RESPIRATORY HYPERSENSITIVITY TO DIPHENYLMETHANE-4,4′-DIISOCYANATE IN GUINEA PIGS: COMPARISON WITH TRIMELLITIC ANHYDRIDE

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Published evidence demonstrates successful induction and elicitation of respiratory hypersensitivity in guinea pigs by the known human respiratory allergens trimellitic anhydride (TMA) and diphenylmethane-4,4′-diisocyanate (MDI). From these data it is apparent that TMA-related respiratory hyperresponsiveness can be elicited readily in guinea pigs upon inhalation challenge with the free chemical. Despite the interlaboratory variability in methodological procedures used for the sensitization as well as elicitation of response and the wide range of concentrations of TMA employed for challenge exposures (6–57 mg/m^3 air), TMA had been unequivocally identified as a benchmark respiratory sensitizer by measurements of the respiratory rate during challenge. The protocols were duplicated to examine the respiratory sensitizer MDI. In intradermally sensitized guinea pigs, changes in immediate-onset-like respiratory response were observed when MDI challenge concentrations exceeded ~30 mg MDI/m^3 air. Collective experimental evidence suggests that the respiratory responses observed upon challenge with TMA were markedly more pronounced and easier to identify than those recorded following challenge with MDI or MDI conjugate. In contrast to TMA, irritant concentrations of MDI had to be used to elicit any respiratory response and the differentiation of irritant and allergic responsiveness became increasingly difficult. Despite the absence of unequivocal changes in breathing patterns upon MDI challenge, MDI-sensitized animals displayed elevated anti-MDI immunoglobulin G_1 (IgG_1) antibodies, and a significant influx of eosinophilic granulocytes in the bronchial wall and lung-associated lymph nodes. Therefore, it is believed that the robustness of this animal model to identify low-molecular-weight agents as respiratory sensitizer is increased when several endpoints are considered. These are (1) positive respiratory response upon challenge with the hapten, and if negative, also challenge with the conjugate of the hapten; (2) an influx of eosinophilic granulocytes; and (3) increased specific IgG_1 response. Furthermore, it appears that particles in the range of approximately 2–6 μm evoke more consistent respiratory response upon challenge exposure than particles in the 1–2 μm range.

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Diphenylmethane-4,4′-diisocyanate (MDI) and trimellitic anhydride (TMA) have been implicated in multiple occupational immunologic syndromes, of which rhinitis, occupational asthma, and hypersensitivity pneumonitis types of reactions are important examples. Inhalation challenge with either chemical in sensitized individuals may induce an immediate, late, or dual asthmatic response and an increase in airway responsiveness (Baur, 1995; Backman et al., 1996; Hayes et al., 1992c; Keskinen et al., 1988; McGrath et al., 1983; Newman-Taylor, 1987; Patterson et al., 1979; Tansar et al., 1973; Vandenplas et al., 1993; Zeiss et al., 1980; Zammit-Tabona et al., 1983; Venables, 1989). In several laboratories, these chemicals have been evaluated in the guinea pig respiratory hypersensitivity model using either the free chemicals or their homologous protein conjugates for elicitation of respiratory response. The guinea pig displays an extreme sensitivity in systemic as well as passive cutaneous anaphylaxis models (Verdier et al., 1994). Despite this sensitivity, in many instances, sensitized guinea pigs did not fully display surrogate immediate-onset type asthmatic responses from challenge with MDI or MDI conjugate, but did when challenged with the TMA or TMA conjugate. It remains puzzling that the physiological effects (change in breathing patterns upon challenge with the hapten) of the potent respiratory-tract sensitizer TMA could be satisfactorily modeled in the guinea pig, while those of the free or conjugated MDI could not, in spite of the presence of MDI-induced allergic bronchial inflammation and anti-MDI antibodies, important features in occupational asthma.

The guinea pig model for assessment of pulmonary allergenicity of low-molecular-weight chemicals utilizes the inhalation, intranasal, intratracheal, or (intra)dermal routes of exposure to the hapten for sensitization, and inhalation of the hapten and/or hapten–protein conjugate for the elicitation phase of the response (Briatico-Vangosa et al., 1994; Blaikie et al., 1995; Sun & Chung, 1997; Griffiths-Johnson & Karol, 1991; Hayes & Newman-Taylor, 1995; Hayes et al., 1992a, 1992b, 1992c; Karol & Thorne, 1988; Karol, 1983, 1988, 1994; Pauluhn & Eben, 1991; Pauluhn, 1994a, 1994b; Pauluhn & Mohr, 1994; Sarlo & Clark, 1992; Sarlo & Karol, 1994; Sugawara et al., 1993; Welinder et al., 1995). The particular advantages and disadvantages of this animal model have been reviewed extensively (Bratico-Vangosa et al., 1994; Sarlo & Karol, 1994; Karol, 1994; Pretolani & Vargaftig, 1993; Verdier et al., 1994). Though sensitization by inhalation may duplicate occupational exposure best, injection models have been proposed because irritant chemicals may act both as a “sensitizer” and as a toxic irritant and the resultant inflammation of the airways may complicate the interpretation of results obtained during the elicitation phase. The role of irritation in the induction of pulmonary immune response to chemicals and the resultant confounding factors have been discussed elsewhere (Briatico-Vangosa et al., 1994; Kimber et al., 1996).

Induction of respiratory hypersensitivity to TMA can readily be achieved in the guinea pig bioassay as a result of single or repeated inhalation exposure(s) or intradermal injection(s). Characteristic immediate-onset respiratory responses occur following challenge to either the TMA dust or the TMA–protein conjugate. Moreover, published evidence suggests that for TMA, low-dose intradermal induction regimens appear to evoke more vigorous anaphylactic respiratory response upon challenge with the free or TMA–protein conjugate when compared to high-dose ones (Hayes et al., 1992a, 1992b, 1992c; Pauluhn, 1994a). It had been demonstrated that the repeated induction exposure (3 h/day for 5 consecutive days) to TMA was less successful at achieving a high incidence of unequivocal changes in breathing pattern when compared to the low-dose intradermal injection regimen (Pauluhn & Eben, 1991). From the comparison of reports demonstrating the high incidence of typical respiratory responses observed following inhalation challenge with TMA (free or conjugated), it appears that it is more difficult to duplicate these findings with MDI. These conflicting results obtained have prompted additional studies with MDI taking into account the protocols used for TMA or in previous studies with MDI (Pauluhn & Mohr, 1994; Rattray et al., 1994). Emphasis was directed at analyzing factors related to the selection of the "optimal" MDI challenge concentration and the reproducible generation of MDI challenge atmospheres. Delayed-onset reactions have only been addressed in protocols using MDI as inciting agent to allow comparison of pulmonary reactions delayed in onset and the extent of influx of eosinophilic granulocytes in bronchial tissues. Furthermore, for comparison of the effectiveness of the various induction protocols, serological data were not addressed in detail, as the degree of haptenation of the protein by the chemical can alter the results of serological assays, and protein conjugates of the same chemical prepared in the same manner by different laboratories have detected different titers of antibody in the same serum samples (Botham et al., 1988). It appears to be generally accepted that an antibody response provides the potential for the elicitation of a pulmonary response to occur; however, there is no clear relationship between antibody titer and pulmonary responsiveness or indeed the severity of any pulmonary response (Blaikie et al., 1995; Pauluhn & Eben, 1991; Lushniak et al., 1998). Therefore, the objective of this study was to analyze factors addressing the physiological pulmonary response, that is, the change in respiratory rate, upon challenge with MDI or TMA and to analyze to what extent methodological or substance-specific factors could affect this endpoint.

MATERIALS AND METHODS

Test Materials

Monomeric diphenylmethane-4,4′-diisocyanate (MDI, >99% pure) and trimellitic anhydride (TMA) were from Bayer AG, Leverkusen, Germany,

and from Aldrich Chemicals, Steinheim, Germany, respectively. Corn oil was supplied by Caesar and Loretz GmbH, Hilden, Germany.

Animals and Maintenance

Female Dunkin-Hartley guinea pigs (Crl:(HA)BR), with an initial weight range of ~250–350 g, were obtained from Charles River, Sulzfeld, Germany. Animals were acclimatized for a period of 1–2 wk, randomized, and housed four per cage. Guinea pigs were allowed food and tap water ad libitum, except during exposures. A 12-h on/off light cycle was maintained in the animal housing room. Temperature was 23 ± 2°C, and relative humidity was in the range of 40–70%.

Sensitization to and Challenge with TMA

The individual animal data shown in this study utilized a protocol to sensitize guinea pigs to and challenge them with TMA that is largely consistent with the methods described previously (Pauluhn & Eben, 1991; Pauluhn, 1994a). Briefly, 3 groups of 8 guinea pigs each were used, and animals of the control, low, and high dose group received either a single injection on day 0 (low dose) or single injections on days 0, 2, and 4 (high dose), respectively. In all groups corn oil served as vehicle and the injection volume was 100 µl. The concentration of TMA in corn oil was 0.3% (w/v). The vehicle control received three injections of the vehicle alone. Starting with day 21, animals were challenged for 30 min with a mean concentration of 25 ± 1.2 mg TMA/m^3 air. TMA was dispersed as a dry powder using a digitally controlled Wright dust feeder (BGI, Inc., Waltham, MA). A cyclone was used to remove larger particles. TMA concentrations were sampled in the breathing zone area and determined by filter analysis. Further technical details are consistent with those described subsequently. This study subpart was directed toward the identification of respiratory responses upon challenge with the free chemical. No emphasis was made to duplicate the induction of the immunoglobulin G_1 (IgG_1) antibody response or to demonstrate that exposure to free TMA dust in sensitized guinea pigs is accompanied by an increase in airway eosinophilia as described elsewhere (Hayes et al., 1990, 1992a, 1992c; Obata et al., 1992).

Sensitization to and Challenge with MDI

Intradermal Sensitization Groups of guinea pigs (n = 8) received a single intradermal injection (day 0; 400 µl) or repeated injections (days 0, 2, and 4; 3 × 100 µl) of 0.3% (w/v) MDI in dehydrated corn oil as vehicle. The relative locations on the flanks used for repeated injection were cranial, thoracic, and caudal. Control animals (n = 12) received vehicle alone under otherwise identical conditions. Prior to each injection the MDI content of the solution was verified analytically. An additional group of guinea pigs (n = 16) was sensitized by intradermal injections (days 0, 2, and 4; 3 × 2 injections of 100 µl/day) of 5% (w/v) MDI in dehydrated corn oil as vehi-

cle and challenged with the homologous protein conjugate of MDI. Concurrent control animals (n = 16) received the vehicle alone.

Inhalation Sensitization Groups of guinea pigs (n = 16) were sensitized with 132 mg/m^3 MDI by a single 15-min nose-only inhalation exposure. Concurrent control animals received identical exposure to dry air.

Challenge

All challenge exposures were nose only, as described in the section on inhalation exposure technique.

MDI Starting on day 21, guinea pigs were challenged with MDI aerosol using 2 different challenge protocols. Intradermally sensitized animals were challenged for 30 min with 36 ± 5.3 mg/m^3 MDI. Animals sensitized by the brief, high-level inhalation protocol were challenged consecutively with increasing concentrations of MDI in targeted steps of 5, 15, and 35 mg/m^3 MDI (actual concentrations: 5.1 ± 1.8, 14.8 ± 2.9, and 37.2 ± 5.9 mg MDI/m^3). The duration of each step was 20 min.

MDI Conjugate Data addressing challenge exposures using the MDI conjugate have been published previously (Pauluhn & Mohr, 1994). The mean concentration of conjugate in the vicinity of the breathing zone of animals was 35 ± 2.8 mg/m^3 air (filter analyses) with an MMAD of 1.6 µm and GSD of 2.2.

Atmosphere Generation

Atmospheres of MDI for inhalation exposures were generated using the modified condensation aerosol generation system illustrated in Figure 1 (Rapaport & Weinstock, 1955; Liu et al., 1966), consisting of a two-nozzle BGI collison nebulizer type MRE (BGI Incorp., Waltham, MA), with a 2-L glass flask between the collison nebulizer and the evaporator to prevent overloading of the evaporator. The collison nebulizer containing the solid MDI (approximately 30–50 g) was immersed in a digitally controlled heated oil bath at approximately 80°C. The flask temperature was allowed to stabilize and a measured airflow (digitally controlled mass flow controller; Hastings HFC-C Mass Flow Controllers, Teledyne Hastings-Raydist, Hampton, VA) was then passed through the collison nebulizer (~3.5 L/min, ~70 kPa dispersion pressure). The MDI atmosphere then passed into the tubing system of the evaporator to initially allow heating to approximately 180°C. The temperature of the aluminum block was measured and controlled digitally. The evaporator consisted of an aluminum-jacketed glass tube 45 cm long. The chimney consisted of a glass tube, 115 cm long with an internal diameter of about 2.5 cm. Before entering the evaporator, the atmosphere was diluted further with dry conditioned air while maintaining a constant total air flow by applying the extraction/dilution cascade depicted in Figure 1. The evaporator/chimney system served the purpose of reducing the particle size and producing a more monodisperse aerosol.

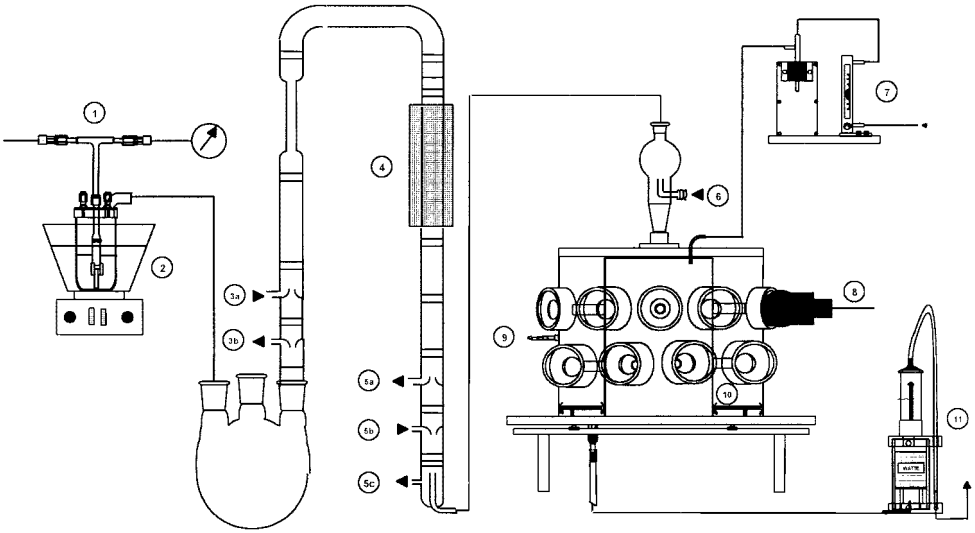

FIGURE 1. MDI condensation aerosol generator and directed-flow inhalation chamber used for MDI-challenge: 1, Compressed air supply; 2, collison nebulizer in oil bath; 3, first dilution unit; 4, digitally controlled heating chimney; 5, second dilution unit followed by condensation tube and final dilution of MDI atmosphere (6) dilution to attain the targeted total airflow rate of approximately 21 L/min and entrainment of MDI atmosphere into the inhalation chamber; 7, aerosol photometer (real-time monitoring); 8, sensor for temperature and humidity measurement; 9, sampling location ("breathing zone sampling"); 10, animal exposure port; 11, cotton-wool aerosol filter + HEPA filter/exhaust (connection to vacuum).

In relation to previously published studies (Pauluhn & Mohr, 1994), the MDI condensation aerosol generator described herein was significantly improved to meet the specific requirements of this study, that is, to generate temporally stable aerosol atmospheres. Airflows through the collison nebulizer were most precisely measured and supplied by a digitally controlled mass flow controller. By increasing the dispersion pressure through the nebulizer, the particle size distribution of primary aerosol was smaller and more monodisperse when compared to previous experiments. An additional optimization of the generator was achieved by reversing the flow direction of the aerosol atmosphere released from the collison nebulizer (approximate concentration of primary atmosphere 1500–2000 mg MDI/m^3 air) into the heating chimney in a top-to-bottom direction. In previous experiments the reverse direction was used. The particular advantage of the current modification is that no reflux of condensed MDI back into the heating system can occur. During previous studies it was observed that a reflux of minute amounts of condensed MDI could result in short-term peak concentrations of aerosol, which might provoke irritant-related changes in breathing pattern, which could be misconstrued as specific airway response. The quantification of such short-term peak concentration is beyond the analytical resolution of the system developed.

Inhalation Exposure Technique

The guinea pigs were exposed nose only to atmosphere as reported previously (Pauluhn, 1994c; Pauluhn & Mohr, 1994), in order to minimize rebreathing of exhaled test atmosphere and prevent hydrolytic degradation of isocyanate. Stability of the aerosol atmosphere was monitored continuously using a RAS-2 aerosol photometer (MIE, Bedford, MA), and no temporal fluctuations were apparent during the course of these experiments. Chamber humidity and temperature were approximately 15% and 20–25°C, respectively.

Atmosphere Analysis Breathing zone samples of aerosol for MDI analysis were taken from the chamber atmosphere. For gravimetric determinations, glass-fiber filters were used (SM 13400, Sartorius, Göttingen, Germany). For isocyanate-specific determinations, two in-line connected tubes packed with glass-powder-filled sampling tubes containing the derivatizing agent *N*-4-nitrobenzyl-*N*-*n*-propylamine were used, according to the method published by Dunlap et al. (1976). The resultant urea derivative was subsequently extracted using acetonitrile (Baker, HPLC gradient grade) and analyzed by high-performance liquid chromatography (HPLC). Gravimetric and nitroreagent determinations provided virtually identical results. Analysis of particle-size distributions of the MDI atmospheres were determined using a critical-orifice, low-pressure AERAS stainless steel cascade impactor (HAUKE, Gmunden, Austria). Throughout the concentrations tested, the mass median aerodynamic diameter (MMAD) was in the range of 1.0–1.4 μm and the geometric standard deviation (GSD) ranged from 1.1 to 1.7.

Measurement and Analysis of Immediate-Onset Pulmonary Responses

Challenge-induced changes in respiratory rate were measured and analyzed as described previously (Pauluhn, 1997). Briefly, after acclimatization to the nose-only plethysmograph, the baseline respiratory rate was measured for approximately 15 min. Measurements were continued during the 30-min challenge period, followed by postchallenge measurements of at least 30 min. Measurements were made with four guinea pigs simultaneously. Signals were averaged during logging periods of 20 s. The analysis of data was as follows: For each animal, the individual baseline data collected during the 15-min prechallenge period were used to calculate the baseline mean + 3SD. All data collected were normalized to the mean of this prechallenge period (= 100%). In order to allow a more quantitative comparison of effect, the area under the curve ("intensity" of response) was calculated. Any response exceeding the mean + 3SD during the subsequent challenge and postchallenge periods was classified as a positive response. Data shown in figures were smoothed by a low-pass filter in order to eliminate high-frequency breathing periods.

Measurement of Delayed-Onset Pulmonary Responses

Measurements of delayed-onset effects were made on animals sensitized by intradermal injection, including controls, commencing after cessation of measurements of the immediate-onset response following challenge. Delayed-onset respiratory hypersensitivity responses were monitored on unrestrained guinea pigs using four water-jacketed, temperature-controlled (thermostat Julabo UC-5B/5) whole-body plethysmographs (average chamber temperature 22.5°C; duration of measurement ~19 h; chamber volume 2.4 L; bias airflow rate 2 L/min). During the monitoring period, guinea pigs were kept on bedding material (low-dust wood shavings); diet and tap water were provided ad libitum. Signals (respiratory rate) were averaged during logging periods of 1 min, and a positive response was indicated by an increased respiratory rate (> ~130 breaths/min) during a period of at least 30 min.

Necropsy and Histopathology In studies with MDI using the single intradermal injection (day 0; 400 µl) or repeated injections (days 0, 2, and 4; 3 × 100 µl) of 0.3% (w/v) MDI, including the respective vehicle control group, the lung, trachea, and lung-associated lymph nodes (LALN) were preserved for histopathological examinations. Animals were exsanguinated 1 day after challenge. Lungs were fixed by instillation of buffered formaldehyde. All tissue blocks were embedded in paraffin (paramat-wax), and ~5-µm-thick sections were cut from each tissue block. Tissue sections were stained with hematoxylin and eosin according to Lillie and Mayer (Lillie & Fullmer, 1976). For assessment of eosinophilic granulocytes, tissues were stained with hematoxylin–eosin–azure according to Tan and Bethel (1992) and examined by light microscopy using procedures consistent with those described by Murlas and Roum (1985). The intensity of the influx of eosinophilic granulocytes into the airways was scored as follows: minor (influx of few cells into the bronchial epithelium), mild (marked influx of cells into the bronchial epithelium), moderate (as for mild, with two to three subepithelial infiltrates), and marked (as for mild, with high density infiltrates in epithelial/subepithelial tissue with extension into the tracheal musculature and focal infiltrates present at the alveolar level). The number of eosinophilic granulocytes per millimeter of trachea was enumerated.

Enzyme-Linked Immunosorbent Assay (ELISA)

For serological analyses blood was drawn from guinea pigs by cardiac puncture approximately 21 days following initiation of exposure. Serum was prepared and stored at –20°C until analyzed. Plastic microtiter plates (Nunc Immunoplate type II, Nunc, Copenhagen, Denmark) were coated with 50 µl per well of 5 µg/ml MDI–guinea pig serum albumin conjugate (MDI-GPSA) in 0.5 *M* sodium carbonate/bicarbonate buffer (pH 9.6) by overnight incubation at 4°C. Various dilutions of guinea pig serum in phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween) were added in duplicate (100-µl aliquots) and the plates were incubated for 30 min at 37°C. Plates were washed 3 times in PBS-Tween and 100 µl of rabbit anti-guinea pig IgG_1 antibody (Miles Scientific, Slough, UK), diluted 1 in

2500 in PBS-Tween added to each well. Plates were again incubated for 30 min at 37°C and washed prior to addition of a peroxidase-labeled goat anti-rabbit IgG antibody (Miles Scientific), diluted 1 in 5000 in PBS-Tween. Following a further 30-min incubation at 37°C, the plates were again washed and substrate (*o*-phenylenediamine and urea hydrogen peroxide) added. Reactions were terminated after approximately 10 min by addition of 50 µl of 0.5 *M* citric acid per well. Absorbance was measured at 450 nm using an automatic reader (Multiskan, Flow Laboratories, Irvine, Ayrshire, UK). The mean optical density for the reagent blank wells for each plate was calculated, as were the means of each duplicate serum sample. A reading was regarded as positive if it was higher than twice the reagent blank for the particular plate. The titer of each serum was the highest dilution of serum that gave a positive reading.

Preparation and Characterization of Conjugate

MDI-GPSA was prepared as described previously (Rattray et al., 1994). Briefly, approximately 200 mg GPSA was dissolved in 20 ml 0.05 *M* sodium borate buffer (pH 9.4). Approximately 60 mg MDI was added and the solution stirred at 4°C for 30 min. Glass vessels were used throughout, as MDI reacts with plastic. The solution was dialyzed successively against phosphate-buffered saline (PBS, pH 7.2) and distilled water for a period of approximately 48 h at 4°C. The lyophilized conjugate was stored at –20°C until use. This conjugate was used for the enzyme-linked immunosorbent assay (ELISA) of the single (1 × 400 µl) and repeated intradermal injection (3 × 100 µl) studies, while the hapten–protein conjugate used for the ELISA and challenge exposure in the high-dose intradermal study (6 × 5% MDI) used for reference purposes was prepared in a different laboratory (for details see Pauluhn & Mohr, 1994). The degree of substitution of the MDI conjugate was assessed using a method based upon the determination of free amino groups by reaction with 2,4,6-trinitrobenzene sulfonic acid (TNBS) as described previously (Rattray et al., 1994). The degree of substitution was 15–20:1 for moles hapten:moles protein.

Statistical Evaluation of Data

Quantitative histopathological data were analyzed by one-way analysis of variance and the Tukey–Kramer post hoc test. Quantal histopathological findings (incidence of airway eosinophilia) were compared with the concurrent control using the pairwise Fisher test with $R \times C$ chi-square test (Gad & Weil, 1982).

RESULTS

MDI: Induction by Intradermal Injection

Following intradermal induction, slight to moderate skin reactions occurred (hyperemia of skin areas not covered by hair, inflammation,

solidification, and coloration of injection sites), which were more pronounced in animals injected repeatedly. Other clinical parameters were not markedly different from the concurrent vehicle control group(s).

For elicitation of specific respiratory hypersensitivity, guinea pigs of the intradermal induction groups were exposed for 30 min to an analytically determined concentration of approximately 36 (±15%) mg MDI/m^3 air on or close to day 21. The results obtained upon challenge with MDI in the concurrent vehicle control, in the 3 × 100 µl (0.3%) group, and in the 1 × 400 µl (0.3%) group are summarized in Table 1. Representative examples of immediate-onset changes of respiratory rate upon challenge with MDI are shown in Figure 2. As can be seen from Figures 2–4, the changes in respiratory rate were observed when the concentration of MDI exceeded approximately 30 mg/m^3. As demonstrated by the response observed in naive guinea pigs challenged with higher concentrations of MDI aerosol (Figure 3), changes in respiratory pattern appear to occur as a result of respiratory tract irritation. Measurements for delayed-onset responses after challenge with MDI revealed a transient increase in respiratory rate in some animals. In the control, 1 × 0.3%, and 3 × 0.3% groups, 1/12, 1/8, and 0/8 animals displayed increased respiratory rates, respectively. Thus, measurements for delayed-onset effects did not indicate any conclusive difference between naive and sensitized guinea pigs.

Animals sensitized according to the high-dose intradermal injection regimen (2 injections/day of 5% MDI on days 0, 2, and 4; abbreviated as id: 6 × 5%) were challenged with 35 mg MDI conjugate for 30 min on or close to day 21 (reevaluation of data; Pauluhn & Mohr, 1994). As summarized in Figure 3, the intensity of respiratory response in naive and sensitized animals was virtually indistinguishable.

MDI: Induction by Inhalation Exposure

Following brief, high-level inhalation exposure (132 mg MDI/m^3 air for a duration of 15 min), guinea pigs experienced a reduced motility on the day of exposure and were normal during the entire postexposure period. Upon challenge, these guinea pigs appeared to be mildly more responsive to the MDI aerosol when subsequently challenged with 5, 15, and 37 mg MDI/m^3 air (each for 20 min). The changes of respiratory rate displayed by naive guinea pigs upon challenge with MDI demonstrate that with increasing concentrations of MDI, the respiratory pattern becomes increasingly unstable (Figure 4) or mimicks positive responsiveness by increased respiratory rate. As illustrated in Figure 4, the quality of response differs from animal to animal and the responses observed in control and sensitized guinea pigs become indistinguishable in regard to the pattern as well as the intensity of response. The results of the quantitative analysis of respiratory response are summarized in Figure 3 and the individual animals' data shown supports what has been illustrated in Figure 4, namely, that concentrations in the range of ~37 mg MDI/m^3 air appear to evoke irri-

TABLE 1. Summary of Findings of Single and Repeated Intradermal Injection Studies

Animal number	Group number	Respiratory response MDI	Influx of eosinophilic granulocytes			ELISA (1/dilution)
			Trachea (eosinophils/mm)	Lung	LALN	
1	Sham control	n.a.	125.7	+	–	<10
2		n.a.	100.6	–	–	<10
3		n.a.	99.0	–	–	<10
4		n.a.	46.9	–	–	<10
5		n.a.	138.3	(+)	–	<10
6		n.a.	74.0	(+)	n.e.	<10
7		n.a.	91.9	–	–	<10
8		n.a.	69.6	–	–	<10
9	Vehicle control	–	33.1	–	–	<10
10		–	66.9	(+)	–	<10
11		–	39.0	–	–	<10
12		+	74.2	–	–	<10
13		+	86.0	–	n.e.	<10
14		+	116.3	(+)	–	<10
15		–	131.0	–	–	<10
16		–	115.8	–	–	<10
17		–	85.2	–	–	<10
18		+	93.3	–	–	<10
19		+	121.7	+	+	<10
20		–	100.6	+/++	+	<10
21	MDI 1 × 0.3% (400 µl)	–	121.3	++	++	20,480
22		–	89.6	++	++	1280
23		–	130.0	++	++	20,480
24		–	142.7	++	++	2560
25		–	96.9	++	++	10,240
26		+	63.3	+/++	+/++	5120
27		–	115.0	++	++	10,240
28		–	76.0	+	+	2560
29	MDI 3 × 0.3% (100 µl)	–	108.3	++	++	5120
30		+	149.8	++/+++	++/+++	5120
31		–	135.6	++	++	10,240
32		–	79.0	++	++	5120
33		+	104.2	+/++	+/++	1280
34		–	148.8	++	++	20,480
35		–	91.5	++	++	>40,960
36		+	106.9	++	++	10,240

Note. n.a., Not applicable (no MDI challenge); LALN, lung-associated lymph node; n.e., not examined (organ missing). For MDI challenge, –, no response observed; +, mild immediate-onset response during exposure to approximately 36 mg MDI/m^3 air, i.e., change of respiratory rate exceeding ±3SD of the prechallenge exposure period. Lung/stain, hematoxylin–eosin–azure: (+), minor influx of eosinophils (slightly more eosinophilic granulocytes than normal); +, mild influx of eosinophils (epithelial influx of eosinophilic granulocytes); ++, moderate influx of eosinophils (epithelial, subepithelial, and adventitial influx of eosinophilic granulocytes); +++, marked influx of eosinophils (epithelial, subepithelial, and adventitial influx of eosinophilic granulocytes).

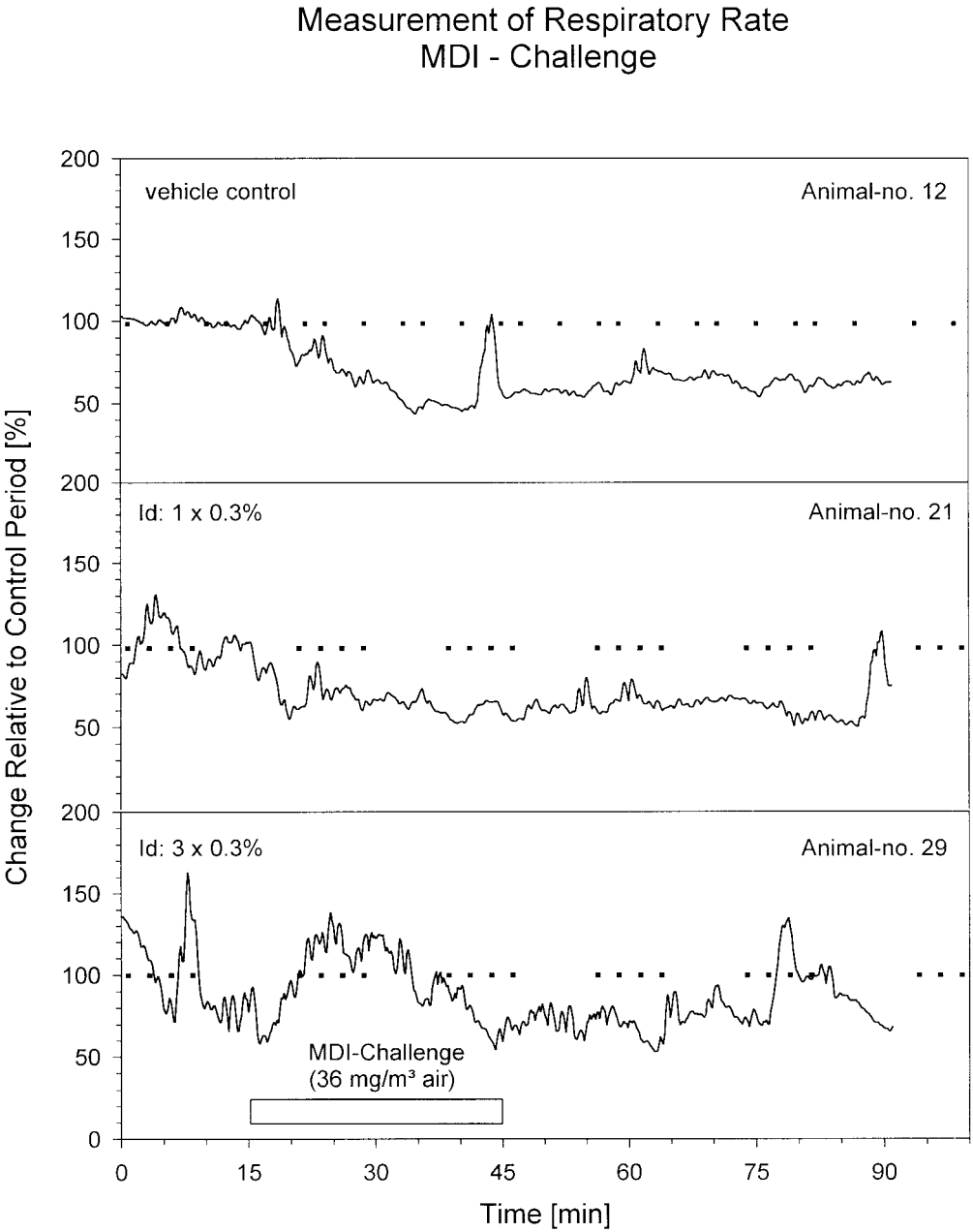

FIGURE 2. Change of respiratory rate during a challenge with a target concentration of 36 mg MDI/m^3 air (duration of challenge: 30 min). Guinea pigs were sensitized by single 1 × 0.3% and repeated 3 × 0.3% intradermal injections. For group assignment of animals see Table 1. Respiratory response data were normalized to the mean of the 15-min prechallenge exposure period (=100%). Before and after challenge, the guinea pigs were exposed to conditioned air.

tant-related respiratory response. Taking into account the background intensity of changes (dotted line in Figure 3), 5 out of 16 and 9 out of 16 guinea pigs of the control and MDI induction groups, respectively, responded. Thus, the group receiving the brief, high-level exposure appears to be more responsive than the concurrent control group.

Antibody Response to MDI

IgG_1 antibody determinations revealed high anti-MDI antibody titers in animals sensitized by either the single (1 × 0.3%) or the repeated (3 × 0.3%) intradermal induction regimen. As summarized in Table 1 and Figure 5, antibody titers showed little difference between the 3 × 0.3% (injection volume: 100 µl) and the 1 × 0.3% (injection volume: 400 µl) groups. IgG_1 anti-MDI antibody titers in the 1 × 132 mg/m^3 inhalation

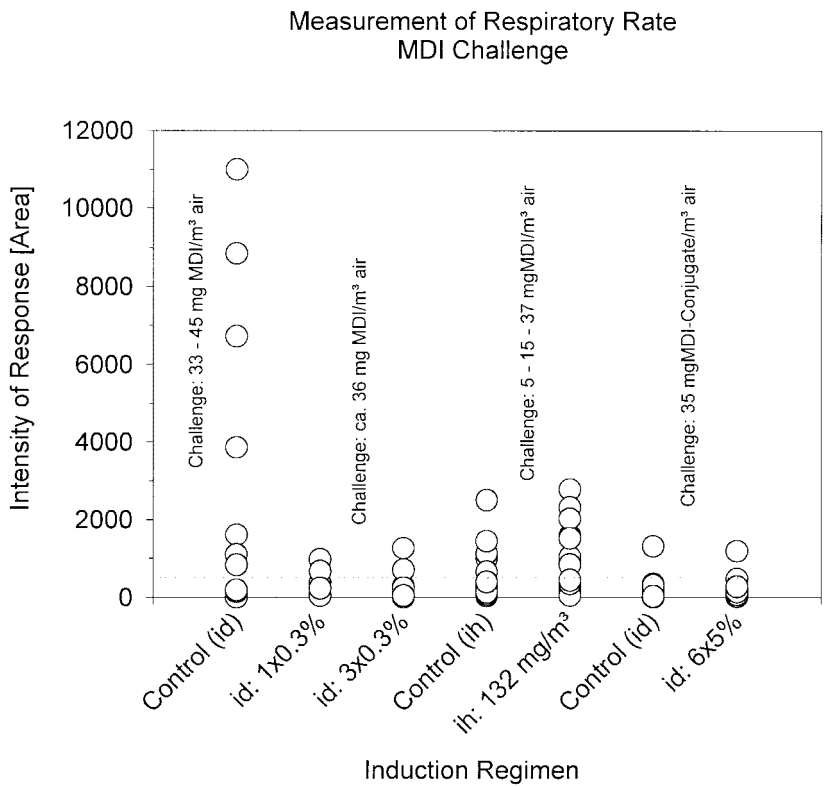

FIGURE 3. Analysis of intensity (area under the curve exceeding 3SD of the individual prechallenge period) of respiratory rate of guinea pigs sensitized to and challenged with MDI or the homologous protein conjugate of MDI. The challenge periods were 30 min. Key: id, induction by intradermal injection of either 1 × 0.3% in 400 µl vehicle or 3 × 0.3% in 100 µl vehicle; ih, induction by a brief high-level inhalation exposure of 15 min. Controls received either 1 × 400 or 3 × 100 µl vehicle. The dotted line represents the background intensity of changes.

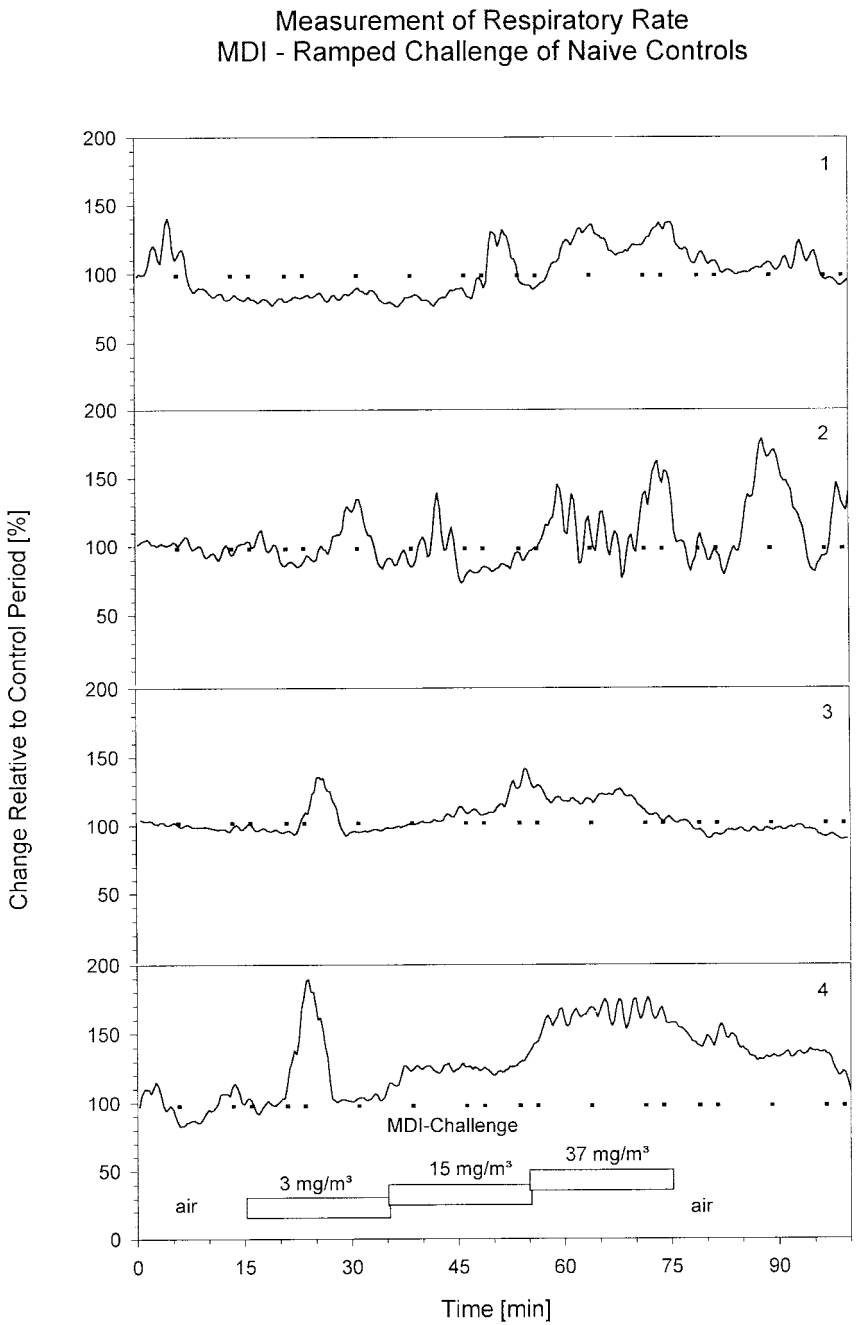

FIGURE 4. Change of respiratory rate during a stepped challenge with target concentrations of 3, 15, and 37 mg MDI/m^3 air (consecutively each concentration for 20 min) of 4 naive guinea pigs. Respiratory response data were normalized to the mean of the 15-min prechallenge exposure period (=100%). Before and after challenge the guinea pigs were exposed to conditioned air.

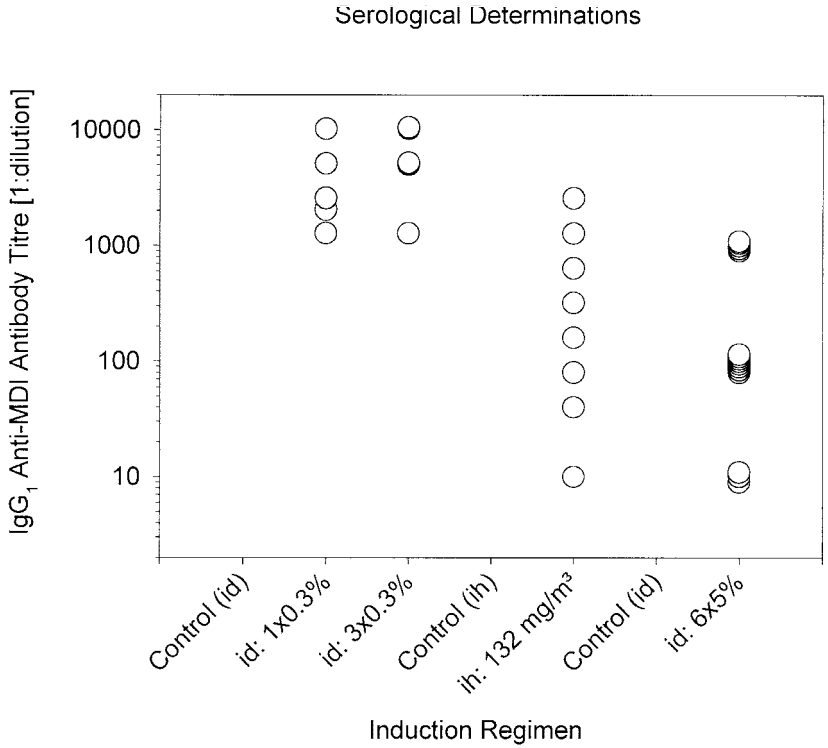

FIGURE 5. Determination of IgG_1 anti-MDI antibody using an ELISA assay on blood collected by cardiac puncture at sacrifice. In the 6 × 5% intradermal injection study and the remaining studies the homologous protein conjugate was synthesized in different laboratories. Key: id, induction by intradermal injection of either 1 × 0.3% in 400 µl vehicle or 3 × 0.3% in 100 µl vehicle; ih, induction by a brief high-level inhalation exposure of 15 min. Controls received either 1 × 400 or 3 × 100 µl vehicle.

induction group were observed in 15 out of 16 animals but were lower and of greater variability than in guinea pigs sensitized by intradermal injection (Figure 5). In the brief, high-level inhalation induction group (6 × 5%, injection volume: 100 µl) the anti-MDI antibody titers were markedly lower than in the previous group. This finding appears to be related to the different methodology of conjugate synthesis used in that study (Pauluhn & Mohr, 1994). Thus, the lack of any conclusive dose response in anti-MDI antibody levels relative to the studies just shown are likely to be related to differences of the conjugates used in this assay. In both laboratories, the antibody titers of all naive guinea pigs were below twice the optical density of the reagent blanks.

Histopathology

Evaluation of tissue sections of guinea pigs sensitized by 3 × 0.3% (injection volume: 100 µl) and 1 × 0.3% (injection volume: 400 µl) intradermal injections to MDI indicated a marked increased influx of eosino-

philic granulocytes in the lung (bronchial airways) and LALN (Table 1). The incidence of elevated levels of eosinophilic granulocytes in MDI-sensitized guinea pigs was significantly different from the vehicle control group ($p < .01$, Fisher's exact test), whereas the enumeration of eosinophils in the tracheal airway resulted in 88.6 ± 31.3, 104.3 ± 27.5, and 115.5 ± 26.3 eosinophilic granulocytes/mm trachea (mean ± SD), respectively. The mild effect observed in the trachea did not reach statistical significance ($p > .05$, one-way ANOVA).

TMA: Intradermal Induction and Inhalation Challenge

A similar intradermal sensitization protocol was also used for the strong respiratory allergen TMA. Following either single or repeated intradermal administrations of TMA, a high incidence of unequivocal immediate-onset responses was observed upon challenge with approximately 25 mg TMA/m^3. As shown in Figure 6, no or minimal responses were observed in the guinea pigs of the concurrent vehicle control group challenged with the same concentration. Clear induction-dependent pulmonary effects upon challenge with TMA could be demonstrated in animals sensitized to TMA using either the 1 × 0.3% or 3 × 0.3% injection regimen (Table 2). Although the incidence of response occurring during challenge with TMA was almost the same, the magnitude of response that occurred after challenge with TMA was markedly more pronounced following the single injection regimen (most guinea pigs on the low-dose injection regimen died in anaphylactic shock). Due to the absence of significant respiratory responses in the guinea pigs of the vehicle control group and the presence of vigorous respiratory responses with ensuing mortality in the TMA-sensitized animals, a mathematical analysis of data was not performed.

DISCUSSION

The studies described in this article have shown and confirm that the intradermal route of induction sensitizes guinea pigs to MDI, as defined as an elevation of anti-MDI IgG_1 antibodies and an influx of eosinophilic granulocytes in the airways of the lung and lung-associated lymph nodes (LALN). In this context, induction of anti-MDI antibody titer, appreciable differences between the 3 × 100 µl (0.3%) and the 1 × 400 µl (0.3%) injection regimen could not be ascertained. Attempts to sensitize guinea pigs using a brief high-level inhalation exposure (1 × 132 mg MDI/m^3 air for 15 min) resulted in markedly lower antibody titers. A significantly increased influx of eosinophilic granulocytes in airways and lung-associated lymph nodes was observed in guinea pigs induced by intradermal injections when compared with the concurrent control group (guinea pigs induced by inhalation and by the high-dose intradermal injection protocol, 6 × 5%, were not examined). An association between an increased airway responsiveness upon challenge with MDI and an increased influx of eosinophilic granulocytes or increased levels of IgG_1 anti-MDI antibody titers could not be established (Table 1).

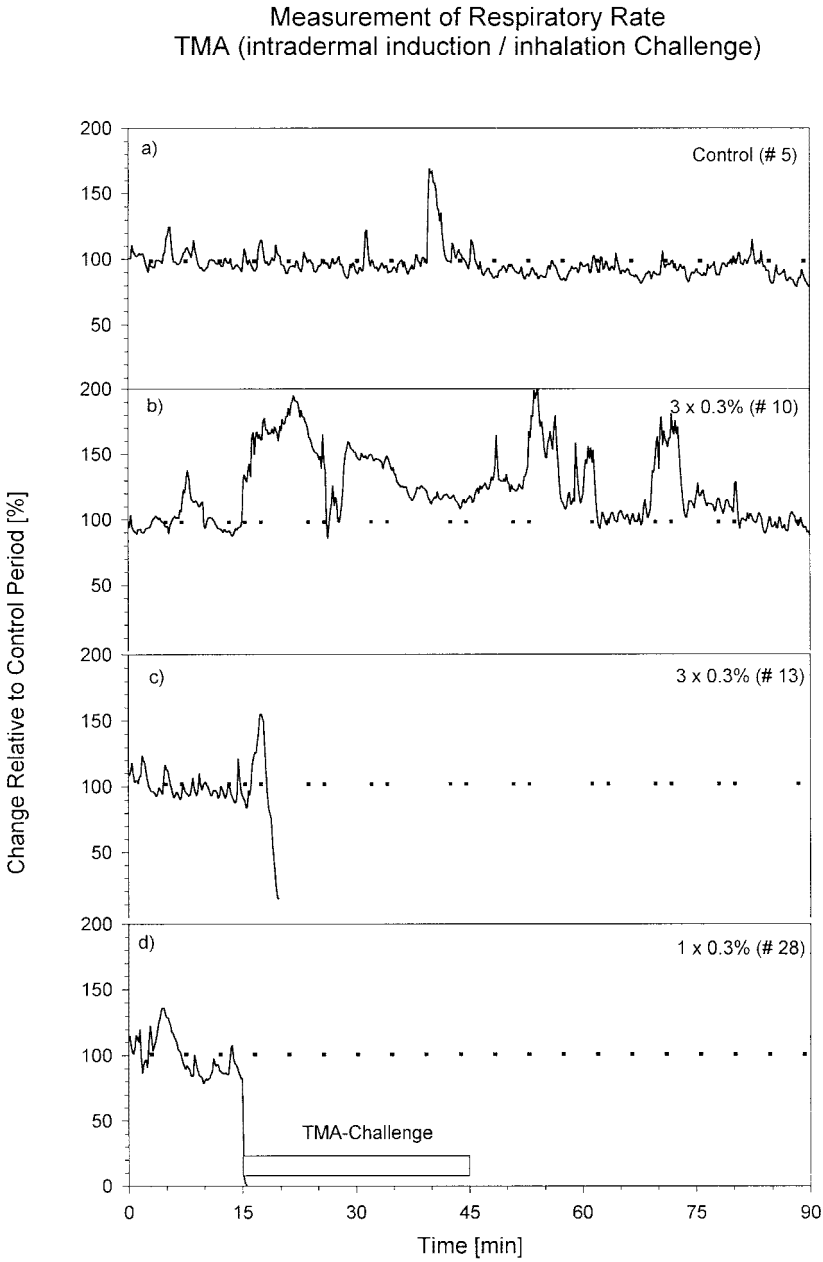

FIGURE 6. Change in respiratory rate during challenge with 25 mg TMA/m^3 air for 30 min (particle-size distribution, see Figure 9). Individual respiratory response data were normalized to the mean of pre-challenge exposure period (=100%). (a) Vehicle control (3 × 100 µl corn oil). (b) Animal induced by intradermal injection with TMA (0.3%, 3 × 100 µl on 3 different days), which experienced a moderate respiratory response upon challenge with TMA. (c) Animal induced by intradermal injection with TMA (0.3%, 3 × 100 µl on 3 different days), which experienced a severe respiratory response upon challenge with TMA. (d) Animals induced by intradermal injection with TMA (0.3%, 1 × 100 µl on day 0), which experienced a severe respiratory response upon challenge with TMA. (#), Animal numbers.

When animals were challenged with mildly irritant concentrations of MDI, conclusive immediate, or delayed-onset responses were not observed. Slight isolated atypical changes in respiratory pattern occurred in all groups challenged with MDI (or TMA) and hence appeared not to be indicative of specific allergic hypersensitivity reactions. Thus, previous and current investigations demonstrate that concentrations of MDI that exceed approximately 30 mg/m^3 cause an increase in respiratory rate in both naive and sensitized animals. The type of change in breathing pattern observed—namely, an increase in respiratory rate—is a common finding for lower-respiratory-tract irritants in rodents (Bos et al., 1992) and renders the differentiation of irritant and allergic response most difficult when irritant

TABLE 2. Summary of Immediate-Onset Responses Following Sensitization and Challenge with TMA or TMA–Protein Conjugate

Compound induction regimen	Induction concentration (w/v)	Hapten challenge (mg/m^3 air)	Response (#/#)	Conjugate challenge (mg/m^3 air)	Response (#/#)	Ref.[c]
Control	Vehicle	42	0/8	—	2/8	1
id (6×)	5–10%	45	8/8	—	7/8	1
id (3×)	0.3%	25	8/8[a]	—	n.d.	2
id (1×)	0.3%	25	7/8[b]	—	n.d.	2
id (1×)	Vehicle	—	—	3	0/5	3
id (1×)	30%	—	—	0.9–2.4	8/22	3
id (1×)	30%	—	—	7	1/8	3
id (1×)	30%	—	—	15	6/8	3
id (1×)	Vehicle	8	0/8	—	—	3
id (1×)	Vehicle	35	0/7	—	—	3
id (1×)	Vehicle	53	0/8	—	—	3
id (1×)	30%	6	4/8	—	—	3
id (1×)	30%	52	7/8	—	—	3
id (1×)	30%	44	6/8	—	—	3
id (1×)	30%	12	Pos.	—	—	4
id (1×)	30%	—	—	it	Weakly pos.	5
id (1×)	0.3%	—	—	it	Pos.	5
id (4×)	0.1%	—	—	it	Pos.	5
id (1×)	Vehicle	8–17	0/8	—	—	6
id (1×)	0.003%	8–17	2/7	—	—	6
id (1×)	0.01%	8–17	2/8	—	—	6
id (1×)	0.03%	8–17	4/8	—	—	6
id (1×)	0.1%	8–17	2/8	—	—	6
id (1×)	0.3%	8–17	4/8	—	—	6
id (1×)	Vehicle	51	0/8	—	—	6
id (1×)	5%	7	6/8	—	—	6
id (1×)	5%	11	8/8	—	—	6
id (1×)	5%	23	7/8	—	—	6
id (1×)	5%	40	8/8	—	—	6
id (1×)	5%	57	7/8	—	—	6

TABLE 2. Summary of Immediate-Onset Responses Following Sensitization and Challenge with TMA or TMA–Protein Conjugate (*Continued*)

Compound induction regimen	Induction concentration (w/v)	Hapten challenge (mg/m^3 air)	Response (#/#)	Conjugate challenge (mg/m^3 air)	Response (#/#)	Ref.[c]
Control	Sham	36	0/8	16	0/8	1
ih (1 × 15 min)	290 mg/m^3	31	7/8	—	—	1
ih (4 × 15 min)	180–310 mg/m^3	44	6/8	~40	6/8	1
ih (5 × 3 hr)	3 mg/m^3	36	2/8	24	5/8	1
ih (5 × 3 hr)	14 mg/m^3	33	3/8	40	2/8	1
ih (5 × 3 hr)	51–118 mg/m^3	44	6/8	25	6/8	1
Control	Sham	—	—	5–18	0/8	3
ih (5 × 3 hr)	2 mg/m^3	—	—	5–18	0/8	3
ih (5 × 3 hr)	14 mg/m^3	—	—	5–18	0/8	3
ih (5 × 3 hr)	109 mg/m^3	—	—	5–18	0/8	3

Note. Controls received vehicle during sensitization; id, intradermal; ih, inhalation exposure; it, intratracheal instillation. #/#: Number of animals responding/number of animals challenged. Values in brackets (first column): number of injections or exposure.

[a]3/8 Died during challenge.

[b]6/8 Died during challenge.

[c]Refs.: 1, Pauluhn and Eben (1991); 2, current study; 3, Botham et al. (1989); 4, Hayes et al., (1992a); 5, Hayes et al. (1992b); 6, Blaikie et al. (1995).

concentrations of MDI are used for challenge. Likewise, none of the MDI-induced animals experienced such vigorous changes in respiratory patterns following challenge with MDI resembling those observed upon challenge with TMA (Figure 6). Most interestingly, TMA has been identified unequivocally as a respiratory sensitizer by a great variety of experimental protocols (Table 2), whichever induction protocol was used or concentration of TMA was selected for challenge exposure (Figure 7). In none of the studies depicted in Figure 7 did naive guinea pigs demonstrate any respiratory responses, whereas the outcome of MDI-challenged naive guinea pigs is variable (Figure 8) and appears to be associated with the various ways of measuring and defining a weakly positive respiratory response. In this context it is difficult to comprehend that challenge exposures of naive guinea pigs up to 55 mg MDI/m^3 air are tolerated without any effect on breathing patterns (Blaikie et al., 1995; see Table 3), taking into account that on mice an RD50 (due to pulmonary irritation) of 32 mg MDI/m^3 air has been described (Weyel & Schaffer, 1985).

As evident from the various publications addressing this type of examination, the analysis of immediate-onset pulmonary sensitivity is subject to variability, since the definition of positive response as well as their categorization varies from one laboratory to another (Huang et al., 1993; Karol, 1983; Sarlo & Clark, 1992; Botham et al., 1988, 1989; Blaikie et al., 1995; Pauluhn, 1997). The challenge regimens that were employed in studies shown in Tables 2 and 3 utilized either the whole-body or nose-

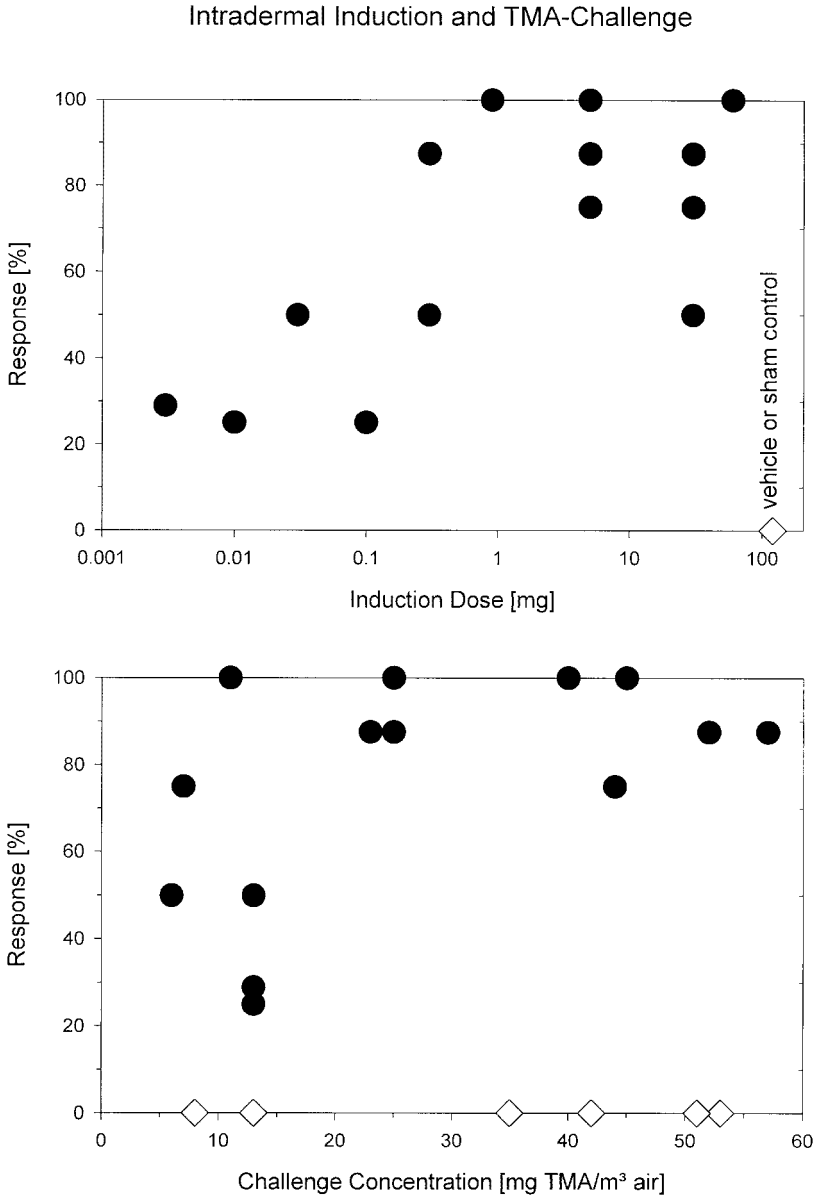

FIGURE 7. TMA. Summary of respiratory response data following intradermal induction (for details see Table 2). Upper panel: Respiratory response as a function of induction dose. Lower panel: Respiratory response as a function of challenge concentration of TMA. Open symbols: vehicle controls.

only mode of inhalation exposure. Changes in respiratory rates and patterns were recorded either optically, by pressure and flow plethysmography, or by signs of respiratory distress. Most laboratories used challenge periods of approximately 15–30 min followed by postchallenge measurements of respiratory rate of 15–45 min. Thus, methodological differences in

the sensitivity of detecting mild respiratory tract irritation may be the reason for the wide range of concentrations used for challenge exposures. For TMA, however, this variable plays apparently a negligible role, since respiratory response resembles more an all-or-nothing response (Figure 6).

When the same type of analysis that has been used for TMA data is applied to published data of MDI (Table 3), it appears that the atmos-

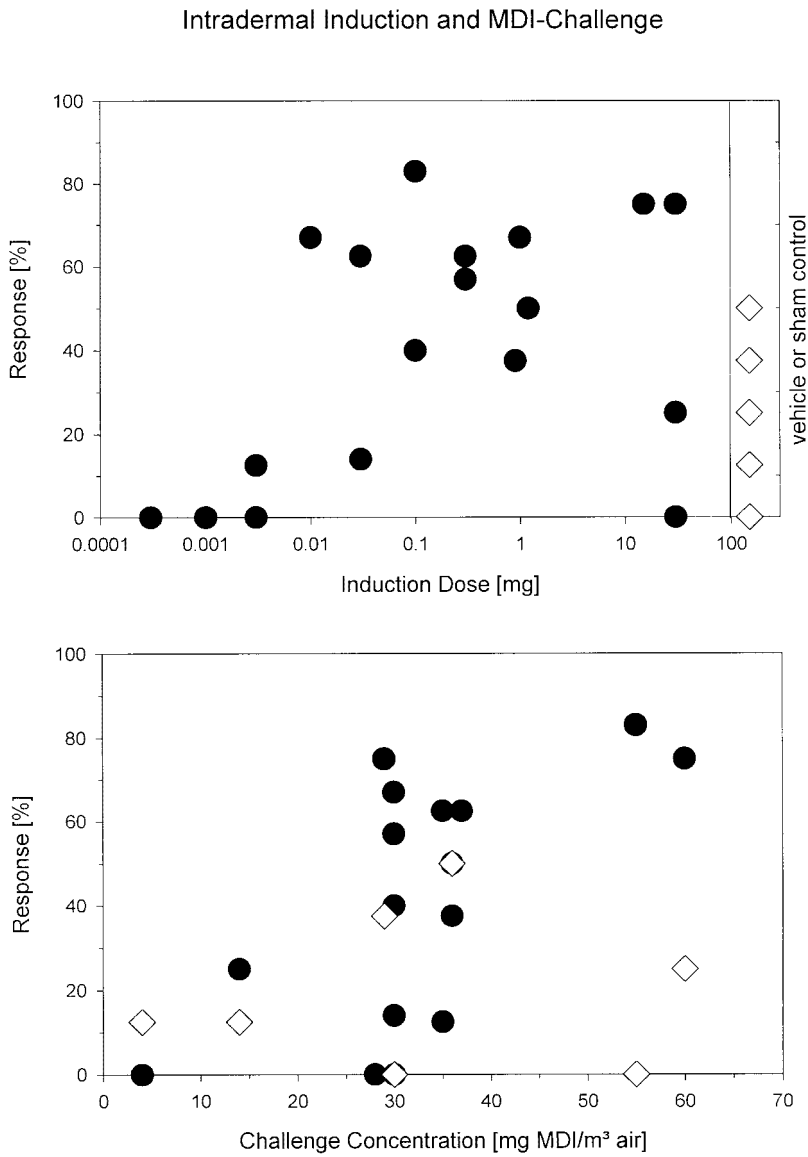

FIGURE 8. MDI. Summary of respiratory response data following intradermal induction (for details see Table 3). Upper panel: Respiratory response as a function of induction dose. Lower panel: Respiratory response as a function of challenge concentration of MDI. Open symbols: vehicle controls.

TABLE 3. Summary of Immediate-Onset Responses Following Sensitization with MDI and Challenge to MDI or MDI-Protein Conjugates

Compound induction regimen	Induction concentration (w/v)	Hapten challenge (mg/m^3 air)	Response (#/#)	Conjugate challenge (mg/m^3 air)	Response (#/#)	Ref.[a]
Control	Vehicle	4	1/8	—	—	3
id (6 × 100 µl)	5%	4	0/8	—	—	3
id (6 × 100 µl)	Vehicle	14	1/8	—	—	3
id (6 × 100 µl)	5%	14	2/8	—	—	3
id (6 × 50 µl)	Vehicle	29	3/8	—	—	3
id (6 × 50 µl)	5%	29	6/8	—	—	3
id (6 × 100 µl)	Vehicle	60	2/8	—	—	3
id (6 × 100 µl)	5%	60	6/8	—	—	3
id (6 × 100 µl)	Vehicle	—	—	35	2/16	3
id (6 × 100 µl)	5%	—	—	35	6/16	3
id (1 × 400 µl)	Vehicle	36	6/12	—	—	5
id (1 × 400 µl)	0.3%	36	4/8	—	—	5
id (3 × 100 µl)	0.3%	36	3/8	—	—	5
id (1 × 100 µl)	Vehicle	30	0/8	—	—	1
id (1 × 100 µl)	0.0003%	28	0/6	—	—	1
id (1 × 100 µl)	0.003%	35	1/8	—	—	1
id (1 × 100 µl)	0.03%	37	5/8	—	—	1
id (1 × 100 µl)	0.3%	35	5/8	—	—	1
id (1 × 100 µl)	Vehicle	18–42	0/16	—	—	6
id (1 × 100 µl)	Vehicle	55	0/8	—	—	6
id (1 × 100 µl)	0.0003%	18–42	0/6	—	—	6
id (1 × 100 µl)	0.001%	18–42	0/7	—	—	6
id (1 × 100 µl)	0.003%	18–42	0/7	—	—	6
id (1 × 100 µl)	0.01%	18–42	10/15	—	—	6
id (1 × 100 µl)	0.03%	18–42	1/7	—	—	6
id (1 × 100 µl)	0.1%	55	10/12	—	—	6
id (1 × 100 µl)	0.1%	18–42	6/15	—	—	6
id (1 × 100 µl)	0.3%	18–42	4/7	—	—	6
id (1 × 100 µl)	1.0%	18–42	4/6	—	—	6
to (1 × 400 µl)	Vehicle	31	0/8	—	—	1
to (1 × 400 µl)	10%	26	2/8	—	—	1
to (1 × 400 µl)	30%	29	2/8	—	—	1
to (1 × 400 µl)	100%	36	3/7	—	—	1
Control	Air	35	1/7	—	—	1
ih (5 × 3 hr)	19–24 mg/m^3	35–44	0/16	—	—	1
ih (5 × 3 hr)	19 mg/m^3	2.5	2/4 del	Not specif.	2/4	2
ih (3 × 3 hr)	72 mg/m^3	2.5	0/3	Not specif.	2/3	2
ih (1 × 15 min)	Sham	12	2/8	36	1/8	3
ih (1 × 15 min)	135 mg/m^3	12	6/8	36	7/8	3
ih (1 × 15 min)	360 mg/m^3	12	4/8	36	5/9	3
ih (1 × 15 min)	Air	5–15–37	5/16	—	—	4
ih (1 × 15 min)	132 mg/m^3	5–15–37	9/16	—	—	4

Note. id, Intradermal; ih, inhalation exposure; to, topical; del, delayed-onset response. #/#, Number of animals responding/number of animals challenged.

[a]Ref.: 1, Rattray et al. (1994); 2, Karol and Thorne (1988); 3, Pauluhn and Mohr (1994); 4, 5, current study; 6, Blaikie et al. (1995).

pheric concentrations of MDI used for challenge exposure, as well as their temporal stability, are more critical for the elicitation of response when compared with the intradermal dose used for induction (Figure 8). The variability in response of naive guinea pigs when challenged to MDI aerosol in mildly irritant concentrations exceeding approximately 30 mg MDI/m^3 (see Table 3) suggests that there were apparently some individual variations in the susceptibility of the animals to the irritant response to MDI. This variability has also been observed by other authors (Sugawara et al., 1993). Accordingly, comparison and interpretation of respiratory response becomes exceptionally demanding when wide ranges of concentrations below and above the irritant concentration as well as particles of variable size are used for challenge (see Table 3) and, due to the longer sampling periods from small inhalation chambers, fluctuations of the MDI aerosol generator may not readily be recognized.

For inhalation challenge with TMA or MDI additional aspects have to be considered, namely, the dependence on the particle size distribution of site-specific deposition within the respiratory tract. TMA is generated as a dust by dispersion, whereas monomeric MDI aerosol is commonly generated by a combination of dispersion/condensation techniques, with the latter favoring generation of monodisperse aerosol distributions. In comparison to the generation and characterization of TMA dust atmospheres, the generation of temporally stable MDI atmospheres, including their characterization employing specific derivatization techniques, appears to be particularly more demanding for MDI (for review see Levine et al., 1995) than for TMA, which is determined gravimetrically by filter analysis.

Another consideration that may be important is that for TMA dust it is likely that the width of the particle-size distribution of the polydisperse solid aerosol favors deposition throughout the entire, and particularly the upper, respiratory tract, whereas the "finer" monodisperse MDI condensation aerosol is deposited preferentially within the lower respiratory tract. This notion is supported further by the change in respiratory rate shown in Figure 4. Accordingly, small variations in procedures related to aerosol generation may lead to significant differences in particle-size distributions for MDI, while for the polydisperse TMA dust this possibility appears to be of minor concern (Figure 9). For intrinsically irritant aerosols in a species known to have abundant smooth bronchial musculature, the location of deposition of MDI aerosol within the lung is of paramount importance, since stimulation of irritant receptors in the lower or upper respiratory tract or irritation of bronchial airways is known to affect the breathing pattern. Thus, particularly for MDI, temporal fluctuations of challenge concentrations in the range of 30–40 mg/m^3 air may evoke an irritant-related increase in respiratory rate, which is likely to be falsely classified as an immediate-onset respiratory response. This situation is complicated even further because the guinea pig is known to change its breathing pattern from nasal to oronasal breathing when exposed to irritant concentrations of isocyanates

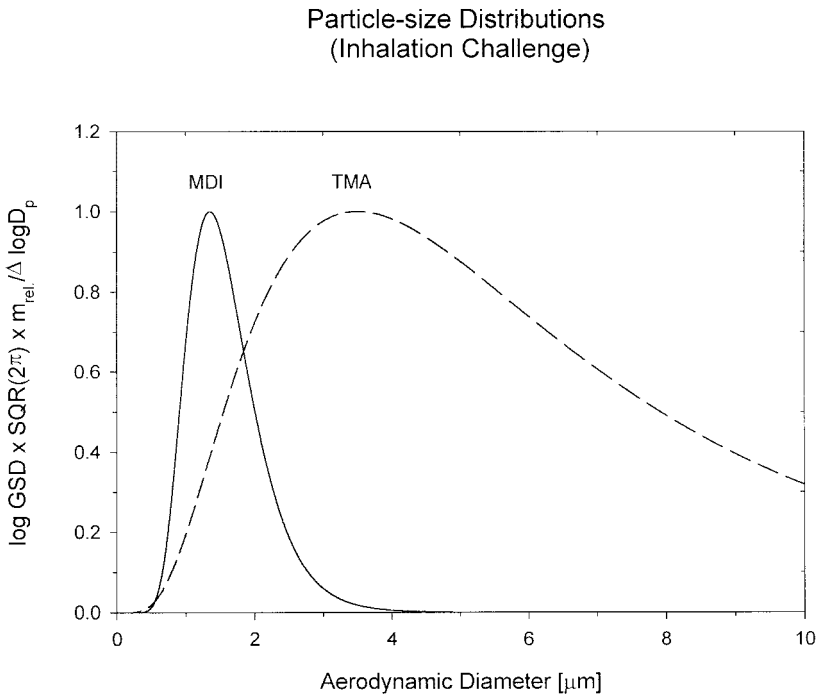

FIGURE 9. Comparison of normalized particle size distribution of MDI and TMA used for challenge of guinea pigs.

(Dodd et al., 1986). Accordingly, the different particle-size distributions are likely to have a qualitative and quantitative impact on the respiratory pattern of guinea pigs exposed to irritant concentrations of MDI. Notionally, it appears that particles in the range of approximately 2–6 μm evoke more consistent respiratory response upon challenge exposure than particles in the 1–2 μm range. This interpretation is supported by findings of Blaikie et al. (1995) where guinea pigs were intradermally sensitized to (0.1%) and challenged with MDI using "smaller" and "larger" MDI aerosol ("small": 18–42 mg MDI/m^3 air, MMAD 1.0–1.6 μm; "large": 55 mg MDI/m^3 air, MMAD 2.2 μm, SD ± 2.2). A positive pulmonary response upon challenge was reported to occur in 40% and 83% of the guinea pigs challenged with "smaller" and "larger" MDI particles, respectively. However, due to the variability in challenge concentrations, this interpretation needs to be confirmed in future studies.

Additionally, a possible explanation of these differences between MDI and TMA is that the isocyanate moieties on MDI may be more reactive than acid anhydrides under physiological conditions with nucleophilic groups, including humidity and low-molecular-weight nucleophilic scavengers present in the epithelial lining fluids of the airways and those bound to (specific) proteins that can form immunogenic and potentially allergenic

conjugates. Although it is beyond the scope of this analysis to attempt any assessment of the relative proportions of MDI and TMA deposited in the various locations of the airways and that penetrating the lung, the considerations made earlier suggest that they may differ considerably from one laboratory to another. Nonetheless, the differentiation of allergic and irritant-related respiratory response becomes increasingly complex when overtly irritant concentrations of MDI are used for challenge. Therefore, the equivocal outcome of MDI challenge exposures appears to be associated with the irritant potency of MDI.

In summary, no similarity of respiratory patterns was evident in guinea pigs challenged with mildly irritant concentrations of MDI when compared with similar studies with TMA. It appears that for MDI, due to its pulmonary irritation potential, irritant and allergic respiratory reactions are quite difficult to distinguish experimentally. Despite these shortcomings, MDI-sensitized animals—that is, those demonstrating increased specific IgG_1 anti-MDI antibodies—displayed a significant influx of eosinophilic granulocytes in the bronchial walls and lung-associated lymph nodes, and this MDI-specific allergic inflammation is considered to be mimicking an important component of occupational asthma. Accordingly, in order to classify a low-molecular-weight substance as respiratory sensitizer for evaluation of response, several endpoints need to be considered: (1) positive respiratory response upon specific challenge exposures, (2) an influx of eosinophilic granulocytes in bronchi and LALN, and (3) immunological response.

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