

Respiratory Response to Toluene Diisocyanate Depends on Prior Frequency and Concentration of Dermal Sensitization in Mice

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Received on January 21, 2004; accepted on April 20, 2004

Occupational asthma is the principal cause of work-related respiratory disease in the industrial world. In the absence of satisfactory models for predicting the potential of low molecular weight chemicals to cause asthma, we verified that dermal sensitization prior to intranasal challenge influences the respiratory response using toluene diisocyanate (TDI), a known respiratory sensitizer. BALB/c mice received TDI or vehicle (acetone/olive oil) on each ear on three consecutive days (days 1, 2, and 3; 0.3 or 3% TDI) or only once (day 1, 1% TDI). On day 7, the mice received similar dermal applications of vehicle or the same concentration of TDI as before ("boost"). On day 10, they received an intranasal dose of TDI (0.1%) or vehicle. Ventilatory function was monitored by whole body plethysmography for 40 min after intranasal application, and reactivity to inhaled methacholine was assessed 24 h later. Pulmonary inflammation was assessed by bronchoalveolar lavage and histology. Mice that received an intranasal dose of TDI without having received a prior dermal application of TDI did not exhibit any ventilatory response or inflammatory changes compared to vehicle controls. In contrast, mice that had received prior application(s) of TDI, even if only on day 7, exhibited the following: ventilatory responses, compatible with bronchoconstriction, immediately after intranasal application with TDI; enhanced methacholine responsiveness 24 h later; and pulmonary inflammation characterized by neutrophils. This was, however, not the case in mice that received the highest dermal amount of TDI (3% on days 1, 2, and 3). These findings suggest that respiratory response to TDI depends on prior frequency and concentration of dermal sensitization in mice.

Key Words: occupational asthma; animal model; diisocyanates; BALB/c mice; dermal sensitization; whole body plethysmography; BALF.

Occupational asthma is the principal cause of work-related respiratory disease in the industrial world (Chan-Yeung and Malo, 1994; Tarlo, 2000). It may be caused by exposure to low molecular weight agents such as diisocyanates, acid

anhydrides, reactive dyes, and many other chemicals (Agius, 2000; Bernstein *et al.*, 1999). The pathogenesis of chemical-induced asthma is less clear than that of classical (IgE-mediated) allergen-induced asthma (Busse and Rosenwasser, 2003; Holt *et al.*, 1999). Animal models are needed not only to understand how chemicals may induce asthma, but also to improve our ability to identify and predict which (new) chemicals pose a risk of asthma in exposed workers. A good animal model of chemical-induced asthma would be one in which an inhalation exposure to the offending agent results in airway narrowing, bronchial inflammation, and nonspecific airway hyperresponsiveness in exposed, sensitized animals (Busse and Lemanske, 2001; Maddox and Schwartz, 2002).

Toluene diisocyanate (TDI) is a chemical that is widely used in industry and is a well-known cause of occupational asthma (Bernstein *et al.*, 1999). Scheerens *et al.* (1996 and 1999) succeeded in inducing tracheal hyperreactivity and inflammation using protocols involving dermal application and subsequent intranasal administration of TDI. However, in these experiments, some respiratory responses seemed irritant in nature since they also occurred in nonsensitized animals. Matheson *et al.* (2001 and 2002) used TDI and reported mild inflammation in the trachea but not in the lung. Herrick *et al.* (2002) succeeded in creating a mouse model for occupational asthma using HDI (hexamethylene diisocyanate), in which the sensitized mice showed a strong inflammatory response, with eosinophils, in the lung and airways.

We have shown that an intranasal administration of TDI led to methacholine hyperresponsiveness only in mice that had been previously dermally sensitized with TDI (Vanoirbeek *et al.*, 2003). Our dermal sensitization protocol was based on the local lymph node assay (LLNA), which has been extensively used and validated, to predict the ability of chemicals to cause dermal sensitization and, hence, allergic contact dermatitis (Kimber and Weisenberger, 1989; Kimber *et al.*, 1986). Although the LLNA was not designed to identify respiratory sensitizers, it seemed reasonable to expect that administration via the airways of a chemical to which the animal had been sensitized using a well-validated method would elicit a

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respiratory response (Arts *et al.*, 1998; Pauluhn *et al.*, 2002a,b; Scheerens *et al.*, 1999). However, our initial experiments yielded an incomplete “asthma phenotype,” because there was neither detectable bronchial obstruction nor airway inflammation in the sensitized and challenged animals. In the present study, we have refined our experimental protocol, mainly with regard to the dose-response relationships of dermal sensitization, and this has enabled us to obtain a more satisfactory mouse model of TDI-induced asthma.

MATERIALS AND METHODS

Chemicals. Toluene-2,4-diisocyanate (98%; Fluka, CAS 584-84-9) was obtained from Aldrich Chemical Co. (Milwaukee, WI). The vehicle (AOO) used to dissolve the TDI consisted of a mixture of 2 volumes of acetone (VWR, Louvain, Belgium) and 3 volumes of olive oil (extra virgin, Carbonell, Spain). Concentrations of TDI are given as percentages (v/v).

Animals. Male BALB/c mice (approximately 20 g and 6 to 7 weeks old) were obtained from Harlan (Horst, Netherlands). They were kept in a conventional animal house with 12 h dark/light cycles. They 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.

Experimental protocols. The mice received dermal applications of 20 μ l of vehicle or 20 μ l TDI on each ear, either on three consecutive days (days 1, 2, and 3; 0.3 or 3% TDI) or only once (day 1, 1% TDI). On day 7, they received similar dermal application of either vehicle or TDI (in the same concentration as for the initial application) on both ears (20 μ l per ear). On day 10, they received, under light anesthesia using diethyl ether, intranasal instillations of 10 μ l of vehicle or 10 μ l TDI (0.1%) in each nostril.

In all experiments, treatment with TDI is coded as 1 and treatment with vehicle is coded as 0. Thus, the 1/1/1 group consists of mice that were dermally sensitized, dermally boosted, and intranasally challenged with TDI, while the 0/0/0 control group consists of mice that received the AOO vehicle on all occasions. Initially, all other possible combinations were included, i.e., 1/1/1, 1/1/0, 1/0/1, 1/0/0, 0/1/1, 0/1/0, 0/0/1, and 1/1/1. Later, only the four most relevant groups were studied, i.e., 1/1/1, 1/0/1, 0/1/1, and 0/0/0. Although not all animals of an experiment could be studied on the same day, there were always animals from all treatment groups included on each study day. In a separate experiment to evaluate the effect of the vehicle, control mice remained untreated (n) or they received three daily applications of 20 μ l on each ear of pure acetone (A) or pure olive oil (OO). On day 7, mice received a dermal application with 0.3% TDI or AOO vehicle and, on day 10, they received an intranasal instillation of 0.1% TDI or AOO vehicle.

Ventilatory function measurements: early phase response. Before the intranasal instillation, the ventilatory function of each mouse was recorded, in resting condition, for 10 min in a whole body plethysmograph (EMKA Technologies, Paris, France). After the intranasal instillation, mice were placed in the whole body plethysmograph (time point 0 min) and their ventilatory function was followed for 40 min. Every 30 s, the mean of all ventilatory parameters was recorded. Ventilatory function, expressed as enhanced pause (Penh; Hamelmann *et al.*, 1997), was plotted against time, and the area under the curve (AUC) was calculated for each mouse.

Measurement of airway responsiveness to methacholine. On day 11, 24 h after the intranasal instillation, reactivity to methacholine was assessed in the whole body plethysmograph according to the procedure of Hamelmann *et al.* (1997). Briefly, the Penh was calculated for each mouse under resting conditions (baseline) and after nebulizing incremental doses of methacholine (0, 10, 25, 50, and 100 mg/ml) aerosolized for 1 min. An average Penh over 30 s was calculated during 3 min (six measurements). The mean of these six values was used for each

condition. For each mouse, Penh was plotted against methacholine concentration (from 0 to 100 mg/ml) and the AUC was calculated.

Autopsy-bronchoalveolar lavage (BAL)-histology. After the methacholine challenge (day 11), mice were deeply anesthetized by intraperitoneal injection of pentobarbital (90 mg/kg) and sacrificed. A blood sample was drawn from the inferior vena cava. The left bronchus was clamped and the lung was removed from the thorax, weighed (wet weight), dried (for 24 h at 75–85°C), and weighed again (dry weight). The right lung was lavaged three times with 0.4 ml sterile saline (0.9% NaCl) at room temperature. The recovered fluid was pooled and centrifuged (1500 \times g for 10 min). For differential cell counts, 250 μ l (100,000 cells/ml) of the resuspended cells were spun (1400 \times g for 6 min; Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried, and stained (Diff-Quik method), and 3 \times 100 cells were identified as macrophages, eosinophils, neutrophils, or lymphocytes.

After the lavage, the right lung was instilled with 10% formaldehyde until full inflation of all lobes, as judged visually. An experienced pathologist, who evaluated lung injury without knowledge of the animals' treatment, examined slices (5 μ m sections) from all lung lobes.

Mouse total IgE. The OptEIA mouse IgE set from Pharmingen (San Diego, CA) was used to measure total serum IgE (diluted 1/70). Measurements were performed according to the manufacturer's instructions, with the use of anti-mouse IgE, biotinylated anti-mouse IgE, and horseradish peroxidase conjugate.

Data analysis. The mice from the 0/0/0 groups of the 3-day dermal application experiments [3% ($n = 4$) and 0.3% ($n = 6$) TDI] were pooled ($n = 10$) for statistical analysis. All data were analyzed using the nonparametric Kruskal-Wallis test (GraphPad Prism 3.01). A level of $p < 0.05$ was considered significant. Nonparametric Spearman correlation test and linear curve fitting were performed using SPSS 9.0 to describe correlations among early phase response, response to methacholine challenge, neutrophil influx in BAL, and total serum IgE in the different groups exhibiting a response (1/1/1, 1/0/1, and 0/1/1).

RESULTS

The early ventilatory responses immediately after nasal instillation are presented in Figure 1, where the left panels show the average values of Penh as a function of time, 10 min before (baseline) and 40 min after intranasal instillation, and the right panels show the individual and group mean values for the AUC of Penh against time after intranasal instillation. The results of the methacholine tests, done 1 day later, are presented in Figure 2, with average responses to methacholine shown in the left panels and the individual and group mean values of the AUC of Penh against methacholine concentration in the right panels. Figure 3 presents the results of the cellular composition in BAL.

Dermal Application of 3% TDI on Days 1, 2, and 3

In this protocol, mice received dermal applications of 3% TDI (or vehicle) on days 1, 2, and 3, followed by a similar application of 3% TDI (or vehicle) on day 7 and then intranasal instillation with 0.1% TDI (or vehicle) on day 10.

None of the animals that received intranasal instillations of the vehicle (groups 0/0/0, 0/1/0, 1/0/0, and 1/1/0) showed changes in Penh over the course of the following 40 min (Fig. 1A). The response of the animals that received instillations of 0.1% TDI without any prior dermal application of TDI did not differ from the response of those that received vehicle intranasally. Of the

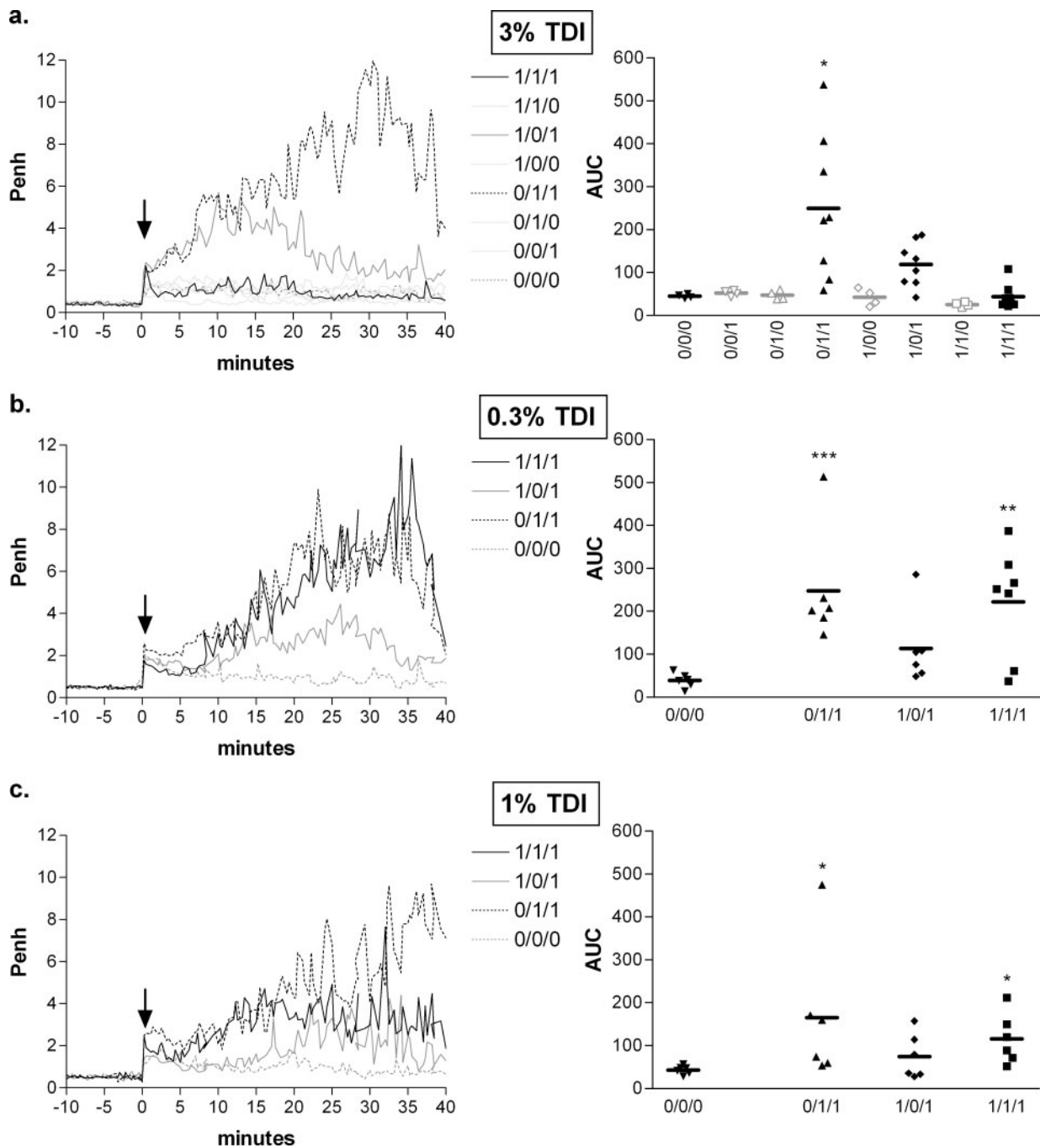


FIG. 1. Early ventilatory response to intranasal administration of TDI in mice having undergone three types of pretreatment. Ventilation, expressed as enhanced pause (Penh), was measured by whole body plethysmography before and after the intranasal instillation of vehicle or 0.1% TDI. Left panels show mean values of Penh measured during 10 min before and 40 min after instillation (arrow); right panels show individual values of the area under the curve (AUC) of Penh against time between 0 and 40 min. Experimental groups are identified by three numbers (e.g., 0/0/1), where 0 and 1 represent administration of vehicle (acetone:olive oil) and TDI, respectively; the first number identifies the agent used in the initial dermal applications, the second number identifies the agent applied dermally on day 7, and the third number identifies the agent instilled intranasally on day 10. The three pretreatment protocols are as follows: (A) dermal applications of vehicle or 3% TDI on days 1, 2, 3, and 7 ($n = 4-8$ per group); (B) dermal applications of vehicle or 0.3% TDI on days 1, 2, 3, and 7 ($n = 6-7$ per group); and (C) dermal applications of vehicle or 1% TDI on days 1 and 7 ($n = 6$ per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0/0/0 control.

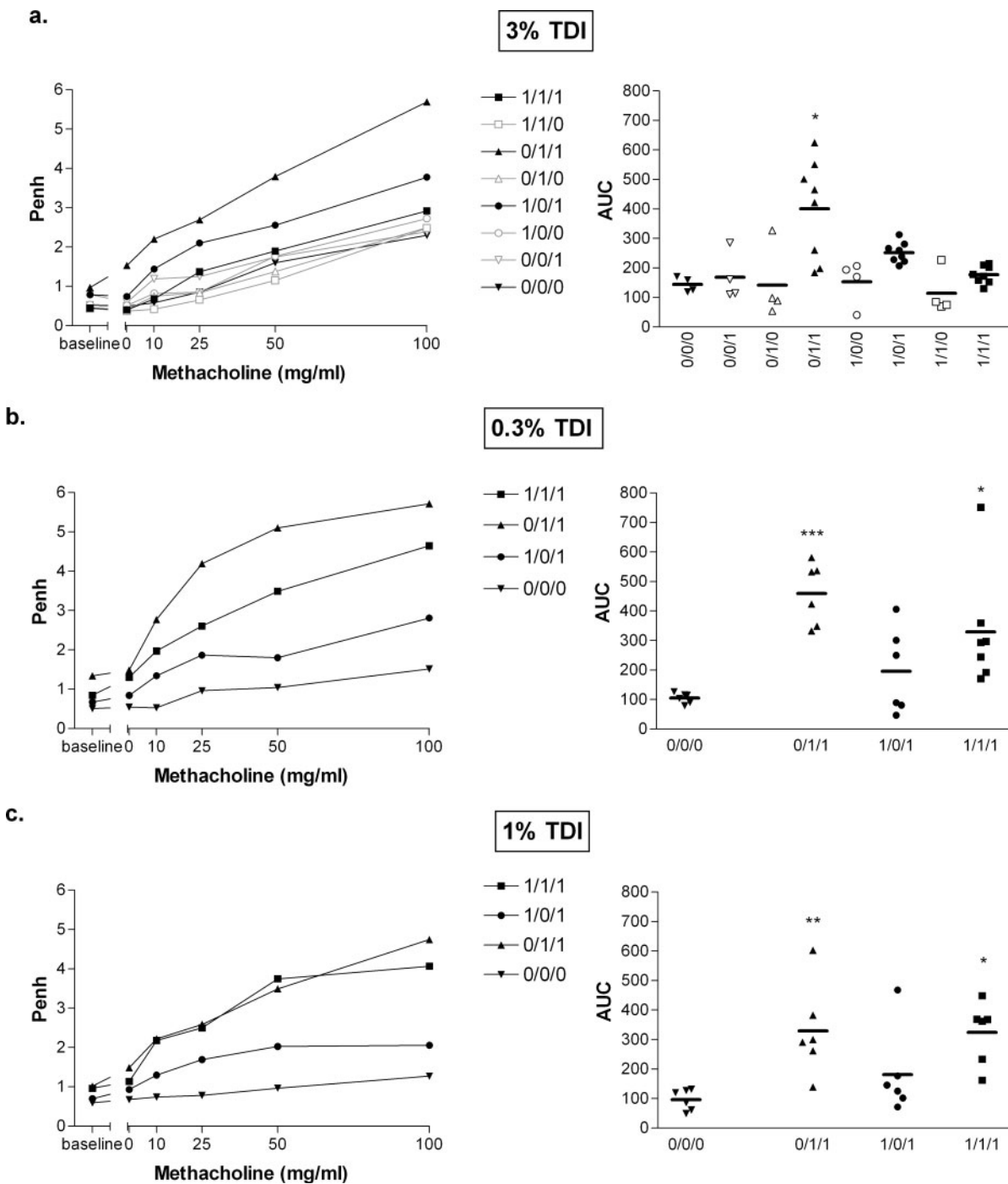


FIG. 2. Methacholine responsiveness 1 day after intranasal administration of TDI in mice having undergone three types of pretreatment. Ventilation, expressed as Penh, was measured by whole body plethysmography during exposure for 1 min to increasing concentrations of methacholine. Left panels show mean values of Penh; right panels show individual values of the AUC of Penh against methacholine concentrations between 0 and 100 mg/ml. These data were obtained from the same mice as those in Figure 1. Experimental group legends are the same as in Figure 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0/0/0 control.

other groups receiving TDI intranasally, only the 0/1/1 and 1/0/1 groups showed a 5.5-fold ($p < 0.05$) and 3-fold ($p > 0.05$) increase in Penh, respectively, whereas Penh did not change in the 1/1/1 group compared to the 0/0/0 and 0/0/1 controls.

There were no significant differences in methacholine responsiveness, when measured 1 day after intranasal instillation, among the four groups that had received vehicle (Fig. 2A). Among those that had received TDI, only the 0/1/1 group

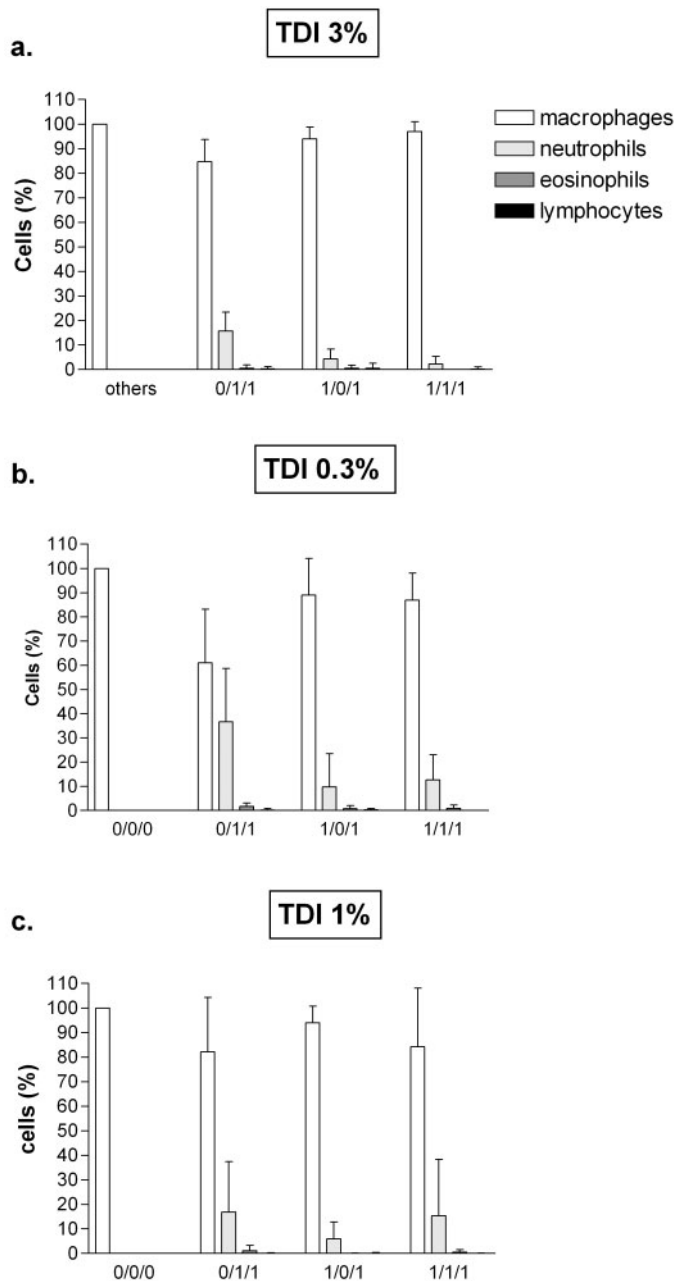


FIG. 3. Differential cell counts in BAL 1 day after intranasal administration of TDI in mice having undergone three types of pretreatment. Cells were obtained by bronchoalveolar lavage of the right lung and identified on cytopins as macrophages, neutrophils, lymphocytes, or eosinophils. These data were obtained from the same mice as those in Figures 1 and 2. Experimental group legends are the same as in Figure 1.

showed a significantly higher (3.5-fold) reactivity compared to the control group (0/0/0). The mice of the 1/0/1 and 1/1/1 groups showed nonsignificant 2- and 1.5-fold increases, respectively.

Only macrophages were found in the BAL of groups 0/0/0, 0/0/1, 1/1/0, 1/0/0, and 0/1/0 (Fig. 3A). However, there were increased numbers of neutrophils (15%) and eosinophils

TABLE 1
Total Serum IgE

Groups	3% TDI	0.3% TDI	1% TDI	AOO
0/0/0	2.27 ± 0.12	2.48 ± 0.13	2.06 ± 0.23	2.36 ± 0.27
0/0/1	2.29 ± 0.09			
0/1/0	2.20 ± 0.30			
0/1/1	2.18 ± 0.20	2.63 ± 0.26	2.03 ± 0.19	2.31 ± 0.38
1/0/0	3.38 ± 0.14***			
1/0/1	3.32 ± 0.28***	3.13 ± 0.14***	2.43 ± 0.24*	
1/1/0	3.47 ± 0.44***			
1/1/1	3.80 ± 0.09***	3.60 ± 0.08***	3.57 ± 0.26***	
A/1/1				2.31 ± 0.37
OO/1/1				2.42 ± 0.24
n/1/1				2.30 ± 0.35

Total serum IgE is expressed in log ng/ml. TDI, toluene diisocyanate; AOO, acetone/olive oil (2:3); A, acetone; OO, olive oil; n, no treatment. Experimental groups are identified by three numbers (e.g., 0/0/1); 0 stands for treatment with the vehicle (AOO) and 1 stands for treatment with TDI (in AOO). The first number identifies the agent used in the initial dermal applications, the second number identifies the agent applied dermally on day 7, and the third number identifies the agent instilled intranasally on day 10. Empty cells indicate that the test group was not included in the experiment.

* $p < 0.05$, *** $p < 0.001$.

(1%) in the BAL of the 0/1/1 group and increased numbers of neutrophils in the 1/0/1 and 1/1/1 groups (4.5 and 2.5%, respectively).

Concentrations of total serum IgE were significantly increased, compared to the 0/0/0 controls, in the four groups that had received dermal applications of TDI on days 1, 2, and 3 (1/1/1, 1/1/0, 1/0/1, and 1/0/0) and were not changed in those that had not received these three applications (0/1/1, 0/1/0, 0/0/1; Table 1).

Dry and wet left lung weights did not differ among treatment groups (data not shown). Histology of the lungs revealed a minor influx of eosinophils around the blood vessels of the 0/1/1 and 1/0/1 groups (Fig. 4). In the other groups (1/1/1, 1/1/0, 1/0/0, 0/1/0, 0/0/1, and 0/0/0), no histological changes were found.

Dermal Applications of 0.3% TDI on Days 1, 2, and 3

This protocol was identical to the previous one, except that the concentration of TDI was 10-fold lower for all skin applications (i.e., on days 1, 2, 3, and 7). Based on the findings of the previous experiment, we limited the test and control groups to the four most relevant groups (i.e., 1/1/1, 1/0/1, 0/1/1, and 0/0/0).

Directly after the intranasal administration of TDI, both the 1/1/1 and the 0/1/1 groups exhibited increased values of Penh (Fig. 1B). The mean AUCs for these two groups were significantly increased (6-fold) compared to the control group 0/0/0. The 1/0/1 group also showed an increased ventilatory response after intranasal administration of TDI; although the mean AUC was increased 3-fold, this was not significantly different from the 0/0/0 control.

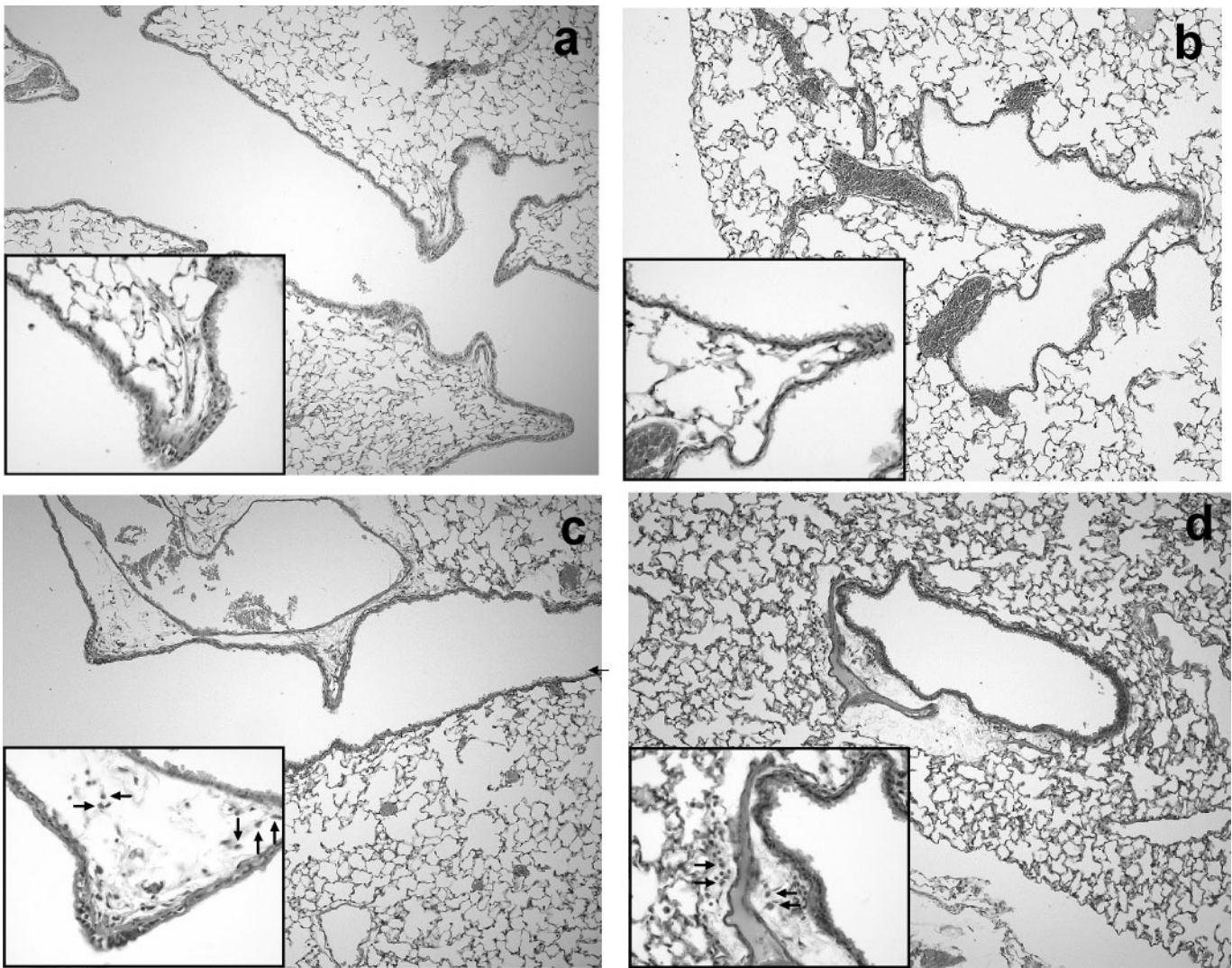


FIG. 4. Histopathology of the lung 1 day after intranasal administration of TDI in mice; (A) 0/0/0, (B) 0/0/1, (C) 0/1/1 (dermal treatment with 3% TDI), and (D) 1/1/1 (dermal treatment with 0.3% TDI). Experimental group legends are the same as in Figure 1. The alveoli, airways, and vessels have a normal appearance in all animals, but there is (C and D) discrete eosinophil infiltration (arrows) in perivascular spaces of vessels accompanying airways. Histological assessment was done without knowledge of the animal treatment. Hematoxylin-eosin stain. Original magnification is $\times 100$; original magnification of insets is $\times 400$.

Figure 2B shows the increased responsiveness to methacholine in groups 0/1/1, 1/1/1, and 1/0/1. The mean AUCs of the 0/1/1 and 1/1/1 groups are both significantly increased, by 4-fold and 3-fold, respectively, compared to the 0/0/0 group. The mean AUC of the 1/0/1 group is also increased 2-fold, but this was not statistically different from the 0/0/0 control.

In the BAL of the 0/1/1 group, 36% neutrophils and 1% eosinophils were found (Fig. 3B). A less pronounced influx of neutrophils was found in the 1/1/1 (13%) and 1/0/1 (10%) groups. Wet and dry left lung weights, taken as indices of lung injury, were significantly increased in mice from the 0/1/1 group [228 ± 20 mg/100 g body weight (bw) and 55 ± 2 mg/100 g bw, respectively] compared to the 0/0/0 control (194 ± 14 mg/100 g bw and 47 ± 4 mg/100 g bw, respectively); but, the wet-to-dry weight ratios did not differ. The wet left lung weight

of the 1/1/1 group (218 ± 14 mg/100 g bw) was also significantly increased compared to the 0/0/0 control group.

Total IgE in the serum was significantly increased in the 1/1/1 and 1/0/1 groups compared with the 0/0/0 group (Table 1).

Histological evaluation showed a minor influx of eosinophils around the blood vessels only in the lungs of mice from the 0/1/1 and the 1/1/1 groups (Fig. 4).

Dermal Application of 1% TDI on Day 1

In this protocol, instead of three successive dermal applications on days 1, 2, and 3, only one dermal application was given on day 1. The concentration of TDI applied on the skin on day 1 or on day 7 was 1%. These experiments were also limited to the four most relevant groups, (i.e., 1/1/1, 1/0/1, 0/1/1, and 0/0/0).

On day 10, increased Penh values (Fig. 1C) were observed immediately after intranasal administration of TDI in the 0/1/1, 1/1/1, and 1/0/1 groups. The 0/1/1 and 1/1/1 groups had a 3.5-fold significantly increased mean AUC of Penh compared to the 0/0/0 control, whereas the 1/0/1 group had a nonsignificant 2-fold increase compared to the control.

All three groups receiving intranasal TDI (0/1/1, 1/0/1, and 1/1/1) had an increased responsiveness to methacholine (Fig. 2C). Of these, only the 0/1/1 and 1/1/1 groups showed a significantly increased mean AUC (6-fold and 5.5-fold, respectively) compared to the control mice.

The BAL of the 0/0/0 group only contained macrophages (>99%). The BAL of both the 0/1/1 and 1/1/1 groups contained increased percentages of neutrophils (17 and 15%, respectively; Fig. 3C). The 1/0/1 group only showed a minor influx of neutrophils (5%).

Total serum IgE was significantly increased in the 1/1/1 and 1/0/1 groups compared to the 0/0/0 control (Table 1).

No changes, in comparison to the control group, were found in dry and wet left lung weights. Histologic evaluation of the lungs revealed no changes among the groups (not shown).

Consistency Among the Various End Points Measured

Because each animal provided more than one physiological end point, it was possible to look for correlations, at the individual mouse level, among the magnitude of the early ventilatory response, the magnitude of the methacholine responsiveness, the degree of neutrophil influx, and total serum IgE (Table 2). Only

the mice from the groups showing some response (i.e., 0/1/1, 1/0/1, and 1/1/1) were included in these calculations, because the inclusion of the groups showing no changes would have yielded significant but rather meaningless correlations. Table 2 shows that there were significant mutual correlations among the early ventilatory response, the methacholine responsiveness, and the neutrophil influx in BAL. In other words, mice showing pronounced early responses on day 10 were also those having high methacholine responsiveness and high neutrophil inflammation on day 11. None of these parameters were correlated with total IgE in serum.

The Effect of the Vehicle

The vehicle used to dissolve TDI was composed of acetone:olive oil (2:3). As described above, intranasal instillation of this vehicle did not cause ventilatory reactions. However, because of the surprising but consistent results observed in animals from group 0/1/1, we were concerned that the vehicle might itself affect or even cause sensitization. So, we repeated the experiment concerning group 0/1/1 (using 0.3% TDI on day 7) and included three more groups given applications of only acetone (A) or only olive oil (OO) on days 1, 2, and 3, or three sham applications (n).

Directly after the intranasal administration of TDI, the 0/1/1, A/1/1, and the n/1/1 groups exhibited increased Penh values (Fig. 5A). The mean AUCs for these three groups were significantly increased (7-, 5.5-, and 3.5-fold, respectively) compared to the control group 0/0/0. The OO/1/1 group also showed an increased ventilatory response after intranasal administration of TDI; although the mean AUC was increased 2.5-fold, this was not significantly different from the 0/0/0 control. These results were similar for the methacholine challenge (Fig. 5B). In the BAL of the 0/1/1 and A/1/1 groups, 20 and 15.5% of neutrophils, respectively, were found (Fig. 5C). A less pronounced influx of neutrophils was found in the n/1/1 and OO/1/1 groups (6 and 4%, respectively). Total IgE in the serum did not change in any group compared with the 0/0/0 group (Table 1).

DISCUSSION

We have developed a murine model that reproduces several important features of chemical-induced asthma in humans, namely the occurrence of bronchoconstriction, nonspecific airway hyperresponsiveness, and bronchial inflammation following exposure to an agent to which the subject has been previously sensitized. Because all end points were measured in each animal, we could show that there was a remarkable consistency, within individual animals, between the ventilatory response observed in the first 40 min after challenge and methacholine responsiveness on the next day, as well as between these functional parameters and inflammatory changes in the lungs (Table 2). While these associations of different end points were convincing,

TABLE 2
Nonparametric Spearman Correlations for Data from Mice of Groups 1/1/1, 1/0/1, and 0/1/1

	Early phase	Methacholine	Neutrophils	Total IgE
3% TDI (n = 12)				
Early phase	1.00	0.60*	0.69*	-0.25
Methacholine		1.00	0.84**	0.06
Neutrophils			1.00	-0.12
Total IgE				1.00
0.3% TDI (n = 19)				
Early phase	1.00	0.73**	0.64**	-0.36
Methacholine		1.00	0.77**	-0.40
Neutrophils			1.00	-0.38
Total IgE				1.00
1% TDI (n = 18)				
Early phase	1.00	0.72**	0.70**	0.08
Methacholine		1.00	0.74**	0.07
Neutrophils			1.00	-0.09
Total IgE				1.00

Early phase, AUC of Penh against time; methacholine, AUC of Penh against methacholine concentration; neutrophils, percentage of neutrophils in bronchoalveolar lavage fluid; total IgE, total serum IgE.

* $p < 0.05$ (two-tailed), ** $p < 0.01$ level (two-tailed).

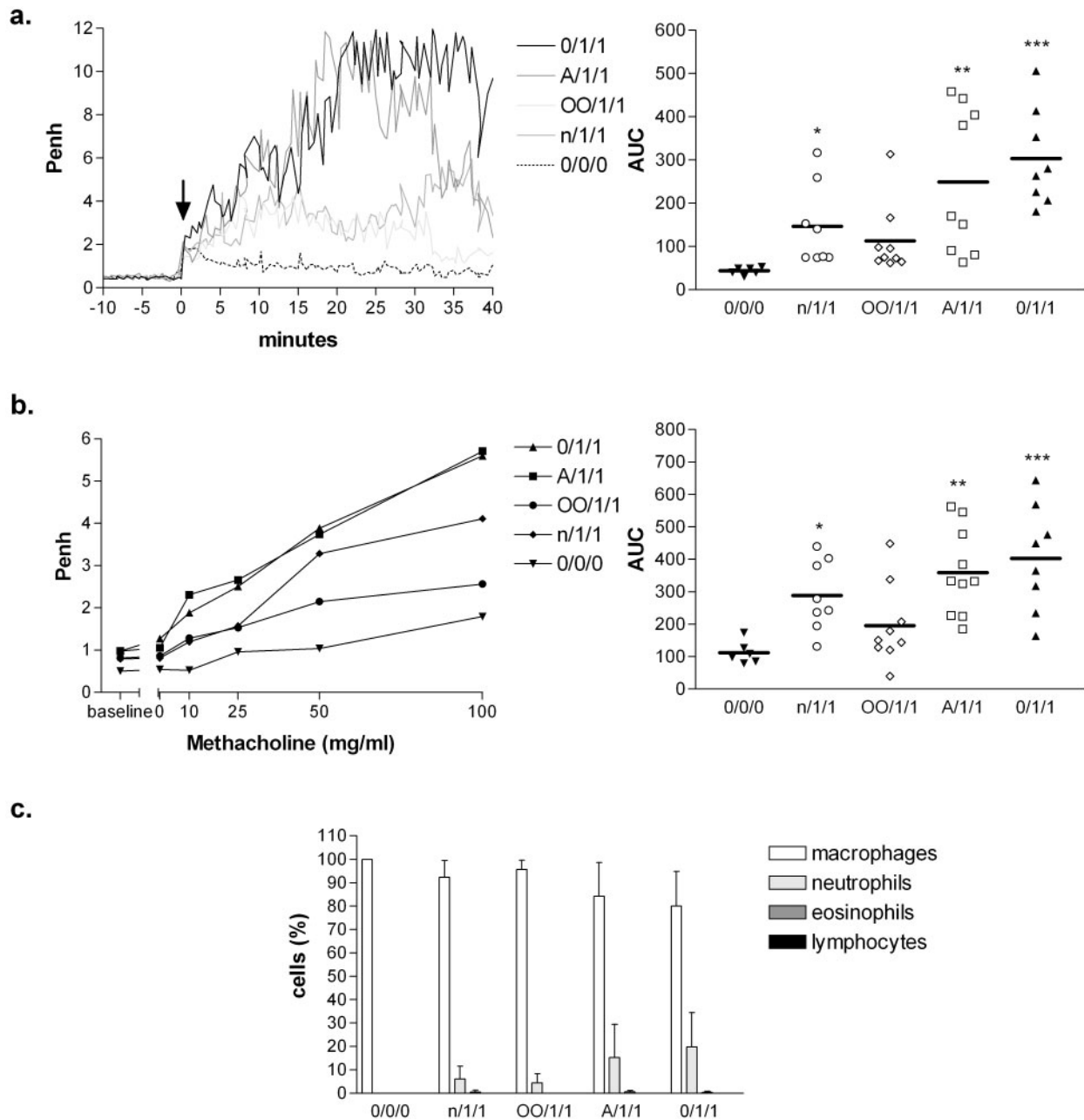


FIG. 5. The effect of the dermal application of various vehicles on the response to intranasal administration of TDI in mice. On days 1, 2, and 3, mice received dermal applications, on each ear, of 20 μ l acetone (A), olive oil (OO), or acetone:olive oil (AOO, indicated as 0), or they remained untreated (n). Mice received dermal applications, on each ear, of 20 μ l of AOO or 0.3% TDI (in AOO) on day 7 and intranasal instillations, in each nostril, of 10 μ l of AOO or 0.1% TDI (in AOO) on day 10. The (A) early ventilatory response, (B) methacholine responsiveness, and (C) BAL cell distribution were assessed as in Figures 1, 2, and 3, respectively ($n = 6-9$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0/0/0 control.

this does not necessarily imply that the different phenomena were causally related, i.e., the neutrophil influx does not necessarily cause the bronchial hyperresponsiveness.

We are confident that the pulmonary reactions were not simply due to an irritant effect of the intranasal administration of 0.1% TDI, because this treatment did not lead to ventilatory changes or

airway inflammation in animals that had not been previously exposed to TDI (group 0/0/1). Consequently, our results demonstrate that respiratory responses to TDI only occurred if a previous dermal contact with TDI had taken place. However, our results also show that the frequency and concentration of dermal sensitization to TDI affects the subsequent response to intranasal

exposure to TDI in a complex way. Before addressing these relationships between dermal sensitization and respiratory reactions, we must acknowledge that our model of chemical-induced asthma is not yet fully accomplished.

Limitations of the Model

Firstly, we did not actually measure bronchoconstriction, but we assessed changes in ventilation by unrestrained whole body plethysmography. This technique, and particularly the use of Penh as an index of airway resistance, has been heavily criticized (Bates and Irvin, 2003; Lundblad *et al.*, 2002). While changes in Penh do not accurately reflect changes in airway caliber, there is little doubt that increases in Penh do reflect alterations in ventilatory pattern that are compatible with airway obstruction. Unrestrained whole body plethysmography has been validated for evaluating methacholine responsiveness in mice (Hamelmann *et al.*, 1997) and it has been widely used in experimental asthma research (Klink and Meade, 2003). Regardless of the validity of Penh as a measure of airway resistance, it is valid to state, thanks to our inclusion of appropriate control groups, that early ventilatory responses and increases in methacholine responsiveness occurred after TDI administration only in animals that had been previously sensitized. We infer that these ventilatory changes are of an obstructive character, but the site of the obstruction remains unknown.

Secondly, our mode of administration of TDI by intranasal instillation may be criticized as being nonphysiological. Intranasal administration has been shown to be effective to administer agents to the lower respiratory tract in mice (Southam *et al.*, 2002). Intranasal application has the advantage of speed and convenience over intratracheal instillation, which requires a deeper anesthesia, and over inhalation exposure, which is more complex to perform. The substantial changes found in the BAL of some groups (sometimes even with increases in lung weights) also suggest that the site of the reactions was not confined to the upper airways. Nevertheless, we plan to verify the practical relevance of our model by exposing the animals by inhalation.

Thirdly, the cellular composition of the BAL in our model was not entirely typical of an immunologically mediated asthmatic reaction, since there were no increases in lymphocytes and mainly an influx of neutrophils, which was sometimes accompanied by small increases in eosinophils in the most-responding animals. Histology also revealed only some perivascular infiltrate by eosinophils. The predominantly neutrophilic pattern of inflammation with only minor histological changes found here may be due to the fact that the animals were only challenged once and then examined 24 h later. It remains to be determined how the inflammation evolves with time and how it is affected by repeated exposure. However, a predominance of neutrophils has also been observed in other diisocyanate-based models of asthma (Matheson *et al.*, 2001) and even in ovalbumin models of asthma (Makela *et al.*, 2003; Whitehead *et al.*,

2003). Moreover, it has been established that, in humans, eosinophils are not always increased (Anees *et al.*, 2002) and that neutrophilia may be an important feature of occupational asthma (Park *et al.*, 1999a; Wisnewski and Redlich, 2001).

Influence of the Frequency and Concentration of Sensitization on the Response to a Subsequent Respiratory Challenge

Our initial sensitization procedure was based on existing, well-validated recommendations for achieving optimal sensitization in the mouse LLNA (Basketter *et al.*, 2002; Kimber *et al.*, 2001). This is why TDI, dissolved in a suitable vehicle (Dearman *et al.*, 1992, 1996), was applied on the ears on 3 successive days. The initial concentration of 3% was chosen because it gave a strong response in the LLNA, both in our research (Mandervelt *et al.*, 1997; Vanoirbeek *et al.*, 2003) and that of others (Hilton *et al.*, 1995). A 3-day dermal sensitization had also proven successful to provoke a respiratory response in a first series of experiments with TDI and another respiratory sensitizer (Vanoirbeek *et al.*, 2003). However, in that experiment the asthmatic response was mild and incomplete in that there was no detectable early response and no airway inflammation.

Consequently, we formulated the simple hypothesis that animals might need to receive a boost to achieve stronger respiratory reactions. We added a dermal application of 3% TDI on day 7 in between the sensitization sequence on days 1, 2, and 3 and the intranasal challenge on day 10. This led to the expected full asthmatic response, however, not in the expected group. The mice that had been sensitized to TDI and then boosted with TDI (1/1/1) hardly responded; those that had been sensitized to TDI but did not receive a boost (1/0/1) responded moderately (as before); surprisingly, the most pronounced reactions occurred in mice from group 0/1/1, i.e., in mice that we thought had not been sensitized to TDI (although they received a single dermal application 3 days prior to the challenge). Because the concentration of TDI applied on the skin could have been too high, possibly inducing a state of tolerance, the experiment was repeated with a tenfold lower concentration (0.3%). This now gave a full response in most animals of the sensitized and boosted group (1/1/1), a mild to moderate response in the sensitized but not boosted animals (1/0/1), and again a strong response in the animals that had only received a dermal application of TDI 3 days prior to challenge (0/1/1). To simplify the protocol and to avoid possible cumulative effects of applying the test compound and vehicle three times, we decided to give almost the same initial amount of TDI in one application (1% TDI). The same picture emerged for all groups, suggesting that the outcome is not affected by giving a single dermal application dose or fractionating it over 3 successive days, at least not with the sensitization dose administered. A single administration also attenuated any possible cumulative effect of applying the vehicle three times.

Overall, these results confirm that dermal application of TDI confers heightened sensitivity of the respiratory tract to TDI. Our data also show that the concentration and intensity of such

dermal sensitization are critical. There were no apparent differences in the (moderate) responses to intranasal TDI challenge between mice that had undergone a “strong” sensitization (3% TDI for 3 days) and those that had undergone a “light” sensitization (either 0.3% TDI for 3 days or 1% TDI on a single day), if no dermal boost was given (group 1/0/1). However, if a dermal boost was given (group 1/1/1), the respiratory response was blunted in the strongly sensitized animals, whereas it was enhanced in the lightly sensitized animals. At this stage, it is not possible to disentangle whether the blunting of the response was caused by the high amounts of TDI given on the skin in the first 3 days or by the high dose (3% TDI) used for the dermal boost. The blunted respiratory response could be explained by immunological tolerance suppressing the immune response in general, or by an attraction of the immunological effector cells away from the respiratory mucosa towards the dermal sites of application.

The occurrence of concentration-dependent reactions is in agreement with observations of Scheerens *et al.* (1996), who also noted that dermal treatment with lower TDI concentrations (1% to 0.1%) was more effective in leading to an increased total cell count and neutrophilic inflammation 24 h after intranasal challenge. Similar findings were reported by Herrick *et al.* (2002) in studies on the effect of HDI (1% to 0.1%). It is difficult to speculate on the cause of such a concentration-dependent effect to the exposure agent. However, it is likely that, as with protein antigens (Kurts *et al.*, 1999), immunological tolerance plays a role.

As expected, the insertion of a boost in our experimental protocol led to an increased respiratory response (at least if dermal sensitization had not been too strong). However, to our surprise, the single application of TDI on day 7 to animals that had not been previously sensitized (group 0/1/1) led to the most pronounced and consistent response to an intranasal administration of TDI on day 10. The latency between sensitization on day 7 and a positive challenge on day 10 is much shorter than would be expected on the basis of conventional immunologic theory, where the delay in mounting a primary response to an antigen is generally thought to take at least 4–7 days (Janeway *et al.*, 1999).

We first ruled out the possibility that the respiratory response was an artifact caused by sensitization to the vehicle in an experiment (Fig. 5) in which sham-exposed mice (n/1/1) responded in the same way as vehicle-exposed mice (0/1/1) or acetone-exposed mice (A/1/1). The reason for the absence of response in the olive oil-exposed mice (OO/1/1) is not clear, but it does suggest that (a putative) sensitization to this component of the vehicle is not responsible for the respiratory response. Moreover, we do not think olive oil downregulates the response, because there is almost no difference between the responses of groups n/1/1 and OO/1/1. However, acetone (when applied purely or in combination with olive oil) may enhance the response, possibly by drying the skin and allowing a better skin penetration of TDI. If olive oil would downregulate the response, the 0/1/1 group would have had a lower response than the A/1/1 group.

The short latency between the first dermal contact with TDI and the respiratory reaction could be explained by a different behavior of TDI compared to protein antigens. Such short latency was also observed by Thorne *et al.* (1987), who observed increased ear thickness upon application of isocyanates 3 days after dermal sensitization (on the flank) of guinea pigs. Furthermore, Saint-Mezard *et al.* (2003) showed that a single, dermal application of 2,4-dinitrofluorobenzene (DNFB) can induce contact dermatitis already on the fifth day. We are in the process of assessing the changes in lymphocytes and cytokines in draining lymph nodes and BAL to discover the mechanisms of these intriguing effects in group 0/1/1.

One mechanism that does not appear to be critical in the response is the level of total IgE in serum. All mice that had undergone the initial dermal-sensitizing protocol (1/1/1, 1/0/1, 1/1/0, and 1/0/0 groups) showed an increased total IgE. There was no difference in total IgE between mice challenged intranasally with TDI and mice given vehicle. This could result from the short delay between the respiratory challenge (day 10) and the blood sampling (day 11). Nevertheless, total IgE was not increased in the 0/1/1 group and yet this was the most reactive group. This apparent independence of the respiratory response from serum IgE levels is in agreement with our own previous results and those of others (Herrick *et al.*, 2002; Scheerens *et al.*, 1999). Furthermore, these findings reflect observations of people with TDI-induced asthma (Park *et al.*, 1999b). However, the involvement of immunoglobulins cannot be ruled out completely. The possibility remains that their cell surface binding and local tissue absorption render them undetectable in serum.

As previously discussed (Kimber and Dearman, 2002; Mapp *et al.*, 1994; Redlich *et al.*, 2002), the role of IgE in the pathophysiology of chemical-induced asthma is still unclear. We can only speculate about the underlying cellular and humoral mechanisms of respiratory changes following dermal sensitization observed in our murine and other models. As discussed by Redlich *et al.* (2002), in the case of HDI, it seems that both Th1-type and Th2-type cytokines may be involved, although the sensitizing dose may influence the response and the Th1/Th2 balance (Herrick *et al.*, 2002). Further studies are required to understand the bases for respiratory changes observed in our model. The pattern of cytokines produced by cells of draining lymph nodes and those produced by skin cells has to be considered as a potential determining element.

In summary, we have described a mouse model for TDI-induced asthma that exhibits several features of occupational asthma, namely an early ventilatory response after intranasal challenge, methacholine hyperresponsiveness, and airway inflammation in previously sensitized animals. Further work is underway to refine the model and determine its immunologic mechanisms. Such a model should allow us to further study the pathogenesis of chemical-induced asthma. Also, this model could perhaps be used for assessing the respiratory sensitization potential of chemicals by analogy with tests that have been developed to assess the risk of allergic contact dermatitis.

ACKNOWLEDGMENTS

We thank the Belgian Federal Science Policy Office (PS/01/43) and FWO-Vlaanderen (project: 7.0024.00) for funding this research.

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