

Airway Responses in Brown Norway Rats Following Inhalation Sensitization and Challenge with Trimellitic Anhydride

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Trimellitic anhydride (TMA) is a cause of asthma in man. Dose-dependent TMA-specific IgE, histopathology, and airway responses after sensitization by inhalation were examined in the Brown Norway rat. Rats were exposed to 0.04, 0.4, 4, or 40 mg/m³ TMA aerosol for 10 min, once a week, over 10 weeks. All lower exposures were, subsequently, rechallenged to 40 mg/m³ TMA aerosol. All rats received a sham exposure 1 week prior to the first TMA exposure. Following the sham exposure and weekly after each TMA exposure, TMA-specific IgE and both early-phase airway response (EAR) and late-phase airway response (LAR) were measured using enhanced pause (Penh). All rats sensitized by 40 mg/m³ TMA developed specific IgE, EAR, and LAR to one or more of the challenges to 40 mg/m³ TMA. TMA of 4 mg/m³ induced a much lower, but stable, specific IgE response. EAR and LAR were observed only after a 40 mg/m³ TMA rechallenge in this group, but it was much larger than that observed in the 40 mg/m³ TMA-sensitized and challenged group. Exposure-dependent histopathological changes noted included eosinophilic granulomatous interstitial pneumonia, perivascular eosinophil infiltrates, bronchial-associated lymphoid tissue hyperplasia, and peribronchiolar plasma cell infiltrates.

Key Words: trimellitic anhydride; inhalation; airway response; IgE.

Trimellitic anhydride (TMA) is widely used in industry for the synthesis of plasticizers for polyvinyl chloride resins, as a reactant in wire and cable insulation enamels and polyester powder coating resins for powder coatings. It is a crystalline solid at room temperature. The worldwide annual production of TMA is 100,000,000 kg; the majority of which (65%) is produced in the United States, according to a report by Organization for Economic Co-operation and Development (OECD SIDS, 2002). TMA and several other organic acid anhydrides (OAAs) including phthalic anhydride (PA), tetrachlorophthalic anhydride, and methyltetrahydrophthalic anhydride are a group of low molecular weight and highly reactive

chemicals. These anhydrides have been demonstrated to induce IgE-mediated asthma and rhinitis (Grammer *et al.*, 2002; Topping *et al.*, 1986; Zeiss *et al.*, 1977).

Inhalation of OAA is a relevant exposure route in the occupational setting. TMA and other OAA-induced asthma studies in workers in, approximately, the past 25 years have assumed that the workers' sensitizing exposure was mainly through inhalation (Barker *et al.*, 1998; Baur *et al.*, 1995; Bernstein *et al.*, 1982; Drexler *et al.*, 1994; Grammer *et al.*, 2002; Patterson and Harris, 1985; Wernfors *et al.*, 1986; Zeiss *et al.*, 1977). This may be true, but the experimental work in animal models suggests dermal exposure as a viable route of sensitization (Botham *et al.*, 1989; Zhang *et al.*, 2004).

Successful animal models of OAA-induced (IgE) sensitization have been developed using intradermal injection or topical skin exposure with the chemicals suspended in oil or liquid paraffin (Arakawa *et al.*, 1993; Botham *et al.*, 1989; Hayes *et al.*, 1992; Warbrick *et al.*, 2002; Zhang *et al.*, 1998). Airway responses have been induced after inhalation challenge with TMA-protein conjugates (Botham *et al.*, 1989; Hayes *et al.*, 1992). Recently, we found that Brown Norway (BN) rats developed specific IgE and IgG after topical skin exposure to dry TMA powder (Zhang *et al.*, 2002a, 2004), and both early-phase airway response (EAR) and late-phase airway response (LAR) in dermally sensitized rats were also noted after inhalation challenge with dry TMA powder (Zhang *et al.*, 2004).

Attempts to develop OAA-asthma models with sensitization via respiratory tract exposure have been problematic. Botham *et al.* (1988) were able to immunologically sensitize guinea pigs by inhalation exposure to TMA aerosol but were unable to elicit a respiratory response (change in respiratory rate) following chemical-protein conjugate challenge. Sarlo *et al.* (1994) reported PA aerosol-induced sensitization but not respiratory reactivity to PA aerosol challenge. Those problems may have been due to the regime of exposure, selection of animal species, and indices reflecting responses after airway challenge. Pauluhn *et al.* (2002) reported that TMA topically sensitized rats produced a more pronounced EAR upon TMA challenge than those sensitized by the inhalation route.

The present study examines respiratory sensitization and airway responses to repeated inhalation challenge with

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TMA-aerosol atmospheres in the BN rat model. Immunological (IgE), pulmonary inflammatory, and respiratory responses (EAR and LAR) are reported.

METHODS

Animals

Female, inbred BN rats (150–175 g) were from Charles River Laboratories (Wilmington, MA). All rats were obtained from rooms free of “rat respiratory virus” and other specific pathogens. Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, fed Purina rat chow, and water *ad libitum*, supplied with high efficiency particulate air-filtered air, and kept on a standard 12:12 light:dark cycle. Rats were acclimated in the facility for 1 week prior to use.

Dry TMA Powder Preparation and Aerosol Generation

TMA (Acros Organics, Fair Lawn, NJ) flakes were ground in a water-cooled analytical mill (IKA WORKS, Inc., Wilmington, NC). TMA aerosol was generated using a Wright Dust Feed Mechanism (Messrs. L. Adams Ltd, London, England) into a 20-l plastic settling chamber at the flow rate of 5 l/min. Humidified air (5 l/min) was combined to dilute the TMA concentration and keep the humidity at 40–50% prior to introduction into the nose-only exposure chamber (CH Technologies, Westwood, NJ).

Airway Exposure/Challenges to TMA and Monitoring of Airway Responses

TMA aerosol inhalation exposure was conducted with 4 groups ($n = 8$ per group for the two higher doses and $n = 4$ per group for the two lower doses) of BN rats that were placed in the nose-only exposure chamber at concentrations of 40, 4, 0.4, and 0.04 mg/m³, respectively, starting on day 0 and every 7 days, thereafter, for 10 weeks (exposure/challenge = 1× per week). Under this protocol, the distinction between sensitization exposure and TMA inhalation challenge is the immunological state of the animal rather than the exposure route or dose. All rats, except for the 40 mg/m³ TMA group, were rechallenged with 40 mg/m³ of TMA aerosol, for 10 min (in the nose-only chamber), 2 weeks after the final inhalation exposure to TMA. Chamber concentrations were measured using a real-time, continuous dust monitor (model 1.108, GRIMM Technologies, Inc., Douglasville, GA) and gravimetrically using a 0.45- μ m, 37-mm HAWP filter (Millipore Corporation, Bedford, MA). Rats were immediately moved to whole-body plethysmograph chambers (Buxco Electronics, Troy, NY) after 10 min of TMA exposure, to monitor enhanced pause (Penh) (Hamelmann *et al.*, 1997), for 12–20 h. Normal Penh values (used as control) from all rats were also recorded before the day 0 TMA inhalation exposure. Indices were recorded every 30 s. Arithmetic means of peak values from every 30 min for the first hour and every 1 h after that were used to quantify the responses. Penh area under the curve (AUC) was also determined, as well as starting point, ending point, and duration of the LAR. EAR is an increase in Penh ≥ 0.9 (3 SDs above the mean peak Penh value) that is apparent within 30 min of challenge and is usually resolved by 1 h. LAR manifests 2–4 h postchallenge as an increase in Penh > 0.9 and last for several hours.

Pathologic Analyses of the Lungs

At the end of each experiment, approximately 24 h following airway challenge, rats were euthanized with pentobarbital (100 mg/kg) ip and bronchoalveolar lavage fluid (BALF) obtained. BALF was used for eosinophil enumeration using a Coulter Multisizer (Coulter Corporation, Hialeah, Florida) for total cell count per milliliter and microscopic differential analysis (total cell count/ml \times percent eosinophils). Lungs were excised, perfused, and fixed in 10% phosphate-buffered formalin for the preparation of hematoxylin and eosin (H&E)-stained slides.

Lung sections from the left lung lobes were histopathologically examined by a board-certified veterinary pathologist. Semiquantitative histopathology scores

were assigned for consistent histopathologic changes as previously described (Hubbs *et al.*, 1997). Briefly, the severity (intensity) and distribution (extent) of changes are each evaluated with a range of 0 (none) to 5 (greatest involvement) producing a cumulative histopathology score with a potential range of 0–10.

ELISA for TMA-Specific IgE Analysis

Sera sampling. Blood samples were collected from the tail vein (< 12 h) before each exposure and 2 weeks after the final exposure. The first sera, collected prior to TMA exposure, were used as controls. Sera were stored at -80°C until assayed.

Preparation of TMA-rat serum albumin conjugate. The procedure was described previously (Zhang *et al.*, 2004). In brief, rat serum albumin (RSA) was dissolved (3 mg/ml) in half-saturated sodium borate buffer (pH 9.4). TMA was dissolved in acetone (10 mg/100–200 μ l) and added slowly, dropwise to the RSA solution while stirring. The final reaction solution contained TMA-RSA conjugate and the molar ratio was 60:1. The TMA-RSA solution was stirred for 30 min and then dialyzed against distilled water. TMA-RSA was lyophilized and stored, desiccated, at -20°C until use.

The ELISA. The procedures were previously described (Zhang *et al.*, 2004). Microtitration plate wells were coated with TMA-RSA (100 μ l, 0.15 mg/ml carbonate buffer), washed (PBS-tween, 3 \times), and then blocked with 5% heat-inactivated horse serum (Sigma Chemical Company, St Louis, Missouri) in carbonate buffer. Rat sera were serially diluted in PBS and added to the wells and incubated for 1 h at 37 $^{\circ}\text{C}$ and washed. Sheep anti-rat IgE (Cat. No. 64-352, ICN Biomedicals, Inc., Costa Mesa, California) was added to the wells and incubated for 1 h at 37 $^{\circ}\text{C}$, plates were washed, and HRP-donkey anti-sheep IgG (Cat. No. 67541, ICN Biomedicals, Inc.) was added to each well for 1 h at 37 $^{\circ}\text{C}$. Plates were developed using the substrate tetramethylbenzidine (100 μ l/well, Sigma Chemical Company) in the dark, at room temperature, for 30 min. Optical density (OD) was read at 630 nm (ELX808 Microplate reader, Bio-Tek Instruments, Inc., Winooski, Vermont). The OD values of the sera (dilution 1:50) were considered positive if they were greater than 0.05 and also greater than the mean OD values of control sera plus threefold SD (mean +3 SD). Quantitative analyses expressed as “relative units” were performed for the sera with positive specific IgE; antibody-positive sera from previous rats that had been sensitized to TMA (Zhang *et al.*, 2002a) were pooled and used as a reference standard serum. The pooled sera were diluted serially to develop a standard curve to which each sample was referenced. The absorbance from a 1:100 dilution of the reference sera was assigned a value of 100, thus the reference sera had a concentration of 10^4 units of activity of IgE.

Data Analyses

All IgE data were log transformed to stabilize the variance across time. Differences in the time patterns of IgE response between low and high TMA doses were tested using repeated measures ANOVA using SAS PROC Mixed software (SAS Institute, 1999). In order to accomplish this, we tested for a day-by-group interaction, and when this was found to be significant ($p = 0.006$), we performed *post hoc* comparisons between groups by day.

In order to perform this analysis, it was necessary to select the appropriate covariance structure for the correlated measures over time to be included in the ANOVA. This was accomplished by examining correlation of log IgE data points between times separately for each group and by testing a series of covariance structures including the unstructured, autoregressive, and compound symmetric models to determine the best fit for these data. The autoregressive model with unequal variances for TMA dose groups provided the best fit covariance structure, minimizing both the Akaike’s information criteria and Bayesian information criteria statistics (Brown and Prescott, 1999).

Data were described as mean \pm standard error (SEM). Comparisons for two groups were made with the Student *t*-test. One-way ANOVA with *post hoc* Tukey tests were used for experiments in which more than two groups were compared. Man-Whitney *U*-test was used for data quantified as percentage of total cell count. *P* values < 0.05 were considered significantly different.

The pathology scores for the 0.04, 0.4, 4, and 40 mg/m³ of TMA exposure concentration (all challenged with 40 mg/m³) were evaluated with the Exact Kruskal-Wallis test. Where statistical significance was identified, the Exact Wilcoxon test was used to compare the different exposure groups.

RESULTS

Specific IgE to TMA

TMA-specific IgE was detected by day 7 in the 40 and 4 mg/m³ TMA exposure groups. Only 1/4 rats had measurable specific IgE in the group exposed to 0.4 mg/m³ of TMA, and no specific IgE was detectable in rats from the lower exposure groups (0.04 mg/m³). While antibody titers were considerably higher in the 40 (vs. 4) mg/m³ TMA exposure group (Fig. 1), TMA-specific IgE progressively decreased after day 42 in this group.

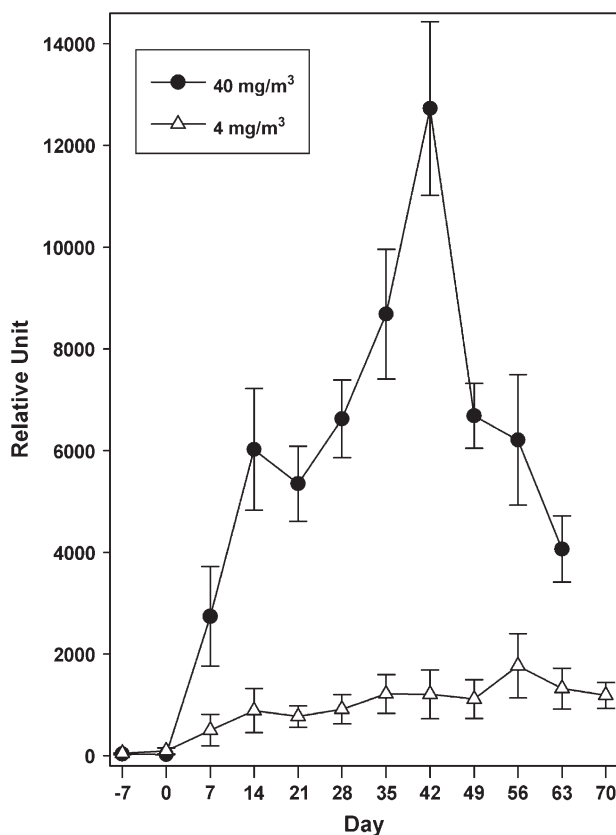


FIG. 1. Rat serum-specific IgE analyses by ELISA ($n = 8$ per group). Sera collection started on day -7 , TMA exposure (inhalation for 10 min) started on day 0, and the procedures repeated every 7 days thereafter through day 63 or 70. IgE levels increased significantly ($p < 0.05$) by day 7 and reached a peak by day 42 in rats sensitized to 40 mg/m³ TMA. In rats exposed to 4 mg/m³ TMA, IgE increased significantly ($p < 0.05$) by day 21 and reached the peak after day 35. IgE levels are significantly higher in rats exposed to the higher concentration of TMA than rats exposed to the lower concentration of TMA.

Airway Responses after Inhalation Exposure and Final Challenge with TMA

During the 10-week exposure period, EAR and LAR were noted only in rats exposed to 40 mg/m³ TMA. No respiratory changes were found before TMA exposure and after exposure on day 0 and 7. On day 14, 6/8 of the rats challenged with 40 mg/m³ TMA developed both EAR and LAR; however, these responses were inconsistent from week to week from each animal (Table 1). No significant respiratory changes were noted in groups before and after exposure to lower concentrations of TMA over the 10 weekly exposures. On day 77, all the lower exposure groups were challenged with 40 mg/m³ TMA for 10 min. All eight rats from the 4 mg/m³ TMA group and only one rat from the 0.4 mg/m³ TMA group responded with both EAR and LAR to the challenge (AUC 1439.2, the same rat with measurable specific IgE). No airway responses were noted in the rats from the 0.04 mg/m³ TMA group following the 40 mg/m³ TMA challenge. The AUC of the LAR from the 4 mg/m³ TMA group challenged with 40 mg/m³ was 1120 ± 134 . In comparison, the highest LAR AUC noted from each animal exposed over 10 weeks to 40 mg/m³ was 592 ± 89 . The repeated high-dose exposure produced a weaker LAR than the lower dose sensitization—high-dose challenge protocol ($p = 0.0027$). There was no pattern (increasing or decreasing Penh AUC) of the LAR with repeated exposure observed in the high-dose group and, in fact, LAR Penh was inconsistently manifested following challenges in this group. Figure 2A is the LAR and EAR indicated by Penh on days -7 , 49, and 63 from rats sensitized and challenged to 40 mg/m³ TMA. Figure 2B illustrates the lack of EAR and LAR responses in the 4 mg/m³ TMA exposure group until subsequently challenged to 40 mg/m³. It must be noted that LAR onset, duration, and time of peak response varies from rat to rat, and thus, the figure demonstrates the average AUC but blunts the actual peak responses. Table 2 lists onset, duration, peak, and AUC of LARs noted from the 40 and 4 mg/m³ exposure groups following challenge with 40 mg/m³ TMA.

Pathologic findings. Table 3 lists the number of total cells recovered from each BALF and the cell differential. Eosinophils, but not neutrophils, were found in the lavage of animals that were sensitized with and responded to TMA challenge. Rats exposed to 4 mg/m³ TMA (and challenged to 40 mg/m³ TMA) had significantly higher percentage of eosinophils, than that in rats exposed to 40 mg/m³ of TMA. The total BALF eosinophils recovered was not statistically different. Lower total BALF cells in this group may be due to inflammatory changes such as activated “sticky” macrophages causing poor recovery of these cells.

The principal histopathologic changes in the lungs of TMA-exposed rats were eosinophilic granulomatous interstitial pneumonia, eosinophil perivascular infiltrates, peribronchiolar plasma cell infiltrates, and hyperplasia of bronchus-associated lymphoid tissue (BALT). Some rats demonstrated airway

TABLE 1
Prevalence of Rats Developing EAR (E) and/or LAR (L) after Each Exposure to 40 mg/m³ of TMA

Rat No.	Day 14		Day 21		Day 28		Day 35		Day 42		Day 49		Day 56		Day 63		Day 70	
	E	L	E	L	E	L	E	L	E	L	E	L	E	L	E	L	E	L
1	+	+			+		+	+	+	+	+	+	+		+	+		
2									+	+	+	+	+		+	+		+
3	+	+	+		+				+	+	+	+	+		+	+		+
4			+						+		+	+				+		+
5	+	+	+		+				+	+	+	+	+		+	+		+
6	+	+			+				+		+	+	+		+			+
7	+	+			+				+	+	+	+	+		+	+		+
8	+	+	+		+	+	+	+	+	+	+	+	+		+	+		+

Note. + denotes EAR or LAR following TMA challenge observed. Absence of response to TMA challenge is denoted by the absence of the + sign.

epithelial cell hypertrophy consistent with mucous metaplasia. However, the consistent and predominant histopathologic alterations in this study were not in the airways but instead were localized to the deep lung. Eosinophilic granulomatous interstitial pneumonia was characterized by interstitial infiltration by histiocytic macrophages and eosinophils (Fig. 3A). Lesser numbers of giant cells, neutrophils, and lymphocytes contributed to the interstitial inflammation in some lungs. Many foci of intense eosinophilic granulomatous inflammation did not have a demonstrated pattern near airways. Frequently,

the interstitium between foci of intense inflammation was involved but to a lesser extent. At 0.4 mg/m³ TMA, two of the four rats had eosinophilic granulomatous interstitial pneumonia. All rats inhaling 4 or 40 mg/m³ had eosinophilic granulomatous interstitial pneumonia, although the severity of the response was slightly, but not significantly, reduced in rats inhaling 40 mg/m³. The pathology scores for eosinophilic granulomatous interstitial pneumonia in rats inhaling 40 mg/m³ were significantly higher than in rats inhaling 0.4 mg/m³. Perivascular eosinophils were frequently observed in TMA-exposed rats and may represent vascular eosinophils migrating to the inflamed parenchyma. Peribronchiolar infiltrates of plasma cells were seen adjacent to occasional small bronchioles in all rats inhaling 40 mg/m³ and two rats inhaling 4 mg/m³ (Fig. 3B). The pathology scores for these plasma cell infiltrates were significantly greater in rats inhaling 40 mg/m³ TMA than in rats inhaling 4 or 0.4 mg/m³ TMA. These plasma cell infiltrates are unusual in the rat lung. Other evidence of antigenic stimulation seen in this study included hyperplasia of BALT. Hyperplasia of BALT was observed in all rats exposed to TMA but did not demonstrate a dose-response relationship. Eosinophilic granulomatous interstitial pneumonia, eosinophil perivascular infiltrates, peribronchiolar plasma cell infiltrates, and hyperplasia of BALT were present in TMA-exposed rats but not observed in the two controls (Fig. 4).

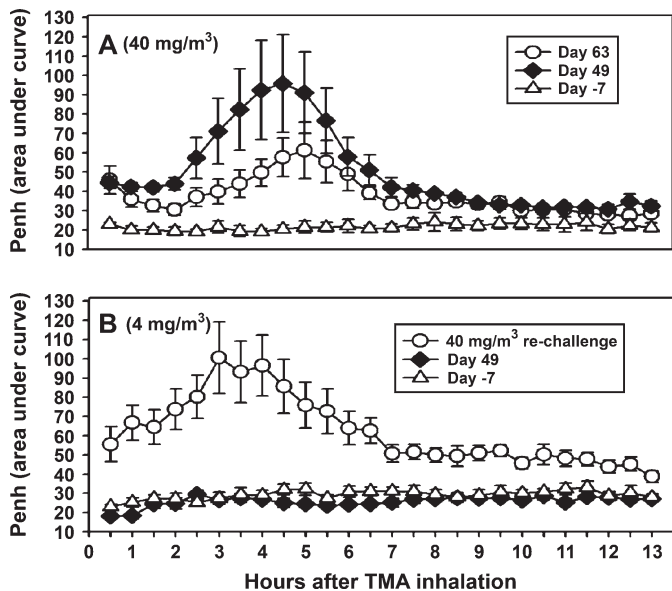


FIG. 2. Airway response after inhalation sensitization and challenge with 40 mg/m³ TMA (Fig. 2A) and 4 mg/m³ with a subsequent 40 mg/m³ TMA rechallenge (Fig. 2B). Eight rats per group were exposed to clean air on day -7 and to 4 or 40 mg/m³ TMA every 7 days through day 70. The 4 mg/m³ TMA group was then rechallenged with 40 mg/m³ TMA on day 84 (2 weeks after the last 4 mg/m³ TMA exposure). AUC averaged over each hour of the Penh response from selected, representative days are presented. EAR and LAR were only noted following challenge with 40 mg/m³ TMA.

DISCUSSION

The utility of the dermal route of sensitization followed by inhalation challenge in the development of animal models of OAA asthma has been well documented (Ban *et al.*, 2001; Derman *et al.*, 2000; Pauluhn, 2003; Zhang *et al.*, 2002a, 2004); however, as described in the introduction, protocols using both sensitization and challenge by the inhalation route have been unsatisfactory with respect to induction of airway responses (Botham *et al.*, 1988; Sarlo *et al.*, 1994). The BN rat model was used in the present study to examine specific airway

TABLE 2
LARs Following 40 mg/m³ TMA Aerosol Challenge in TMA Airway Sensitized Rats

Sensitization route (dose)	Starting point	Ending point	Duration	Peak Penh	AUC
Inhalation (4 mg/m ³)	2.1 h ± 0.1	10.7 h ± 1.1*	8.6 h ± 1.0*	3.2 ± 0.4	1120 ± 134*
Inhalation (40 mg/m ³)	2.8 h ± 0.3	5.3 h ± 0.2	2.5 h ± 0.5	2.6 ± 0.9	338 ± 184

Note. Starting or ending points denote the hours that the LARs started or ended after airway challenge with TMA aerosol. Duration is the time that the LARs lasted. Peak Penh, maximal Penh during the entire LAR; AUC, area under the curve of Penh.

*Significantly different from the 40 mg/m³ inhalation sensitization, $p < 0.05$.

reactivity following sensitization by the respiratory tract route. We found that inhalation exposure to TMA aerosol induces (1) the production of measurable specific IgE following a single exposure (specific IgE noted in serum prior to second 40 mg/m³ exposure), (2) specific airway reactivity (Penh, EAR, and LAR), (3) pulmonary allergic inflammatory pathology, and (4) an attenuated specific IgE response with repeated high-dose aerosol exposure. The results also suggest that potential physiological compensatory mechanisms such as receptor or mediator down regulation may be triggered by repeated high dose (40 mg/m³ TMA) as physiological airway responses to TMA challenge were both inconsistent from week to week from the same animals and muted compared to the lower repeated inhalation dose group (4 mg/m³ repeated exposure with a single 40 mg/m³ challenge).

Dose-dependent specific antibody production has been demonstrated in animal models following TMA inhalation (Botham *et al.*, 1988; Leach *et al.*, 1989; Zeiss *et al.*, 1987). Leach *et al.* (1989) used a 13-week inhalation exposure regimen at the concentration of 0, 2, 15, and 50 µg/m³ TMA for 6 h/day, 5 days/week using Sprague-Dawley (S-D) rats. Tolerance with respect to both pulmonary pathology and serum-specific antibody was reported. The S-D rat is a very poor producer of IgE and may be considered a more "Th-1"-responsive rat than the BN rat. Data from the present work showed a hapten exposure versus specific IgE dose-response relationship, and the antibody levels peaked by 5 weeks following inhalation with tolerance noted in the high-exposure group. TMA-induced hypersensitivity diseases noted from the occupational setting include (1) allergic (IgE-mediated, type I) asthma/rhinitis, (2) late respiratory systemic syndrome (LRSS), and (3) pulmonary disease anemia (PDA) (Zhang *et al.*, 2002b). The mechanisms of the LRSS and PDA are not well understood and thought to possibly involve type II, III, and IV hypersensitivity mechanisms. Responses noted by Leach *et al.* (1989) may reflect TMA-induced LRSS or PDA, while that from the BN rat reported may be predominantly, but not solely, IgE mediated.

In the present work, histopathological changes in the lungs were examined following the 10-week exposure protocol. Hyperplasia of BALT noted in 2/4 animals exposed to the lowest (0.04 mg/m³) TMA dose may indicate local airway immune responses to the antigen. Eosinophilic granulomatous

interstitial pneumonia was found in all rats inhaling 4 or 40 mg/m³ and in 2 rats inhaling 0.4 mg/m³ TMA; however, the severity of eosinophilic granulomatous pneumonia did not correlate with a physiological (Penh) response in an individual animal. There was a slight, statistically insignificant decrease in severity of eosinophilic granulomatous pneumonia in the 40 mg/m³ TMA group when compared with the 4 mg/m³ TMA by histopathology. The eosinophilic granulomatous pneumonia observed in BN rats in our study may represent a response to chronic interstitial inflammation following inhalation of TMA. TMA has been reported to cause chemical pneumonitis in workers (Rice *et al.*, 1977). The fact that TMA produces several hypersensitivity diseases may have contributed to the lack of correlation of pathology with specific IgE serum levels or airway reactivity. It also points to the complexity of low molecular weight (LMW) hypersensitivity diseases and that findings from protein allergen hypersensitivity studies may not be generalized to LMW-induced asthma.

The multifocal peribronchiolar plasma cell infiltration was dose dependently associated with TMA exposure concentration. Plasma cells increased in the lung while circulating levels of TMA-specific IgE declined. These plasma cells may not be secreting the IgE isotype, or possibly, more IgE is being sequestered in the lung. Further work is needed to investigate the inverse relationship between circulating specific IgE and lung plasma cell content.

TABLE 3
Differential of BALF Cells (mean ± SEM) Following
40 mg/m³ Challenge

Group (mg/m ³)	Total (per ml)	Eosinophils (%)	Macrophages (%)	Lymphocytes (%)
0.04	(28.34 ± 3.33) × 10 ⁴	0.50 ± 0.29	98.75 ± 0.75	0.75 ± 0.48
0.4	(41.42 ± 14.99) × 10 ⁴	16.50 ± 16.18	82.25 ± 15.76	1.25 ± 0.75
4	(17.80 ± 2.92) × 10 ⁴	42.38 ± 4.00*	55.50 ± 4.20	2.13 ± 0.29
40	(34.02 ± 5.26) × 10 ⁴	20.38 ± 3.75**	77.63 ± 3.54	2.00 ± 0.46

*Eosinophils %: the data from 4 mg/m³ is significantly higher than that from other groups.

**Eosinophils % significantly different than 0.04 mg/m³ (Mann-Whitney, $p < 0.05$).

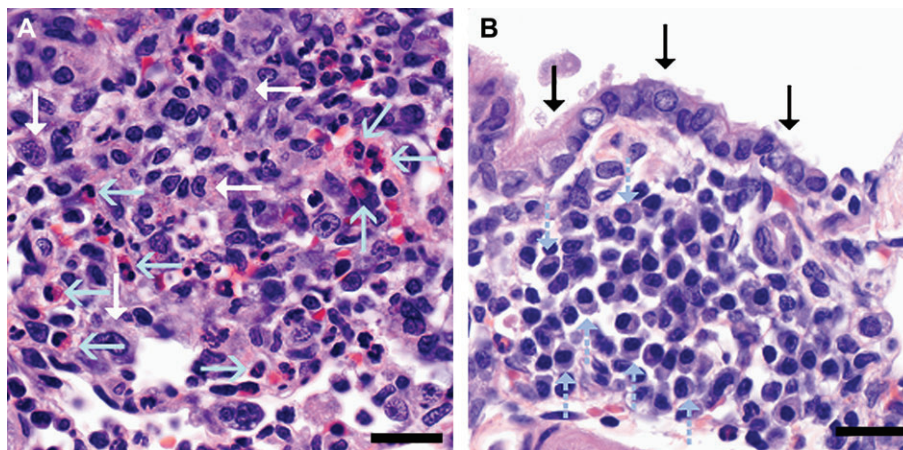


FIG. 3. Photomicrographs of H&E-stained sections of the TMA-exposed lung. (A) Eosinophilic granulomatous pneumonia in a rat inhaling 4 mg/m^3 TMA. Large numbers of epithelioid macrophages (white arrows) are infiltrating alveoli, frequently forming foci comprised of confluent sheets of macrophages admixed with large number of eosinophils (blue arrows). Bar = $20 \mu\text{m}$. (B) In a rat inhaling 40 mg/m^3 TMA, large numbers of plasma cells (blue dashed arrows) are in the lamina propria beneath the lining epithelium (black arrows).

Exposure to and sensitization by TMA in workers has been considered to be mainly through aerosol inhalation; however, dermal exposure has also been of concern. Attempts to model TMA asthma-like responses in animal models of airway exposure reported during the past 30 years have been problematic. The importance or relative contribution of dermal versus inhalation routes of sensitization to TMA asthma in the occupational setting cannot be gleaned from the present study. The short inhalation exposure used for sensitization and challenge would more reflect a short excursion/accidental release versus permissible 8-h exposure limits (National Institute for Occupational Safety and Health (NIOSH) recommended exposure limit = 0.04 mg/m^3 time weighted average).

It is very difficult to directly compare the dermal and inhalation routes of sensitization. Multiple exposure variables (exposure time, dose, exposure pattern, and surface area with respect to skin) may impact both sensitization and subsequent elicitation of immunologically mediated physiological and inflammatory responses. Our previous studies employed dermal sensitization to TMA powder in the BN rat model (Zhang *et al.*, 2002a, 2004). In these studies, dermal sensitization was achieved by application of dry TMA powder, once a week for up to 5 weeks. It is apparent that both routes can induce immunological sensitization and subsequent pulmonary responses upon aerosol challenge; however, significant differences exist using the specific inhalation versus dermal sensitization protocol. The aerosol concentration that produced consistent immunological sensitization (4 mg/m^3 TMA) in the present study was insufficient to produce an airway physiological response. The higher challenge dose (40 mg/m^3 TMA) was required to produce EAR and LAR in the 4 mg/m^3 sensitization group, and these responses were considerably greater than those seen from rats both sensitized and challenged to 40 mg/m^3 TMA, even though circulating TMA-specific IgE was much higher in the latter. The maximal LAR response observed in

the 4 mg/m^3 sensitized, 40 mg/m^3 TMA-challenged group was comparable to that seen in the 20 mg dermal-sensitized (4 weekly dermal exposures), 40 mg/m^3 TMA inhalation-challenged group. Much lower TMA aerosol challenge doses

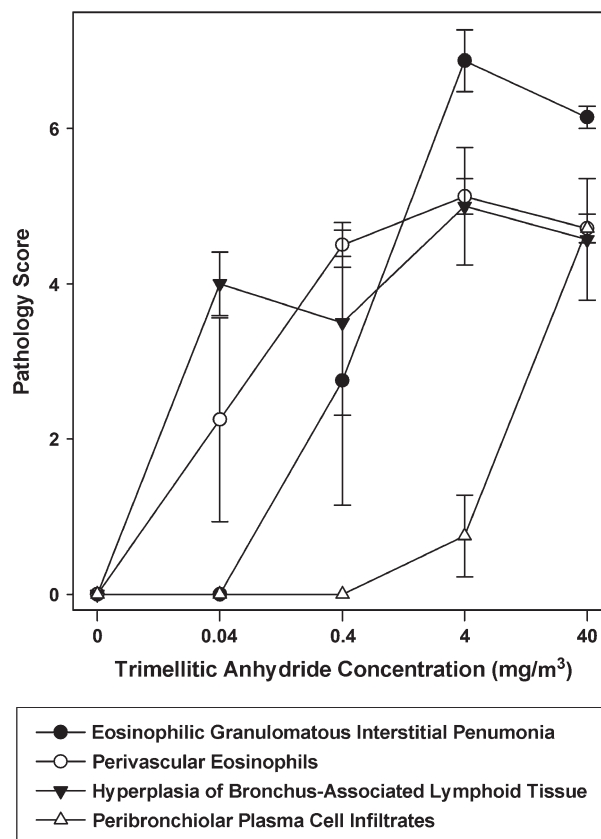


FIG. 4. Semiquantitative pathology scores for the principal histopathologic changes observed in the lungs of TMA-exposed rats. Scores are based on both severity and distribution.

were required to produce an EAR or LAR following the dermal sensitization protocol (Zhang *et al.*, 2004; 1 mg/m³ for EAR and 5 mg/m³ for LAR), when compared to inhalation challenge dose of 40 mg/m³ required in the present study. A striking difference between the two sensitization protocols is the maximal circulating TMA-specific IgE levels. The dermal sensitization protocol produced specific IgE titers that are approximately 20-fold higher than that obtained through inhalation sensitization (259,668 ± 27,014 vs. 12,724 ± 4,828 relative TMA-specific IgE units, respectively). The TMA-specific IgE titer peaked by day 42 in higher inhalation TMA dose of the present study, while the peak was observed by day 28 with the dermal sensitization protocol. The IgE titer on day 35 of weekly dermal dosing was significantly lower than day 28; however, this further dermal dosing was not done in this previous study to be able to verify attenuation of the dermal exposure-induced IgE response.

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