

Is Toluene Diamine a Sensitizer and is there Cross-Reactivity between Toluene Diamine and Toluene Diisocyanate?

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Toluene diamine (TDA) is formed when toluene diisocyanate (TDI), a potent sensitizer, comes in contact with an aqueous environment. The sensitizing capacity of TDA and the cross-reactivity between TDI and TDA are unknown. TDA (5–25%) and TDI (0.3%), dissolved in acetone/olive oil (AOO) (4:1) were tested in the mouse local lymph node assay (LLNA). To determine the capacity of TDA to elicit an asthmatic response and to determine the cross-reaction with TDI, a locally developed experimental mouse model of chemical-induced asthma was used. On days 1 and 8, BALB/c mice received 20 μ l of TDI (0.3%), TDA (20%), or AOO (4:1) on each ear. On day 15, they received an intranasal instillation of TDI (0.1%), TDA (0.5%) or AOO (3:2). The EC₃ of TDA in the LLNA is 19%. In the model of chemical-induced asthma, TDI induced a ventilatory response [increased Penh after challenge; increased airway hyperreactivity (AHR)], inflammatory changes (bronchoalveolar lavage neutrophils), and immunological changes (increased CD19⁺ lymphocytes, IL-4 and total serum IgE), whereas TDA did not show any of these responses. Mice sensitized with TDI and challenged with TDA also did not show any airway or inflammatory response, although they had increased levels of total serum IgE. Mice sensitized with TDA and challenged with TDI did not show any response. According to the classification of sensitizers in the LLNA, TDA is a weak dermal sensitizer. In the experimental mouse model of chemical-induced asthma, TDA does not act as a respiratory sensitizer, at the concentration used. No cross-reactivity between TDI and TDA was found.

Key Words: TDI; TDA; occupational asthma; murine model; cross-reactivity.

Diisocyanates are low molecular weight chemicals widely used in industry for the production of, for example, polyurethane foams, varnish, paint, and isolation material (Bello *et al.*, 2007; Wisniewski and Redlich, 2001). They constitute one of the principal causes of occupational asthma (Chan-Yeung and Malo, 1994; Chan-Yeung *et al.* 2003). Diisocyanates contain two highly reactive isocyanate groups ($-\text{N} = \text{C} = \text{O}$), which can react with $-\text{OH}$, $-\text{SH}$, and $-\text{NH}_2$ groups of endogenous

proteins (Vandenplas *et al.*, 1993). It is known that diisocyanates are potent sensitizers and are able to bind to albumin, tubulin, keratin, and glutathione in the skin or mucosal surfaces (Bello *et al.*, 2007; Kimber *et al.*, 1996; Lange *et al.*, 1999; Matheson *et al.*, 2001, 2005; Wisniewski *et al.*, 1999, 2000, 2005).

When the isocyanate groups ($-\text{N} = \text{C} = \text{O}$) of toluene diisocyanate (TDI) come into contact with an aqueous environment, amine groups ($-\text{NH}_2$) are formed spontaneously, with release of CO_2 (Shanmugam *et al.*, 2001). In industry, toluene diamine (TDA) is used as an intermediate product for the formation of TDI (Lind *et al.*, 1996). It is described in the literature that 2,4-TDA is an animal carcinogen, whereas 2,6-TDA is not, although it is mutagenic (Shanmugam *et al.*, 2001). TDA is a crystalline powder, which is also used as paint for fur and leather (Lind *et al.*, 1996; Shanmugam *et al.*, 2001).

In an attempt to elucidate the chemical mechanisms of sensitization to TDI we wanted to investigate the possible cross-reactivity between TDI and TDA in our experimental mouse model of chemical-induced asthma. However, because it is unknown whether TDA is a dermal sensitizer, we first used the local lymph node assay (LLNA) to characterize the dermal sensitizing capacity of TDA (Basketter *et al.*, 2002; Kimber *et al.*, 1989). Following the LLNA, we set out to test whether TDA was able to elicit an asthmatic response after dermal sensitization, using our experimental mouse model of chemical-induced asthma, developed and validated by Vanoirbeek *et al.* (Tarkowski *et al.*, 2007; Vanoirbeek *et al.*, 2004, 2006a, 2008). Finally, we checked our initial aim, whether cross-reactivity between TDI and TDA can occur. We, therefore, dermally sensitized mice with TDI and intranasally challenged them with TDA and vice versa. Our results showed that TDA is a weak dermal sensitizer, that it did not elicit a respiratory response after dermal sensitization and that it did not show any sign of cross-reactivity with TDI.

MATERIALS AND METHODS

Reagents

Toluene-2,4-diisocyanate (98%, CAS 584-84-9) (MW: 174.15), toluene-2,4-diamine (98%, CAS 95-80-7) (MW: 122.17), acetyl- β -methylcholine

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(methacholine), acetone, and diethyl ether were obtained from Sigma-Aldrich (Bornem, Belgium). Pentobarbital (Nembutal) was obtained from Sanofi Santé Animale (CEVA, Brussels, Belgium). For dermal applications both in the LLNA and the experimental mouse model of chemical-induced asthma, the vehicle (AOO) consisted of four volumes of acetone (A) and one volume of olive oil (OO) (Selection de Almazara, Carbonell, Madrid, Spain). For the intranasal instillation the vehicle (AOO) consisted of a mixture of two volumes of acetone and three volumes of olive oil. Concentrations of TDA are given as percent (wt/vol) and of TDI as percent (vol/vol) in AOO.

Animals

Male BALB/c mice (approximately 20 g, 6 weeks old) were obtained from Harlan (Horst, Netherlands). The mice were housed in a conventional animal house with 12-h dark/light cycles. They were housed in plastic cages with filter tops and received lightly acidified water and pelleted food (Trouw Nutrition, Gent, Belgium) *ad libitum*. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Local Lymph Node Assay

The LLNA was performed according to the standard protocol described by Kimber *et al.* (1989). Briefly, the mice (five animals per test group) received dermal applications of 25 μ l of TDA (5–25%); TDI (0.3%), or AOO vehicle alone, on each ear, daily for 3 consecutive days. The area around the exposure site was monitored for any signs of irritancy. Five days after the first dermal application, all mice were injected intravenously via the tail vein with (^{3} H)-thymidine [^{3} HTdR], 2 Ci/mmol from ICN Pharmaceuticals (Asse, Belgium) in 250 μ l of phosphate-buffered saline (pH 7.2). Five hours later, the mice were sacrificed by an overdose of pentobarbital (90 mg/kg) and the draining auricular lymph nodes were excised, pooled for each experimental group, and weighed (lymph node weight). A single-cell suspension of lymph node cells (LNCs) was prepared, and LNCs were washed twice. ^{3} HTdR incorporation was measured by β -scintillation counting (Beckman LS 5000CE, Irvine, CA) and expressed as disintegrations per min (dpm). The stimulation index (SI) was calculated as the ratio of ^{3} HTdR incorporation by lymphocytes from treated animals relative to that from concurrent vehicle-treated controls. The EC₃ value was determined via linear interpolation of the SI (Kimber *et al.*, 2002).

Mouse Model of Chemical-Induced Asthma

Groups of animals and treatment protocol. On days 1 and 8, the animals received a dermal application (20 μ l) with 20% TDA, 0.3% TDI, or vehicle on the dorsum of both ears. On day 15, they received, under light diethyl ether anesthesia, an intranasal instillation (10 μ l/nostril) of 0.5% TDA, 0.1% TDI (challenge), or vehicle. Mice were sacrificed 48 h after the last challenge. Each group consisted of 10 animals, of which five underwent a methacholine provocation using the whole-body plethysmography followed by an autopsy, whereas the other five underwent methacholine provocation using the invasive FlexiVent technique before sacrifice. In all experiments, treatment with TDI is indicated as 1, treatment with TDA is indicated as 2, and treatment with vehicle is indicated as 0. Thus, the 1/1 group consists of mice that received dermal applications of TDI (days 1 and 8, first digit) and an intranasal instillation of TDI (day 15, second digit), whereas the 0/0 control group consists of mice that received the AOO vehicle on all occasions. The following groups were studied 0/0, 0/1, 1/1, 1/2, 0/2, 2/2, and 2/1.

Early ventilatory response and airway hyperreactivity. The ventilatory function of each mouse was first recorded, in resting condition and unrestrained, for 5 min in a whole-body plethysmograph (EMKA Technologies, Paris, France). Immediately after the intranasal instillation, mice were placed again in the whole-body plethysmograph (zero time point) and their ventilatory parameters were measured for 40 min. Every 30 s, the mean enhanced pause (penh) was calculated. Then, the area under the curve (AUC) of Penh against time between 0 and 40 min was calculated for each individual mouse and this figure was used for statistical analysis.

In five mice per group, reactivity to methacholine was assessed by whole-body plethysmography. Briefly, Penh was calculated for each mouse under resting

condition (baseline) and after inhalation, during 1 min, of nebulized methacholine (successively 0, 5, 10, 25, and 50 mg/ml). An average Penh over 30 s was calculated during 3 min (six measurements), and the mean of these six values was used for each concentration. For each mouse, Penh was plotted against methacholine concentration (from 0 to 50 mg/ml) and the AUC was calculated.

In another five mice per group, airway hyperreactivity responses were measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). Airway resistance (*R*) was assessed after increasing exposures to methacholine. Mice were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged with methacholine aerosol, generated with an in-line nebulizer and administered directly through the ventilator for 5 s, with increasing concentrations (0, 0.625, 1.25, 2.5, 5, and 10 mg/ml). Airway resistance (*R*) was measured using a “snapshot” protocol each 20 s for 2 min. The mean of these six values was used for each methacholine concentration, unless the coefficient of determination of a measurement was smaller than 0.95. For each mouse, *R* was plotted against methacholine concentration (from 0 to 10 mg/ml) and the AUC was calculated (Vanoirbeek *et al.*, 2009).

Pulmonary inflammation (Bronchoalveolar lavage). Two days (48 h) after the intranasal instillation, mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight). Blood was first sampled from the retro-orbital plexus, and the mice were then killed by section of the abdominal vessels. Serum samples were obtained from centrifugation of whole blood at room temperature (14,000 $\times$ g, 10 min) and stored at -80°C awaiting further analysis. The lungs were lavaged, *in situ*, three times with 0.7 ml of sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cells) and the bronchoalveolar lavage (BAL) fluid was centrifuged (1000 $\times$ g, 10 min). The supernatant was frozen (-80°C) until further analyses. For differential cell counts, 250 μ l of the resuspended cells (100,000 cells/ml) were spun (1400 $\times$ g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried, and stained (Diff-Quik method, Medical Diagnostics, Düringen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils, and lymphocytes.

Levels of tumor necrosis factor- α (TNF- α), and macrophage inflammatory protein-2 (MIP-2) were measured in undiluted BAL fluid by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (TNF- α : Biosource, Nivelles, Belgium; MIP-2: R&D Systems, Abingdon, UK). Lower limits of detection were 3.0 and 1.5 pg/ml for TNF- α and MIP-2, respectively.

LNCs analysis. Retro-auricular and superficial cervical lymph nodes were also obtained from the same mice. The lymph nodes from two to three mice were pooled and kept on ice in RPMI-1640 (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 μ m) (BD Bioscience) and rinsing with 10 ml of tissue culture medium (RPMI-1640). Cells were counted using a Bürker hemocytometer. Lymphocytes were washed three times and suspended (10⁷ cells/ml) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mg/ml streptomycin, 100 IU/ml penicillin, 1 mM sodium pyruvate, nonessential amino acids). Five hundred thousand cells were stained with anti-CD3⁺ (allophycocyanin), anti-CD4⁺ (APC-Cy7), anti-CD8⁺ (PerCP-Cy5.5), and anti-CD25⁺ (phycoerythrin), or received a single staining with anti-CD19⁺ (PE) labeled antibodies, according to standard procedures, and with control samples being labeled with isotype match control antibodies (BD Biosciences). Flow cytometry (Facsarray, BD Biosciences) was performed using at least 10⁵ cells.

Cells were also seeded into 48-well culture plates at a density of 10⁶ cells/ml and incubated in complete RPMI-1640 medium for 18 h without or with 2.5 μ g/ml

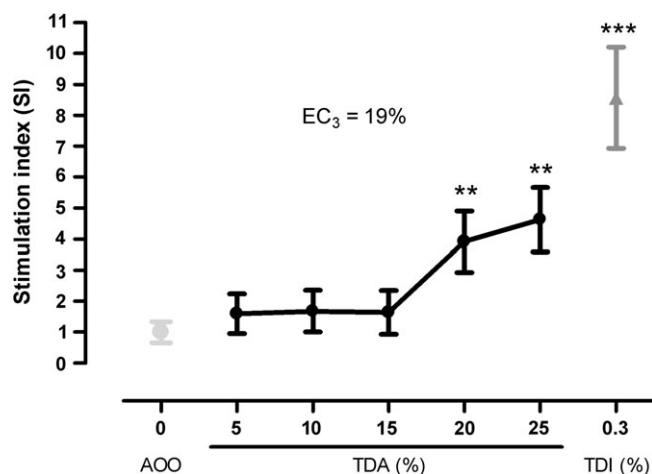


FIG. 1. SI in the LLNA. The SI was calculated as the ratio of thymidine incorporation by lymphocytes from treated mice relative to that from concurrent vehicle-treated controls. The two auricular lymph nodes of each mouse were collected individually. The symbols represent mean $\pm$ SD ($n = 4-6$). ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

of concanavaline A (ConA) (Sigma-Aldrich). Cells were then centrifuged ($1000 \times g$, 10 min) and supernatants were stored at -80°C . Concentrations of interleukin-4 (IL-4) and interferon-gamma (IFN- γ) were measured in undiluted supernatant by standard ELISA techniques, according to the manufacturer's instructions (Biosource, Nivelles, Belgium). Lower detection limits were 5 and 1 pg/ml for IL-4 and IFN- γ , respectively.

Total serum IgE, IgG₁ and IgG_{2a}. The OptEIA Mouse IgE and IgG_{2a} set from Pharmingen (BD Biosciences) was used to measure total serum IgE (diluted 1/70) and total serum IgG_{2a} (diluted 1/10,000). Measurements were performed according to the manufacturer's instructions. For measurement of total serum IgG₁ (diluted 1/250,000), plates were coated using purified anti-mouse IgG₁, a standard was created using purified mouse IgG₁ and the further measurements were performed according to manufacturer's (BD Pharmingen) instructions with the use of biotinylated anti-mouse IgG₁ and avidin horseradish peroxidase conjugate.

Data Analysis

Data are shown as means and SD. All data were analyzed using the nonparametric Kruskal-Wallis test with a Dunnett's *post hoc* test (Graphpad Prism 4.01, Graphpad Software, Inc, San Diego, CA). A level of $p < 0.05$ (two tailed) was considered significant.

RESULTS

Local Lymph Node Assay

Figure 1 shows the results of the stimulation index in the LLNA, for various concentrations of TDA, and for TDI (0.3%). Applying 5, 10, or 15% TDA on the ears of the mice entirely negative results, but at higher concentrations (20 and 25%), significant increases in SI were obtained, compared with the vehicle-treated mice. Nevertheless, the SI values were much lower than those obtained with 0.3% TDI. From the SI of TDA we calculated the EC₃ value, which was 19%.

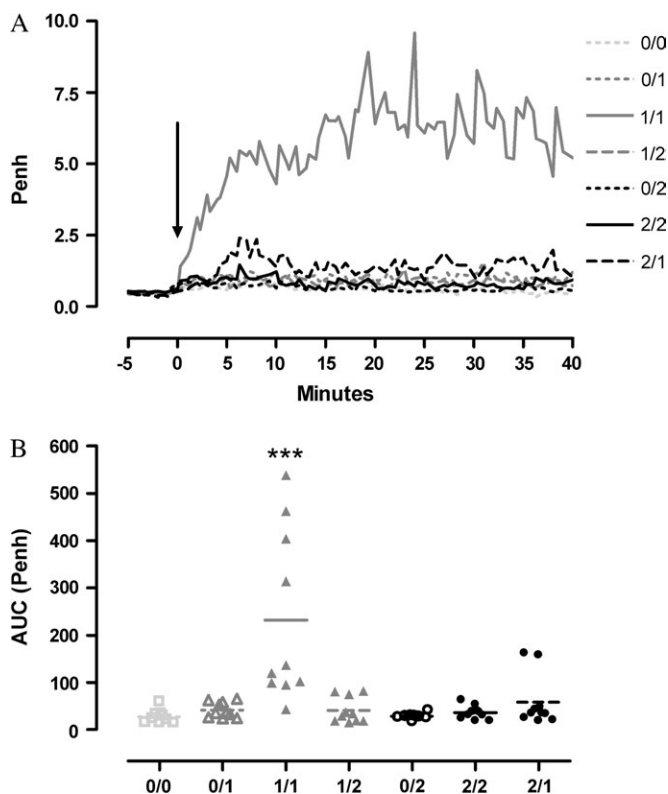


FIG. 2. Early ventilatory response (Penh) after intranasal challenge. Panel (a) shows mean values of Penh during 5 min before and 40 min after instillation (indicated with arrow), and panel (b) show individual values of the AUC of Penh against time between 0 and 40 min. Experimental groups are identified by two symbols (e.g., 1/1). 0, 1, and 2 represent administration of vehicle (AOO), TDI, or TDA, respectively; the first symbol identifies dermal sensitization on days 1 and 8 (0.3% TDI or 20% TDA), whereas the second symbol identifies the intranasal instillation on day 15 (0.1% TDI or 0.5% TDA). $n = 9-10$ per group. *** $p < 0.001$ compared with the 0/0 control group.

Mouse Model of Chemical-Induced Asthma

Based on the data of the LLNA, we decided to dermally treat the mice in our experimental mouse model of chemical-induced asthma (days 1 and 8) with TDA at a concentration of 20%.

Ventilatory function. Figure 2a shows the early ventilatory responses immediately after intranasal instillation. Figure 2b shows the AUC of the individual mice and the group averages. Mice dermally treated with TDI are the only group showing an immediate response upon challenge with TDI, compared with the control group (0/0). Both TDA-sensitized and TDA-challenged (2/2) and the TDI-TDA cross-treated groups (1/2 and 2/1) did not show any relevant changes in Penh after intranasal challenge.

Figures 3a and 3c show the methacholine responsiveness 46 h after the intranasal instillation (day 17), whereas Figures 3b and 3d show the AUC of the individual mice and the group average. Both the measurement performed using the whole-body plethysmograph (Penh, Figs. 3a and 3b) and the FlexiVent

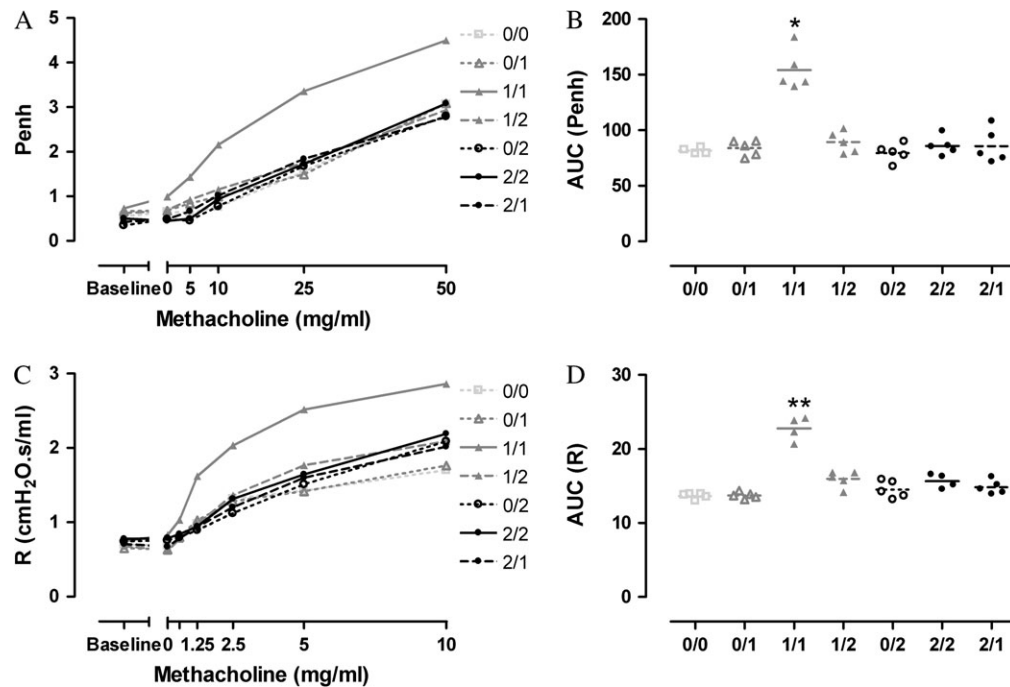


FIG. 3. Methacholine (Mch) responsiveness. Airway reactivity was assessed 46 h after the intranasal challenge. AHR was measured by whole-body plethysmography (a and b) (Penh), and by forced oscillation technique (c and d) (resistance, *R*). (a and c) Mean values of Penh and *R*; (b and d) individual values of the AUC of Penh and *R* against methacholine concentrations between 0 and 50 mg/ml for Penh and between 0 and 10 mg/ml for *R*. Experimental groups are as in Figure 1. $n = 4-5$ per group. * $p < 0.05$ and ** $p < 0.01$ compared with the 0/0 control group.

(*R*, Figs. 3c and 3d) show that the TDI-sensitized and TDI-challenged mice are the only group exhibiting significant increases in methacholine sensitivity, compared with the vehicle-treated control group.

Pulmonary inflammation (BAL). Figure 4 shows the total BAL cell count, and a differentiation between the total number of macrophages and neutrophils in BAL, 48 h after the intranasal challenge. No eosinophils or lymphocytes were found. Both the total number of macrophages and neutrophils were significantly increased in completely TDI-treated mice. In all other treatment groups no changes were found.

BAL cytokine analysis revealed that MIP-2 and TNF- α concentrations were barely increased in the TDI-treated mice (data not shown).

LNCs analysis. Figure 5 shows the total amount of CD3⁺ (T lymphocytes), CD3⁺CD4⁺ (Th-lymphocytes), CD3⁺CD4⁺CD25⁺ (activated—regulatory Th-lymphocytes), CD3⁺CD8⁺ (Tc-lymphocytes), and CD19⁺ (B lymphocytes) cells in auricular and cervical lymph nodes. In the auricular lymph nodes of TDI-sensitized (1/1 and 1/2) mice, a significant increase was found in both T and B lymphocytes (Fig. 5a). The increase in T lymphocytes was due to a significant increase in all three subpopulations measured (Th, Treg, and Tc-lymphocytes). Auricular lymph node subpopulations of TDA-sensitized and TDA-challenged mice (2/2) were clearly changed, but they only showed a significant increase in CD19⁺ positive B-cells

(Fig. 5a). Auricular lymph nodes of TDA-sensitized, but TDI-challenged mice (2/1) did show significant increases in CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, and CD19⁺ lymphocytes.

Both the cervical lymph nodes of completely TDI-treated mice (1/1) and TDA-sensitized and TDI-challenged (2/1) mice showed an increase in total number of CD3⁺, CD3⁺CD4⁺,

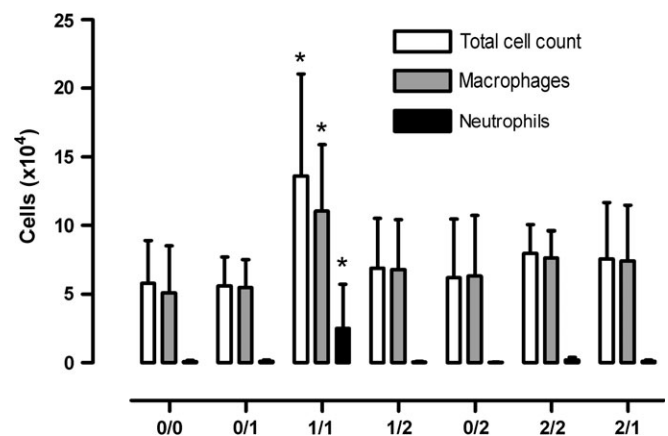


FIG. 4. Total cell count and total numbers of macrophages and neutrophils in BAL. Cells were obtained by BAL of the whole lung 48 h after the intranasal challenge. Figure shows the mean and SD of the total number of cells in the BAL and the total number of macrophages and neutrophils in BAL. Experimental groups are as in Figure 1. $n = 9-10$ mice per group. * $p < 0.05$ compared with the 0/0 control group.

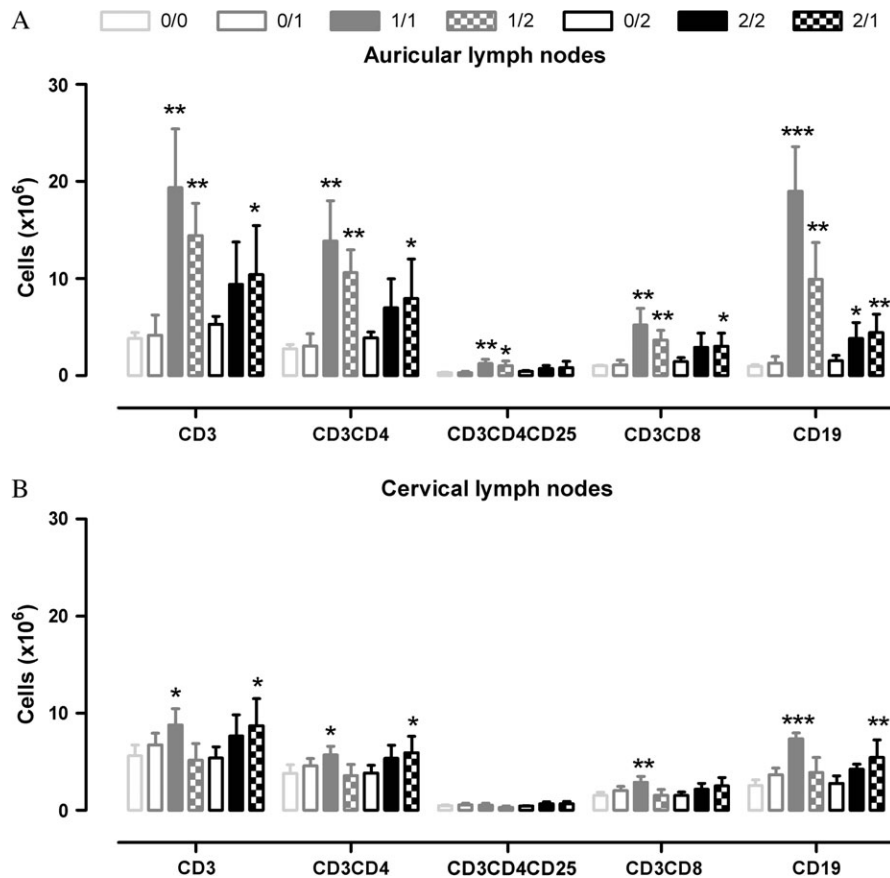


FIG. 5. Lymphocyte subpopulations in auricular and cervical lymph nodes. The lymph nodes of two to three mice were pooled. Five hundred thousand auricular lymphocytes (a) and cervical lymphocytes (b) were stained with anti-CD3⁺ (T lymphocytes), anti-CD3⁺CD4⁺ (Th-lymphocytes), anti-CD3⁺CD4⁺CD25⁺ (Treg-lymphocytes) and anti-CD3⁺CD8⁺ (Tc-lymphocytes) or were stained with a single anti-CD19⁺ (B-lymphocytes). Experimental groups are as in Figure 1. $n = 3-4$ experiments per group. Bars show mean and SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the 0/0 control group.

CD19⁺ lymphocytes (Fig. 5b). The completely TDI-treated mice also showed a significant increase in CD3⁺CD8⁺ lymphocytes (Fig. 5b).

Figures 6a and 6c show the levels of *in vitro* release of IL-4 and IFN- γ , in supernatants of auricular lymphocytes cultured for 18 h with ConA stimulation, respectively, whereas Figures 6b and 6d show the levels of *in vitro* release of IL-4 and IFN- γ , in supernatants of cervical lymphocytes cultured in the same conditions, respectively. Auricular lymphocytes of TDI-sensitized mice (1/1 and 1/2) both produced significantly increased levels of IL-4 (Fig. 6a), but only completely TDI-treated mice (1/1) showed a significantly increased level of IFN- γ (Fig. 6c). In cervical lymphocytes only TDI-sensitized and TDI-challenged mice (1/1) show an increased level of both IL-4 (Fig. 6b) and IFN- γ (Fig. 6d), compared with the lymphocytes of control mice.

Total serum IgE, IgG₁, and IgG_{2a}. Figure 7 shows the concentration of total serum IgE, IgG₁, and IgG_{2a}. TDI-sensitized mice (1/1 and 1/2) show a significant increase in total serum IgE and IgG₁, compared with the vehicle-treated

mice. TDA-treated mice did not show any difference. Nevertheless, TDA-sensitized, but TDI-challenged mice (2/1) did show a significant increase in total serum IgG₁.

DISCUSSION

The present study shows that TDA is a weak dermal sensitizer, with an EC₃ of 19% in the LLNA and that, in an experimental mouse model of chemical-induced asthma TDA does not elicit a ventilatory, nor inflammatory response. TDA application and challenge did change the composition of the auricular lymph node subpopulations slightly, but it did not result in increased cytokine production after stimulating these lymphocytes with ConA, nor did TDA application induce an enhanced production of total serum IgE. TDI on the other hand responded as previously described (Tarkowski *et al.*, 2007; Vanoirbeek *et al.*, 2004, 2008, 2009), like a typical respiratory sensitizer, with increased airway reactivity to methacholine, neutrophil inflammation in BAL, increased number of T- and B-lymphocytes and production of both Th2 and Th1 cytokines.

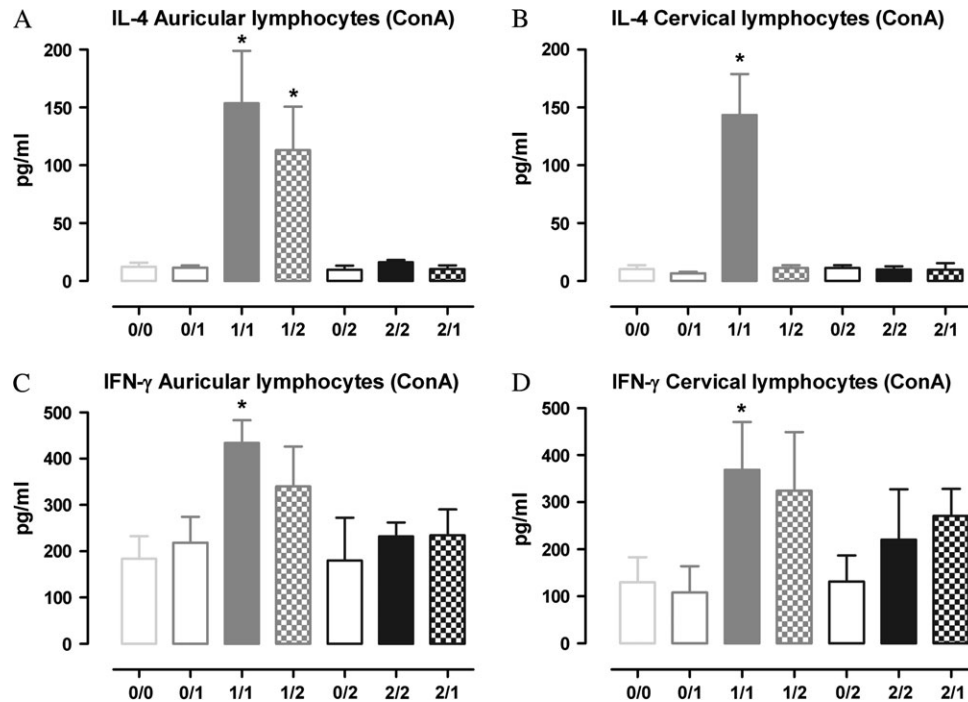


FIG. 6. IL-4 and IFN- γ secretion in auricular and cervical lymphocyte supernatant. Auricular (a and c) and cervical (b and d) lymph nodes of two to three mice were pooled, and the obtained LNCs were cultured (18 h) with ConA (2.5 μ g/ml). Levels of IL-4 (a and b) and IFN- γ (c and d) were measured in supernatant of both auricular and cervical lymphocytes. Experimental groups are as in Figure 1. $n = 3-4$ experiments per group. Bars show mean and SD. * $p < 0.05$ compared with the 0/0 control group.

When we challenged TDI-sensitized mice with TDA or vice versa, we could not find the same ventilatory, inflammatory, and immune responses as in completely TDI-treated mice.

Before discussing these findings further, it is important to clarify a semantic issue concerning dermal or respiratory sensitizers. Strictly speaking, such qualification should be given according to the route of initial sensitization. However, we (Tarkowski *et al.*, 2007; Vanoirbeek *et al.*, 2004, 2006a, 2008)—and others (Dearman and Kimber, 2001; Farraj *et al.*,

2007; Pauluhn 2008a,b; Redlich and Herrick, 2008; Sailstad *et al.*, 2003; Selgrade *et al.*, 2006)—use the term “respiratory sensitizer,” as opposed to “dermal sensitizer,” to indicate the site of the response after elicitation, rather than the route of sensitization. Experience has shown that almost all chemical sensitizers can lead to primary sensitization when they are applied to the skin (Briatico-Vangosa *et al.*, 1994). It is not known whether this also applies when sensitizers are applied to the respiratory mucosa. Although this is an important question,

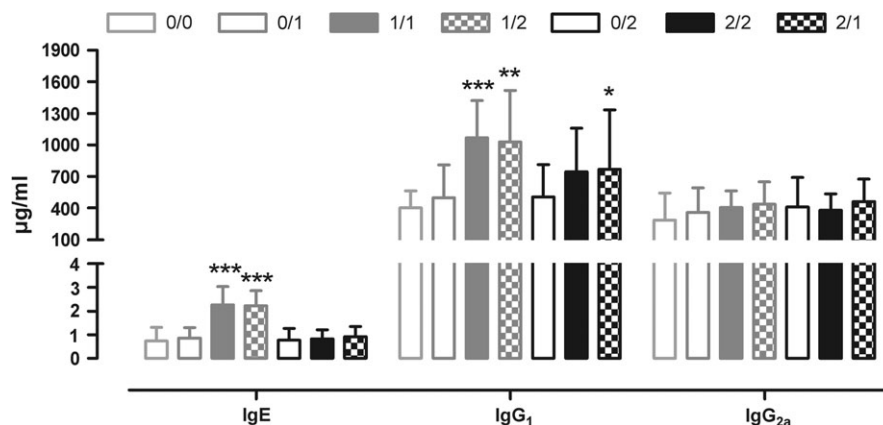


FIG. 7. Total serum immunoglobulins. Blood was collected 48 h after the intranasal challenge. Total serum IgE, IgG₁, and IgG_{2a} were measured using standard ELISA assays. Experimental groups are as in Figure 1. $n = 9-10$ mice per group. Bars show mean and SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the 0/0 control group.

this is not the issue of the present study. Admittedly, it would be more appropriate to label such chemicals as sensitizers “with potential respiratory reaction after respiratory challenge,” but this is too cumbersome. Obviously, this is not only a semantic problem, but it is also relevant in terms of prevention.

One of the purposes of this study was to investigate the potential of TDA to cause sensitization via dermal application. This information was not available from the literature, but was necessary to further study the potential of TDA to induce a respiratory effect in our model of chemical-induced asthma, and to investigate possible cross-reactivity with TDI. Kimber *et al.* (2003), proposed a classification to categorize contact allergens according to their potency as determined by their EC₃ value in the LLNA. In this approach, four categories are used. (1) Extreme skin sensitizers are defined as agents with an EC₃ value less than 0.1%, (2) strong sensitizers have an EC₃ value between 0.1 and 1%, (3) moderate sensitizers have an EC₃ value between 1 and 10%, and (4) weak sensitizers have an EC₃ value above 10% (Gerberick *et al.*, 2001). From the literature, it is known that TDI is a strong dermal sensitizer, with an EC₃ of 0.1% (van Och *et al.*, 2000). We established that TDA has an EC₃ of 19% and can therefore be classified as a weak dermal sensitizer.

The validity of the LLNA to assess the sensitizing potential of chemicals is well established. The LLNA is now an accepted hazard identification method for identifying dermal sensitizers. The method is based on the immunological events that occur during the induction phase of contact sensitization. Contact allergens are identified as a function of lymphocyte proliferative responses provoked in draining lymph nodes following open, epicutaneous application of the test material (Kimber and Weisenberger, 1989; Kimber *et al.*, 1986), with the degree of lymphocyte proliferation correlating closely with the skin sensitizing potency of the chemical (Kimber and Dearman, 1991). In the present experiment we followed the recommendations for performing the murine LLNA, but instead of using CBA/Ca mice as described in OECD guideline 429, we used BALB/c mice. We did this to use the same strain as in our previous studies on respiratory responses after dermal sensitization and intranasal challenge. It has been shown that the BALB/c strain is also responsive to the LLNA (van Och *et al.*, 2000).

In our model of chemical-induced asthma, the response to TDA proved to be entirely negative with regard to functional endpoints (early response after intranasal challenge and AHR) and inflammatory endpoints (BAL cell count). On the other hand, the lymph node subpopulations of TDA-sensitized mice showed substantial increases in T- and B-lymphocytes, but unlike in TDI-sensitized mice these lymphocytes were unable to secrete IL-4 or IFN- γ after *in vitro* stimulation with ConA. Furthermore, TDA did not induce an increase in total serum IgE. Taken together, these data indicate that dermally applied TDA is capable of eliciting an immune response but that it is

unable to elicit a phenotype typical for asthma in mice after a single intranasal challenge. We do not exclude that under other exposure scenarios (multiple challenges) TDA could yield ventilatory and inflammatory responses and act as a respiratory sensitizer. TDI on the other hand demonstrates its sensitizing potency, as shown before (Tarkowski *et al.*, 2007; Vanoirbeek *et al.*, 2006b, 2008). This is a further validation that this test is able discriminate between inducers and noninducers of an asthma phenotype in mice. Previously, this has already been proved in a validation study using trimellitic anhydride and dinitrochlorobenzene (Vanoirbeek *et al.*, 2006a). Dermal sensitization with TDI leads to increased numbers of Th-, Treg-, Tc-, and B-lymphocytes in auricular lymph nodes, resulting in increased *in vitro* production IL-4 and IFN- γ by these T lymphocytes, along with increased total serum IgE levels. When TDI-sensitized mice were challenged with TDI, they also showed the same changes in cervical lymph nodes. But if TDI-sensitized mice were challenged with TDA, we did not find any respiratory, inflammatory nor any change of subpopulation in the cervical lymph nodes, and cervical lymphocytes also did not produce IL-4 and IFN- γ when stimulated *in vitro* with ConA. On the other hand, TDA-sensitized mice, which were challenged with TDI, also did not induce respiratory and inflammatory changes, except for some minor increases in lymphocyte subpopulation of auricular and cervical lymph nodes. These changes are likely due to the weak dermal sensitization caused by TDA. Unfortunately, we did not attempt to identify antigen-specific responses by testing TDI- or TDA-protein conjugates *in vitro*, which further assesses possible cross-reactivity between TDI and TDA.

Knowledge on TDA is very limited, not only with regard to human exposure, but also with regard to *in vitro* and *in vivo* laboratory experiments. Skarping *et al.* (1991) investigated TDI exposure in human volunteers by measurement of TDA in urine. At that time, the authors concluded that the analysis of TDA in urine was an appropriate method for biological monitoring of exposure to TDI. Sakai *et al.* (2002) optimized the analysis of urinary metabolites of TDA after exposure to TDI. A recent paper, Austin (2007) confirms that urinary TDA measurement is a useful indication of the contribution of skin absorption to total TDI exposure, thereby suggesting that skin protection when handling uncured polyurethane foam may not receive sufficient consideration (Austin, 2007). This was also the key message of Bello *et al.* and Redlich *et al.*, which both suggested that skin exposure probably has a greater influence on the development of certain occupational lung diseases than previously expected (Bello *et al.*, 2007; Redlich and Herrick, 2008). Pons *et al.* (1999) investigated the effect of TDA on the viability and growth of human lung fibroblast cells *in vitro*. Their results show that both TDI and 2,4-TDA can contribute to damage of the lung epithelium, which is associated with TDI-induced asthma, whereas 2,6-TDA might either trigger epithelial damage or induce cell proliferation that could contribute to subepithelial fibrosis. We could not confirm this

in our model, because histological analysis (data not shown) did not reveal any indication of epithelium damage in our completely TDA-treated mice.

In conclusion, we can classify TDA on the basis of the EC₃ value as a (very) weak dermal sensitizer. Furthermore, we can conclude that TDA does not elicit airway, inflammatory, nor immunological responses in our experimental mouse model of chemical-induced asthma in the concentrations we used. Also, we could not find any cross-reactivity between TDI and TDA. This suggests that the isocyanate groups ($-N = C = O$) on TDI play a crucial role (for binding to endogenous proteins in the skin and lung) to cause dermal sensitization and, subsequently, an asthma like response upon intranasal challenge. This is in contrast to the more stable amine groups ($-NH_2$) on TDA, which have less potency to bind to proteins and elicit allergic responses.

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