

Dermal Exposure to 3-Amino-5-mercapto-1,2,4-triazole (AMT) Induces Sensitization and Airway Hyperreactivity in BALB/c Mice

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A cluster of occupational asthma (OA) cases associated with occupational exposure to 3-amino-5-mercapto-1,2,4-triazole (AMT) and *N*-(2,6-difluorophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide (DE498) in a herbicide producing plant was previously reported by the National Institute for Occupational Safety and Health. Due to the limited toxicological data available for these chemicals, murine studies were undertaken to evaluate the toxicity and sensitization potential of these two agents. No signs of systemic toxicity as evaluated by body and selected organ weights or irritancy were observed following dermal exposure to concentrations up to 25% (w/v) AMT in BALB/c mice. DE498 tested negative for sensitization potential in both the TOPKAT QSAR model and *in vivo* in the Local Lymph Node Assay (LLNA), while AMT tested positive in both TOPKAT QSAR and the LLNA. Evaluation of the potential for AMT to induce contact hypersensitivity using the MEST yielded negative results. Cytokine evaluation and phenotypic analysis of draining lymph node (DLN) cells demonstrated an increase in IL-4 and IgE+B220+ cells 4 and 10 days post initial exposure, respectively. Following dermal exposure 7 days a week for 35 days, animals exposed to up to 25% AMT demonstrated a dose-dependent elevation in total serum IgE and an increase in airway hyperreactivity upon methacholine challenge. Following intratracheal challenge with AMT, pulmonary histopathology revealed a dose-dependent suppurative and histiocytic alveolitis in these animals. These studies indicate that DE498 does not induce sensitization following dermal exposure; however, AMT was identified as a sensitizer with the potential to induce airway hyperreactivity.

Key Words: 3-amino-5-mercapto-1,2,4-triazole (AMT); *N*-(2,6-difluorophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide (DE498); airway hyperreactivity; dermal sensitization; IgE.

Occupational exposure to xenobiotic agents represents a significant cause of asthma, with approximately 28% of all adult cases reported to be related to workplace exposure

(NIOSH, 1996). A cluster of eight occupational asthma (OA) cases, potentially caused by occupational exposure to *N*-(2,6-difluorophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide (DE498) and 3-amino-5-mercapto-1,2,4-triazole (AMT), was investigated by the National Institute for Occupational Safety and Health (NIOSH; Hnizdo and Sylvain, 2003). No information could be found in the literature on respiratory health effects due to exposure to AMT or DE498. Consequently, this project was undertaken to evaluate the toxic and sensitization potential of the two agents.

AMT is a low-molecular-weight chemical used (1) in the synthesis of organic chemicals, (2) in the processing of silver halide photographic materials, (3) as an antioxidant for aluminum and lubricating oils, and (4) as a viscosity index improver and dispersant. To meet these usage needs, 250,000 pounds of AMT was imported into the United States in 1993 (U.S. EPA, 1998). Despite the wide potential for exposure to this chemical, there is little toxicity data available. A single study reported a decrease in thyroid peroxidase and thyroxine (T_4) concentrations, with a concurrent increase in thyroid weight and thyroid stimulating hormone (TSH) levels in Fischer rats following oral exposure to AMT (Takaoka *et al.*, 1994). Therefore, the Environmental Protection Agency has listed AMT on the Toxic Substance Control Act (TSCA) for further evaluation of its toxicity potential (1998).

DE498 (trade name; Flumetsulam) is a broad-spectrum herbicide acting by inhibiting acetolactate synthase (ALS), an enzyme necessary for branched-chain amino acid (leucine, isoleucine, and valine) synthesis (Kleschick *et al.*, 1992). By preventing protein synthesis, DE498 effectively inhibits the growth and development of broadleaf weeds (Frear *et al.*, 1993). Few studies have been performed regarding toxicity induced by DE498 in mammalian systems.

As a result of the widespread use of AMT and DE498, the lack of toxicity data available on the chemicals, and the occurrence of OA in a plant where there was exposure to these chemicals, the following studies were undertaken to evaluate the potential of AMT or DE498 to induce irritancy, sensitization, and/or systemic toxicity. Further studies investigated the mechanism by which AMT induced sensitization and airway hyperreactivity (AHR).

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MATERIALS AND METHODS

Animals. Female BALB/c and CBA mice, obtained from Taconic Laboratories (Germantown, NY) at 6–8 weeks of age, were quarantined for at least one week after arrival. Animals were maintained under NIH animal care guidelines and housed with a 12-h light/dark cycle. Target temperature and humidity ranges were 18–26°C and 30–70%, respectively. Food (Agway ProLab 3500 diet) and tap water were available ad libitum. Mice were individually identified and assigned to homogenous weight groups prior to the first exposure. Animals were weighed immediately before sacrifice in all studies.

Chemicals. 3-Amino-5-mercapto-1,2,4-triazole (AMT; 99% pure; Fig. 1A) was obtained from Acros Organics (NJ) and from the factory where the cases of OA occurred. AMT was tested at concentrations of 1, 5, 10, 15, and 25% (w/v) in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), based on limits of solubility. DE498 (factory obtained; Fig. 1B) was tested at concentrations of 5, 15, 25, and 45% (w/v) in DMSO, based on limits of solubility. Toluene 2,4-diisocyanate (TDI; 99.6% pure) and α -hexylcinnamaldehyde (HCA; 85% pure) were used as positive controls for the Local Lymph Node Assay (LLNA) and phenotypic analysis assays; 2,4-dinitrofluorobenzene (DNFB; purity > 99.4%) was used as a positive control for the mouse ear swelling test (MEST). Positive controls were dissolved (w/v for all chemicals) in either DMSO or acetone (99.99% pure). All chemicals used for positive controls or vehicles were obtained from Sigma.

TOPKAT computational analysis. TOPKAT 6.0, a qualitative structural activity relationship (QSAR) computer model, designed by Accelrys, Inc. (San Diego, CA), was used to evaluate the sensitization and/or irritancy potential of the test chemicals. The valence structures of the test chemicals were analyzed in five dimensions of the optimal prediction space (OPS)[®] and compared to the known database of sensitizers and irritants in the Skin Sensitization and Ocular Irritancy (Draize) modules, respectively.

Twenty-eight day toxicology study. Prior to the initiation of exposure, BALB/c mice (study 1, $n = 10$ /group; study 2, $n = 5$ /group) were weighed, and the hair was clipped from their dorsal thoraco-lumbar region. Animals were dosed on the clipped site for 28 consecutive days with 50 μ l of either vehicle or test chemical. On day 29, animals were weighed, sacrificed by CO₂ asphyxiation, and blood was collected by cardiac puncture. In study 1, blood from five animals per group was collected into EDTA tubes for hematology analysis, and blood from the remaining five animals per group was collected into serum separator tubes for chemistry analysis. In the second study, sera were evaluated for serum chemistries, corticosterone, triiodothyronine (T₃), and thyroxine (T₄) levels. In the second study, to reduce stress which could modulate corticosterone levels, animals were housed individually in covered cages 24 h prior to sacrifice. Mice were sacrificed within one minute of handling the cage, and blood collected promptly after CO₂ asphyxiation. In both studies, following blood collection, the animals were necropsied, and organ weights (brain, thymus, lungs, spleen, liver, adrenal glands, and kidneys) were taken. Organ/brain and organ/body ratios were evaluated as indicators of target organ toxicity.

Local Lymph Node Assay (LLNA). The LLNA was used for an *in vivo* confirmation of sensitization potential, and was performed according to the

protocol recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), with minor modifications (ICCVAM, 1999). Briefly, CBA or BALB/c mice ($n = 5$ /group) were dosed with test chemical, vehicle, or positive control on the dorsal surface of each ear for 3 consecutive days. On day 6 mice were intravenously injected with 20 μ Ci ³H-thymidine (Perkin Elmer, MA). Five h after injection, mice were sacrificed, and a single-cell suspension was prepared from pooled right and left draining lymph nodes (DLNs) of each animal. The uptake of ³H-thymidine was determined using a β scintillation counter, and a stimulation index (SI) was determined for each experimental group by dividing the group mean by the mean of the vehicle controls. A chemical was classified as positive if at least one concentration reached a SI of ≥ 3 with the results being statistically significant and dose-responsive.

Mouse ear swelling test (MEST). The MEST, used to evaluate contact hypersensitivity inducing potential, followed the procedure described by Hayes *et al.* (1998), with slight modifications. On days 1–3 (induction phase), BALB/c mice were dosed on the clipped dorsal thorax with 50 μ l of vehicle, test chemical, or positive control. On day 8 the thickness of the right ear pinna of each mouse was measured prior to chemical challenge, using a modified engineer's micrometer (Mitutoyo Corporation, Japan). Mice were then challenged with 25 μ l of vehicle, test chemical, or positive control chemical on the dorsal surface of the right ear (see Table 1 for dosing concentrations). The thickness of the right ears of all mice were measured 24 and 48 h after challenge, and percentage ear swelling was calculated as follows: [(Mean post-treatment ear thickness / mean pre-treatment ear thickness) $\times$ 100] – 100. The response of the background control group was compared to the vehicle group as an indicator of the irritancy of the challenge concentration. Test article groups were compared to the background control for significance and dose response, and the positive control was compared to the background positive control (Table 1).

Combined irritancy and phenotypic analysis. Prior to the initial exposure, the ears of mice ($n = 5$ /group) were premeasured, and blood was collected from the lateral tail vein to establish baseline IgE levels. Mice were then exposed to 25 μ l of AMT (ranging from 2.5 to 25%), vehicle (DMSO), or positive control (2.5% TDI) for 4 consecutive days on the dorsal surface of each ear. Twenty-four h after the final dose, the thickness of the ears of the mice was measured to evaluate irritancy by calculating percentage of ear swelling as described for the MEST. Mice were rested for 6 days and then sacrificed on day 10. Cardiac puncture was performed following CO₂ euthanasia to collect sera, and both DLNs were removed and placed in 2 ml PBS. To further evaluate sensitization potential, phenotypic analysis of DLN cells was conducted as described previously, gating on viable nonred blood cells with results expressed as percentage of gated cells (Manetz and Meade 1999). Cells were stained using monoclonal antibodies against IgE (FITC; clone R-35-72) and B220 (PE; clone RA3-6132). The Fc block used was anti-mouse CD32/16 (clone 2.4 G2). All antibodies were purchased from PharMingen (San Diego, CA). Sera were used for analysis of total serum IgE by ELISA as described below.

Cytokine evaluation. The dosing protocol for cytokine evaluation consisted of 3 days dermal exposure (as in the LLNA) followed by collection of the DLNs for mRNA analysis 24 h following the final exposure. This time point was chosen based on the studies by Manetz and colleagues (2001), which demonstrated the potential for differentiation of T-cell versus IgE-inducing sensitizers following this dosing regime. Twenty-four h after the last exposure, mice were sacrificed by CO₂ asphyxiation, and both DLNs were excised aseptically. Lymph nodes were flash frozen with liquid nitrogen and stored at –80°C. Total RNA was isolated according to Qiagen's (Valencia, CA) protocol for total RNA isolation with DNase treatment. Reverse transcription was performed with a TaqMan Reverse Transcription kit in a GeneAmp PCR System 9700 version 1.25 (Applied Biosystems, NJ) according to manufacturer's instructions. Copy DNA levels were measured by real-time polymerase chain reaction (RT-PCR) using TaqMan reagents (IL-4, -5, -10, and -12p40, GAPDH, and IFN- γ) as prescribed by manufacturer's instructions (Applied Biosystems). Biomarkers chosen to evaluate the Th2 response included IL-4,

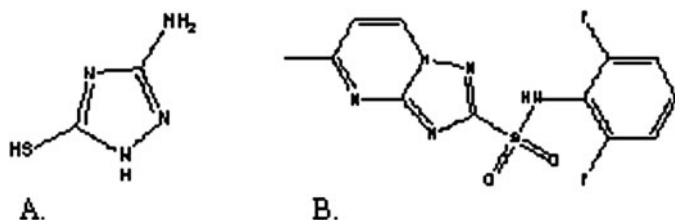


FIG. 1. Structural representations of (A) 3-amino-5-mercapto-1,2,4-triazole and (B) DE498.

TABLE 1
Concentrations of Chemicals Used for MEST

Group ID	Induction	Challenge
Vehicle	DMSO	DMSO
Background control ^a	DMSO	25% AMT
5% AMT ^b	5% AMT	25% AMT
10% AMT ^b	10% AMT	25% AMT
25% AMT ^b	25% AMT	25% AMT
Background positive control	DMSO	0.15% DNFB
Positive control ^c	0.15% DNFB	0.15% DNFB

Note. Dose concentrations used for MEST experiments. AMT and DNFB percentages are in w/v and v/v, respectively. The group mean percentage ear swelling for *a*, *b*, and *c* were compared to the vehicle, background control, and background positive control groups, respectively, for statistical significance.

5, and 10 (Mosmann *et al.*, 1986), while Th1 biomarkers included IFN- γ and IL-12 (Finkelman *et al.*, 1988; Trinchieri, 1994).

Data is expressed as fold increase over vehicle, calculated by the following formula: $2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct(\text{Sample}) - \Delta Ct(\text{Vehicle})$. The $\Delta Ct = Ct(\text{GAPDH}) - Ct(\text{target})$, where Ct = cycle threshold as defined by manufacturer's instructions (Applied Biosystems).

Total serum IgE analysis. To evaluate the time course of total serum IgE production, BALB/c mice ($n = 5/\text{group}$) were dosed with 25 μl of test article (5, 15, or 25% AMT) or vehicle (DMSO) on the dorsal surface of each ear 5 days a week for 68 days. Mice were tail bled prior to exposure (day 0) to obtain baseline total serum IgE levels, and every two weeks thereafter to monitor alterations in total serum IgE levels.

Total serum IgE was quantified following the procedure described by Manetz and Meade (1999) with minor modifications. A rat α -mouse IgE monoclonal Ab (B1E3) was used as the capture antibody at a concentration of 6 $\mu\text{g}/\text{ml}$. B1E3 hybridomas were generously provided by Daniel Conrad (Virginia Commonwealth University). Samples were applied to the plates at an initial dilution of 1:40 and serially diluted (1:2) down eight wells. A PharMingen (Torrance, CA) purified mouse IgE clone IgE-3 (anti-TNP), was used as the standard, at a starting concentration of 5000 ng/ml and serially diluted (1:2) through 12 wells. The secondary antibody, biotinylated anti-mouse IgE, clone R35-92 (PharMingen), was used at a concentration of 2 $\mu\text{g}/\text{ml}$. A 1:400 dilution of streptavidin alkaline phosphatase (SAP, Sigma-Aldrich Cat# S-2890) was used, followed by detection with substrate [*p*-nitrophenyl phosphate tablets (Sigma-Aldrich) added to substrate buffer]. Plates were read on a Beckman Vmax model plate reader at wavelengths of 405 and 650 nm, and data were analyzed with softmax 3.1.1 ELISA software. Plates were analyzed when the standard reached an OD of at least 1.5, but did not exceed 2.1. Test samples were quantitated by comparison to the standard curve.

Airway hyperreactivity. In the initial study, BALB/c mice ($n = 10/\text{group}$) were dosed with 25 μl of test article (5, 15, or 25% AMT) or vehicle on the dorsal surface of each ear, 7 days a week, throughout the study. Total serum IgE was evaluated on days 0, 22, and 36. Nonspecific airway hyperreactivity was evaluated on day 21, while specific airway challenge was performed on days 28 and 35. Airway hyperreactivity was assessed in mice as described previously (Howell *et al.*, 2002) with minor modifications. For nonspecific challenge, baseline enhanced pause (PenH) values were obtained in Buxco whole body plethysmography chambers over a 5-min period. Mice were then challenged by nebulizing increasing concentrations (10, 25, and 50 mg/ml) of methacholine (MCH; Sigma) into the chambers. At each concentration of MCH, PenH was assessed over a 5-min period with exposure occurring for the first 3 min. For antigen-specific challenge, mice were lightly anesthetized using isoflurane (Abbott Laboratories, 99.9%) inhalation and exposed by intratra-

cheal (IT) aspiration of 250 μg AMT / 50 μl PBS suspension. Prior to challenge, a 5-min baseline PenH was assessed for each animal. Following challenge, animals were immediately returned to the chambers and PenH was assessed over the following 6 h. Twenty-four h following the final IT challenge, mice were sacrificed by CO₂ asphyxiation, and blood was collected following transection of the abdominal aorta. Lungs were fixed by insufflation of 1 ml 10% buffered formalin via the trachea and placed in 10 ml of 10% buffered formalin for histopathology analysis. Hematoxylin and eosin (H&E), eosinophil (0.5 Chromotrope 2R), mucin (Mayers Mucicarmine), and mast cell staining (Toluene Blue) were performed.

A second study was performed to fill in the dose response curve for AHR. BALB/c mice ($n = 15/\text{group}$) were dosed with 25 μl of test article (5, 10, 15, 20, or 25% AMT) or vehicle on the dorsal surface of each ear, 7 days a week, throughout the study. All animals were tail bled prior to dosing and on day 22 to evaluate total serum IgE levels. On day 35, five animals per group were challenged with MCH as previously described. An additional five animals per group were challenged by intratracheal aspiration with AMT on days 28 and 35. The remaining five animals per group received only dermal exposure. All animals were sacrificed on day 36 (IT challenged animals were sacrificed 24 h post final IT exposure) by CO₂ asphyxiation, and blood was collected following transection of the abdominal aorta. Immediately after sacrifice, lungs were insufflated with 10% formalin for histology analysis.

Statistics. One-way analysis of variance (ANOVA) was performed to analyze variability between at least three experimental groups. If a *p* value of 0.05 was achieved, Dunnett's posttest was performed to compare test groups to their appropriate control. Differences were considered statistically significant at *p* < 0.05. To evaluate dose response relationships, linear regression was calculated. The values are considered statistically significant at *p* < 0.05. When only two groups were analyzed, a *t* test was performed, with differences considered significant at *p* < 0.05.

RESULTS

AMT, but Not DE498, Was Identified as a Sensitizer Using TOPKAT QSAR

As an initial screen, DE498 and AMT were evaluated for irritation and sensitization potential using TOPKAT QSAR analysis. Both AMT and DE498 fit zero out of five dimensions of the optimal prediction space (OPS)[®] in the irritancy-screening assay, and thus, based on this analysis both were considered negative for irritancy potential. Using the sensitization module, DE498 was negative for sensitization, fitting zero out of five dimensions of the ops[®], resulting in a classification of a nonsensitizer. In contrast, AMT fit four out of five dimensions of the ops[®], testing positive for sensitization with QSAR modeling.

AMT, but Not DE498, Tested Positive for Sensitization in the Local Lymph Node Assay

While efforts are underway to develop sensitive and specific QSAR models for the evaluation of chemical sensitization potential, these have not been accepted as stand-alone methods. Therefore, to confirm the QSAR results, AMT and DE498 were tested *in vivo* for the potential to induce sensitization using the LLNA at concentrations ranging from 1 to 45%. With factory-obtained AMT, a dose-responsive increase in proliferation was observed (*p* < 0.05) in CBA mice, with stimulation indices (SI) of 6.20 and 8.86 at the 15% and 25% AMT

concentrations, respectively (Fig. 2A). Given that subsequent studies to evaluate the IgE-inducing potential of AMT were to be conducted using BALB/c mice, studies were conducted to compare the LLNA response of this strain with the response in CBA mice. Figure 2B shows a representative study of two experiments in which a dose-responsive ($p < 0.05$) increase in lymph node proliferation was induced in BALB/c mice following exposure to AMT. The 15 and 25% dose groups were significantly elevated ($p < 0.01$) over the DMSO control with SIs of 3.45 and 4.08, respectively, identifying AMT as a sensitizer. In contrast, DE498 tested negative for sensitization potential in the LLNA, as demonstrated by a representative study (one of two studies) where exposure to up to 45% DE498 did not induce an increase in lymph node cell proliferation in BALB/c mice as compared to control animals (Fig. 2C).

No Toxicity Was Observed in BALB/c Mice following 28-Day Dermal AMT Exposure

Based on the results of the initial sensitization assays, all further studies were focused on AMT. In order to assess systemic and target organ toxicity, body weight, organ weight, serum chemistry, and hematology analyses were conducted following 28-day dermal exposure to the vehicle or increasing concentrations of the test article. All mice appeared clinically normal throughout the study, with no significant differences in body weights occurring between vehicle and AMT-exposed mice (data not shown). In addition, no effects were seen on brain, thymus, lung, liver, adrenal gland, kidney, or spleen weights or organ-to-body-weight and organ-to-brain-weight indices. Likewise, there were no significant differences in hematology or serum chemistry parameters, including serum

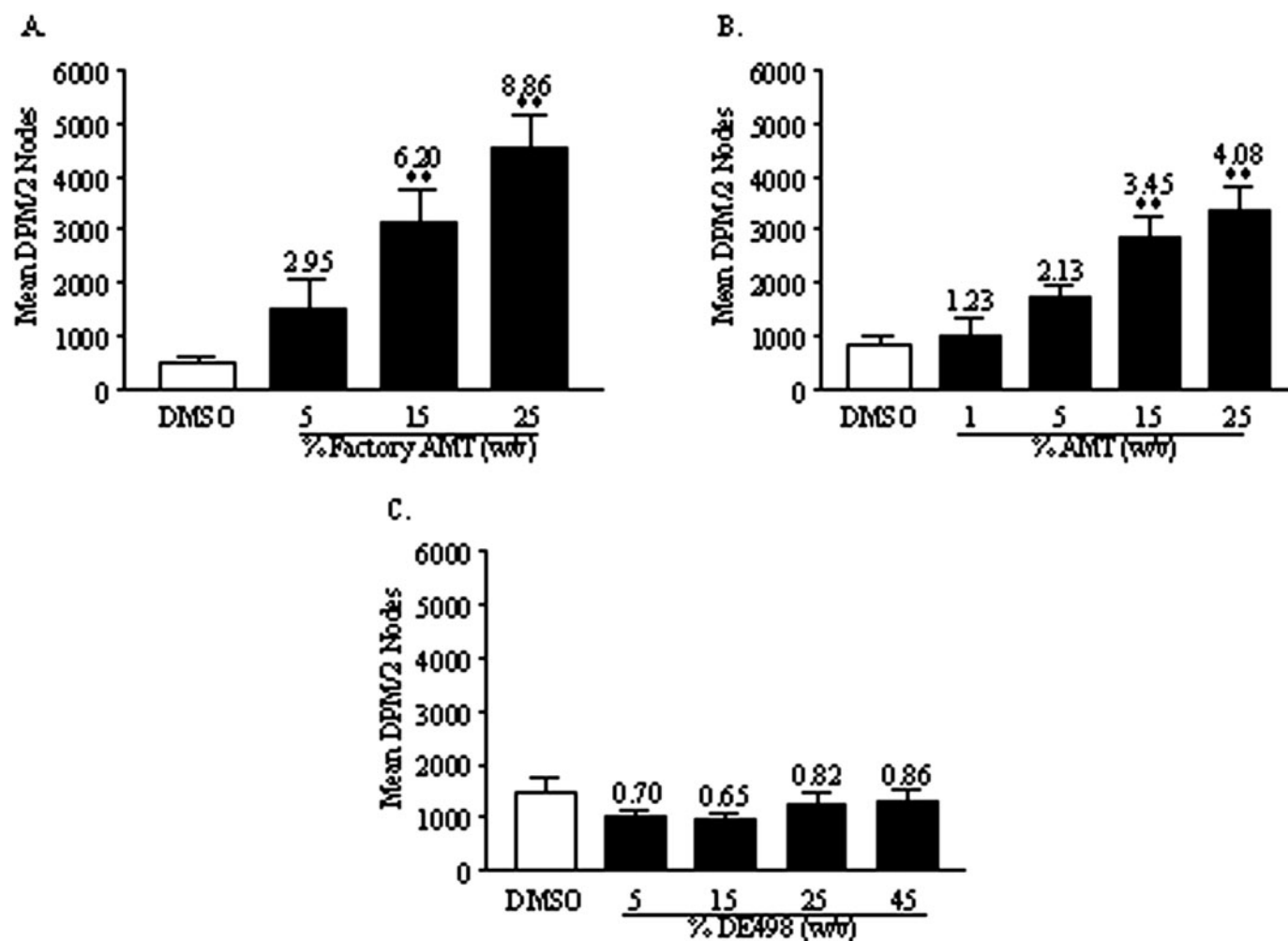


FIG. 2. Evaluation of sensitization potential of AMT and DE498 by the LLNA. Animals were dosed with (A) AMT obtained from Sigma (BALB/c mice), (B) AMT obtained from the factory (CBA mice), and (C) DE498 (BALB/c mice). Bars represent the dose group mean DPM $\pm$ SE for five animals per group. The positive controls for each study were (A) 30% HCA in DMSO, SI = 6.1, (B) 2.5% TDI in acetone, SI = 13.4, and (C) 30% HCA in acetone, SI = 4.3. **indicates $p < 0.01$ as compared to the vehicle control.

corticosterone, T_3 , and T_4 levels as measured in AMT-exposed mice compared to the vehicle-exposed mice (data not shown).

AMT Was Negative for Contact Hypersensitivity Inducing Potential as Evaluated by the MEST

Given that both IgE and T-cell mediated sensitizers may test positive in the LLNA, as a means to further elucidate the mechanism of sensitization, contact hypersensitivity potential was further evaluated using the MEST. None of the test concentrations induced a significant increase in ear swelling over the BC group at either 24 or 48 h post challenge. The positive control group demonstrated a 49% and 85% increase in ear swelling at 24 and 48 h, respectively. Results are shown in Figure 3.

AMT-Exposed Mice Had Increased Numbers of B220+ and IgE+B220+ Cells in Draining Lymph Nodes

The Combined Irritancy and Phenotypic Analysis assay was conducted to evaluate the potential of AMT to induce an irritant response *in vivo* and to begin to evaluate the potential for AMT to induce an IgE-mediated hypersensitivity response. Mice were dosed for 4 days on the dorsal surface of each ear, and lymph nodes were excised on day 10. Irritancy was assessed by measurement of ear thickness 24 h following the last exposure. In concordance with the QSAR data, AMT did not induce irritancy at concentrations of up to 25% in these studies (Data not shown). At concentrations of 2.5 ($4.1 \pm 0.24 \times 10^8$ cells), 10 ($3.9 \pm 0.11 \times 10^8$ cells), and 25% ($5.0 \pm 0.35 \times 10^8$ cells) AMT, a statistically significant ($p < 0.05$) increase in B220+ cells was observed as compared to the vehicle control ($3.1 \pm 0.27 \times 10^8$ cells). A similar increase in these cell populations was seen when data was analyzed as percentage of total viable cells (Fig. 4). A dose-dependent ($p < 0.01$) increase in IgE+B220+ cells was observed following AMT exposure, reaching statistical significance in the 25% dose group ($2.5 \pm 0.55 \times 10^8$ cells) as compared to the vehicle control group ($0.33 \pm 0.21 \times 10^8$ cells). On day 10 post initial exposure, no exposure-related changes were seen in total serum IgE levels (343.4 ± 30.41 ng/ml; 25% AMT) as compared to the vehicle control mice (374.3 ± 89.16 ng/ml; vehicle). Data shown is representative of two studies.

Three-Day Dermal Exposure to AMT Induced an Increase in IL-4 mRNA in BALB/c Mice

To further evaluate the potential for AMT to induce a Th2 response, the cytokine profile of lymph node cells draining the site of chemical exposure was evaluated. Following 3 days of dermal exposure, DLNs were excised, and messenger RNA was processed and quantitated by RT-PCR for analysis of IL-4, -5, -10, and -12p40 and IFN- γ . AMT exposure resulted in an increase in IL-4 mRNA reaching statistical significant in the 15% dose group ($p < 0.05$; Fig. 5). This pattern of expression repeated in two subsequent experiments. Levels of IL-5, -10,

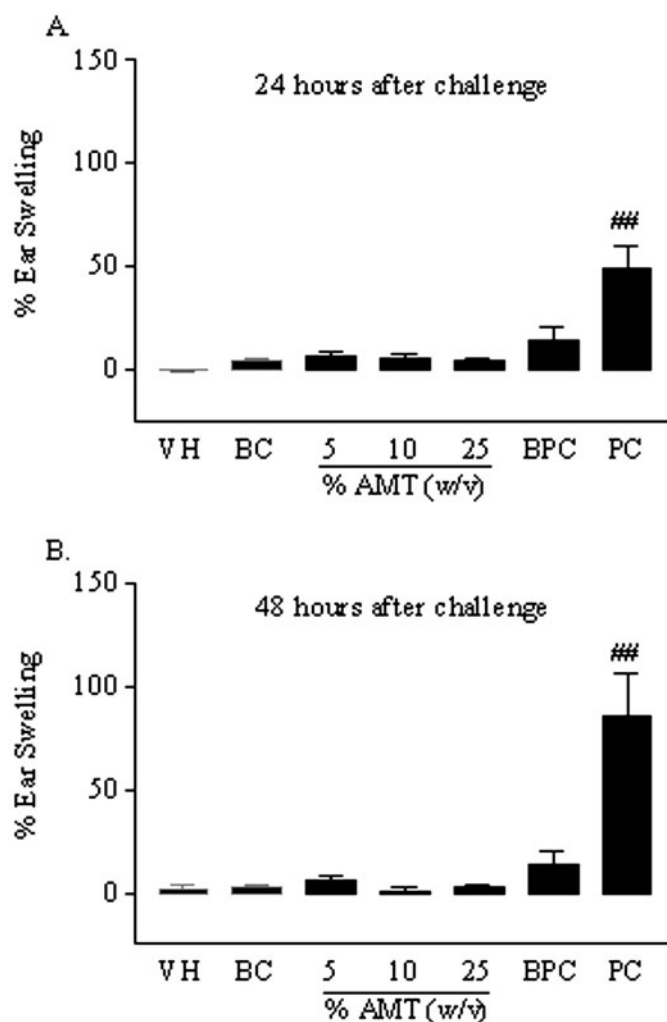


FIG. 3. Evaluation of hypersensitivity response to AMT by the MEST. Bars represent mean percentage ear swelling (A) 24 and (B) 48 h post challenge. Abbreviations represent: VH, vehicle; BC, background control; BPC, background positive control; PC, positive control. See Table 1 for dose group identification. ## indicates $p < 0.0001$ as compared to the BPC. Results shown are a combination of two independent studies (vehicle and test groups, $n = 16$; PC and BPC, $n = 8$).

and -12p40 and IFN- γ were not modulated following exposure to AMT as compared to the DMSO control (data not shown).

AMT Induced a Time- and Dose-Dependent Increase in the Levels of Total Serum IgE

To evaluate the dose and time course response of serum IgE, mice were exposed to AMT dermally 7 days/week for 68 days. A dose-dependent increase ($p < 0.05$) in IgE was observed by day 26 of exposure, reaching significance in the 25% AMT dose group ($p < 0.01$; 3838 ± 509 ng/ml vs. 1255 ± 459 ng/ml DMSO). Forty days into the study, IgE levels in all dose groups were statistically elevated over the vehicle control group, and from this point no significant differences in IgE levels were observed between AMT dose groups (Fig. 6).

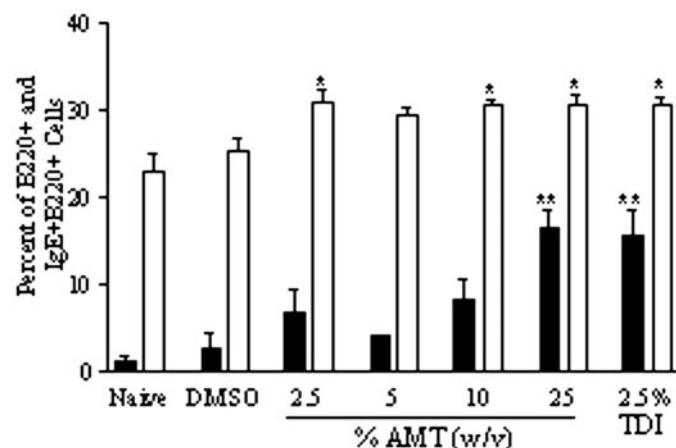


FIG. 4. Evaluation of the local IgE response in BALB/c mice following 4 days exposure to AMT. Black bars represent IgE+B220+ cells, and empty bars represent B220+ expressing viable nonred blood cells (% of gated cells), where $n = 5$. * indicates $p < 0.05$ as compared to the vehicle control group. ** indicates $p < 0.01$ as compared to the vehicle control group.

Nonspecific Airway Hyperreactivity Is Elevated and Suppurative Histiocytic Alveolitis Is Observed following 35-Day Dermal Exposure to AMT

In the initial study to evaluate the effect of dermal AMT exposure on AHR, animals were dosed 7 days a week with DMSO, 5, 15, or 25% AMT, and on day 21 nonspecific AHR was evaluated via MCH challenge. Animals previously exposed dermally to 15% AMT demonstrated an increase in AHR at all three MCH concentrations as compared to the vehicle control group, while the 25% dose group was not significantly different from the vehicle animals (Fig. 7). On days 28 and 35, mice were challenged via intratracheal instillation with 250 μ g AMT/50 μ l PBS, and antigen-specific airway hyperreactivity was assessed over a 6 h period. IT exposure to AMT in previously sensitized animals had no effect on PenH values (data not shown).

Twenty-four h after the second IT challenge, lungs were removed, fixed in formalin, and prepared for histopathological evaluation of cellular infiltration and mucin production. Lesions appeared to be more disseminated and of greater severity in the AMT exposed dose groups, as compared to the dermal vehicle control/IT AMT exposed animals. Suppurative and histiocytic alveolitis was observed in all dose groups (DMSO, 2/5; 5% AMT, 4/5; 15% AMT, 5/5; 25% AMT, 5/5); however, the pathology was more pronounced in the 15 and 25% AMT sensitized animals. Representative sections of the vehicle and 15% AMT exposed groups are shown in Figure 8. Mayers Mucicarmine staining did not reveal an increase in mucin production in any of the dose groups. Likewise, a 0.5% Chromotrope 2R stain did not demonstrate treatment-related increases in eosinophils (data not shown).

To further analyze the dose response relationship, animals were dosed 7 days a week for 35 days with DMSO, 5, 10, 15,

20, or 25% AMT. Nonspecific AHR was evaluated on day 35 ($n = 5$), and specific AHR was evaluated on days 28 and 35 ($n = 5$). Nonspecific AHR was elevated following MCH challenge in the 10, 20, and 25% AMT dose groups (Fig. 9). Consistent with the first study evaluating antigen-specific AHR in animals dermally exposed to 5, 15, or 25% AMT, antigen-specific AHR was not detected in any of the dose groups in this expanded dose response study (data not shown). Lung pathology was similar to that observed in the first study, with a dose-responsive increase in suppurative histiocytic alveolitis (data not shown).

DISCUSSION

Although adverse effects on the respiratory system have classically been assumed to be induced by inhalation exposure, more recent data have supported the potential for dermal exposure to lead to respiratory tract sensitization. To illustrate, the low-molecular-weight chemical, trimellitic anhydride (TMA) has been shown to induce a dose- and time-responsive increase in TMA-specific IgE after epicutaneous sensitization (Zhang *et al.*, 2002b), with the subsequent demonstration of an early and late phase airway hyperreactivity response upon TMA inhalation challenge (Zhang *et al.*, 2002a). Another study evaluating pulmonary effects following low-molecular-weight chemical exposure demonstrated an increase in nonspecific AHR *in vivo* following IV serotonin challenge and *ex vivo* following carbachol challenge to isolated tracheal preparations in animals previously dermally exposed to TDI (Scheerens *et al.*, 1999). Similarly, dermal protein exposure has been shown to induce pulmonary sensitization. After dermal exposure to nonammoniated latex proteins, BALB/c mice demonstrated a dose-dependent increase in total and latex-specific IgE. Furthermore, an increase in nonspecific and specific airway hy-

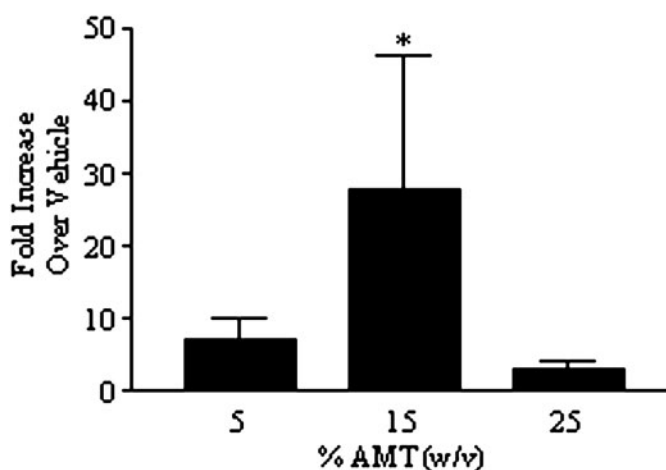


FIG. 5. Evaluation of the mRNA profile of DLN cells following 3-day dermal AMT exposure to BALB/c mice. Results are expressed as the group mean fold increase over the vehicle control, calculated by the $2^{-\Delta\Delta C_t}$ method ($n = 5$). * indicates $p < 0.05$ as compared to the vehicle control group.

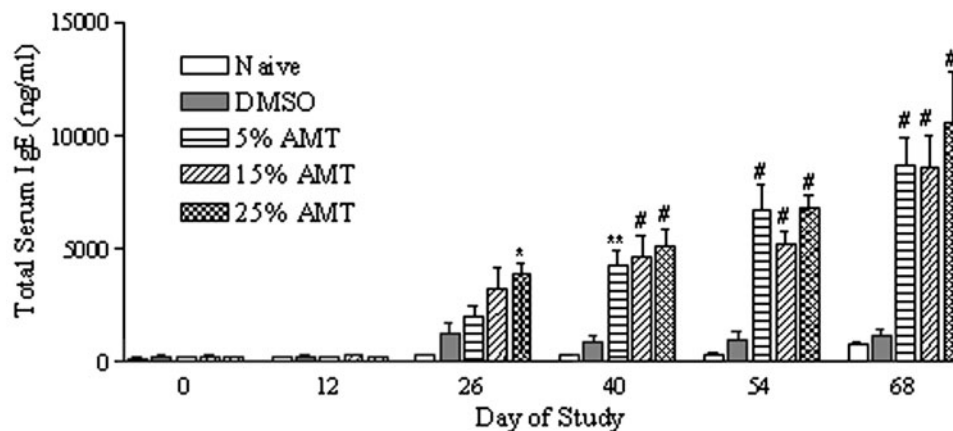


FIG. 6. Time and dose response evaluation of the total IgE production in BALB/c mice following dermal exposure to AMT. Mice were dosed 5 days/week for 68 days and total serum IgE was assessed on the days shown. * indicates $p < 0.05$ as compared to the vehicle control on appropriate day. ** indicates $p < 0.01$ as compared to the vehicle control on appropriate day. # indicates $p < 0.001$ as compared to the vehicle control on appropriate day.

perreactivity was observed following methacholine and latex challenge, respectively (Howell *et al.*, 2002). Due to potential for both respiratory and dermal exposure, AMT and DE498 were evaluated for the potential to induce sensitization through a dermal exposure mechanism.

Initial screening using QSAR yielded negative results for irritancy for both AMT and DE498. Earlier studies using the Draize ocular irritancy rabbit test reported slight irritation at 24 h post DE498 challenge with resolution by 48 h (Kleschick *et al.*, 1992). The absence of any indication of irritancy in the TOPKAT evaluation is incongruent, given that the training set for the irritation module for the TOPKAT program is based on Draize test results (Accelrys, Inc.). No previous evaluation of the irritancy potential of AMT was found in the literature.

Both computer and animal modeling were utilized to characterize the sensitization potential of these chemicals. In the QSAR model proposed by Ashby *et al.* (1995), chemicals are grouped into categories based on their major reactive subgroups as well as their sensitization potential as evaluated in the LLNA. In this model, chemicals with NH_2 reactive groups, classified as electrophiles, tended to test positive in the LLNA,

whereas sulphonates with large side groups lost reactive potential due to decreased ability to translocate across the stratum corneum. Based on this classification, one would predict AMT to be positive in this model, as it contains a NH_2 side group, while DE498 may be hindered from penetrating the stratum corneum. In another QSAR model, Karol *et al.* (1996) associated biological activity with major biophores, or fragments of the molecules statistically associated with sensitization based on the LLNA. The four biophores associated with contact sensitivity include (1) nitrogen-to-carbon double bonds or nitrogen-to-nitrogen double bonds, (2) substituted aromatic structures, (3) sulfur and sulfur-containing moieties, and (4) electrophiles. Based on this QSAR analysis, the 5-mercapto or 3-amino side groups would be potential reactive sites for AMT. In DE498, the 3-mercapto group of AMT is incorporated into a sulfonamide structure, while the 5-amino group of AMT is incorporated into a pyrimidine base. Lacking these reactive moieties, the DE498 was negative for sensitization potential in the TOPKAT QSAR model and the LLNA.

Although originally developed and validated as a method to detect contact sensitizers (ICCVAM, 1999; Kimber *et al.*,

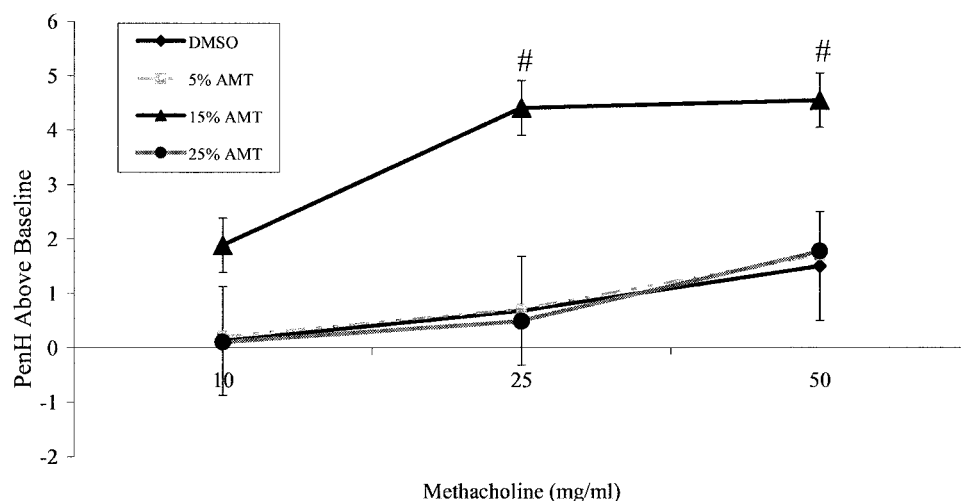


FIG. 7. Evaluation of nonspecific AHR in BALB/c mice following dermal exposure to AMT. Evaluation of AHR by MCH challenge after 21 days dermal exposure to AMT, with results showing the dose group means $\pm$ SE at each MCH concentration. # indicates $p < 0.001$ as compared to the vehicle control.

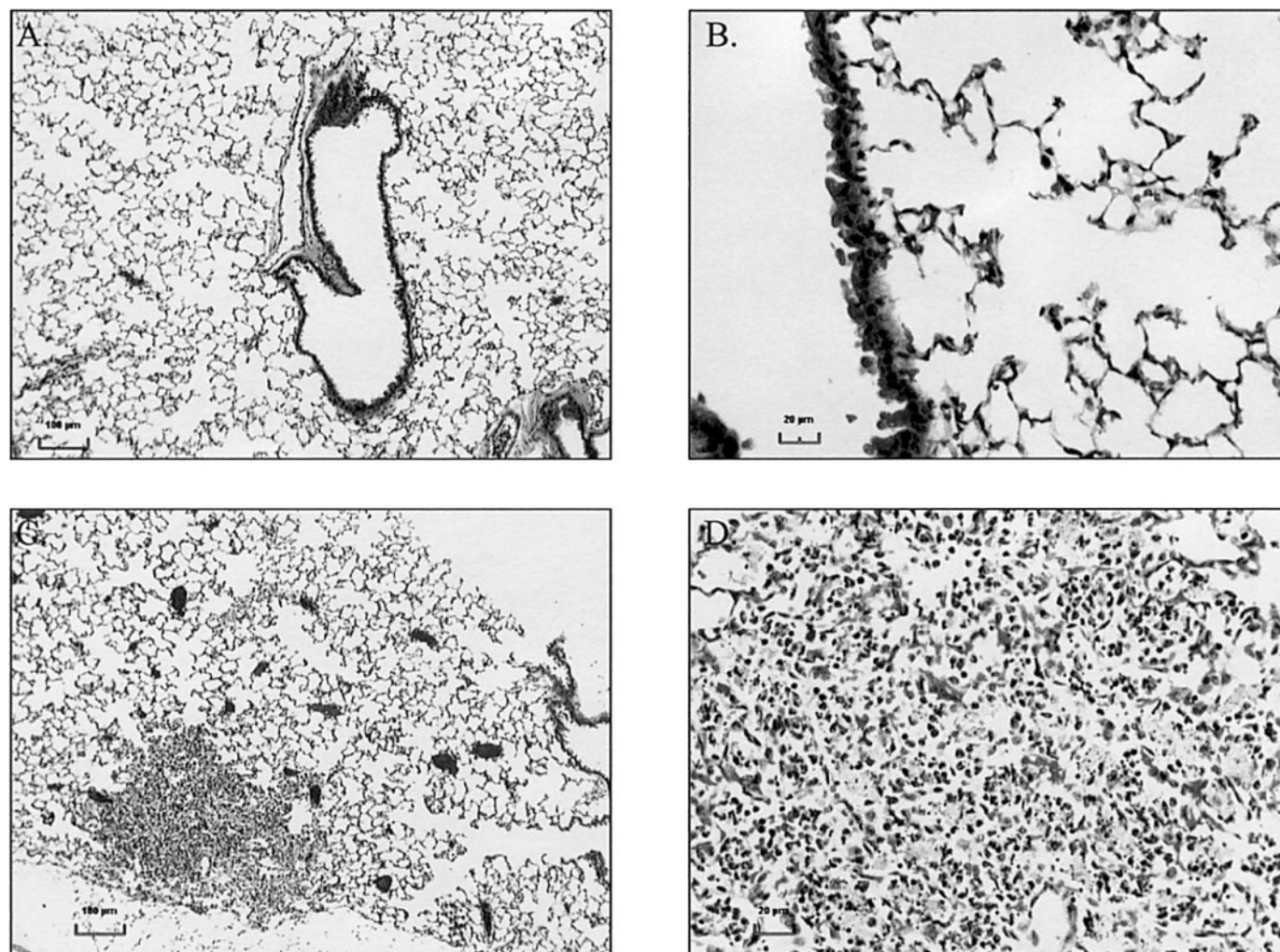


FIG. 8. Lung histopathology following dermal exposure to either vehicle or 15% AMT, with IT AMT challenge on days 28 and 35. The bronchiole and alveoli are shown in the (A) 10 $\times$ H&E slide and (B) 40 $\times$ H&E slides of mice exposed dermally to vehicle while (C) is a 10 $\times$ H&E and (D) is a 40 $\times$ H&E representation of mice dermally sensitized with 15% AMT.

1986), the LLNA has also been shown to produce positive results following exposure to chemicals that induce an IgE-mediated response. Examples of respiratory sensitizers testing positive in the LLNA include phthalic anhydride (Dearman and Kimber, 1992), TMA (Dearman and Kimber, 1992; Manetz and Meade, 1999), and TDI (Manetz and Meade, 1999; Woolhiser *et al.*, 1998). Initial data, which yielded a positive response in TOPKAT QSAR (based on the GPMT) and LLNA, suggested the potential for AMT to induce a DTH reaction. Yet, AMT was negative in the MEST, another murine assay for contact sensitivity. Disparate results seen in the LLNA versus the MEST could in part be due to the phase in which the response was measured. A positive result in the LLNA is based on the initial sensitization phase, while a positive result in the MEST is dependent on the elicitation phase. In a study by Howell *et al.* (2000), the LLNA was able to identify contact sensitivity to potassium dichromate at lower concentrations

(0.25%) as compared to the MEST (requiring an induction concentration of 0.5%). Higher induction doses of acrylate compounds were also required to induce a positive response in the MEST as compared to the LLNA (Hayes and Meade, 1999). Many chemicals have also been shown to elicit both a contact and IgE-mediated hypersensitivity response. Dearman *et al.* (1992) demonstrated both a DTH and IgE-mediated hypersensitivity response in BALB/c mice following the dermal application of 50% TMA. Glutaraldehyde has been demonstrated to induce contact dermatitis in both health care workers and workers in the cosmetic industry (Goncalo *et al.*, 1984; Kiec-Swierczynska and Krecisz, 2001), while other reports have shown respiratory sensitization and the induction of occupational asthma upon glutaraldehyde exposure (Quirce *et al.*, 1999; Stenton *et al.*, 1994).

The potential for AMT to induce sensitization through an IgE-mediated mechanism was supported by local induction of

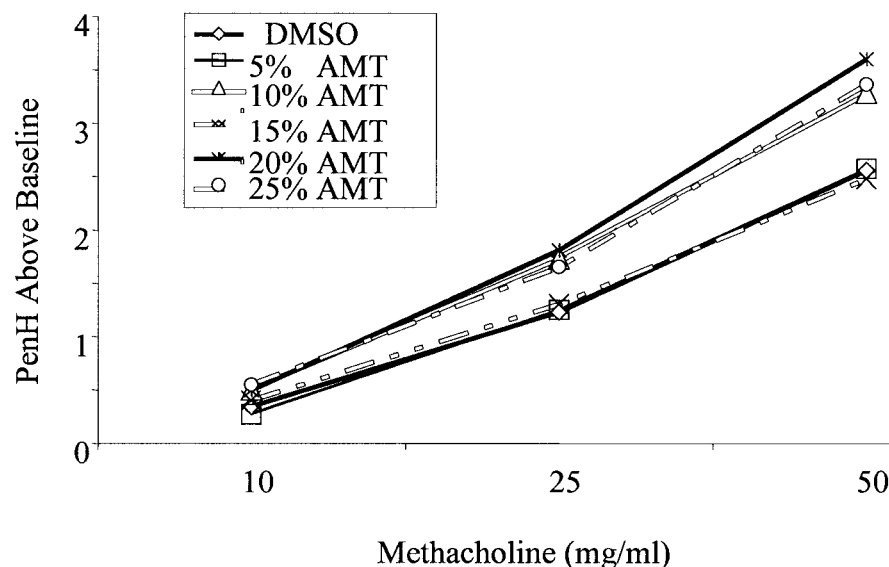


FIG. 9. Evaluation of nonspecific AHR in BALB/c mice following dermal exposure to AMT at concentrations of 5, 10, 15, 20, and 25%. Evaluation of AHR by MCH challenge after 35 days dermal exposure to AMT, with results showing the dose group means $\pm$ SE at each MCH concentration.

IL-4 mRNA and production of IgE in the DLNs (as measured by an increase in IgE+B220+ lymph node cells). The evaluation of IL-4 mRNA was chosen over the measurement of protein levels, in that PCR is more sensitive than the ELISA, and ConA is not required to induce measurable levels of transcript with PCR, whereas this mitogen has been shown to be required to induce quantifiable levels of IL-4 protein. Results obtained by Ryan *et al.* (1998) demonstrated similar trends in IL-4 mRNA and protein levels following exposure to TMA, while DNCB, a contact allergen, did not induce increased expression of either IL-4 mRNA or protein. In addition, Warbrick *et al.* (1998) demonstrated that TMA induced mRNA and protein levels of IL-4 and IL-10, while DNCB elicited an increase in IFN- γ mRNA and protein levels. Further support for the association of increased IL-4 with the development of respiratory sensitizers is provided by Dearman *et al.* (1996), who showed that TMA, cyanuric chloride, and diphenylmethane diisocyanate induce a Th2 cytokine profile, while DNCB, isoeugenol, and formaldehyde induce a Th1 cytokine profile. Manetz and Meade (1999) demonstrated an increase in IgE+B220+ cells in DLNs following exposure to the IgE-mediated allergens (TDI, TMA), as compared to an increase in B220+ cells in the absence of increased IgE+B220+ cells following exposure to contact allergens. In addition to the local Th2 response, following 26 days of dermal exposure to AMT, an increase in total serum IgE was observed.

The increase in nonspecific AHR to MCH challenge in mice dermally exposed to AMT is consistent with the findings from humans exposed to AMT. From the cluster of OA cases potentially caused by exposure to AMT, of the six cases for whom NIOSH was able to obtain medical records, five tested positive for nonspecific bronchial hyperreactivity. Following removal from AMT exposure, all three of the subjects who were reevaluated by NIOSH no longer had an increase in

nonspecific bronchial hyperreactivity (Hnizdo and Sylvain, 2003).

Two observations in these studies are inconsistent with an IgE-mediated mechanism of sensitization. Animals challenged IT with AMT demonstrated a lack of antigen-specific AHR, although the absence of this response could possibly be related to ineffective pulmonary distribution of the chemical following IT administration of a suspension. Additionally, histopathology was inconsistent with an IgE-mediated mechanism, in that there was an absence of eosinophilia and lack of increased mucin present in the lungs.

Taken together, these studies show a lack of dermal sensitization potential for DE498. Computer and animal modeling demonstrate that AMT is not an irritant or systemically toxic at the concentrations tested, yet has the potential to induce sensitization following dermal exposure. Although the mechanism is not yet defined, the development of dermal sensitization and subsequent pulmonary hyperreactivity in BALB/c mice suggests the potential for AMT to induce occupational asthma.

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REFERENCES

- Ashby, J., Basketter, D. A., Paton, D., and Kimber, I. (1995). Structure activity relationships in skin sensitization using the Murine Local Lymph Node Assay. *Toxicology* **103**, 177–194.
- Dearman, R. J., and Kimber, I. (1992). Divergent immune responses to

- respiratory and contact chemical allergens: Antibody elicited by phthalic anhydride and oxazolone. *Clin. Exp. Allergy* **22**, 241–250.
- Dearman, R. J., Mitchell, J. A., Basketter, D. A., and Kimber, I. (1992). Differential ability of occupational chemical contact and respiratory allergens to cause immediate and delayed dermal hypersensitivity reactions in mice. *Int. Arc. Allergy Immunol.* **97**, 315–321.
- Dearman, R. J., Smith, S., Basketter, D. A., and Kimber, I. (1996). Classification of chemical allergens according to cytokine secretion profiles of murine lymph node cells. *J. Appl. Toxicol.* **17**, 53–62.
- Finkelman, F. D., Katona, I. M., Mosmann, T. R., and Coffman, R. L. (1988). IFN- γ regulates the isotypes of Ig secreted during *in vivo* humoral immune responses. *J. Immunol.* **140**, 1022–1027.
- Frear, D. S., Swanson, H. R., and Tanaka, F. S. (1993). Metabolism of flumetsulam (DE-498) in wheat, dorn, and barley. *Pestic. Biochem. Physiol.* **45**, 178–192.
- Goncalo, S., Menezes Brandao, F., Pecegueiro, M., Moreno, J. A., and Sousa, I. (1984). Occupational contact dermatitis to glutaraldehyde. *Contact Dermatitis* **10**, 183–184.
- Hayes, B. B., Gerber, P. C., Griffey, S. S., and Meade, B. J. (1998). Contact hypersensitivity to dicyclohexylcarbodiimide and diisopropylcarbodiimide in female B6C3F1 mice. *Drug Chem. Toxicol.* **21**, 195–206.
- Hayes, B. B., and Meade, B. J. (1999). Contact sensitivity to selected acrylate compounds in B6C3F1 mice: Relative potency, cross reactivity, and comparison of test methods. *Drug Chem. Toxicol.* **22**, 491–506.
- Hnizdo, E., and Sylvain, D. C. (2003). NIOSH Health Hazard Evaluation Report: ChemDesign Corporation. National Institute for Occupational Safety and Health, Cincinnati. HETA # 2000-0096-2976.
- Howell, M. D., Manetz, T. S., and Meyers, D. A. (2000). Comparison of murine assays for the identification of chemical sensitizers. *Toxicol. Meth.* **10**, 1–15.
- Howell, M. D., Weissman, D. N., and Meade, B. J. (2002). Latex sensitization by dermal exposure can lean to airway hyperreactivity. *Int. Arc. Allergy Immunol.* **128**, 204–211.
- Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). (1999). The Murine Local Lymph Node Assay: A test method for assessing the allergic contact dermatitis potential of chemical/compounds. NIH Pub No. 99-4494.
- Karol, M. H., Graham, C., Gealy, R., Macina, O. T., Sussman, N., and Rosenkranz, H. S. (1996). Structure-activity relationships and computer-assisted analysis of respiratory sensitization potential. *Toxicol. Lett.* **86**, 187–191.
- Kiec-Swierczynska, M., and Krecisz, B. (2001). Occupational allergic contact dermatitis in hairdressers due to glutaraldehyde. *Contact Dermatitis* **44**, 185–186.
- Kimber, I., Mitchell, J. A., and Griffin, A. C. (1986). Development of a Murine Local Lymph Node Assay for the determination of sensitizing potential. *Food Chem. Toxicol.* **24**, 585–586.
- Kleschick, W. A., Gerwick, B. C., Carson, C. M., Monte, W. T., and Snider, S. W. (1992). DE-498, a new acetolactate synthase inhibiting herbicide with multicrop selectivity. *J. Agric. Food Chem.* **40**, 1083–1085.
- Manetz, T. S., and Meade, B. J. (1999). Development of a flow cytometry assay for the identification and differentiation of chemicals with the potential to elicit irritation, IgE-mediated, or T Cell-mediated hypersensitivity responses. *Toxicol. Sci.* **48**, 206–217.
- Manetz, T. S., Pettit, D. A., and Meade, B. J. (2001). The determination of draining lymph node cell cytokine mRNA Levels in BALB/c mice following dermal sodium lauryl sulfate, dinitrofluorobenzene, and toluene diisocyanate exposure. *Toxicol. Appl. Pharmacol.* **171**, 174–183.
- Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T Cell clone I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348–2357.
- NIOSH (1996). National Occupational Research Agenda. U.S. Department of Health and Human Services, Cincinnati. DHHS Pub No. 96–115
- Quirce, S., Gomez, M., Bombin, C., and Sastre, J. (1999). Glutaraldehyde-induced asthma. *Allergy* **54**, 1121–1122.
- Ryan, C. A., Dearman, R. J., Kimber, I., and Gerberick, G. F. (1998). Inducible interleukin 4 (IL-4) production and mRNA expression following of mice to chemical allergens. *Toxicol. Lett.* **94**, 1–11.
- Scheerens, H., Buckley, T. L., Muis, T. L., Garssen, J., Dormans, J., Nijkamp, F. P., and van Loveran, H. (1999). Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and *in vivo* airway hyperresponsiveness three hours after intranasal challenge. *Am. J. Respir. Crit. Care Med.* **159**, 1074–1080.
- Stenton, S. C., Beach, J. R., Dennis, J. H., Keaney, N. P., and Hendrick, D. J. (1994). Glutaraldehyde, asthma, and work—a cautionary tale. *Occup. Med.* **44**, 95–98.
- Takaoka, M., Teranishi, M., and Manabe, S. (1994). Structure-activity relationships in 5-substituted 3-amino-1,2,4-triazoles-induced goiters in rats. *Toxicol. Pathol.* **7**, 429–434.
- Trinchieri, G. (1994). Interleukin-12: A cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of t-helper cells type 1 and cytotoxic lymphocytes. *Blood* **84**, 4008–4027.
- U.S. Environmental Protection Agency (U.S. EPA) (1998). Federal Register: Forty-Second Report of the TSCA Interagency Testing Committee; Notice, pp. 42553–42559. Environmental Protection Agency, Washington, DC.
- Warbrick, E. V., Dearman, R. J., Basketter, D. A., Gerberick, G. F., Ryan, C. A., and Kimber, I. (1998). Analysis of cytokine mRNA expression following repeated exposure of mice to chemical contact and respiratory allergens. *J. Appl. Toxicol.* **18**, 205–213.
- Woolhiser, M. R., Hayes, B. B., and Meade, B. J. (1998). A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. *Toxicol. Meth.* **8**, 245–256.
- Zhang, X. D., Fedan, J. S., Lewis, D. M., and Siegel, P. D. (2002a). Airway Responses after specific challenge of rats sensitized via skin exposure to trimellitic anhydride (TMA). *Toxicologist* **66**, 1184 (Abstract).
- Zhang, X. D., Murray, D. K., Lewis, D. M., and Siegel, P. D. (2002b). 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. *Allergy* **57**, 620–626.