Asthmalike biphasic airway responses in Brown Norway rats sensitized by dermal exposure to dry trimellitic anhydride powder

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Background: Trimellitic anhydride (TMA) can induce specific IgE and occupational asthma. The significance of dermal exposure to TMA in immunologic sensitization and on subsequent airway responses is not clearly known. An animal model displaying both an early-phase airway response (EAR) and a late-phase airway response (LAR) after sensitization and subsequent inhalation challenge to a low-molecular-weight chemical has not been previously reported.
Objective: The present study investigated EAR and LAR after TMA inhalation challenge in Brown Norway rats sensitized by skin exposure to TMA dry powder.
Methods: Twenty milligrams of dry TMA powder was applied to the skin of each clipped rat's dorsum on days 0, 7, 14, and 21 and occluded overnight with surgical tape. Rats were challenged for 10 minutes with 0.2 to 40 mg/m^3 of TMA aerosol after day 35. Enhanced pause (an index of airway resistance) was recorded overnight in a whole-body plethysmography system. Specific IgE and pulmonary eosinophilia were also measured.
Results: Concentration-dependent responses to TMA were observed: provocation with 0.2 mg/m^3 produced no response; 1 mg/m^3 induced only EAR; and 5 mg/m^3 and 40 mg/m^3 induced both EAR and LAR. Specific IgE was positive; airway eosinophilic inflammation was observed.
Conclusion: TMA powder applied to the skin can lead to both immunologic sensitization and subsequent dose-dependent biphasic airway responses after TMA aerosol challenge. (J Allergy Clin Immunol 2004;113:320-6.)

Key words: *Trimellitic anhydride, dermal exposure, enhanced pause, airway challenge, late-phase airway response*

Mechanisms of allergy

Organic acid anhydrides (OAAs), such as trimellitic anhydride (TMA), are reactive, low-molecular-weight chemical compounds used in the manufacture of resins, dyes, adhesives, polymers, and printing inks. They are used in the production of agricultural chemicals and pharmaceuticals and are also used as plasticizers. TMA is a crystalline solid at room temperature. Clinical symptoms after exposure include direct irritation to mucosa and skin and immune-related allergic manifestations, such as rhinitis and asthmatic symptoms.[1,2]

Abbreviations used
EAR: Early-phase airway response
LAR: Late-phase airway response
OAA: Organic acid anhydride
Penh: Enhanced pause
TMA: Trimellitic anhydride

Specific IgE to TMA has been found in TMA-exposed workers.[3] Sensitized workers developed allergic airway responses (an early-phase airway response [EAR], a late-phase airway response [LAR], or both) after bronchial provocation with TMA.[4,5] Individuals can be exposed to dry TMA powder and/or dust through inhalation or skin contact, but whether skin exposure to TMA can be a mode of sensitization for subsequent asthmatic responses after inhalation of TMA dust is not known. Previous studies using animal models demonstrated the production of specific antibodies after exposure by airway inhalation, intradermal injection, or skin painting with OAA (in acetone-oil solvent) and subsequent development of EAR after inhalation challenge to either free OAA or OAA-protein conjugates.[6-11] A study of LAR in guinea pigs sensitized to TMA by intradermal injection did not obtain conclusive results, possibly because of the index used to monitor airway responses.[12]

To simulate workplace conditions and test the hypothesis that asthmalike responses might occur on the basis of the sensitization to TMA by the dermal route, dry TMA powder was applied topically to the skin. We previously reported that such exposure can lead to a time- and dose-dependent IgE antibody response in Brown Norway rats.[13] Here the results of inhalation challenge with TMA powder from rats sensitized by dermal TMA exposure are reported. Whole-body plethysmography was used to examine the appearance of EAR and LAR in response to inhaled TMA aerosol.

METHODS

Animals

Male Brown Norway rats (each weighing 150-175 g; Charles River Laboratories, Wilmington, Mass) were acclimated for 1 week in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

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Chemicals

TMA was from Acros Organics (Fair Lawn, NJ). Methohexital sodium (brevital sodium) was from Jones Pharma (St Louis, Mo). TMA flakes were ground in an analytical mill (IKA WORKS, Wilmington, NC). More than 90% of the particles had diameters of <4 μm, as determined by a Coulter Multisizer II (Coulter Corporation, Hialeah, Fla).

Sensitization

Rats were dermally exposed to dry TMA powder at a dose of 20 mg (covering an area of 4.5 cm^2) on days 0, 7, 14, and 21, as previously described.[13] Briefly, a patch of fur on the dorsum was clipped with scissors; this was done carefully to prevent skin irritation and trauma. Dry TMA powder was applied to the clipped area. TMA exposed areas were occluded with a nonabrasive dermal surgical tape for approximately 20 hours, after which the application skin area was washed with water to remove any residual TMA. Sham-exposed rats (0 mg of TMA) were used as controls. Sera were collected after the final airway challenge for specific IgE analyses.

TMA aerosol generation

TMA aerosol was generated by means of a Wright Dust Feed Mechanism (Messrs. L. Adams Ltd, London, United Kingdom) through use of ground TMA powder. The aerosol was generated into a 20-L plastic settling chamber at a flow rate of 5 L/min. Humidified air (5 L/min) was combined to dilute the TMA concentration and keep the humidity at 40% to 50% before introduction into a nose-only exposure chamber (CH Technologies, Westwood, NJ). Chamber concentrations were measured through use of a continuous monitor (model 1.108; GRIMM Technologies, Douglasville, Ga) and gravimetrically through use of a 0.45-μm, 37-mm HAWP filter (Millipore, Bedford, Mass).

Airway challenge and pathologic analyses of the lung

Sensitized and nonsensitized control rats (n = 8 for each group) received one inhalation challenge to clean air (on day 28) and two 10-minute inhalation challenges (on days 35 and 42) to a 40-mg/m^3 TMA aerosol. After challenge, rats were immediately moved to the whole-body plethysmograph chambers (Buxco Electronics, Troy, NY) for monitoring for 16 to 20 hours. Nose-only exposure was used to avoid deposition of the TMA powder on the animals' fur and in the chamber. Animals in the whole-body plethysmograph chambers were conscious and unrestrained; food and water were provided ad libitum. Enhanced pause (Penh)[14] and respiratory rate were recorded every 30 seconds throughout the entire monitoring period. Rats were killed 24 hours after the last challenge and bronchoalveolar lavage fluid was obtained for eosinophil enumeration through use of the Coulter Multisizer II and microscopic differential counts. Lungs were then fixed with 10% buffered formalin. The lungs were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin.

A separate group of sensitized rats (n = 8) were challenged at a concentration of 0, 0.2, 1, 5, or 40 mg/m^3 of TMA aerosol (for the last dose, only 4 animals were tested because 8 rats had already been challenged to that dose in the foregoing study), as per the previously detailed procedure. Arithmetic means of peak Penh values for every 30 minutes during the first hour and for every 1 hour after that were used to quantify the responses. EAR and LAR of sensitized rats were defined by comparison of their Penh data with the data from nonsensitized TMA challenged control rats.

ELISA for TMA-specific IgE analysis

ELISA procedures have been described previously.[13] Quantification of the IgE response in "relative units" was performed by reference to a standard curve generated through use of serial dilution of pooled rat sera positive for TMA-specific IgE and their corresponding ELISA optical densities.

Data analyses

Data were described as means ± SEMs. Comparisons for 2 groups were made with a Student *t* test. One-way ANOVA with a post hoc Tukey test was used for experiments in which more than 2 groups were compared. *P* values of <.05 were considered significantly different.

RESULTS

Airway challenge with 40 mg/m^3 of TMA

Penh. There was no significant difference in Penh baseline values between sensitized rats (16 hours' measurements; mean ± SEM = 0.4 ± 0.06; n = 8) and controls (16 hours' measurements; mean ± SEM = 0.4 ± 0.03; n = 7) after challenge with clean air. An alteration in the respiratory waveform was noted within 30 minutes (EAR period) after TMA aerosol challenge in TMA-sensitized rats. This waveform was typified by an extended expiratory phase—ie, the pause, which was reflected by a significant increase in Penh values (≥0.9, which is greater than 3 SDs above the mean peak Penh value for the control animals) in TMA-sensitized, TMA-challenged rats. The duration of the early Penh increase in sensitized rats lasted 0.5 minutes to several minutes and was noted in all 8 rats. The change in Penh was not noted in nonsensitized control rats after TMA aerosol challenge. The Penh values of TMA-sensitized, TMA-challenged rats quickly declined to control levels after EAR and before LAR. From 1 hour until 3 hours after challenge, maximal Penh values of sensitized rats were not significantly different from those of the controls (Fig 1).

The increase in Penh was observed again in sensitized rats from approximately 2 to 4 hours after TMA challenge (LAR period). As denoted by an increase in Penh (≥0.9), LAR was observed in 7 of 8 sensitized rats. The LAR lasted for approximately 6 to 12 hours in 4 rats, but in 3 rats the Penh values were still elevated at the end of the 16-to-20–hour monitoring period. During this LAR period, the maximal Penh values of the sensitized rats (range, 1.2-4.7) were significantly higher than the values of the controls. The duration of LAR was also significantly longer than that of EAR within the sensitized group (Figs 1 and 2).

Respiratory rate. The respiratory rate of nonsensitized control rats during the 16-hour period was 148 ± 10 breaths per minute, and no significant difference was observed in the respiratory rate between nonsensitized and sensitized rats after challenge with clean air. TMA aerosol challenge caused a decrease in respiratory rate in control rats throughout the monitoring period. A decrease in the respiratory rate was noted immediately after challenge in sensitized rats, but the respiratory rate then

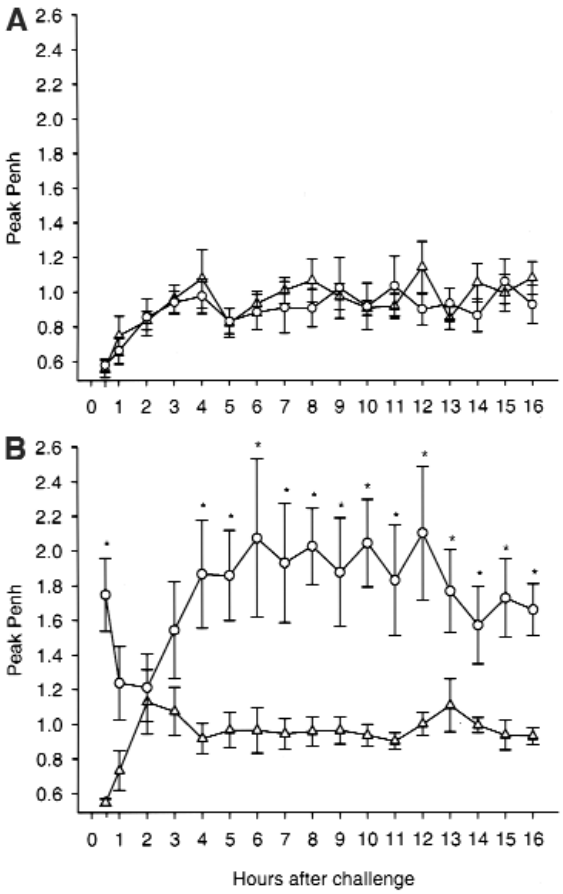

FIG 1. Penh from nonsensitized controls *(triangles)* and TMA-sensitized rats *(circles)* after 10 minutes with air challenge **(A)** and challenge with 40 mg/m^3 of TMA **(B)**. Each point is the peak value (mean ± SEM of 8 rats) recorded during the hour indicated. TMA challenge caused an early- and late-phase increase in Penh from sensitized rats. In *B*, points at hour 0.5 showed the Penh increase of the early phase and points after 4 hours showed the Penh increase of the late phase in sensitized rats. *Significantly different from TMA-challenged, nonsensitized controls ($P < .05$; *t* test).

TABLE I. Respiratory rate (mean ± SEM) after challenge

Time after challenge	Clean air	TMA (40 mg/m^3)
0-0.5 h		
Control rats	211 ± 16	182 ± 11
Sensitized rats	223 ± 17†	138 ± 14*
0.5-2 h		
Control rats	159 ± 5†	127 ± 11
Sensitized rats	146 ± 8	118 ± 7
2-16 h		
Control rats	137 ± 4†	123 ± 4
Sensitized rats	130 ± 3	150 ± 7*

*Significantly different between sensitized and control rats with the same challenge at the same time period ($P < .05$; *t* test).
†Significantly different between TMA and air challenge in the same rats at the same time period ($P < .05$; *t* test).

increased steadily throughout the 2-to-16–hour monitoring period, even when LAR Penh values returned to normal (Table I and Fig 2).

Specific IgE and bronchoalveolar lavage fluid cells. TMA-specific IgE in the sensitized group was (9.1 ± 1.1) × 10^4 (mean ± SEM) relative units; in the control group the value was 594 ± 127 (representing nonspecific binding). The numbers and differentials of bronchoalveolar lavage fluid cells from sensitized and control rats 24 hours after TMA challenge are presented in Table II. The TMA-challenge–induced allergic inflammatory response was predominantly eosinophilic in nature. The difference between the 2 groups for IgE and eosinophils was statistically significant ($P < .05$; *t* test). No correlation was seen between specific IgE levels, eosinophil numbers, and Penh values from individual sensitized, TMA-challenged rats.

Pathologic findings in the lung. Alveolar spaces were clear in control rats, with occasional eosinophils within the alveolar septae. There were occasional peribronchial lymphoid aggregates. In contrast, the lungs of TMA sensitized rats showed numerous eosinophils and numerous small granulomatous lesions characterized by aggregates of epithelioid histiocytes. There was increased mucus present in the bronchus and small airways, but large mucinous plugs were not seen (possibly because of bronchoalveolar lavage prior to fixation).

Effect of repeated TMA aerosol challenge. A second TMA aerosol challenge of 40 mg/m^3 was administered 1 week after this initial challenge. The second challenge resulted in a significant decrease in the time required for the appearance of EAR and LAR. The peak of the Penh in LAR and EAR did not differ between the first and second TMA aerosol challenges. LAR resolved much faster after the second challenge (Table III). One rat that did not develop LAR after the first challenge developed LAR after the second challenge.

Dose-response studies

As shown in Table IV, Penh changes in peak, duration, and response pattern of both EAR and LAR were challenge dose–dependent. A threshold of approximately 1 mg/m^3 was noted for EAR. Late and dual airway responses were not observed below 5 mg/m^3. Both the peak Penh and the duration of LAR increased with increasing TMA dose for airway challenge.

DISCUSSION

We previously reported that dermal exposure to dry TMA powder resulted in allergic sensitization of Brown Norway rats, as evidenced by the production of IgE and IgG antibodies. The antibody response was both time- and dose-dependent, the peak response being seen 3 to 4 weeks after exposure.[13] In the present work we extended those observations by evaluating the airway responses of animals challenged by TMA aerosol. Both EAR and LAR were observed in the sensitized animals.

Other researchers have reported the induction of EAR, but not of LAR, in animals sensitized by intradermal injection and challenged with OAA.[6,7,9,10,12] In addition, a recent study reported nonspecific methacholine airway hyperresponsiveness in Brown Norway rats (sensitized by

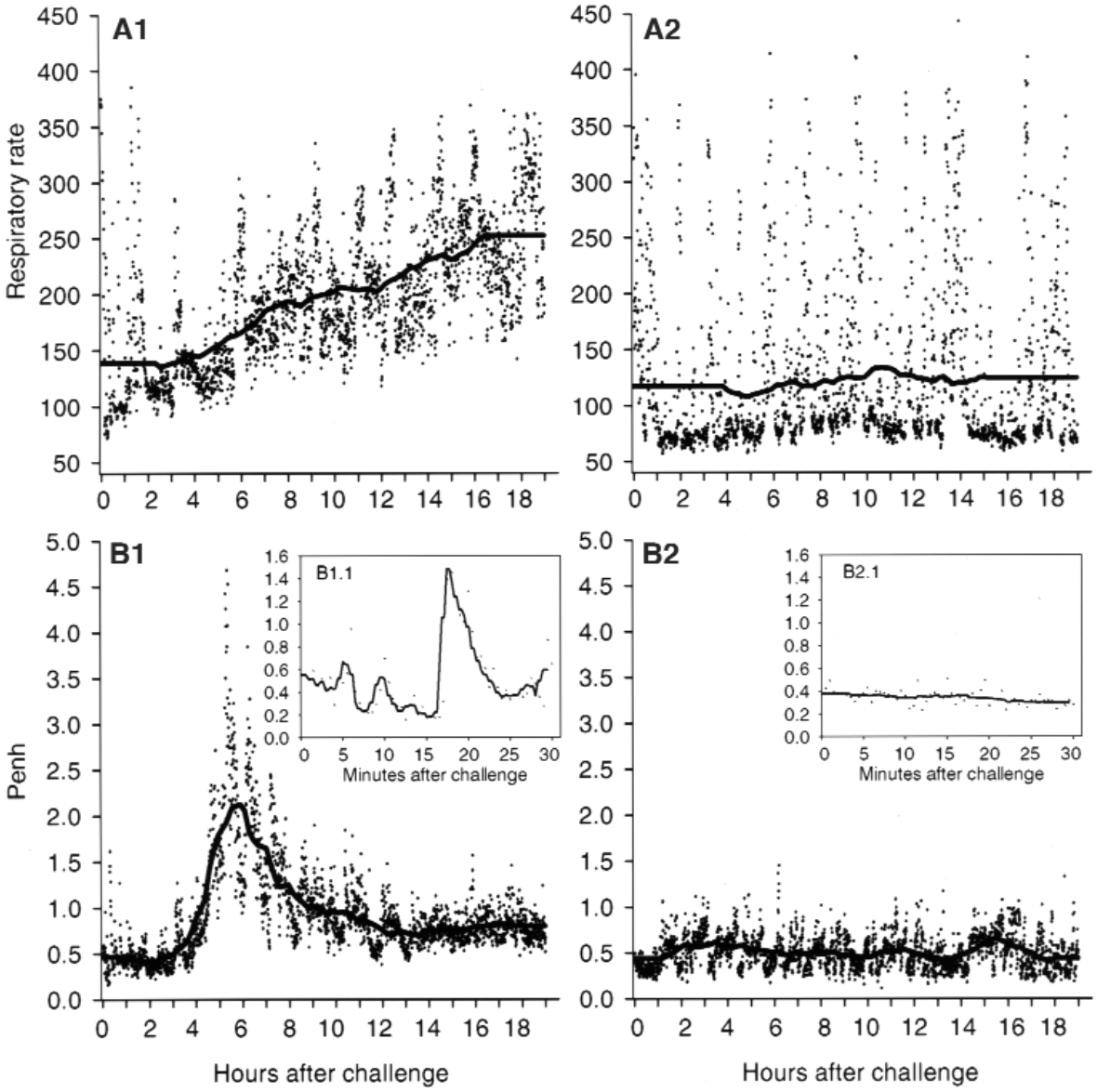

FIG 2. Representative respiratory rates and Penh values from TMA-sensitized and nonsensitized control/TMA aerosol–challenged rats.The respiratory rate steadily increased 2 hours after TMA challenge in the sensitized rat **(A1)** but not in the control rat **(A2)**. Penh increased during the early phase (the first 30 minutes after challenge) and the late phase (2 hours after challenge) in the sensitized rat **(B1** and **B1.1)** but not in the control rat **(B2** and **B2.1)**. Data were collected at 0.5-min intervals (indicated by the *dots*).The curves *(solid lines)* represent the running average of 10 data points.

skin painting with TMA in acetone and olive oil) 24 hours after specific challenge.[11] In the present work, both EAR and LAR were observed in sensitized Brown Norway rats after specific inhalation challenge with TMA. Specific IgE was elevated in the sensitized animals, and eosinophilic airway inflammation was noted after challenge to TMA aerosol. Furthermore, a relationship was found between airway responses and the concentration of TMA used for inhalation challenge. Thus this animal model, with both EAR and LAR, more closely mimics the occupational asthma associated with TMA exposure.[15,16]

Penh is an empirical index derived from breathing patterns reflecting changes of the waveform during expiration. Hamelmann, et al[14] made an extensive study of airway responses in ovalbumin-sensitized mice. Penh was measured after methacholine inhalation in that study, and increases in Penh correlated with increases in pulmonary resistance, intrapleural pressure, serum specific IgE, and lung eosinophilic inflammation. Other studies in rats, mice, and piglets demonstrated that allergic airway responses could be measured by increases in Penh through use of specific allergen challenge.[17-20]

TABLE II. Differential of bronchial lavage fluid cells (mean ± SEM) after challenge

Rats	Total	Eosinophils (%)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)
Sensitized	$(1874.6 \pm 210) \times 10^3$	63.63 ± 2.54	26.56 ± 1.75	7.75 ± 1.07	2.06 ± 0.72
Control	$(254 \pm 32.5) \times 10^3$	2.38 ± 0.5	94.06 ± 0.89	2.75 ± 0.44	0.81 ± 0.13

The cell counts for all cell types in sensitized rats were significantly greater than the counts for the controls ($P < .05$; *t* test). Red blood cells were not evaluated.

TABLE III. Penh after repeated airway challenge with 40 mg/m³ of TMA

	EAR			LAR			
	Start point	Peak	AUC	Start point	End point	Peak	AUC
1st TMA	10 min ± 3.5	1.8 ± 0.2	21.2 ± 2.1	3.9 h ± 1.1	16.3 h ± 2.3	2.9 ± 0.4	958 ± 123
2nd TMA	1.3 min ± 0.6*	2.1 ± 0.3	47.2 ± 9.5*	1.2 h ± 0.2*	6.5 h ± 0.8*	3.2 ± 0.4	375 ± 63*

Values are means ± SEMs.
Peak, Maximal Penh value observed; *AUC*, Penh area under the curve.
*Statistically different from the first challenge ($P < .05$; *t* test).

TABLE IV. Challenge dose of TMA and airway response

	EAR period	LAR period		Response pattern		
Dose (mg/m³)	Peak Penh*	Peak Penh*	Duration (h)	EAR only	LAR only	EAR and LAR
0	0.6 ± 0.05	1.6 ± 0.1	—	0/8	0/8	0/8
0.2	0.7 ± 0.04	1.6 ± 0.1	—	0/8	0/8	0/8
1	2.3 ± 0.4	1.9 ± 0.2	—	8/8	0/8	0/8
5	2.5 ± 0.3	5.1 ± 0.8	8.6 ± 1.3	0/8	1/8	7/8
40	4.3 ± 1.0	7.2 ± 1.7	14.5 ± 0.8†	0/4	0/4	4/4

The EAR period and the LAR period are 0 to 30 minutes and 2 hours after challenge, respectively. The peak Penh is the maximal value of enhanced pause. *Duration* is the time from the start point (Penh increases) to the end point (Penh down to normal) of LAR. In the response pattern, the denominator is the total number of rats and the numerator is the number of rats giving a positive response.
*Significantly different ($P < .05$; ANOVA). Tukey test: For the EAR period, all were significantly different ($P < .05$) except for the groups of 1 versus 5 mg and 0 versus 0.2 mg. For the LAR period, the following means were significantly different: 40 mg versus 0, 0.2, and 1 mg; 5 mg versus 0, 0.2, and 1 mg.
†Significantly different from the 5-mg group ($P < .05$; *t* test).

The experimental challenge design in the present study used a nose-only chamber with transfer immediately into the plethysmograph chambers. Approximately 3 minutes lapsed between end of challenge and initiation of respiratory function monitoring. There is an acclimatization period after transfer of the animals from the nose-only holding tubes to the plethysmograph; this might have affected the initial values. It was felt that the advantages of the present method outweighed these limitations; aerosol concentrations were monitored and precisely controlled, little to no residual TMA was carried over to the plethysmograph chambers, and exposure was limited to the respiratory tract.

An effect of TMA aerosol concentration on airway irritation versus airway specific response was reported by Arts et al.[11] Respiratory rate and/or breathing pattern became normal immediately after cessation of exposure to levels as high as 100 mg/m³ in naive Brown Norway rats, and the difference between asthmalike and irritant responses was clearly observed when 45 to 54 mg/m³ of TMA was used for specific challenge in sensitized and control rats.[11,21] The highest concentration of TMA aerosol used in the present work was 40 mg/m³; at this level no significant difference was found in Penh values between clean air- and TMA-challenged control rats. Similarly, clean air inhalation of TMA-sensitized rats did not elicit Penh increases. We believe that Penh is a consistent marker of TMA-elicited EAR and LAR.

EAR developed within the first 30 minutes after TMA challenge. In Penh from sensitized rats, EAR was evident by an increase after TMA challenge that was not seen in any of the controls challenged with air or TMA or in sensitized rats challenged with air. It was found that in the sensitized rats given a second TMA challenge, the time to onset of EAR and LAR was earlier than after the first challenge and that the duration of LAR was shorter. In human beings, repeated challenge with low doses of allergen given directly to the airways attenuates the LAR response to high-dose allergen challenge.[22]

Respiratory rates were also monitored. Fluctuations in the respiratory rate, which were seen in all animals, were probably related to animal movement. Respiratory rates were initially depressed after TMA challenge in sensitized rats; this is consistent with observations by Pauluhn et al.[23] After an initial decrease, the respiratory rates rose throughout the remainder of the monitoring period, even when the Penh returned to control levels. This suggests that airway resistance and respiratory rate are regulated independently, as reported by Hamelmann et al.[14]

Guinea pig models have been developed to investigate OAA-induced asthma, but only EAR was reported in these studies, which used a variety of pulmonary physiological measures, including respiratory rate, lung resistance, and plethysmograph pressure.[6-10,24] Respiratory rate was used as an index of airway response in guinea pigs sensitized to TMA, and LAR could not be confirmed.[12] On the basis of our results in the present study, we consider it difficult to detect LAR by measuring respiratory rate.

Pathology studies in airway and bronchoalveolar lavage fluid after TMA challenge revealed eosinophilic inflammation, which is consistent with the reports by Arts et al.[11,25]

The relationship between TMA challenge dose and airway response in sensitized rats was also examined in the present study. A dose-dependent change in the functional parameters was observed after challenge. The LAR duration from the 40-mg/m^3 challenge lasted longer than that from the 5-mg/m^3 challenge. A study in guinea pigs by Santing et al[26] showed that whether EAR, LAR, or both EAR and LAR develop might depend on the allergen concentration and exposure duration. The present data showed both a threshold and challenge concentration-dependent airway response to specific inhalation challenge.

In conclusion, the present model demonstrates allergic airway responses after dermal sensitization and inhalation challenge to a low-molecular-weight chemical. The dermal exposure pathway might participate in sensitization of workers to this substance; this is in addition to the sensitization that occurs after inhalation.

Our results, such as those of our study of the TMA provocation dose-response relationship, might have occupational health implications. The responses seen in the Brown Norway rat model resembles human TMA asthma with respect to the pulmonary physiology, pathology, and immunology. Further work investigating the mechanism of EAR and LAR, especially the latter, should provide important information concerning OAA-induced asthma.

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