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Original article

Dose-response and time course of specific IgE and IgG after single and repeated topical skin exposure to dry trimellitic anhydride powder in a Brown Norway rat model

Background: Trimellitic anhydride (TMA)-induced occupational asthma is thought to be associated with its ability to acylate proteins and to induce production of TMA-specific immunoglobulin (Ig)E. Though the respiratory tract is considered to be a major exposure route leading to airway sensitization, the potential role of dermal exposure producing asthmatic sensitization is not known. The present study examines the ability of dry TMA powder to sensitize Brown Norway rats when applied, topically, to the skin.
Methods: A patch of hair was carefully clipped with scissors on the rat's back. Dry TMA powder (0.3, 1.25, 5 and 20 mg) was administered on days 0, 7, 14 and 21, and the area occluded with surgical tape overnight after each application. Residual powder recovered from the occluded skin was analyzed by proton nuclear magnetic resonance and was still predominantly TMA. Circulating anti-TMA IgE and IgG were measured by ELISA.
Results: TMA elicited dose-dependent production of specific IgE and IgG. Specific antibodies were detectable 2 weeks after the first TMA exposure and peaked between 3 and 4 weeks.
Conclusion: The data suggest that topical skin exposure to dry TMA powder can induce allergic/immunological sensitization as demonstrated by the production of specific antibodies.

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Key words: Brown Norway rat; IgE; occupational asthma; skin/ dermal exposure; sensitization; trimellitic anhydride.

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Organic acid anhydrides (OAA), such as trimellitic anhydride (TMA), phthalic anhydride, hexahydrophthalic anhydride, methyltetrahydrophthalic anhydride, tetrachlorophthalic anhydride, and maleic anhydride, are widely used industrially to make epoxy and alkyd resins, plasticizers, high temperature polymers and surfactants. OAA products have medical, textile and lubrication applications. OAA are highly reactive, low-molecular-weight chemicals. Specific antibodies to OAA have been found in exposed workers and OAA-induced occupational asthma is thought to be mediated by an IgE-mediated allergic mechanism (1–10, 41).

The pathogenesis of immunologically-induced asthma can be divided into two phases, a primary antigen/hapten exposure leading to antibody production (sensitization) and subsequent aerosol exposures eliciting asthmatic symptoms. The route of exposure leading to OAA sensitization is commonly thought to be inhalation in the workplace (10–13). Allergic sensitization might also result from antigen exposure by other routes, notably dermal contact. However, the exposure conditions (route, concentration, and duration, etc.) required for OAA sensitization are not clearly known (14, 15). Previous studies in animal models have demonstrated that OAA when suspended or dissolved in acetone/oil, and applied to the skin, can induce an IgE response (16, 17). Potential permissive or adjuvant effect of the solvent in these previous studies is of concern. The present work examined the induction of specific antibodies following dermal (topical skin) exposure to dry TMA powder in a Brown Norway (BN) rat model.

Material and methods

Animals

Male, inbred BN rats (150–175 gram) and male Sprague–Dawley rats (12 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were kept in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility, fed Purina rat chow and water, *ad libitum*, and kept on a standard light/dark cycle. Rats were acclimated in the facility for 1 week before use.

Chemicals

TMA, trimellitic acid (TMAcid) and Evans blue were purchased from Acros Organics (Fair Lawn, NJ, USA). Methohexital sodium (Brevital sodium) was from Jones Pharma (Saint Louis, MO, USA). Polyclonal sheep antirat IgE (Epsilon chain, Cat. no. 64–352) and horse radish peroxidase (HRP) conjugated polyclonal donkey antisheep IgG (Cat. no. 67541) were from ICN Biomedicals, Inc. (Costa Mesa, CA, USA). Polyclonal goat antirat IgG (whole molecule, Cat. No. R5005) and HRP-conjugated polyclonal rabbit antigoat IgG (Cat. No. A-3540), rat serum albumin (RSA, fraction V powder), horse serum, phosphate buffered saline (PBS, pH 7.4), phosphate buffered saline with Tween-20 (PBS-Tween, pH 7.4) and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Sigma Chemical Company (Saint Louis, MO, USA). The particle size analyzer Coulter® Multisizer was from Coulter Corporation (Hialeah, FL, USA). Surgical tape (Blenderm™) was obtained from 3M Health Care (St Paul, MN, USA).

Dry TMA powder preparation

TMA flakes were ground in a water-cooled analytical mill (IKA WORKS, Inc., Wilmington, NC, USA). The fine powder was collected and stored in the presence of a desiccant. The particle size distribution was determined using particles suspended in Isoton® (Coulter® balanced electrolyte solution) and measured by Coulter® Multisizer II.

Topical skin exposure to dry TMA powder

BN rats were anesthetized using 40 mg/kg, i.p., methohexital sodium. A patch of fur on the back was carefully clipped with scissors (avoid skin irritation and trauma). Dry TMA powder was applied to the clipped area. Table 1 shows the TMA exposure area sizes. TMA exposed areas were occluded with a nonabrasive dermal surgical tape overnight, after which the skin area of application was washed with water to remove any residual TMA. Controls included: (a) a clipped, occluded, untreated group; (b) powder TMAcid (20 mg) treated group and (c) TMA treated but gauze occluded group (gauze was placed in between the tape and TMA treated skin to test whether the tape occlusion could alter dermal/TMA interaction).

Dose-response and antibody production time course studies following single and multiple TMA applications were conducted. (i) Dose–response relationship after TMA exposure: dry TMA powder at doses of 0, 0.3, 1.25, 5 and 20 mg was applied topically to BN rats ($n = 8$/dose) on days 0, 7, 14 and 21. Blood was collected, intracardially, after anesthetization on day 35 for serum-specific IgE and IgG analyzes. (ii) Time course of antibody formation after single TMA exposure: a single TMA dose (20 mg) was applied to the skin of BN rats ($n = 8$) with blood collected from the tail vein on days 0, 7, 14, 21 and 28 and intracardially on day 35 for serum-specific IgE and IgG analyzes. (iii) Time course of antibody formation after repeated TMA exposure: dry TMA powder (20 mg) was applied to the skin of BN rats ($n = 8$/group) on days 0, 7, 14 and 21. Blood was collected from the tail veins on days 0, 7, 14, 21 and 28 and intracardially on day 35 for serum-specific IgE and IgG analyzes.

Nuclear magnetic resonance (NMR) analysis

Residual TMA powder was collected 20 h after application from the skin and patch material of rats exposed to 1.25, 5 and 20 mg of TMA. The residual powder was analyzed by proton (NMR) for both TMA and its hydrolysis product, TMAcid. The residual powder was dissolved in 1–4 ml of deuterated methanol (CD_3OD), then placed in a dry and sealed NMR tube. ^1H (proton) NMR spectroscopy was performed at 300.131416 MHz on a Bruker Avance DMX 300 NMR spectrometer equipped with either a 10-mm Bruker QNP or a 5-mm Nalorac ZSpec inverse probe. A single pulse sequence was used to acquire 200 scans of 16 k data points at a 6-kHz spectral width. A 4 s recycle delay was used. Chemical shift was referenced internally to the CHD_2 resonance at 3.31 p.p.m., verified by external reference to tetramethylsilane in acetone-D_6 (18).

Preparation of TMA-RSA conjugate

RSA was dissolved (3 mg/ml) in half-saturated sodium borate buffer (pH 9.4), TMA was dissolved in acetone (10 mg/100–200 μl) and slowly added drop-wise to the RSA solution while stirring, the final TMA : RSA molar ratio was 60 : 1. The pH of the solution was maintained at 9.4 by the addition of 2 M sodium hydroxide (NaOH). 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.

TMA specific IgE and IgG

Rat serum-specific IgE and IgG was analyzed by ELISA. Microtitration plate wells (ICN Biomedicals, Inc., Horsham, PA, USA) were coated with TMA-RSA (100 μl, 0.15 mg/ml) in carbonate buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ in 1l water, pH 9.6) and incubated overnight at 4°C. The contents from each well was removed and plates were washed twice with PBS–Tween following each addition/incubation procedure. Non-specific binding was blocked by incubation with 200 μl of 5% heat-inactivated horse serum in carbonate buffer for 2 h at room temperature. Plates were stored at −20°C until use. Rat sera were diluted in PBS (from 1 : 50 to 1 : 10000). Dilute sera (100 μl) were added to the wells and incubated for 1 h at 37°C. Sheep antirat IgE (100 μl, dilution 1 : 5000) was added to the wells and incubated for 1 h at 37°C. HRP-donkey antisheep IgG (100 μl, dilution 1 : 10000) was added to the wells and incubated for 1 h at 37°C. Plates were developed using TMB (100 μl/well) in the dark, at room temperature, for 30 min. Optical density (OD) was read

Table 1. Dose and antibody response relationships of dry trimellitic anhydride (TMA) powder skin exposure. Brown Norway rats ($n = 8$/group) were administered topically with dry TMA powder on day 0, 7, 14 and 21 and occluded overnight with surgical tape after exposure. Sera were collected 2 weeks after the last exposure and specific IgE and IgG were measured using an ELISA. Data shown as relative units. Antibody levels were dose-dependent ($P < 0.05$, Kruskal–Wallis test). There was no significant difference (Mann–Whitney *U*-test) between the two groups given 20 mg of TMA occluded with tape or gauze after TMA application

Dose of TMA applied (mg)	Application size	Prevalence*	IgC relative unit mean ± SE	IgE relative unit median & SE
0.00	0.0	0/8	459 ± 11†	1390 ± 347†
0.30	0.2 × 0.2	4/8	500 ± 71	8031 ± 1200
1.25	0.4 × 0.4	7/8	820 ± 172	26604 ± 11644
5.00	1.0 × 0.6	8/8	4795 ± 2571	86644 ± 25385
20.00	3.0 × 1.5	8/8	19485 ± 5221	231185 ± 32285
20.00‡	3.0 × 1.5	8/8	13837 ± 2227	259668 ± 27014

*Number of animals with measurable specific anti-TMA antibodies.
†Represent nonspecific binding.
‡Occluded with gauze after TMA application.

at 630 nm with a photometer (ELX808 Microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). TMA-specific IgG analysis was conducted in an identical manner, but with the substitution of goat antirat IgG (1 : 1000) and HRP-rabbit antigoat IgG (1 : 25000) for the antirat IgE and HRP antisheep antibodies, respectively. The OD values of the sera (dilution 1 : 50) were considered positive if they were greater than the mean OD values of control sera plus three-fold standard deviation (mean OD + 3 S.D.) and greater than 0.05 OD. Quantitative analyzes expressed as 'relative unit' were performed for the sera with positive specific IgE or IgG: (i) aliquots from all positive sera were pooled and used as a standard serum pool. The standard pool was serially diluted to develop a standard curve to which each sample was referenced. A relative unit value of 100 for IgE and 1000 for IgG was assigned to the reference sera. The standard pool was run on each plate used for antibody analysis. (ii) Individual serum samples were diluted at 1 : 50, 1 : 100, 1 : 1000 and 1 : 10000 for ELISA analyzes to obtain a valid OD value, and by reference to the standard curve, the units of activity for each antibody class was determined.

Passive cutaneous anaphylaxis (PCA) test

PCA was used to confirm the presence of specific IgE and confirm the specificity of the ELISA. Shaven backs of the anesthetized Sprague–Dawley rats were injected intradermally with 100 μl of the antisera/site. The antisera from TMA-exposed BN rats were divided into two portions; unheated and heated at 56°C for 30 min. Heat treatment selectively denatures IgE and prevents its binding to the mast-cell receptor. Seventy-two hours after the injection, the anesthetized Sprague–Dawley rats were challenged i.v. with 1 ml of 1% Evans blue containing 3 mg of TMA-RSA. The outer surface of the skin was examined for a cutaneous response 30 min later. A result was considered to be positive if the blue spot was ≥ 5 mm in diameter.

Data analyzes

Wilcoxin signed ranks test was used for paired two-group samples (before and after TMA exposure to the same group of rats). The Mann–Whitney *U*-test was used for comparisons of two groups and the Kruskal–Wallis test was used for comparisons of more than two groups. *P*-values that were less than 0.05 were considered significant.

Results

TMA particle size

TMA was ground prior to application to the rat skin. Particles' size analysis was performed in Isoton II, and repeated analysis of particles suspended in the solution did not change over time suggesting that surface hydrolysis did not alter the particle size. The particle diameter sizes ranged from < 1.88 μm (lower size limit capability of instrument) to 62.15 μm, with a mean ± SD of 2.77 ± 0.94 μm. Greater than 90% of the particles had diameters < 4 μm and very few of the particles (< 1%) had diameters > 10 μm.

Table 2. Hydrolysis rate of trimellitic anhydride (TMA) recovered from the skin of Brown Norway rats. Rats received dry TMA powder on the topical skin at different doses and occluded with surgical tape overnight. The residual powder was collected and analyzed by proton nuclear magnetic resonance

TMA Dose	Type 1 TMA	Type 2 TMA	TMAcid
Standard*	60%	40%	0%
1.25 mg	91%	9%	0%
5.00 mg	54%	22%	24%
20.0 mg	24%	23%	53%

* TMA stock.

NMR analysis

Residual TMA powder was collected from the occlusion tape and the skin surface 20 h after application. Two chemical forms of TMA and the acid hydrolysis product of TMA (TMAcid) could be identified and relative amounts quantified by NMR (Table 2 and Table 3). At the lowest dose (1.25 mg) the predominant species recovered was Type 1 TMA and no hydrolysis product was noted. The hydrolysis product, TMAcid, tended to increase with increasing dose. There was also a dose-dependent shift in the type of TMA found. The specific differences between Type 1 and Type 2 TMA could not be distinguished by the NMR methodology used.

Specific antibodies

Dose–response Table 1 shows the dose-dependent relationship between TMA exposure and specific antibody response, and the antibody production in the group occluded with gauze after TMA application. Both the concentration and prevalence (i.e., number of animals that produce TMA specific IgE and IgG) of specific antibodies were dose-dependent. TMA specific antibodies were not detectable in a powder application control group given the TMA hydrolysis product, TMAcid.

Time course TMA-specific IgE and IgG was significantly increased ($P < 0.001$) by day 14 in sera from the BN rats given a single or multiple dermal exposures (on days 0, 7, 14 and 21) to TMA. Antibody levels peaked after 2 weeks. Multiple TMA exposure produced higher antibody levels than a single exposure and tended to delay the peak response for approximately 1 week (Fig. 1a–d).

PCA The production of TMA-specific IgE was confirmed using the PCA test. Sera from BN rats exposed to 1.25 mg, 5 mg and 20 mg TMA produced positive PCA reactions in naïve Sprague–Dawley rats. Heat treatment of the sera caused loss of PCA reactivity consistent with an IgE-mediated mast-cell activation. (Fig. 2).

Discussion

The potential for low-molecular-weight chemicals, such as the acid anhydrides, to produce allergic sensitization through dermal exposure has been a health concern for workers exposed to these chemicals. Dearman (19), and Zhang et al. (20) reported the development of specific IgE in mice and rats after topical application of TMA in

Table 3. Chemical shift assignments (in p.p.m) for the protons in TMAcid and in Type 1 and 2 TMA*

Chemical/Proton	H6	H5	H3
TMA Type 1	7.72	8.21	8.45
TMAcid	7.78	8.20	8.39
TMA Type 2	7.86	8.23	8.29

*Peak widths were typically 0.015 p.p.m. H3 is a singlet, while H5 and H6 in every case are split into doublets with J coupling of 8 Hz between them. Relative proton quantities are directly related to peak area, typically to within a few percent.

acetone and olive oil, or after intradermal injection with TMA in dioxane and liquid paraffin. Several studies have also been reported in which OAA, when dissolved in a solvent and applied to the skin, induced the interleukin (IL)-4 production and lymphocyte proliferation using the local lymph node assay (16, 21). These studies suggested the potential of skin to be a route for allergic sensitization by low-molecular-weight chemicals, but all studies, to date, used organic solvents that may have facilitated penetration of chemicals through the stratum corneum. The present study was conducted in order to evaluate the ability of a chemical sensitizer, applied as dry powder, in the absence of solvent, to cause allergic sensitization in the BN rat model.

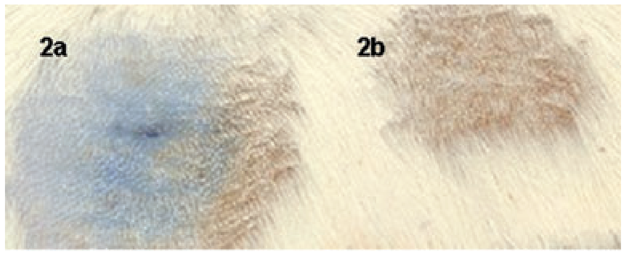

Figure 2. Passive cutaneous anaphylaxis test. Brown Norway rats were topically exposed to dry trimellitic anhydride (TMA) powder. The sera from these rats were injected, intradermally, into naïve Sprague–Dawley rats. Seventy-two hours following injection the rats were challenged by intravenous injection of a solution of TMA–RSA conjugate and Evans blue dye in physiologic saline. The sites with blue colour indicate that the sera from TMA-exposed rats contain specific IgE. Representative reactions following passive transfer of anti-TMA serum are shown in 2a (not treated) and 2b (heat-treated at 56°C for 30 min prior to injection).

TMA was chosen as the sensitizing chemical owing to both its physical/chemical and biological properties. TMA has a low vapour pressure. Occupational exposure to this chemical would, thus, be to a particulate or solution. TMA has been demonstrated to acylate primary amines on proteins acting as a hapten (10). Occupational

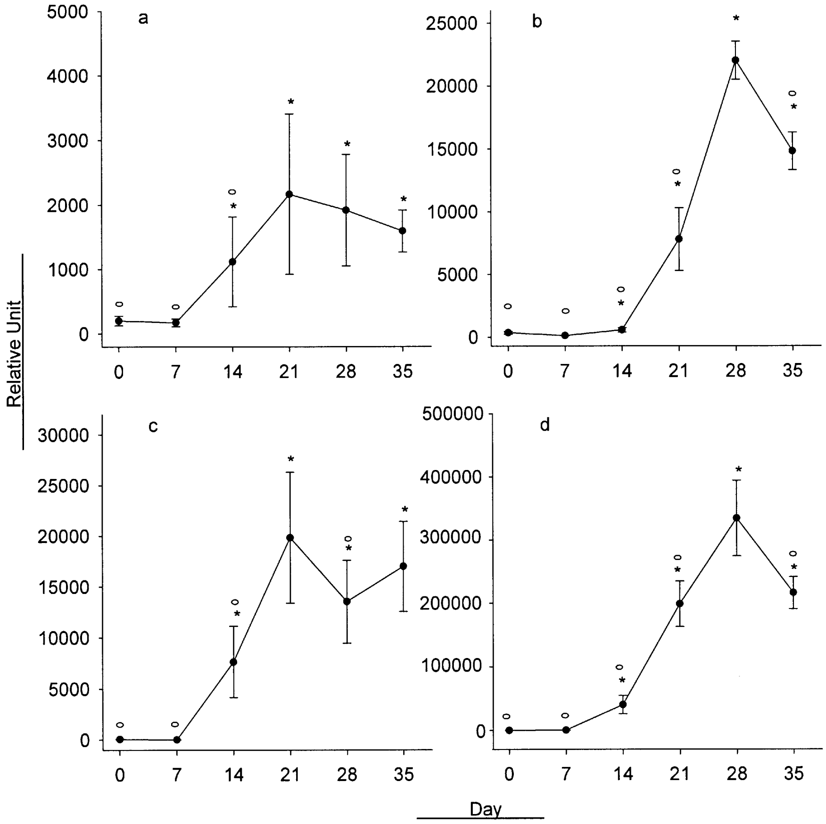

Figure 1. Time course of serum IgE *(a:* single exposure and *b:* multiple exposures. Sera dilution 1/500) and IgG; *c:* single exposure and *d:* multiple exposures. Sera dilution 1/1000). Two groups of rats ($n = 8$/group) were topically exposed to trimellitic anhydride once (a and c) or four times (b and d) on days 0, 7, 14 and 21 at the dose of 20 mg. Sera were taken on days 0, 7, 14, 21, 28 and 35. Antibodies were analyzed by ELISA and expressed as relative units that accorded with optical density (OD). *Different from the relative units on day 0 ($P < 0.05$, Wilcoxin's signed ranks test). °Different from the relative units on day 21 for single TMA exposure or day 28 for multiple TMA exposure ($P < 0.05$, Mann–Whitney *U*-test).

exposure to TMA has been documented to lead to the production of specific IgE and TMA asthma (22). Inhalation has traditionally been considered as a primary route of exposure for low-molecular-weight asthmagens, but the role of dermal exposure or the relation between airway and skin routes in the workplace in sensitization remains in question. It has been reported that workers exposed to airborne epoxy resin compounds, methylhexahydrophthalic anhydride or methyltetrahydrophthalic anhydride not only developed work-related immunological contact urticaria and immediate contact skin reaction, but some also later developed conjunctivitis, rhinitis and asthma (23–25). These clinical cases suggest that skin may be a route for some OAA sensitization; and that the airway response may also occur after challenge following sensitization by the dermal route. The potential for airway and skin (including mucus membrane such as oral and occular mucosa) exposures to OAA exist in the workplace and such exposure can cause direct toxicity such as skin irritation, conjunctivitis, rhinitis, pharyngitis, and bronchitis (26). In addition, the frequently seen, but also frequently ignored, skin mechanical traumas, such as abrasions, cuts, erosions and wounds in the workplace, may greatly increase the probability of sensitization by chemicals (27).

Dearman et al. (16), using a TMA (in acetone-olive oil) dermal exposure mouse model, found an increase in Th2 cytokine production by lymophcytes of culture from draining lymph nodes. The exact mechanism of sensitization is not known, but is thought to be a Th2-driven allergic response. When applied to the skin TMA can act as a hapten either by reacting with proteins at the surface or by penetrating the skin and binding to proteins forming complete antigen (s). This TMA conjugate could then be processed by the antigen-presenting cells (APCs, mainly Langerhans cells), and the cells migrate to the draining lymph nodes for presentation to T lymphocytes. This process would lead to the production of specific antibody by B lymphocytes, and possibly airway reactivity. OAA-specific IgE levels have been shown to be significantly correlated to airway responses in humans (28, 29). Thus, antibody production is one important endpoint in studying OAA-induced sensitization.

OAA dermally sensitized animals have previously been shown to have a positive airway response after antigen challenge (30–32). Airway responses and specific antibody levels were also closely related as shown in a guinea pig model following the sensitization by the dermal route (33). Arts et al. (32), showed in a BN rat model that increased serum IgE after topical sensitization with TMA was associated with inhalation challenge-induced immediate-type specific airway reactivity. In contrast, specific antibodies can be found after airway exposure, but only weak airway responses have been observed following the challenge in these animals (34–36). Inhalation exposure of guinea pigs and rats to TMA has also been reported to induce immunological tolerance (37, 38).

Immunological sensitization by dermal application to TMA dry powder was assessed in the present work by measuring TMA-specific IgE and IgG in the sera (using TMA-RSA as the ELISA capture antigen). The mid-back region of the rat was chosen for the application site and the site was occluded using an air permeable surgical tape to prevent potential exposure by other routes or self-afflicted skin abrasions at the application site. The potential for occlusion to alter the barrier function of the skin was partially evaluated by the addition of gauze under the tape to allow for greater air circulation. The antibody response was not significantly different between occlusions with tape and with gauze.

Dermal application of dry TMA powder in this study was found to produce a dose-dependent specific IgE and IgG response. A single dermal TMA exposure produced a long lasting antibody response. Specific antibodies could be detected by day 14 following the single dose exposure and peaked by day 21. Both IgE and IgG values in rats given multiple exposures were significantly lower at day 35 than at day 28. It is not possible from the present data to determine the cause of this decrease in the specific antibody. Rats were not observed past 35 days and it is not known whether the decrease was the beginning of a downward trend (i.e., the development of tolerance). It must also be noted that the final TMA dermal exposure was given on day 21 and the decrease seen may simply reflect a need for continued stimulation to maintain such high circulating antibody levels.

The dose-dependent pattern of the specific IgG closely resembled that of IgE. Serum levels of specific IgG can be valuable as an index of exposure in surveillance of worker populations (39). Grammer et al. (40), studied 181 subjects exposed to TMA and found that IgG or IgE was predictive of subjects who have or will develop immunologically mediated respiratory disease owing to TMA exposure. Specific IgG is induced after antigen exposure and the absolute amount in the serum is much greater than that of IgE making it more attractive as an index of exposure.

Low-molecular-weight sensitizers are usually very reactive compounds. TMA can react with a number of functional groups on biological molecules and is subject to rapid hydrolysis under aqueous conditions to a carboxylic acid. NMR analyzes of the residual powder collected from the skin after TMA exposure overnight showed that hydrolysis occurred, but a significant percent of the residual powder remained as TMA. Hydrolysis seemed to be related to dose and greater hydrolysis was observed at higher doses. It is possible that an exocrine response increased the amount of water at the application area promoting hydrolysis. It is unlikely that the increased hydrolysis was owing to transudation.

The two types of TMA correspond to two types of anhydride structures formed at vicinal 1 and 2 sites in the TMA product (Table 3). A phthalic anhydride-like species is produced by 1,2 intramolecular carboxylate dehydration, while intermolecular 1,2′, 1,4′, or other anhydride formation produces a second distinct anhydride species. These structural features are consistent with the two types of TMA identified by NMR. H3 and H6 proton chemical shifts are significantly different in Types 1 and 2 TMA, indicating structural differences at the vicinal 1 and 2 carboxylate sites. The isolated 4-carboxylic acid site always produces intermolecular anhydride species, and the adjacent H5 proton chemical shift is similar for both TMA types. Further assignment of molecular structure remains obscure for Type 1 and Type 2 TMA, beyond intra vs. intermolecular 1,2 anhydride formation. The types are distinct spectroscopically, but correspond to distinct features within a dimeric, or oligomeric molecular framework. Both types contain reactive anhydrides, but their chemical properties in this mixed product were not determined. Likewise, their relationship to immunogenicity is unknown. It must be noted that the chemical analyzed was residually collected outside the body and did not provide any insight into the amount or chemical species absorbed.

There were no macroscopic or microscopic signs of inflammation/irritation even at the highest dose (data not shown). However, these histopathologic observations do not rule out the possibility that TMA can induce a dose-dependent transudation, exudation or secretion that may have solubilized or affected the chemical stability of TMA. These observations are consistent with sensitization through healthy intact skin.

In conclusion, OAA have been used for more than half a century. The occurrence of human chemical respiratory allergy following sensitization through skin exposure has been discussed (15). The relative contribution of the respiratory route vs. nonrespiratory route (skin and other mucous membranes) in the development of sensitization to OAA, is not clearly known. The BN rat model presented here may provide insight into the potential contribution of different exposure routes toward allergic sensitization and subsequent airway reactivity. We conclude, using this model, that topical skin exposure to dry TMA powder can induce a dose-dependent IgE and IgG production. The mechanism for TMA skin penetration/reaction requires further clarification, as well as, the effect on the development of TMA respiratory disease.

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