-) no change; (±) nonsignificant increase, $p > 0.05$; (+) significant increase, $p < 0.05$; (++) significant increase, $p < 0.01$; (+++) strong significant increase, $p < 0.001$.

nificant increase of total serum IgE with 10% PCT, and a significant approximate doubling in IgE concentration with 20% PCT. In this mouse IgE test, we could not measure actual concentrations of total IgE but only relative changes in optical density in the final step of the ELISA, as has been done by others (Scheerens *et al.*, 1999). Admittedly, this mode of expression precludes us from having an exact quantification of total serum IgE. Nevertheless, the observed changes correctly reflect the qualitative and probably also the quantitative response to TDI (strong induction of total IgE), oxazolone (no induction), and PCT (marginal to weak induction) in the mouse IgE test when performed as proposed by Hilton *et al.* (1995). Interestingly, blood eosinophils (an endpoint not included in the protocol of Hilton) were also increased with TDI, but not with oxazolone or PCT. In the outbreak of PCT-induced asthma, many patients had an exposure-related increase in blood eosinophils (Breysens *et al.*, 1996). From these mouse IgE tests, we concluded that PCT behaves as a respiratory sensitizer. However, as with the LLNA, the strength of the total IgE response does not appear to reliably reflect the potency of the test agent to cause respiratory sensitization in humans.

Examining validated SAR models of skin (Gealy *et al.*, 1996; Graham *et al.*, 1996) and respiratory sensitizers (Graham *et al.*, 1997), the relationship between PCT and other compounds known to induce allergic responses suggests that PCT may be acting as a sensitizer through mechanisms related to previously tested compounds. Analysis of PCT for dermal sensitization using data based solely on human experience suggested that PCT is not a human allergen. However, it is predicted to be a skin allergen by the GP and human + GP models. This discrepancy is explained by the fact that the human model did not contain the appropriate structural information on which to base a prediction. In other words, the human ACD model did not identify the particular triazine substitution pattern or the specific chlorine substituent on which to derive biophores associated with activity, because compounds with these features were not in the model's learning set. Analysis of PCT for respiratory sensitizing activity indicates that it has the potential to be a respiratory allergen also based on its possession of a chloro-substituted triazine moiety. Overall, the SAR analysis yielded conclusions in agreement with the available human clinical experience and the present experimental findings.

To verify further the relevance of the LLNA and the mouse IgE test in relation to occupational asthma, we evaluated a number of relevant respiratory endpoints after administration of the test agents via the airways. These limited experiments are part of a broader study in which we are trying to develop and validate a model of chemical-induced occupational asthma. We found that TDI-sensitized and PCT-sensitized mice exhibited increased ventilatory responsiveness to methacholine 24 h after an intranasal challenge with TDI and PCT, respectively. This response was clearly dependent on prior sensitization since it was neither observed in control nonsensitized animals receiving the test chemical, nor in sensitized

and nonsensitized mice receiving the vehicle. Various animal models of asthma show that it is valid to use increases in bronchial responsiveness at 24 h after challenge as a physiological endpoint (Matheson *et al.*, 2002; Tomkinson *et al.*, 2001). In humans too, it is known that nonspecific bronchial responsiveness increases after challenge with a sensitizer (Malo *et al.*, 1990; Sastre *et al.*, 2003). Although unrestrained whole-body plethysmography has been validated for evaluating methacholine responsiveness in mice (Hamelmann *et al.*, 1997), and although it has been widely used in experimental asthma research (McGraw 2002; Shen *et al.*, 2003), this method has been strongly criticized as being inadequate for assessing airway mechanics (Bates and Irvin 2003; Lundblad *et al.*, 2002). While an increase in Penh does not correctly reflect bronchial obstruction, whole-body plethysmography can and does assess ventilatory patterns. Consequently, regardless of the physiological basis for the observed ventilatory responses in our experiments, there is no doubt (because our design included all necessary control groups) that methacholine responsiveness increased only in mice that had been previously sensitized and subsequently challenged to TDI or PCT, and not to oxazolone.

Interestingly, the methacholine hyperresponsiveness observed with PCT was obtained following a dermal sensitization protocol that used a concentration of PCT of 3%, i.e., well below the EC₃ of PCT (>10%) in the LLNA. The respiratory response to PCT was also quantitatively more similar to that elicited by TDI than had been the case with the LLNA and the mouse IgE test. These observations, and the absence of a ventilatory response with oxazolone, suggest that the functional respiratory responses to PCT and TDI specifically reflect respiratory immune responses.

Although methacholine hyperresponsiveness was present one day after challenge, there was no detectable early-ventilatory response, and there was no evidence of bronchial inflammation. The absence of inflammatory changes contradicts the theory (Maddox and Schwartz, 2002) that, in asthma, nonspecific bronchial hyperresponsiveness is caused by inflammation. Our findings confirm that this view may not be universally true, as shown by Brewer *et al.* (1999). The limited response found here is probably related to the sensitization protocol used, because we have evidence from subsequent unpublished experiments (Vanoirbeek *et al.*, 2003) that giving a dermal boost of TDI between sensitization and challenge leads to pronounced early increases in Penh, as well as a marked influx of neutrophils (and eosinophils) in bronchoalveolar lavage on the next day.

Besides differences in immunological responsiveness, it is possible that the reason for the absence of pulmonary inflammation in the present experiments relates to the intranasal administration. This may have allowed too little test agent to reach the lower airways to cause inflammation. Intranasal administration is a convenient and also an effective procedure to administer chemical agents to the lungs, when they are dissolved in aqueous solutions (Southam *et al.*, 2002), but this

seems to be much less the case for more viscous suspensions such as acetone/olive oil used here (Ebino *et al.*, 1999). However, we have unpublished evidence that even with this mode of administration pronounced bronchial inflammation can be achieved (Vanoirbeek *et al.*, 2003).

The respective contribution of the nose and lower airway in determining changes in Penh are not known. It is possible that the changes in methacholine responsiveness are to some extent also dependent on nasal responses. We do not have histology of the nasal mucosa to verify whether there was inflammation in the nose. However, regardless of the exact anatomical site of the changes leading to the increase in methacholine responsiveness, it remains that the change in responsiveness was clearly dependent on prior sensitization.

At this stage we can only speculate about the possible mechanisms underlying the respiratory response of TDI- and PCT-sensitized mice following intranasal challenge with TDI and PCT, respectively. Most research into animal models of asthma has been done using high molecular weight agents such as ovalbumin (Lloyd *et al.*, 2001; Tomkinson *et al.*, 2001), and only a limited number of research groups have attempted to produce animal models of chemical-induced occupational asthma in mice (Herrick *et al.*, 2002; Matheson *et al.*, 2002; Redlich *et al.*, 2002; Scheerens *et al.*, 1996) or other animals (Arts *et al.*, 2001; Pauluhn *et al.*, 2000). These have generally involved diisocyanates or acid anhydrides. As discussed by Redlich *et al.* (2002), nobody has been able, so far, to reproduce the full phenotype of either atopic asthma or chemical-induced asthma in experimental animals. At least in the case of hexamethylene diisocyanate, it seems that both Th1-type and Th2-type cytokines may be involved, although the sensitizing dose may influence the response and the Th1/Th2 balance (Herrick *et al.*, 2002). Interestingly, in the protocol involving respiratory challenge, both TDI- and oxazolone-sensitized mice had a significant increase in total serum IgE one day after the challenge, regardless of whether the challenge had been done with vehicle (AOO) or test chemical (TDI or oxazolone). For PCT-sensitized and -challenged mice, there were no significant changes in total IgE. These results do not invalidate those obtained in the mouse IgE-test, where oxazolone gave no increase in IgE, and PCT did cause one. Indeed, the protocol of the mouse IgE test differed considerably from that which involved a respiratory challenge, with regard to sensitization procedure (day 1 *versus* day 1, 2, and 3), challenge time and route (day 8 on the skin *versus* day 10 in the nose), and delay between serum analysis and challenge (day 15 *versus* day 11). Our observations that the changes in hyperresponsiveness did not correlate with those in total serum IgE, are compatible with the findings of others (Herrick *et al.*, 2002; Scheerens *et al.*, 1999) that hyperresponsiveness is independent of IgE levels. The role of IgE and humoral factors in the pathophysiology of chemical-induced asthma is still unclear and being debated (Kimber and Dearman, 2002; Mapp *et al.*, 1994; Redlich *et al.*, 2002). Consequently, other cellular mechanisms must play a role. We propose, according to hypotheses put forward by others (Dear-

man and Kimber, 2001), that the pattern of lymphocyte stimulation caused by the epicutaneous application of TDI and PCT is more conducive to producing a respiratory response upon a subsequent intranasal challenge with the same chemicals than when sensitization is done with oxazolone. Studies are ongoing using cytokine and lymphocyte profiling (Dearman *et al.*, 2003) to address this hypothesis. There are, however, other alternatives, including differences in the ability of the different chemicals to haptenize rapidly with critical proteins and/or to reach appropriate immunological targets in the respiratory tract.

In summary, the novel findings of our study are that we have confirmed the respiratory sensitizing capacity of PCT, an agent that caused an outbreak of occupational asthma. Compared to TDI and oxazolone, PCT proved to be a weak dermal sensitizer (LLNA) and a weak respiratory sensitizer (mouse IgE test) according to conventional test procedures. However, using a novel procedure involving a respiratory (intranasal) challenge, PCT was found to be a strong respiratory sensitizer.

ACKNOWLEDGMENTS

We thank E. K. Verbeken for his work on the lung histology, Jan L. Ceuppens and M. Tarkowski for their advice, the DWTC for funding this research (PS/01/43), and the physician of the factory where the outbreak occurred.

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