

Validation of a mouse model of chemical-induced asthma using trimellitic anhydride, a respiratory sensitizer, and dinitrochlorobenzene, a dermal sensitizer

Jeroen A. J. Vanoirbeek, PhD,^a Maciej Tarkowski, PhD,^{a,b} Hadewijch M. Vanhooren, MSc,^a Vanessa De Vooght, MSc,^a Benoit Nemery, MD, PhD,^a and Peter H. M. Hoet, PhD^a *Leuven, Belgium, and Lodz, Poland*

Background: Occupational asthma can be caused by chemicals. Previously, we established a murine model of immunologically mediated chemical-induced asthma using toluene diisocyanate.

Objective: We sought to verify this model using trimellitic anhydride (TMA), a respiratory sensitizer, and 1-chloro-2,4-dinitrobenzene (DNCB), a dermal sensitizer.

Methods: BALB/c mice received dermal applications (vehicle or chemical) on days 1 and 7. On day 10, they received an intranasal instillation (vehicle or chemical). Whole-body plethysmography (enhanced pause) was used to monitor changes in ventilatory function and methacholine reactivity.

Pulmonary inflammation was assessed by using bronchoalveolar lavage (cells, TNF- α levels, and macrophage inflammatory protein 2 levels). Immunologic parameters included total serum IgE levels, lymphocyte distribution in auricular and cervical lymph nodes, and IL-4 and IFN- γ levels in supernatants of lymph node cells incubated with or without concanavalin A.

Results: Mice dermally treated and intranasally challenged with TMA experienced markedly increased enhanced pause immediately after intranasal challenge and increased methacholine reactivity (24 hours later). Mice similarly treated with DNCB did not show any ventilatory changes. Neutrophil influx and increased macrophage inflammatory protein 2 and TNF- α levels were found in bronchoalveolar lavage fluid in both TMA- and DNCB-treated mice. The proportion of CD19⁺ B cells was increased in auricular and cervical lymph nodes of TMA-treated mice. IL-4 and IFN- γ levels were increased in supernatants of concanavalin A-stimulated auricular and cervical lymph node cells of TMA- or DNCB-treated mice; however, the relative proportions of IL-4 and IFN- γ levels

differed between TMA- and DNCB-treated mice. Serum total IgE levels were increased in TMA-treated mice only.

Conclusion: Both compounds induce a mixed T_H1-T_H2 response, but only TMA induced ventilatory changes.

Clinical implications: In the workplace avoiding skin contact with chemical sensitizers might be advised to prevent chemical-induced asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Trimellitic anhydride, 1-chloro-2, 4-dinitrobenzene, occupational asthma, murine model, T-lymphocytes subtypes, IL-4, IFN- γ , macrophage inflammatory protein 2, TNF- α

Occupational asthma is the most prevalent occupational lung disease in developed countries.¹ More than 250 agents are known to be capable of causing occupational asthma, and more will be recognized in the future.² The prevalence of occupational asthma in various occupational cohort studies depends on the agent or agents to which the workers are exposed, the level of exposure,³ and, to a lesser degree, host susceptibility factors, such as cigarette smoking, atopy, and other genetic factors.⁴ Occupational asthma is characterized by reversible airway obstruction, airway inflammation, and nonspecific airway hyperreactivity causally related to exposure to an agent present in the workplace.⁵

Low-molecular-weight chemical sensitizers are haptens that form complete antigens by combining with proteins. These chemical sensitizers are often classified as skin sensitizers or respiratory sensitizers. Chemicals that are known to cause respiratory allergy include isocyanates (eg, toluene diisocyanate [TDI]), acid anhydrides (eg, trimellitic anhydride [TMA]), reactive dyes, platinum salts, and glutaraldehyde.⁶ 1-Chloro-2,4-dinitrobenzene (DNCB) and oxazolone are typical dermal sensitizers known to induce mainly contact dermatitis.⁷ Dermal sensitizers can be identified by the Magnusson and Kligman guinea pig maximization test⁸ and the murine local lymph node assay (LLNA).⁹ Recently, considerable effort has been devoted to the development of test methods for the prospective identification of chemicals with the potential to cause respiratory sensitization.¹⁰

TMA is a respiratory sensitizer in human subjects,¹¹ but it is also capable of inducing airway irritation in animals.¹²

From ^athe Laboratory of Pneumology, Unit of Lung Toxicology, Katholieke Universiteit Leuven, Leuven, and ^bthe Department of Immunotoxicology, Nofer Institute of Occupational Medicine, Lodz.

Supported by the Belgian Federal Science Policy Office (PS/01/43) and "FWO-Flanders" (project 7.0024.00).

Received for publication May 17, 2005; revised December 31, 2005; accepted for publication January 12, 2006.

Reprint requests: Benoit Nemery, MD, PhD, Katholieke Universiteit Leuven, Laboratory of Pneumology, Unit of Lung Toxicology, Herestraat 49 bus 706, 3000 Leuven, Belgium. E-mail: ben.nemery@med.kuleuven.be.

0091-6749/\$32.00

© 2006 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2006.01.027

Abbreviations used

AOO:	Acetone olive oil
AUC:	Area under the curve
BAL:	Bronchoalveolar lavage
ConA:	Concanavalin A
DNCB:	1-Chloro-2,4-dinitrobenzene
LLNA:	Local lymph node assay
MIP-2:	Macrophage inflammatory protein 2
Penh:	Enhanced pause
TDI:	Toluene diisocyanate
TMA:	Trimellitic anhydride

It has been shown that high doses of TMA, when applied to the skin, preferentially induce a T_H2 response, with increased levels of IL-4, IL-10, and IL-13 and relatively low amounts of the T_H1 cytokine IFN- γ in draining lymph nodes.¹³ Recently it has also been shown that Brown Norway rats dermally sensitized to TMA had asthma-like early and late changes in breathing pattern after airway exposure to TMA.^{12,14,15} DNCB is a typical dermal sensitizer that elicits contact hypersensitivity mediated by T_H1 cytokines, such as IFN- γ and IL-12,¹⁶ and it does not appear to cause occupational asthma or allergic rhinitis.¹⁷

The primary objective of this study was to examine the capability of our previously described mouse model of chemical-induced asthma^{18,19} to discriminate between a respiratory sensitizer (TMA) and a dermal sensitizer (DNCB). Our results show that TMA-sensitized mice exhibit an early ventilatory response after intranasal instillation with TMA and increased airway reactivity 24 hours later. This did not occur in mice that were similarly treated with DNCB. Nevertheless, both TMA- and DNCB-treated mice showed a mixed T_H1 - T_H2 cytokine profile in the draining superficial cervical and retroauricular lymph nodes.

METHODS

A complete description of the materials and methods used is available in the Online Repository at www.jacionline.org.

TMA and DNCB were dissolved in a vehicle (acetone olive oil [AOO]) consisting of a mixture of 2 volumes of acetone and 3 volumes of olive oil. Concentrations of TMA and DNCB are given as percentages (wt/vol) in AOO.

Male BALB/c mice (6 weeks old) received dermal applications of 20 μ L of vehicle or 20 μ L of chemical (5% TMA or 0.2% DNCB) on each ear on days 1 and 7. On day 10, they received, after achievement of light diethyl ether anesthesia, an intranasal instillation of 10 μ L of vehicle or 10 μ L of chemical (1% TMA or 0.02% DNCB) in each nostril. In all experiments treatment with TMA is indicated with a capital T, treatment with DNCB with a capital D, and treatment with the vehicle is indicated as 0. All experimental groups are indicated with 3 symbols: the first and second symbols indicate the dermal treatment on days 1 and 7, whereas the third symbol indicates the intranasal instillation on day 10. Thus the T/T/T group consists of mice that received dermal applications of TMA on days 1 and 7 and intranasal instillations of TMA on day 10, whereas the 0/0/0 control group consists of mice that received AOO

on all occasions. Other groups were 0/0/T, T/0/T, 0/0/D, D/0/D, and D/D/D.

On day 10, the ventilatory function (enhanced pause [Penh]²⁰) of each mouse was first recorded under resting conditions and unrestrained for 5 minutes in a whole-body plethysmograph (EMKA Technologies, Paris, France). Immediately after the intranasal instillation, mice were placed again in the whole-body plethysmograph (0 time point), and their ventilatory parameters were measured for 40 minutes. The area under the curve (AUC) of Penh against time between 0 and 40 minutes was calculated for each individual mouse, and this figure was used for statistical analysis.

On day 11 (ie, 22 hours after intranasal instillation), reactivity to methacholine was assessed by means of whole-body plethysmography according to the procedure of Hamelmann et al.²⁰ Briefly, Penh was calculated for each mouse under resting conditions (baseline) and after inhalations during 1 minute of nebulized methacholine (successively 0, 10, 25, 50, and 100 mg/mL). For each mouse, Penh was plotted against methacholine concentration, and the AUC was calculated.

One day after intranasal instillation, mice were deeply anesthetized by means of intraperitoneal injection of pentobarbital and killed. Blood was first sampled from the retro-orbital plexus, and the mice were then killed by means of section of the abdominal vessels. The lungs underwent lavage, and the recovered fluid was pooled. Total cells were counted, and cells were spun onto microscope slides, air-dried, and stained for enumerating macrophages, eosinophils, neutrophils, and lymphocytes.

Levels of TNF- α and macrophage inflammatory protein 2 (MIP-2) were measured in undiluted bronchoalveolar lavage (BAL) fluid by using a sandwich ELISA.

Retroauricular and superficial cervical lymph nodes were obtained 24 hours after intranasal instillation. The lymph nodes from 3 to 4 mice were pooled and kept on ice. Cell suspensions were obtained by pressing the lymph nodes through a cell strainer. Lymphocytes were washed 3 times and suspended (10^7 cells/mL) in complete tissue culture medium. Five hundred thousand cells were stained with anti-CD3 (phycoerythrin)-labeled, anti-CD4 (fluorescein isothiocyanate)-labeled, anti-CD25 (peridinin chlorophyll [PerCP])-labeled, or anti-CD19 (phycoerythrin)-labeled antibodies, with control samples being labeled with isotype-matched control antibodies. Flow cytometry was performed with at least 10^4 cells. Cells were also seeded into 48-well culture plates and incubated in complete RPMI-1640 medium for 18 hours without or with 2.5 μ g/mL concanavalin A (ConA). Supernatants were stored at -80°C . Concentrations of IL-4 and IFN- γ were measured in undiluted supernatant by using a standard ELISA assay.

Total serum IgE, IgG1, and IgG2a levels were measured with a standard ELISA assay.

Data are shown as individual values with medians or means and SDs. All data were analyzed by using the nonparametric Kruskal-Wallis test with a Dunn multiple comparison post-hoc test (GraphPad Prism 4.01). A *P* value of less than .05 was considered significant.

All experimental procedures were approved by the local ethical committee for animal experiments.

RESULTS

Ventilatory function

Fig 1 shows the early ventilatory responses immediately after intranasal instillation. Mice that had previously received dermal applications of TMA, either once on day 1 (T/0/T) or twice on days 1 and 7 (T/T/T), showed significant increases in Penh after intranasal instillation

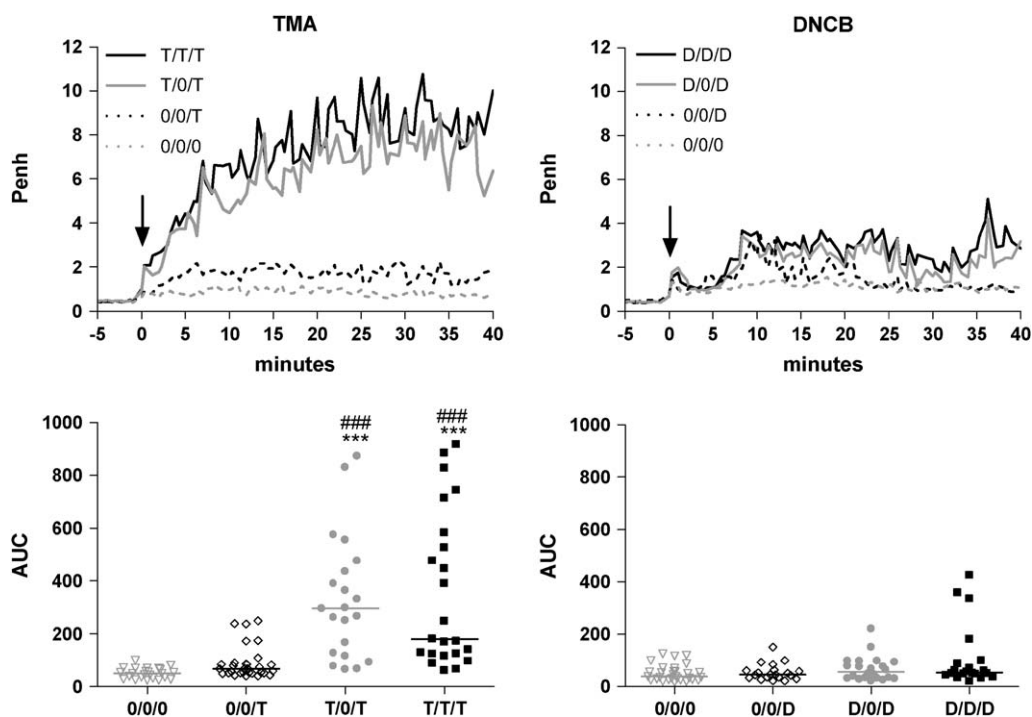


FIG 1. Early ventilatory response (Penh) after intranasal instillation with TMA or DNCB. *Upper panels* show mean values of Penh during 5 minutes before and 40 minutes after instillation (indicated with arrow), and *lower panels* show individual values of the AUC of Penh against time between 0 and 40 minutes. Experimental groups are identified by 3 symbols (eg, T/O/T). O, T, and D represent administration of vehicle (AOO), TMA, or DNCB, respectively; the first 2 symbols identify the agent (5% TMA or 0.2% DNCB) applied dermally on days 1 and 7, and the third symbol identifies the agent (1% TMA or 0.02% DNCB) instilled intranasally on day 10. n = 20-30 per group. ###*P* < .001 compared with the O/O/T control group; ****P* < .001 compared with the O/O/O control group.

with TMA, whereas no significant changes in Penh were observed in the O/O/T control group.

Mice dermally treated with DNCB did not show significant changes in ventilatory function after challenge with DNCB compared with that seen in the relevant control animals.

The mice that had received TMA on the skin (T/O/T and T/T/T) showed increased methacholine responsiveness 22 hours after intranasal challenge compared with that seen in the O/O/T and O/O/O control groups (Fig 2).

Mice treated with DNCB did not show significant changes in methacholine response compared with those seen in the control groups.

Pulmonary inflammation (BAL)

Mice dermally treated with TMA and then intranasally instilled with TMA (T/O/T and T/T/T) had significantly more cells in BAL fluid than the O/O/O control group. Total cell counts in DNCB-treated mice did not differ among the 4 groups (Table I).

Mice dermally sensitized with TMA and intranasally challenged with TMA (T/O/T and T/T/T) had significant increases in neutrophils compared with the O/O/T and O/O/O control groups. DNCB-treated mice (D/O/D and

D/D/D) also showed an increased proportion of neutrophils compared with both the O/O/D and O/O/O control groups.

Fig E1 (available in the Online Repository at www.jacionline.org) shows the changes in MIP-2 and TNF- α concentrations in BAL fluid 24 hours after intranasal instillation. Compared with the O/O/T and O/O/O control groups, the T/T/T group had increased concentrations of MIP-2 and TNF- α in BAL fluid. The T/O/T group had a significant increase in MIP-2 levels compared with both the O/O/T and O/O/O control groups, but TNF- α levels were only statistically increased compared with those in the O/O/O group. In DNCB-treated mice TNF- α levels were increased in the D/O/D and D/D/D groups compared with those in the O/O/O control group.

Lymphocyte subpopulation analysis

A decrease in the proportion of CD3⁺CD4⁺ lymphocytes (T cells) and an increase in the proportion of CD19⁺ lymphocytes (B lymphocytes) was apparent in the auricular lymph nodes of the T/O/T and T/T/T groups of mice. An increase in the proportions of B lymphocytes after TMA treatment in group T/T/T was also significant in cervical lymph nodes compared with that seen in the

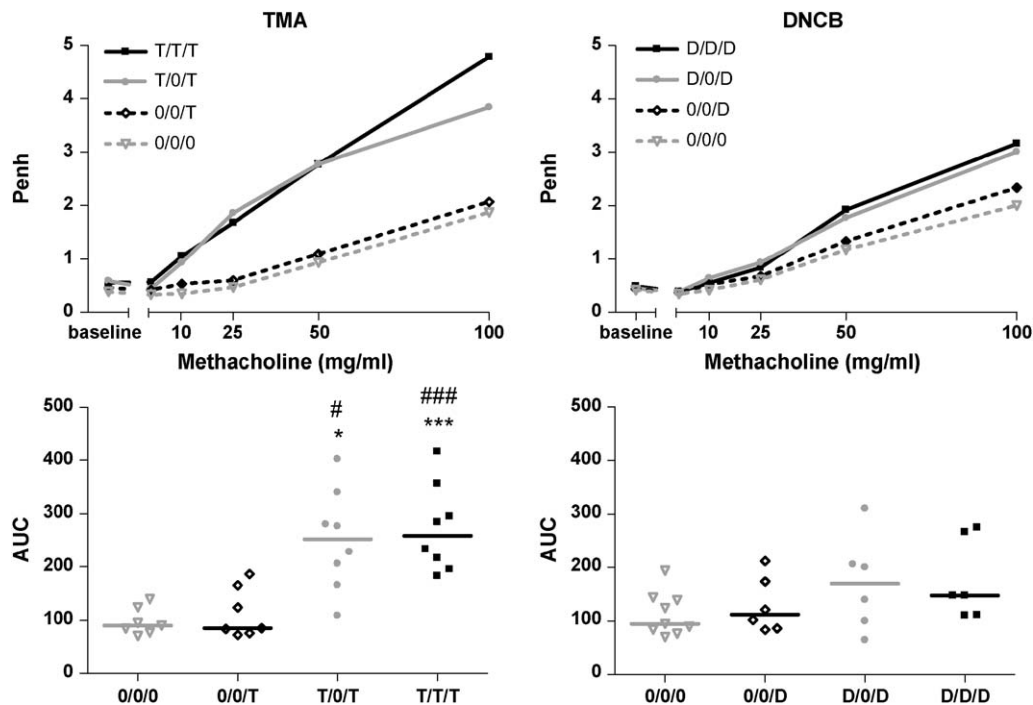


FIG 2. Methacholine responsiveness (Penh) 1 day after intranasal instillation of TMA or DNCB. *Upper panels* show mean values of Penh, and *lower panels* show individual values of the AUC of Penh against methacholine concentrations between 0 and 100 mg/mL. Experimental groups are as in Fig 1. $n = 6-9$ per group. # $P < .05$ and ### $P < .001$ compared with the O/O/T control group; * $P < .05$ and *** $P < .001$ compared with the O/O/O control group.

TABLE I. Total and differential cell counts in BAL fluid 1 day after intranasal instillation of TMA or DNCB

		TMA			
		0/0/0 (n = 24)	0/0/T (n = 30)	T/O/T (n = 21)	T/T/T (n = 23)
Total cell count ($\times 10^4$)		9.1 $\pm$ 3.8	11.4 $\pm$ 3.9	14.3 $\pm$ 5.8 $\ddagger$	14.3 $\pm$ 6.1 $\ddagger$
Differential cell count (%)	Macrophages	98.7 $\pm$ 1.2	97.7 $\pm$ 1.7	95.9 $\pm$ 3.2 $\ddagger$	93.1 $\pm$ 6.3 $\S$
	Neutrophils	0.7 $\pm$ 1.0	1.4 $\pm$ 1.3	3.5 $\pm$ 3.2* $\S$	5.9 $\pm$ 5.9* $\S$
	Eosinophils	0.1 $\pm$ 0.4	0.1 $\pm$ 0.5	ND	ND
	Lymphocytes	0.5 $\pm$ 0.6	0.7 $\pm$ 0.6	0.5 $\pm$ 0.6	0.6 $\pm$ 0.8
		DNCB			
		0/0/0 (n = 30)	0/0/D (n = 20)	D/O/D (n = 22)	D/D/D (n = 20)
Total cell count ($\times 10^4$)		9.3 $\pm$ 2.7	9.5 $\pm$ 4.0	10.1 $\pm$ 3.8	11.4 $\pm$ 4.0
Differential cell count (%)	Macrophages	98.7 $\pm$ 1.2	98.7 $\pm$ 1.0	97.2 $\pm$ 2.0 $\ddagger$	97.4 $\pm$ 1.3* $\ddagger$
	Neutrophils	1.0 $\pm$ 1.2	1.1 $\pm$ 1.0	2.7 $\pm$ 1.8* $\ddagger$	2.5 $\pm$ 1.3* $\ddagger$
	Eosinophils	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2	ND	ND
	Lymphocytes	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2

Cells were obtained by means of BAL 24 hours after intranasal instillation of vehicle or chemical (TMA or DNCB, mean $\pm$ SD). Experimental groups are as in Fig 1.

ND, Not detected.

* $P < .05$ compared with the 0/0/T or 0/0/D control groups.

$\ddagger P < .05$, $\S P < .01$, and $\S P < .001$ compared with the 0/0/0 control group.

0/0/0 group (see Table E1 in the Online Repository at www.jacionline.org). No differences were found in the CD3⁺CD4⁺CD25⁺ lymphocytes (regulatory-activated T cells) of either auricular or cervical lymph nodes (data not shown).

Mice of the D/O/D and the D/D/D groups had a slight increase of CD19⁺ B cells in both auricular and cervical lymph nodes, but this was not statistically different from numbers seen in the control groups (0/0/D and 0/0/0; see Table E1 in the Online Repository at www.jacionline.org).

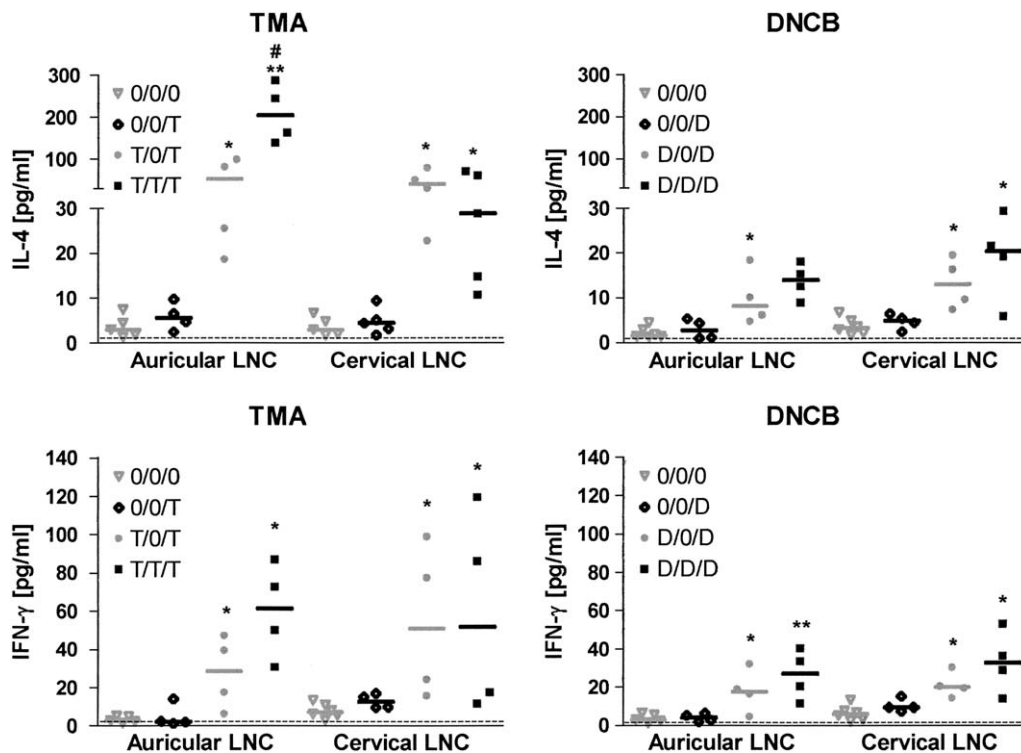


FIG 3. IL-4 and IFN- γ levels in auricular and cervical lymphocyte supernatant 2 days after intranasal instillation of TMA or DNCB. The lymph nodes of 3 to 5 mice were pooled, and the obtained lymph node cells (LNC) were cultured without (shown as a dotted line representing the average spontaneous release) or with ConA (2.5 μ g/mL) stimulation for 18 hours. Experimental groups are as in Fig 1. # $P < .05$ compared with the 0/0/T control group. * $P < .05$ and ** $P < .01$ compared with the 0/0/0 control group.

org). No differences were found in the CD3⁺CD8⁺ and CD3⁺CD4⁺CD25⁺ lymphocytes in either auricular or cervical lymph nodes of DNCB-treated mice compared with those seen in control mice (data not shown).

Lymphocytes of both auricular and cervical lymph nodes showed a spontaneous release (ie, without stimulation of ConA) of IL-4 and IFN- γ just above the detection limit level, and there were no differences in basal release between the groups (Fig 3, data are shown as a dotted line representing the mean of all measurements).

All dermally sensitized mice treated with either TMA (T/0/T and T/T/T) or DNCB (D/0/D and D/D/D) showed significant increases in IL-4 and IFN- γ levels in supernatant of auricular and cervical lymphocytes stimulated with ConA compared with levels seen in the respective 0/0/0 control groups. In all cases the production of IL-4 and IFN- γ was higher after dermal sensitization with TMA than after DNCB, but the ratio of IL-4 and IFN- γ differed between TMA-treated mice (IL-4 > IFN- γ) and DNCB-treated mice (IL-4 < IFN- γ , Fig 3).

Mouse total serum IgE, IgG1, and IgG2a levels

Mice of the T/T/T group showed significantly higher total serum IgE levels than both the 0/0/T and 0/0/0 control groups (Fig 4, A). Levels of total serum IgE did not differ among DNCB-treated mice. All sensitized mice with either TMA or DNCB (T/T/T, T/0/T, D/D/D, and D/0/D)

showed a significantly increased level of total serum IgG1 compared with that seen in the 0/0/0 control mice (Fig 4, B). Mice of the D/D/D groups showed a significantly higher total serum IgG2a level than the 0/0/0 control group (Fig 4, C). Levels of total serum IgG2a did not differ among TMA-treated mice.

DISCUSSION

In this study we demonstrated that intranasal instillation of TMA to dermally sensitized mice led to an early ventilatory response compatible with airway obstruction, increased methacholine responsiveness, and pulmonary inflammation, as we had shown previously with TDI.^{18,19} In contrast, dermal application and intranasal instillation of DNCB did not lead to ventilatory changes and only resulted in a low inflammatory response. These ventilatory changes were accompanied by increased production of cytokines typical for both the T_H1 and T_H2 lymphocyte profile. We conclude that in addition to immunologic analyses and cytokine profiling, ventilatory function studies are necessary to adequately classify the possible target of chemical sensitization.

Before discussing these findings further, it is important to clarify a semantic issue concerning the naming of chemicals as being dermal or respiratory sensitizers.

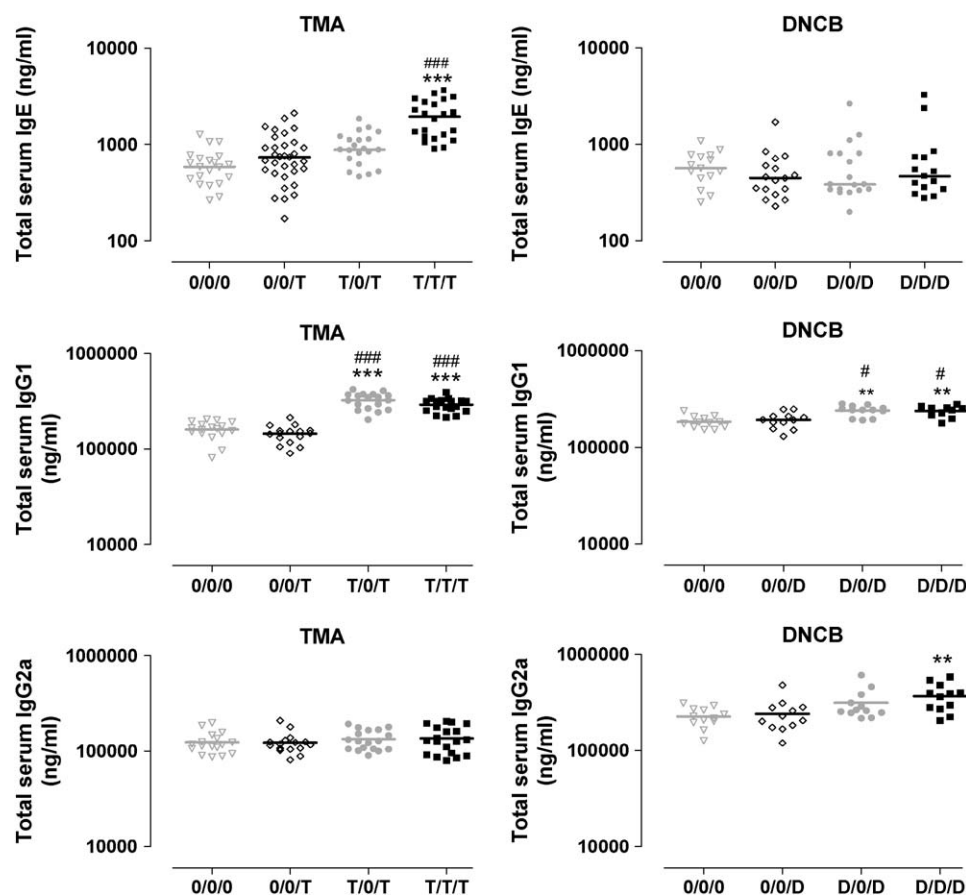


FIG 4. Total serum IgE, IgG1, and IgG2a levels 1 day after intranasal instillation of TMA or DNCB. Experimental groups are as in Fig 1. $n = 20-30$. # $P < .05$ and ### $P < .001$ compared with the 0/0/0 or 0/0/D control group; ** $P < .01$ and *** $P < .001$ compared with the 0/0/T control group.

Strictly speaking, such qualification should be given according to the route of initial sensitization. However, we^{18,19} and others^{10,12,15,17} use the term *respiratory sensitizer*, as opposed to *dermal sensitizer*, to indicate the site of the response after challenge rather than the route of sensitization. Experience has shown that almost all chemical sensitizers can lead to primary sensitization when they are applied to the skin.²¹ It is not known whether this also applies when sensitizers are applied to the respiratory mucosa. Although this is an important question, this is not the issue of the present study. Our objective was to assess the potential of the respiratory tract to be an effector organ after sensitization. Admittedly, it would be more appropriate to label such chemicals as sensitizers “with potential respiratory reaction after respiratory challenge,” but this is too cumbersome. Obviously, this is not only a semantic problem, but it is also relevant in terms of prevention. The possibility that occupational asthma might result from initial sensitization to chemicals or metallic agents through skin contact is an unresolved issue. Occasionally patients present with both allergic contact dermatitis and asthma,^{22,23} but for various reasons, such cases might be more common than generally recognized by either

dermatologists or pulmonologists. Physiologically, it is conceivable that sensitization occurs through the skin but without leading to significant cutaneous manifestations if subsequently the agent is mainly inhaled.

Low-molecular-weight allergens and sensitization

Many chemicals, those already present on the market and those newly produced every year, are potentially hazardous because they can induce sensitization by acting as haptens.

The LLNA is a validated technique for assessing the skin sensitization potential of such chemicals. It is based on measuring in mice the proliferative index of cells of the lymph nodes draining the skin site of the chemical application.⁹ Extension of this assay and its adjustment for evaluating the potential of chemicals to induce respiratory sensitization have been proposed.¹⁷ These models are based on the ability of chemicals to increase serum IgE levels or to induce a T_H2 cytokine profile in cells of the local draining lymph nodes and thus presumably to induce respiratory sensitization. Remarkably, none of the models assess respiratory end points, but they rely on the

paradigm that T_H2 responses are indicative of asthma. Thus TMA has been proved in the murine LLNA and other rodent models to induce lymphocyte proliferation, with an increased serum IgE synthesis and a T_H2 cytokine secretion profile.^{10,24} DNCB has also been proved positive in the murine LLNA.⁷ Most often, no increase in total serum IgE level was found in animals treated dermally with DNCB,¹⁰ although sometimes a minor increase in total serum IgE level has been observed.^{25,26} This difference in obtained results is probably related to the use of different species and protocols. DNCB sensitization is preferentially accompanied by increased production of the T_H1 -dependent cytokines IFN- γ and IL-12.¹⁷ However, the selective production of T_H1 or T_H2 profile of cytokines is not exclusively related to dermal or respiratory sensitizers, respectively. Recently, it has been shown that TMA is also capable of stimulating IFN- γ and that DNCB can induce the T_H2 -dependent cytokine IL-4 as well.¹³ Our previous data were compatible with these observations in that they showed that lymph node cells from TDI-sensitized mice produce increased levels of both T_H1 and T_H2 cytokines after stimulation with ConA (Tarkowski M, et al, submitted for publication). Here we showed in the same model that both TMA (a respiratory sensitizer) and DNCB (a dermal sensitizer) induced a mixed T_H1 and T_H2 cytokine profile in both the auricular and cervical lymph node cells. Although this increase in both cytokine profiles could indicate the lack of polarization of the response to TMA toward T_H2 cytokines or of DNCB toward T_H1 cytokines, it has to be noted that the relative increases in IL-4 compared with IFN- γ levels were opposite for TMA (IL-4 > IFN- γ) and DNCB (IL-4 < IFN- γ).

In addition to cytokine level changes, TMA induced a significant increase of B lymphocytes in auricular lymph nodes and increased IgE and IgG1 production, which is indicative of an activation of T_H2 cells.²⁷ In dermally DNCB-treated mice, only a slight increase in IgG1 levels was found. In contrast to our findings, an increase in B cells in auricular lymph nodes of DNCB-treated mice has been described by others.^{28,29} This discrepancy could be explained by different protocols. Nevertheless, an increase in total serum IgG2a was found in DNCB-treated mice, which can be attributed to the activation of T_H1 cells.²⁷

Ventilatory and pulmonary inflammatory responses

The depiction of individual values in Fig 1 reveals substantial variation in the magnitude of the responses within responding groups. This variability, which has already been observed in our previous experiments¹⁹ (Tarkowski M, et al, submitted for publication) and by other research groups,¹⁴ is not simply due to experimental variation in the measurement of Penh, but it also reflects authentic individual variation in biologic responses. In the T/0/T and T/T/T groups of mice, a clear early ventilatory response after intranasal instillation was found, whereas the ventilatory response in control mice (0/0/T and 0/0/0) did not change.

The absence of response in the 0/0/T and 0/0/D groups proves that an intranasal application of 1% TMA or 0.02% DNCB does not lead to a nonspecific irritant response. On day 11, we found an increase in methacholine reactivity in mice of the T/0/T and T/T/T groups. These early and late ventilatory changes were not found in sensitized and intranasally DNCB-treated mice, thus leading to the conclusion that DNCB (at least at the dose used) is not capable of inducing an airway response after intranasal instillation in sensitized mice (with sensitization being demonstrated by increased IL-4 and IFN- γ levels in draining lymph nodes). Nevertheless, it could be argued that the DNCB-treated mice were not sufficiently or not adequately sensitized to induce an airway response. Until now, DNCB has always been described as being a dermal sensitizer only.^{16,30}

We are aware that unrestrained whole-body plethysmography does not actually measure bronchoconstriction but only gives an indication of changes in ventilatory function. The use of whole-body plethysmography and Penh^{14,31,32} has been heavily criticized as being inadequate for assessing airway mechanics.³³⁻³⁵ Although an increase in Penh does not necessarily reflect bronchial obstruction correctly, it remains that whole-body plethysmography does assess changes in ventilatory patterns. Consequently, regardless of the physiologic basis for the observed ventilatory responses in our experiments, there is no doubt (because our design included all necessary control groups) that early ventilatory responses and increases in methacholine responsiveness occurred in TMA-sensitized and then intranasally instilled mice. We infer that these changes are of an obstructive character. Admittedly, the respective contribution of the nose and lower airways in determining changes in Penh are not known. However, regardless of the exact anatomic site of the changes leading to an early ventilatory response and an increase in methacholine responsiveness, our results clearly indicate that these changes are dependent on prior dermal sensitization. This thus provides a relevant end point to assess respiratory sensitizers. Furthermore, we found clear correlations between early response, methacholine response, total cell count, neutrophil count, MIP-2 level, and TNF- α level in the BAL fluid of TMA-sensitized and TMA-challenged mice (see Table E2 in the Journal Repository at www.jacionline.org); in other words, when we found an increase in one factor, we could expect also an increase in the others. This also indicates that a single intranasal instillation is capable of causing inflammation in the lower airways.

Analysis of lung inflammation showed significant increases in BAL fluid total cell counts (Table I) in TMA-sensitized and intranasally instilled mice (T/0/T and T/T/T) but not in DNCB-treated mice. Further characterization of the BAL cells revealed a significant increase in neutrophils in both TMA- and DNCB-treated mice (Table I). Such influx of neutrophils depending on prior skin sensitization has already been shown with TDI in our model (Tarkowski M, et al, submitted for publication).¹⁹ Moreover, it has also been reported in other rodent

models after exposure to TMA.¹⁴ It seems reasonable to assume that the neutrophil influx in TMA-treated mice is caused by chemotactic activity of MIP-2 that was found to be present at increased levels in BAL fluid. DNCB-treated mice also showed a slight increase in BAL fluid neutrophils, but here we only found a slight nonsignificant increase in MIP-2 levels. Concentrations of TNF- α in BAL fluid were increased in all TMA- and DNCB-sensitized and challenged mice. TNF- α is a cytokine produced by many types of cells, and its release by irritation is well recognized.³⁶ These results confirm the irritant-allergic capacities of both TMA^{37,38} and DNCB.³⁹

Conclusion

In conclusion, clear differences in ventilatory responses were found between the respiratory sensitizer TMA, which caused asthma-like changes, and the dermal sensitizer DNCB, which did not cause asthma-like changes. In terms of lung inflammation, both the respiratory sensitizer (TMA) and the dermal sensitizer (DNCB) showed a response, but TMA had a more pronounced effect than DNCB. In terms of lymph node response, TMA sensitization appeared to increase the participation of B lymphocytes and hence presumably IgE responses, whereas DNCB did not. Nevertheless, after stimulation with ConA, both chemicals induced a mixed T_H1-T_H2 cytokine profile in the auricular and cervical lymphocytes.

REFERENCES

- Bardana EJ Jr. Occupational asthma and allergies. *J Allergy Clin Immunol* 2003;111(suppl):S530-9.
- Lachowsky F, Lopez M. Occupational allergens. *Curr Allergy Asthma Rep* 2001;1:587-93.
- Chan-Yeung M, Malo JL, Tarlo SM, Bernstein L, Gautrin D, Mapp C, et al. Proceedings of the first Jack Pepys Occupational Asthma Symposium. *Am J Respir Crit Care Med* 2003;167:450-71.
- Lombardo LJ, Balmes JR. Occupational asthma: a review. *Environ Health Perspect* 2000;108(suppl 4):697-704.
- Banks DE, Wang ML. Occupational asthma: "the big picture". *Occup Med* 2000;15:335-58.
- Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI. Asthma in the workplace, 2nd ed. New York: Marcel Dekker, Inc; 1999.
- Gerberick GF, Ryan CA, Kern PS, Dearman RJ, Kimber I, Patlewicz GY, et al. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis* 2004;50:274-88.
- Magnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. *J Invest Dermatol* 1969;52:268-76.
- Kimber I, Dearman RJ, Basketter DA, Ryan CA, Gerberick GF. The local lymph node assay: past, present and future. *Contact Dermatitis* 2002;47:315-28.
- Sailstad DM, Ward MD, Boykin EH, Selgrade MK. A murine model for low molecular weight chemicals: differentiation of respiratory sensitizers (TMA) from contact sensitizers (DNFB). *Toxicology* 2003;194:147-61.
- Fawcett IW, Taylor AJ, Pepys J. Asthma due to inhaled chemical agents—epoxy resin systems containing phthalic acid anhydride, trimellitic acid anhydride and triethylene tetramine. *Clin Allergy* 1977;7:1-14.
- Arts J, de Koning M, Bloksma N, Kuper C. Respiratory allergy to trimellitic anhydride in rats: concentration-response relationships during elicitation. *Inhal Toxicol* 2004;16:259-69.
- Vandebriel RJ, de Jong WH, Hendriks JJ, Van Loveren H. Impact of exposure duration by low molecular weight compounds on interferon-gamma and interleukin-4 mRNA expression and production in the draining lymph nodes of mice. *Toxicology* 2003;188:1-13.
- Zhang XD, Fedan JS, Lewis DM, Siegel PD. Asthma-like biphasic airway responses in Brown Norway rats sensitized by dermal exposure to dry trimellitic anhydride powder. *J Allergy Clin Immunol* 2004;113:320-6.
- Pauluhn J. Respiratory hypersensitivity to trimellitic anhydride in Brown Norway rats: analysis of dose-response following topical induction and time course following repeated inhalation challenge. *Toxicology* 2003;194:1-17.
- Dearman RJ, Warbrick EV, Skinner R, Kimber I. Cytokine fingerprinting of chemical allergens: species comparisons and statistical analyses. *Food Chem Toxicol* 2002;40:1881-92.
- Dearman RJ, Kimber I. Cytokine fingerprinting and hazard assessment of chemical respiratory allergy. *J Appl Toxicol* 2001;21:153-63.
- Vanoirbeek JA, Mandervelt C, Cunningham AR, Hoet PH, Xu H, Vanhooren HM, et al. 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. *Toxicol Sci* 2003;76:338-46.
- Vanoirbeek JA, Tarkowski M, Ceuppens JL, Verbeken EK, Nemery B, Hoet PH. Respiratory response to toluene diisocyanate depends on prior frequency and concentration of dermal sensitization in mice. *Toxicol Sci* 2004;80:310-21.
- Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766-75.
- Briatico-Vangosa G, Braun CL, Cookman G, Hofmann T, Kimber I, Loveless SE, et al. Respiratory allergy: hazard identification and risk assessment. *Fundam Appl Toxicol* 1994;23:145-58.
- De Raeve H, Vandecasteele C, Demedts M, Nemery B. Dermal and respiratory sensitization to chromate in a cement floorer. *Am J Ind Med* 1998;34:169-76.
- Meuleman L, Goossens A, Linders C, Rochette F, Nemery B. Sensitization to triglycidylisocyanurate (TGIC) with cutaneous and respiratory manifestations. *Allergy* 1999;54:752-6.
- Dearman RJ, Skinner RA, Humphreys NE, Kimber I. Methods for the identification of chemical respiratory allergens in rodents: comparisons of cytokine profiling with induced changes in serum IgE. *J Appl Toxicol* 2003;23:199-207.
- Farraj AK, Harkema JR, Kaminski NE. Allergic rhinitis induced by intranasal sensitization and challenge with trimellitic anhydride but not with dinitrochlorobenzene or oxazolone in a/j mice. *Toxicol Sci* 2004;79:315-25.
- Warbrick EV, Dearman RJ, Kimber I. Induced changes in total serum IgE concentration in the Brown Norway rat: potential for identification of chemical respiratory allergens. *J Appl Toxicol* 2002;22:1-11.
- Kuby J. Cytokines. In: Allen D, Julet MR, Maass DC, editors. *Immunology*. 3rd ed. New York: W.H. Freeman; 1997. p. 313-33.
- Gerberick GF, Cruse LW, Ryan CA, Hulette BC, Chaney JG, Skinner RA, et al. Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. *Toxicol Sci* 2002;68:420-8.
- Lee JK, Park SH, Byun JA, Kim HS, Oh HY. Evaluation of lymphocyte subpopulations in draining lymph node cells following allergen and irritant. *Environ Toxicol Pharmacol* 2004;17:95-102.
- van Och FM, Van Loveren H, de Jong WH, Vandebriel RJ. Cytokine production induced by low-molecular-weight chemicals as a function of the stimulation index in a modified local lymph node assay: an approach to discriminate contact sensitizers from respiratory sensitizers. *Toxicol Appl Pharmacol* 2002;184:46-56.
- Shen HH, Ochur SI, McGarry MP, Crosby JR, Hines EM, Borchers MT, et al. A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse. *J Immunol* 2003;170:3296-305.
- Whitehead GS, Walker JK, Berman KG, Foster WM, Schwartz DA. Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L32-42.
- Lundblad LK, Irvin CG, Adler A, Bates JH. A reevaluation of the validity of unrestrained plethysmography in mice. *J Appl Physiol* 2002;93:1198-207.
- Mitzner W, Tankersley C, Lundblad LK, Adler A, Irvin CG, Bates JH. Interpreting Penh in mice. *J Appl Physiol* 2003;94:828-32.

35. Bates JH, Irvin CG. Measuring lung function in mice: the phenotyping uncertainty principle. *J Appl Physiol* 2003;94:1297-306.
36. Lisby S, Muller KM, Jongeneel CV, Saurat JH, Hauser C. Nickel and skin irritants up-regulate tumor necrosis factor-alpha mRNA in keratinocytes by different but potentially synergistic mechanisms. *Int Immunol* 1995;7:343-52.
37. Arts JH, Bloksma N, Leusink-Muis T, Kuper CF. Respiratory allergy and pulmonary irritation to trimellitic anhydride in Brown Norway rats. *Toxicol Appl Pharmacol* 2003;187:38-49.
38. Pauluhn J, Eidmann P, Freyberger A, Wasinska-Kempka G, Vohr HW. Respiratory hypersensitivity to trimellitic anhydride in Brown Norway rats: a comparison of endpoints. *J Appl Toxicol* 2002;22: 89-97.
39. Coquette A, Berna N, Vandenbosch A, Rosdy M, De Wever B, Poumay Y. Analysis of interleukin-1alpha (IL-1alpha) and interleukin-8 (IL-8) expression and release in in vitro reconstructed human epidermis for the prediction of in vivo skin irritation and/or sensitization. *Toxicol In Vitro* 2003;17:311-21.