

Late respiratory response and associated eosinophilic inflammation induced by repeated exposure to toluene diisocyanate in guinea pigs

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Objective: The study was designed to establish an animal model of toluene diisocyanate (TDI)-induced late respiratory response and to investigate airway inflammatory cell dynamics in this model.

Methods: Guinea pigs were exposed to 2,4-TDI dissolved in ethyl acetate by nasal application according to three schedules. Schedule 1 consisted of sensitization and multiple challenge (n = 58): 10% TDI was applied once daily for 7 days (sensitization) followed by challenges with 5% TDI once weekly for 4 weeks. Schedule 2 consisted of sensitization only (n = 5): 10% TDI was applied once daily for 7 days. Schedule 3 consisted of single challenge only (n = 12): 5% TDI was applied only once. As controls, ethyl acetate was applied according to the three schedules described above. Each animal was premedicated with metyrapone before each challenge. Bronchoalveolar lavage and histologic examination were performed at various times after the last challenge.

Results: Schedule 1 induced immediate and late respiratory responses at a prevalence of 63% and 56%, respectively. Schedules 2 and 3 induced an immediate response in some animals but no late response. Neither immediate nor late response was observed in control animals. All of the subgroups of schedule 1 that developed late responses (examined at 2, 3, 6, 24 and 168 hours) showed a significant increase of eosinophils in bronchoalveolar lavage fluid and tissue compared with the corresponding control of each (examined at the same time points). Two of the subgroups with late responses (examined at 3 and 6 hours) were compared with their corresponding subgroups, which were given the same treatment, failed to develop late response, and were examined at the same time points; they again proved to have a significant increase of eosinophils in both samples. Subgroups of schedule 1 without late response (30 minutes, 3 and 6 hours), schedule 2 (6 hours), or schedule 3 (2 and 6 hours) did not show significant changes in lavage or tissue cell composition, except for the schedule 1 subgroup examined at 6 hours, which showed a significant increase of eosinophils only in the tissue compared with its control.

Conclusions: Sensitization and multiple challenge with TDI induced immediate and/or late respiratory responses at a high prevalence in guinea pigs. Eosinophilic but not neutrophilic inflammation was involved in the late response. (*J Allergy Clin Immunol* 1996;97:1308-19.)

Key words: Toluene diisocyanate, late respiratory response, eosinophilic airway inflammation, bronchoalveolar lavage, histology, guinea pigs, metyrapone

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Toluene diisocyanate (TDI) is a low-molecular-weight compound widely used as an industrial chemical. TDI is known as one of the most common causes of occupational asthma¹; a prospective study by Butcher et al.² showed that about 5% of industrial workers who were occupationally exposed to this agent had asthma.

In patients with TDI-induced asthma, inhalation challenges with TDI elicit immediate or late asthmatic responses or both,²⁻⁵ and the prevalence of

Abbreviations used

BAL: Bronchoalveolar lavage
E/I: Expiratory time/inspiratory time ratio
TDI: Toluene diisocyanate

the late response is reported to be as high as 70% or more.^{3,5} Fabbri et al.⁴ reported that in patients with a late asthmatic response induced by TDI, bronchoalveolar lavage (BAL) fluid showed an increased number of neutrophils at 2 and 8 hours, and an increased number of eosinophils at 8 hours after inhalation challenges with TDI. This study suggested that airway inflammation is involved in the late asthmatic response induced by TDI, as reported in that elicited by common allergens, such as house dust mite.⁶⁻⁸ However, further studies supporting this view are lacking, probably because human studies are not easy to perform. Thus an animal model of TDI-induced late asthmatic response needs to be developed for investigation of its pathogenesis.

Some animal models of TDI-induced asthma have been described.⁹⁻¹² However, late asthmatic response was not induced in these models. Tanaka et al.¹³ have reported that guinea pigs sensitized and subsequently challenged repeatedly with nasal applications of TDI showed asthma-like symptoms such as exertional breathing, prolonged expiration, and cough immediately after the challenges. In some of the animals, such symptoms lasted or reappeared 2 hours or more after the challenges, suggesting that a late asthmatic response might have developed in the model.

Metyrapone, a cortisol synthesis inhibitor, has been shown to increase the frequency of late asthmatic response in dogs sensitized and challenged with *Ascaris* species.¹⁴ In this study, treatment of the guinea pigs with metyrapone before challenge with TDI was added to the method of Tanaka et al.¹³ to establish a model of TDI-induced biphasic (i.e., immediate and late) respiratory responses. We subsequently investigated airway inflammatory cell dynamics in the model by means of BAL and histologic examination.

METHODS

Materials

Male Hartley guinea pigs, weighing 250 to 300 gm, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were quarantined for 1 week before experimental use. In this study 2,4-TDI, which was obtained from Nacalai Tesque (Kyoto, Japan) was

used to sensitize and challenge guinea pigs. Metyrapone was obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in physiologic saline solution at a concentration of 50 mg/3 ml.

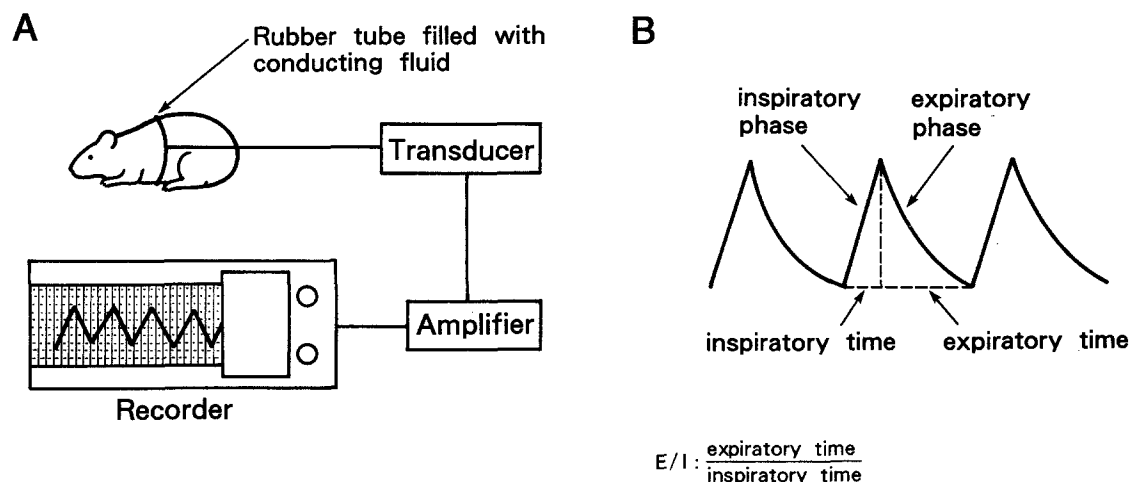
Exposure to TDI

TDI was dissolved in ethyl acetate to a final concentration of 5% or 10% just before use. The solutions were applied softly for several seconds to each side of the bilateral nasal vestibula of conscious guinea pigs with a thin cotton swab (Kawamoto Bandage Material Co., Osaka, Japan) for sensitization (10%) and challenge (5%).¹³ The volume of TDI applied in this way was about 5 μ l for each side of the nasal vestibule. Because ethyl acetate is highly volatile and does not react with TDI, TDI per se can be inhaled with this procedure. In the control animals the solvent (ethyl acetate) alone was applied in the same manner. During the procedure, guinea pigs were held gently by an operator.

Measurement of respiratory responses

Chest wall motion of the guinea pigs caused by inspiration and expiration was monitored and recorded to assess respiratory responses, according to the following method. An extendable rubber tube filled with zinc chloride solution as a conducting fluid (TR-602T; Nihon Kohden, Tokyo, Japan) was wrapped around the rib cage just above the diaphragm of each conscious guinea pig. As the lungs of the animals inflate during inspiration, the tube is stretched and the resistance of the conducting fluid increases. Conversely, it decreases during expiration.¹⁵ These changes in the resistance caused by respiration were conducted to a transducer connected to the rubber tube and then amplified and subsequently recorded graphically as a curve of breathing pattern (Fig. 1).

A prolonged expiratory phase is one of the major respiratory physiologic changes during asthmatic response in guinea pigs,¹⁶⁻¹⁹ as well as in human beings and monkeys.²⁰ Thus in this study, a prolonged expiratory phase, estimated as the increase of expiratory time/inspiratory time ratio (E/I) (Fig. 1, *B*), was adopted as the marker of respiratory responses. A mean E/I value of 10 consecutive breaths was calculated from the recorded breathing pattern (Fig. 1, *B*) by using a gauge, just before and at selected periods (20 and 40 minutes, 2, 3, 4, 5, 6, 8, 24, and 168 hours) after each challenge. The mean E/I in 30 normal, untreated guinea pigs ranged from 1.09 to 1.75 (mean, 1.43; SD, 0.11; SEM, 0.02), and a mean E/I of 2.0 or greater was almost invariably accompanied by labored breathing motion, wheezing, or both in guinea pigs exposed to TDI. We therefore judged a respiratory response to be positive when the mean E/I was 2.0 or greater. Animals with this significant increase of mean E/I at 20 or 40 minutes or both and at any time between 2 and 24 hours after challenge were considered to have immediate and late respiratory responses, respectively.



When the mean value of E/I of 10 consecutive breaths was ≥ 2.0 , respiratory response was judged as positive.

FIG. 1. **A**, Diagram of system used for respiratory monitoring. An extendable rubber tube filled with conducting fluid is attached around the rib cage of a conscious guinea pig. As the lungs of the animal inflate during inspiration, the tube is stretched and the resistance of the conducting fluid increases. Conversely, it decreases during expiration. These changes in resistance caused by respiration are conducted to a transducer connected to the rubber tube and are amplified and subsequently recorded graphically as a curve of breathing pattern. **B**, Example of breathing pattern recorded by the system. E/I value is calculated from the recorded breathing pattern by using a gauge.

Study protocol

Guinea pigs were exposed to TDI according to three different schedules: (1) a sensitization period of 7 consecutive days followed by 4 weekly challenges (sensitization and multiple challenge, $n = 58$), (2) a sensitization period of 7 consecutive days without subsequent challenge (sensitization only, $n = 5$), and (3) a single challenge without preceding sensitization (single challenge only, $n = 12$) (Table I).

Metyrapone, 50 mg/kg, was injected intraperitoneally in each guinea pig 24 hours and 30 minutes before each challenge for the purpose of increasing the prevalence of late respiratory response.¹⁴ In a separate experiment, 18 guinea pigs were sensitized to and subsequently challenged with TDI in the same manner as in the "sensitization and multiple challenge" schedule of this study. In that experiment, 3 ml/kg saline solution, rather than metyrapone, was injected intraperitoneally 24 hours and 30 minutes before each challenge. The total prevalence of immediate respiratory responses in the three challenges was as high as 31 of 54 (57.4%) but that of late response was low (18 of 54, 33.3%). Metyrapone pretreatment was therefore introduced into this study.

The guinea pigs, which were given sensitization and multiple challenge and single challenge only, were subdivided according to the type of respiratory responses observed and the time of BAL and histologic examination after the fourth challenge. The TDI-exposed ani-

mals were thus classified into 11 groups as described below and in Table II.

Groups 1 to 8: Sensitization and multiple challenge

To induce sensitization, 10% TDI was applied once daily for 7 consecutive days. Metyrapone was not administered during this period. Seven days later, the guinea pigs were challenged with 5% TDI after metyrapone administration once weekly for 4 weeks. BAL and a histologic examination were done at various intervals after the fourth challenge. Animals were divided into eight groups according to the type of respiratory response and the time of BAL and histologic examination; group 1 ($n = 8$), during immediate response (30 minutes); group 2 ($n = 7$), during late response (2 hours); group 3 ($n = 6$), no late response (3 hours); group 4 ($n = 7$), during late response (3 hours); group 5 ($n = 9$), no late response (6 hours); group 6 ($n = 9$), during late response (6 hours); group 7 ($n = 7$), during or after late response (24 hours), and group 8 ($n = 5$), after late response (168 hours).

Group 9: Sensitization only

Ten percent TDI was applied once daily for 7 consecutive days in five guinea pigs. After the seventh exposure, E/I was assessed and BAL and a histologic exam-

TABLE I. Sensitization and challenge and the prevalence of respiratory responses

	Sensitization	Challenge*		Prevalence of respiratory responses after each challenge†			
				First	Second	Third	Total
Sensitization and multiple challenges (<i>n</i> = 58) (groups 1 to 8)	10% TDI × 7	5% TDI × 4	I immediate	9/58 (15.5)	8/58 (13.8)	13/58 (22.4)	30/174 (17.2)
			I late	8/58 (13.8)	5/58 (8.6)	5/58 (8.6)	18/174 (10.3)
			Dual	22/58 (37.9)	30/58 (51.7)	27/58 (46.6)	79/174 (45.4)
			Immediate	31/58 (53.4)	38/58 (65.5)	40/58 (69.0)	109/174 (62.6)
			Late	30/58 (51.7)	35/58 (60.3)	32/58 (55.2)	97/174 (55.7)
Sensitization only (<i>n</i> = 5) (group 9)	10% TDI × 7	—	I immediate	3/5 (60.0)	—	—	
			I late	0/5	—	—	
			Dual	0/5	—	—	
Single challenge only (<i>n</i> = 12) (groups 10 and 11)	—	5% TDI × 1	I immediate	8/12 (66.7)	—	—	
			I late‡	0/6	—	—	
			Dual‡	0/6	—	—	

I, Isolated.

*Metyrapone, 50 mg/kg, was injected intraperitoneally 24 hours and 30 minutes before each challenge.

†The numbers in parentheses are the prevalence of respiratory responses indicated by percentage.

‡Prevalence of respiratory responses in the six guinea pigs observed for 6 hours after exposure to TDI.

ination were performed at 6 hours. Metyrapone was not administered.

Groups 10 and 11: Single challenge only

Five percent TDI was applied to nonsensitized metyrapone-pretreated guinea pigs only once. BAL and a histologic examination were performed 2 (group 10, *n* = 6) and 6 hours (group 11, *n* = 6) after the exposure.

The presence or absence of immediate respiratory response was not taken into consideration in the classification of groups 2 to 11.

Each group corresponded to a control set in which the guinea pigs were exposed to ethyl acetate for "sensitization," "challenge," or both, and thereafter BAL and histologic examination were performed according to the same schedule as in the corresponding TDI-exposed animals. Likewise, metyrapone was administered before challenge. Because groups 3 and 4 and groups 5 and 6 were given exactly the same procedure, respectively, these two pairs corresponded to one common control group of each. The number of guinea pigs of each control group is shown in Table II.

BAL and histologic examination

After the fourth challenge, BAL and a histologic examination were performed. Each guinea pig was anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg) and exsanguinated through the jugular vein. A tracheostomy was performed, and the trachea was cannulated. Both lungs were lavaged with 4 aliquots of physiologic saline solution, the volumes of which were 5, 5, 10, and 10 ml, respectively. The return from the first 5 ml aliquot and the returns from the three subsequent aliquots were processed separately. The cellular contents of only the former sample were shown and discussed in this study because there were no major differences between the results from the two samples. The lavage samples were centrifuged at 4° C for 10 minutes at 125 g to pellet cells, and the supernatant fraction was removed. The cell pellets were resuspended, washed, and again resuspended in phosphate-buffered saline. The total number of cells was counted with a hemocytometer. Differential cell counts were determined on centrifuged preparations stained with May-Grünwald-Giemsa stain and nonspecific esterase. A minimum of 500 cells were counted with standard morphologic crite-

TABLE II. Summary of characteristics of the 11 animal groups exposed to TDI

Group	No. of guinea pigs	Sensitization and challenge patterns	Type of respiratory responses and time of BAL and histologic examination after final exposure to TDI			No. of corresponding control guinea pigs
			Immediate	Late	Time	
1	8	Sensitization and multiple challenges	+	Not estimable	30 min	4
2	7		+ or -	+	2 hr	4
3	6		+ or -	-	3 hr	4
4	7		+ or -	+	3 hr	
5	9		+ or -	-	6 hr	7
6	9		+ or -	+	6 hr	
7	7		+ or -	+	24 hr	4
8	5		+ or -	+	168 hr	4
9	5	Sensitization only	+ or -	-	6 hr	4
10	6	Single chal-	+ or -	-	2 hr	4
11	6	lenge only	+ or -	-	6 hr	4

ria to classify the cells into macrophages, lymphocytes, eosinophils, and neutrophils. The total or differential cell number was expressed as 10^4 cells per 1 ml of lavage fluid.

After the BAL, the lungs and trachea were inflated with 4% glutaraldehyde containing 0.05 mol/L cacodylate buffer (pH 7.4) through the tracheal cannula at a pressure of 20 cm H₂O. They were subsequently removed from the body and immersed in fixative. On the next day, the right lung was separated and immersed in 10% formol saline solution for 7 days. Thereafter, the specimen was embedded in paraffin wax. Sections (4 μ m) were cut and stained with hematoxylin-eosin for light microscopic examination.

Because the major pathologic finding in the airways was eosinophil infiltration and because other leukocytes were not prominent in our specimens, only the number of eosinophils was quantified. Six to 10 bronchi were chosen randomly from each guinea pig, and the number of eosinophils in the lamina propria (per 1 mm of basement membrane) was counted at a magnification of $\times 400$. The results were expressed as the mean of the numbers obtained from the six to 10 bronchi. In our preliminary experiments, inflammatory cells, such as lymphocytes or eosinophils, infiltrated the alveolar septa in some guinea pigs sensitized to and challenged with TDI. In this study, animals with such pulmonary involvement were not included.

Examination of BAL fluid and airway tissue inflammatory cells was performed in blinded fashion.

Animal care and use

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis

Data are expressed as median values with the range from lower to upper quartiles in parentheses. To evaluate the volume recovery rate of BAL and the number of inflammatory cells in BAL fluid or bronchial tissue, nonparametric analysis of variance (Kruskal-Wallis method) was used to determine significant variance among the groups. If a significant variance was found, we used the Mann-Whitney U test to analyze significant differences between individual groups. Prevalence of respiratory responses was compared by using the chi square test. A *p* value of less than 0.05 was considered significant. Data were analyzed with a Macintosh computer (Apple Computer Inc., Cupertino, Calif.) with the StatView 4.0 program (Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

Respiratory responses

Table I shows the prevalence of three types of respiratory responses (isolated-immediate, isolated-late, and dual) in the animals exposed to TDI according to the three schedules: sensitization and multiple challenge (groups 1 to 8), sensitization only (group 9), and single challenge only (groups 10 and 11).

In groups 1 to 8, the presence or absence or type, if present, of respiratory responses was reproducible through the first to third challenges in many animals, but this was not so in some. The prevalence of the three types of respiratory responses and that of immediate response (isolated-immediate + dual) and late response (isolated-late +

dual) were not significantly different among the three challenges, although the prevalence of immediate response tended to increase as the challenge was repeated (Table I). In the challenges that induced immediate or late respiratory responses or both, the values for mean $\pm$ SEM of mean E/I at the time when immediate and late responses developed were 2.56 ± 0.11 and 2.48 ± 0.08 , respectively. The total prevalences of immediate and late responses after the three challenges were 109/174 (62.6%) and 97/174 (55.7%), respectively (Table I). This prevalence of late response was significantly ($p < 0.005$) higher than that in the experiment without metyrapone pretreatment described in the study protocol, whereas the prevalence of immediate response was not significantly different between the two experiments. The fourth challenge (not shown in the table) induced isolated-immediate, isolated-late, and dual responses in 14, 8, and 27 guinea pigs, respectively, as far as E/I was assessed.

Some of the late responses observed in these groups persisted for 24 hours after the challenges; all of them ceased by 168 hours. During the sensitization period, some animals showed asthma-like symptoms immediately after the application of TDI, but symptoms ceased within 20 to 40 minutes and did not reappear.

In group 9 immediate response was observed in three guinea pigs after the seventh exposure to TDI. However, no late response was induced during the next 6 hours of observation.

Immediate response was observed in four guinea pigs in each of groups 10 and 11. Thus a single challenge with TDI induced immediate response in eight of 12 animals. However, in the six animals of group 11 in which E/I was assessed for 6 hours after the challenge, no late response was observed.

Neither immediate nor late responses were evident in any of the control animals exposed to ethyl acetate according to the three different schedules.

BAL

The mean $\pm$ SEM of the volume recovery rates of BAL fluid of all the examined guinea pigs exposed to TDI or ethyl acetate was $59.4\% \pm 1.1\%$. The rates were fairly constant and were not significantly different among the groups (data of each group not shown).

The results of the total or differential cell number (10^4 cells/ml) were as follows and are expressed as median values with the range from lower to upper quartiles in parentheses or brackets. The total cell number significantly increased in three of

the five groups that developed late respiratory responses after sensitization and multiple challenge (groups 2, 6, and 7) compared with the corresponding control of each ($16.8 [13.1-26.4]$ vs $5.4 [3.6-7.5]$, $p < 0.009$; $20.3 [16.3-27.4]$ vs $3.6 [3.1-9.9]$, $p < 0.004$; and $20.5 [9.9-25.7]$ vs $3.3 [2.7-7.5]$, $p < 0.03$, respectively). The number also significantly increased in group 4 ($15.7 [8.8-22.9]$) and in group 6 compared with their corresponding TDI-exposed groups without late responses (group 3, $6.3 [4.6-6.7]$, $p < 0.03$ vs group 4; and group 5, $9.4 [5.1-18.3]$, $p < 0.02$ vs group 6), although group 4 did not show a significant increase in the total cell number compared with its control ($9.2 [6.2-10.1]$). In groups 1, 8, 9, 10, and 11, the total cell number was not significantly different compared with the corresponding control of each ($6.9 [4.3-11.5]$ vs $6.8 [4.4-9.5]$, $12.7 [9.0-22.3]$ vs $5.0 [3.2-9.4]$, $5.8 [2.0-13.9]$ vs $6.1 [4.2-6.9]$, $5.4 [4.3-7.0]$ vs $4.9 [3.3-10.0]$, and $3.6 [3.2-9.9]$ vs $7.8 [4.9-9.9]$, respectively).

The numbers of eosinophils from each group are shown in Fig. 2 (p values are described in the figure). All of the groups that developed late responses after sensitization and multiple challenge (groups 2, 4, 6, 7, and 8) showed a significant increase of eosinophils compared with the corresponding control of each ($8.7 [4.6-17.2]$ vs $0.4 [0.1-1.3]$, $4.0 [2.4-10.5]$ vs $0.4 [0.1-1.4]$, $10.5 [5.8-18.6]$ vs $0.5 [0.3-1.2]$, $5.5 [2.9-11.7]$ vs $0.1 [0.0-1.9]$, and $3.9 [3.7-4.1]$ vs $0.5 [0.2-1.0]$, respectively). In groups 4 and 6 the increase was also significant when compared with their corresponding TDI-exposed groups without late responses (group 3, $0.3 [0.2-1.5]$; group 5, $4.0 [0.9-6.6]$, respectively). Among the five groups with late responses, the median value of eosinophil number was highest at 6 hours (group 6); however, differences among the five groups did not reach statistical significance. In groups 1, 9, 10, and 11, no significant increase of eosinophils was observed compared with the corresponding control of each ($1.3 [0.4-3.8]$ vs $0.7 [0.5-0.9]$, $0.4 [0.2-5.4]$ vs $0.3 [0.1-1.8]$, $0.8 [0.7-1.0]$ vs $0.5 [0.3-0.9]$, and $0.9 [0.0-1.8]$ vs $0.7 [0.3-1.0]$, respectively), as well as in groups 3 and 5.

The variance in the numbers of macrophages, lymphocytes, or neutrophils among the groups was not statistically significant as determined by the Kruskal-Wallis method. The numbers of macrophages, lymphocytes, and neutrophils in each group are shown in Table III.

In groups 9, 10, and 11 the numbers of total or differential cells were not significantly different when animals with and without immediate response were compared (data not shown).

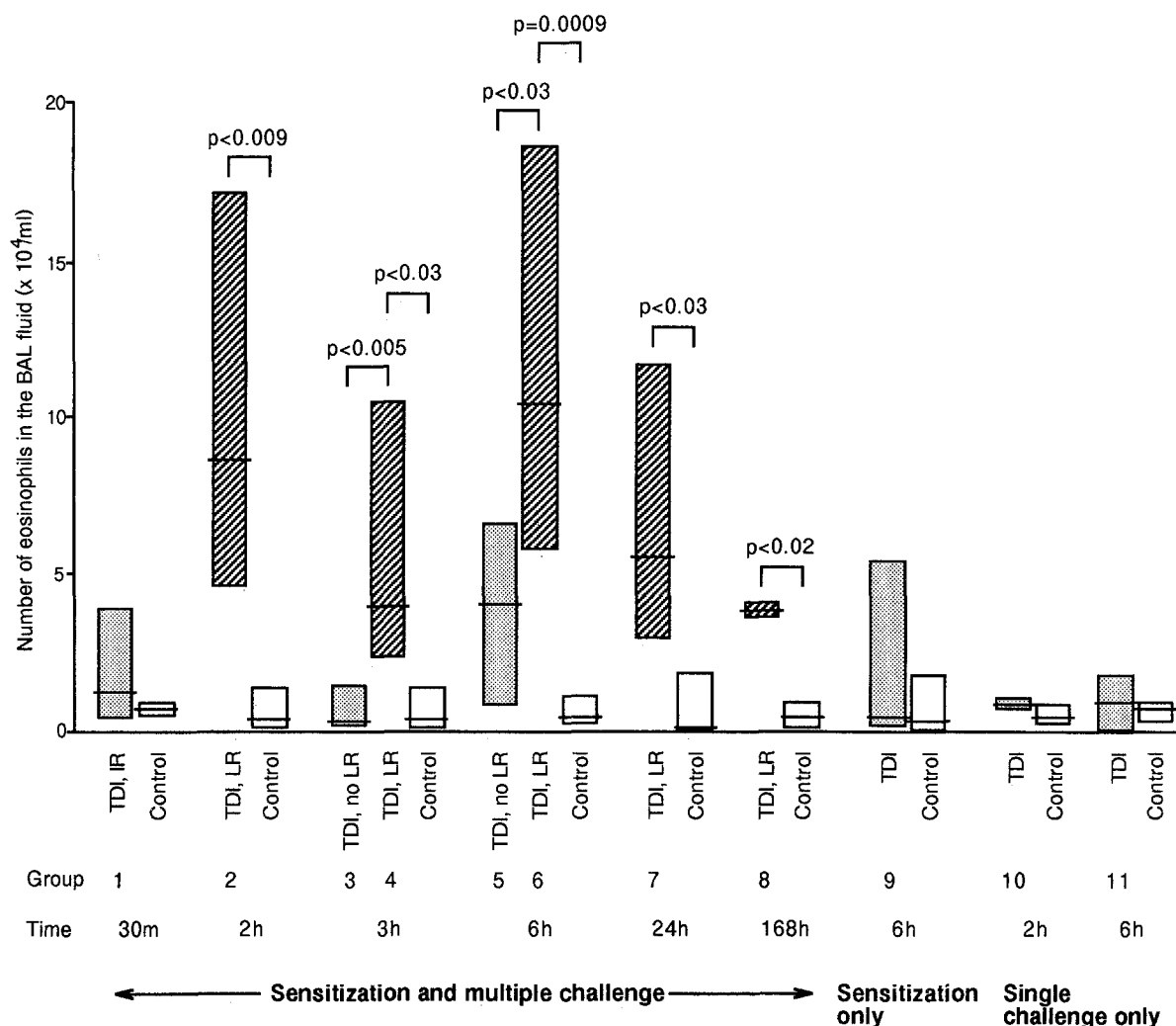


FIG. 2. Number of eosinophils in BAL fluid in the individual groups. *Horizontal lines* are medians; *bars* indicate lower and upper quartiles. Groups with late respiratory response induced by sensitization and multiple challenges with TDI (groups 2, 4, 6, 7, 8; *hatched bars*) have a significantly increased number of eosinophils compared with the control of each (*open bars*). In groups with only immediate response or no response, irrespective of sensitization and challenge patterns (*dotted bars*), eosinophils are not significantly increased compared with the control of each (*open bars*). In groups 4 and 6, eosinophils are also significantly increased compared with their corresponding groups without late response (groups 3 and 5, respectively). *IR*, Immediate respiratory response; *LR*, late respiratory response.

Histologic examination of the airway

In guinea pigs with late respiratory response, marked eosinophil accumulation was observed in the bronchial wall (Fig. 3, *A*). Infiltration of mononuclear cells or shedding of the epithelium was found in some animals with a late response, but these changes were mild. Among the control animals no major pathologic changes were observed (Fig. 3, *B*). Neutrophils were rare in any animal examined.

The number of eosinophils in the bronchial

tissue of each group is shown in Fig. 4 (*p* values are described in the figure). All of the groups that developed late responses after sensitization and multiple challenge (groups 2, 4, 6, 7, and 8) showed a significant increase of eosinophils compared with the corresponding control of each (47.1 [38.0-66.1] vs 6.7 [4.0-10.1], 44.1 [26.1-52.1] vs 10.0 [5.2-10.8], 65.6 [56.2-88.8] vs 7.1 [6.3-9.2], 40.8 [32.6-66.2] vs 5.1 [3.0-15.7], and 31.0 [24.2-34.2] vs 5.5 [3.6-10.1], respectively). In groups 4 and 6 the increase was also significant when compared with their corre-

TABLE III. Variance in numbers of macrophages, lymphocytes, and neutrophils in BAL fluid among groups (10^4 cells/ml)

Group	No. of Macrophages	No. of Lymphocytes	No. of Neutrophils
1	5.9 (3.7-7.1)	0.1 (0.1-0.4)	0.1 (0.1-0.4)
Control of 1	5.7 (3.5-7.9)	0.2 (0.1-0.4)	0.2 (0.2-0.3)
2	7.0 (6.2-9.1)	0.5 (0.4-0.8)	0.4 (0.3-0.7)
Control of 2	6.6 (4.7-9.2)	0.6 (0.3-0.8)	0.3 (0.2-1.2)
3	4.6 (3.9-5.6)	0.2 (0.1-0.5)	0.1 (0.0-0.1)
4	10.0 (3.9-11.0)	0.4 (0.1-2.7)	0.3 (0.1-0.8)
Control of 3 and 4	6.7 (4.5-8.3)	0.5 (0.1-1.6)	0.2 (0.1-0.2)
5	4.9 (3.0-8.0)	0.2 (0.1-0.2)	0.2 (0.1-0.4)
6	8.8 (6.2-11.0)	0.3 (0.2-0.4)	0.1 (0.1-0.5)
Control of 5 and 6	3.0 (2.6-8.1)	0.1 (0.0-0.3)	0.1 (0.1-0.5)
7	8.1 (6.6-12.4)	0.8 (0.5-1.0)	0.3 (0.2-0.9)
Control of 7	2.9 (2.1-8.5)	0.2 (0.1-0.4)	0.3 (0.1-0.6)
8	8.7 (5.0-17.8)	0.1 (0.1-0.4)	0.1 (0.1-0.4)
Control of 8	3.3 (2.0-8.3)	0.3 (0.3-0.4)	0.0 (0.0-0.4)
9	4.6 (1.7-6.7)	0.3 (0.1-0.4)	0.2 (0.0-0.6)
Control of 9	4.1 (3.2-5.4)	0.2 (0.1-0.3)	0.1 (0.1-0.2)
10	4.2 (3.5-5.8)	0.1 (0.1-0.2)	0.1 (0.0-0.1)
Control of 10	3.8 (2.4-8.7)	0.2 (0.2-0.3)	0.2 (0.1-0.5)
11	6.0 (2.6-11.5)	0.1 (0.1-0.1)	0.1 (0.0-0.2)
Control of 11	6.7 (3.5-11.9)	0.4 (0.4-1.0)	0.1 (0.1-0.6)

Values are expressed as medians with ranges from lower to upper quartiles in parentheses.

sponding TDI-exposed groups without late responses (group 3, 22.7 [8.4-28.2]; group 5, 31.8 [17.3-36.3], respectively), although group 5 had a significantly increased number of eosinophils compared with its control. Among the five groups with late responses, the median value of eosinophil number was highest at 6 hours (group 6); however, differences among the five groups did not reach statistical significance. In groups 1, 9, 10, and 11 no significant increase of eosinophils was observed compared with the corresponding control of each (20.3 [7.1-34.3] vs 3.9 [2.9-15.7], 8.5 [6.3-32.4] vs 8.3 [5.4-11.5], 1.5 [1.1-4.9] vs 4.9 [3.6-6.9], and 6.8 [1.2-8.8] vs 3.3 [0.4-16.8], respectively), as well as in group 3.

In groups 9, 10, and 11 the numbers of eosinophils were not significantly different when animals with and without immediate responses were compared (data not shown).

DISCUSSION

A few animal models of TDI-induced asthmatic responses have been described. Karol et al.^{9,10} sensitized guinea pigs with dermal applications or intradermal injections of TDI or inhalations of TDI vapor and challenged them with inhalations of TDI-protein conjugates^{9,10} or TDI vapor.⁹ Bernstein et al.¹¹ passively sensitized guinea pigs with

intravenous injections of guinea pig IgE antisera against TDI-human serum albumin and challenged them with intravenous injections of TDI-protein conjugates. Patterson et al.¹² sensitized and challenged dogs with inhalations of TDI vapor. Although immediate asthmatic response was induced in these models, late response was not mentioned and does not seem to have been observed in any of them. In a study by Tanaka et al.,¹³ guinea pigs were sensitized with a nasal application of 10% TDI once daily for 5 consecutive days and subsequently challenged with 5% TDI several times. Asthma-like symptoms appeared within 10 minutes and occasionally lasted or reappeared several hours after challenges in this model. Recently, Sugawara et al.¹⁸ further studied this model. They sensitized guinea pigs with nasal application of 10% TDI for 5 consecutive days and thereafter challenged them with the same preparation of TDI weekly for 9 weeks. About 35% of guinea pigs displayed asthma-like symptoms after challenges with TDI, although the time that they were observed was not mentioned. E/I was measured in six guinea pigs after the sixth challenge, and a prolongation of E/I at 5 to 10 minutes and at some points between 2 and 21 hours after the challenge was observed. In this study the guinea pigs were treated with metyrapone before each challenge,

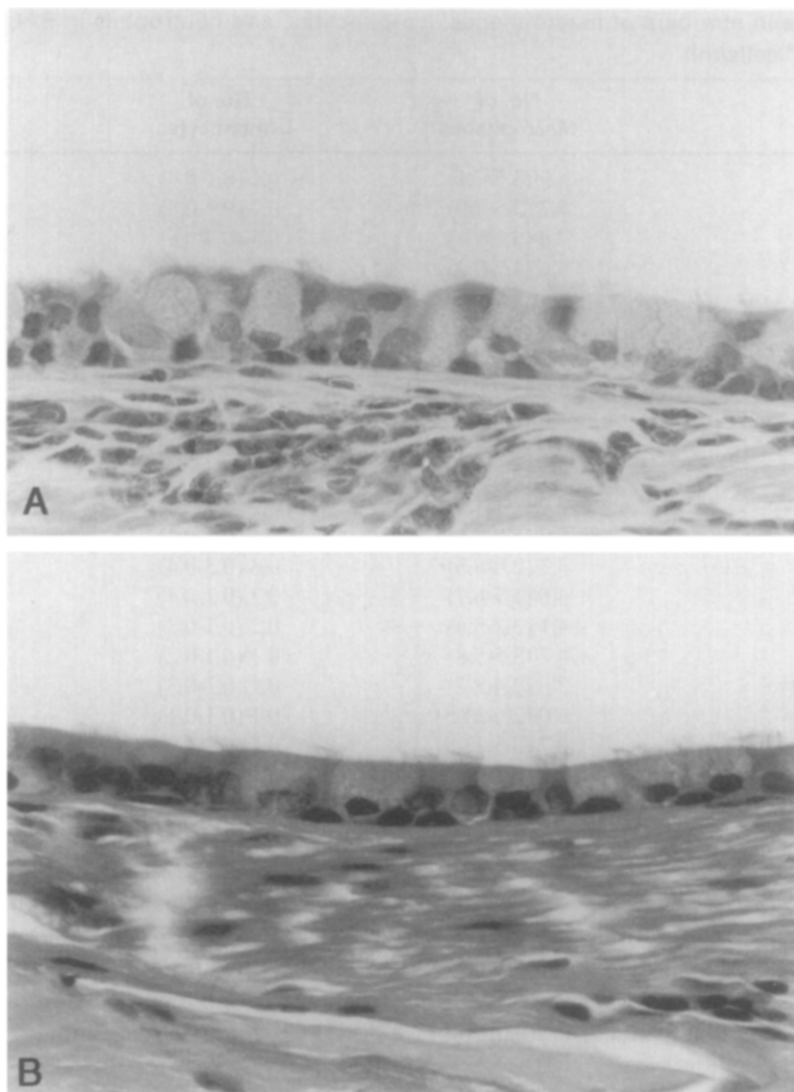


FIG. 3. **A**, Photomicrograph of a section of bronchus from a guinea pig in group 6, sensitized and challenged four times with TDI and killed during a late respiratory response (6 hours after challenge with TDI). There is remarkable infiltration of eosinophils in the epithelium and lamina propria. Neutrophils are rare. **B**, Photomicrograph of a section of bronchus from a guinea pig in the corresponding control of group 6, which was exposed to ethyl acetate. Neither immediate nor late respiratory response was observed in this animal. No major pathologic changes are seen. (Hematoxylin-eosin stain; original magnification $\times 400$.)

and E/I was measured after all challenges in all guinea pigs. This pretreatment significantly ($p < 0.005$) increased the prevalence of late respiratory response from 33.3% to 55.7%. The prevalence of immediate response, which was not influenced by metyrapone, was also high (62.6%). We have thus established an animal model of biphasic respiratory responses induced by TDI at high prevalence.

Airway inflammation is known to be involved in the late asthmatic response. BAL performed in patients with atopic asthma during a late asthmatic

response, which was induced by inhalation challenges with house dust mite⁶⁻⁸ or ryegrass,⁷ revealed an increase in the numbers and/or percentages of eosinophils⁶⁻⁸ or neutrophils⁷ and in the concentrations of eosinophil cationic protein,⁶ major basic protein, histamine, or leukotriene C_4 ⁷ in the lavage fluid. Several experimental studies also support the correlation between late asthmatic response and airway inflammation; that is, guinea pigs^{16, 21} or dogs¹⁴ sensitized and challenged with ovalbumin²¹ or *Ascaris* species^{14, 16} developed late

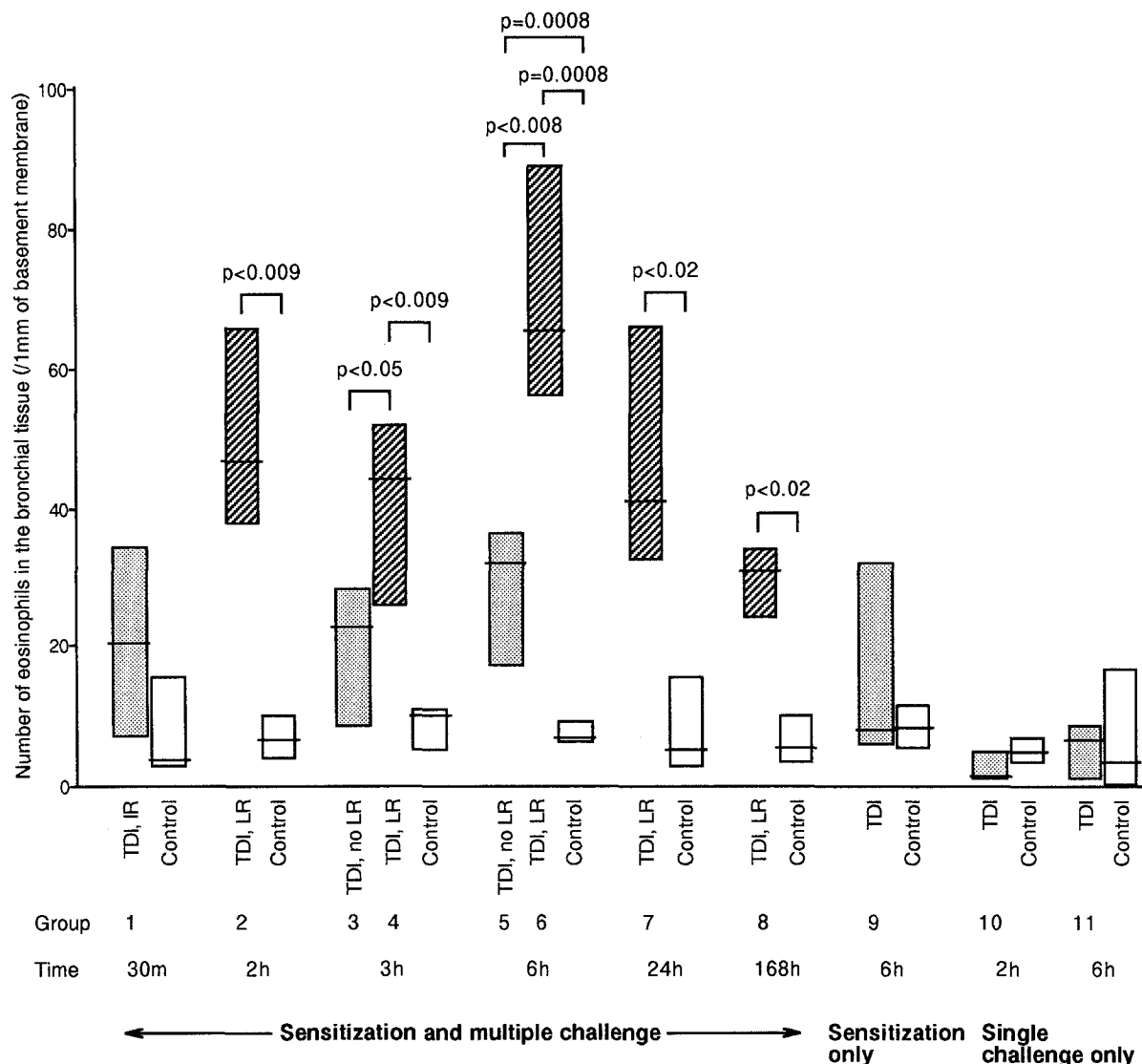


FIG. 4. Number of eosinophils in bronchial tissue in individual groups. Horizontal lines are medians; bars indicate lower and upper quartiles. Groups with late respiratory response induced by sensitization and multiple challenges with TDI (groups 2, 4, 6, 7, 8; hatched bars) have a significantly increased number of eosinophils compared with the control of each (open bars). In groups with only immediate response or no response, irrespective of sensitization and challenge patterns (dotted bars), eosinophils are not significantly increased compared with the control of each (open bars), except for group 5. In groups 4 and 6, eosinophils are also significantly increased compared with their corresponding groups without late response (groups 3 and 5, respectively). IR, Immediate respiratory response; LR, late respiratory response.

asthmatic responses and concomitant BAL fluid or airway tissue eosinophilia^{16, 21} or neutrophilia.^{14, 16, 21}

With regard to isocyanate-induced asthma, Fabri et al.⁴ performed a BAL study in human beings with TDI-induced asthma. In those with late asthmatic responses, an inhalation challenge with TDI induced a significant increase in the number of neutrophils in BAL fluid at 2 hours ($p < 0.01$)

compared with normal control subjects and at 8 hours ($p < 0.001$) compared with individuals with only immediate asthmatic responses. The number of eosinophils also slightly but significantly ($p < 0.05$) increased in the BAL fluid from individuals with late responses at 8 hours compared with those with only immediate response. Airway responsiveness to methacholine significantly ($p < 0.05$) increased at 8 hours in individuals with late re-

sponses. Fabbri et al.⁴ concluded that the late asthmatic response and the associated increase in airway responsiveness induced by TDI may be caused by airway inflammation, as it is in common allergen-induced asthma. In our guinea pig study all of the groups with late respiratory responses induced by sensitization and multiple challenges (examined 2, 3, 6, 24, and 168 hours after challenge) had a significant increase in the number of eosinophils in BAL fluid and the bronchial tissue when compared with their own control groups. In contrast, guinea pigs without late responses despite sensitization and multiple challenges, those sensitized but not challenged, or those challenged singly without sensitization did not show significant changes in the number of eosinophils or other inflammatory cells in BAL fluid or tissue, except for group 5. This group was sensitized and challenged four times with TDI, failed to develop a late response, and showed, at 6 hours, a significant increase of eosinophils in the tissue but not in BAL fluid. However, group 6, which developed late response and was examined at the same time point as group 5, had a significantly larger number of eosinophils than group 5 in tissue and in BAL fluid. Likewise, group 4, which developed late response and was examined at 3 hours had a significantly larger number of eosinophils in both samples than group 3, which was without late response and examined at the same time point. These results were consistent with those of the human study by Fabbri et al.⁴ in that eosinophils were significantly increased in the airways of individuals with late response compared with those without it and that these cells seem to be involved in late response induced by TDI. We therefore believe that this study provides a good model of TDI-induced late respiratory responses and airway inflammation.

In our model, BAL fluid or airway tissue neutrophilia was not observed at any time in contrast to the study by Fabbri et al.,⁴ in which neutrophilia was more prominent than eosinophilia in BAL fluid obtained from human beings with late asthmatic response. There may be several reasons for this discrepancy between the two studies. The first is an interspecies difference. The guinea pig is a relatively "eosinophilic animal"²² in that the eosinophil content of BAL fluid is reportedly 8%²² to 24%²³ of the total number of normal nucleated cells. Additionally, the two species may react differently to TDI. The second reason is that a difference in the route of exposure or concentration of TDI between the two studies might have

generated different results. Interspecies difference is one of the major problems of using animal models. We suggest that this rare guinea pig model of respiratory responses and airway inflammation induced by TDI, an agent that also induces asthma in human beings, may be useful in studying differences between the two species.

Gordon et al.²⁴ and Thompson et al.²⁵ have reported that a single exposure of guinea pigs to high concentrations (1 or 2 ppm) of TDI vapor for 1 hour induced transient airway hyperreactivity and airway mucosal injury or infiltration of neutrophils or eosinophils. Moreover, one of these reports²⁵ and the canine study by Patterson et al.¹² showed that a single exposure to TDI induced some changes in respiratory function, which resembled immediate asthmatic response. It is indicated that a single exposure to TDI may induce airway hyperreactivity, airway inflammation, or immediate asthmatic response. The authors of these studies thought that these phenomena were mediated by nonimmunologic mechanisms,^{12, 24} such as pharmacologic action or an irritation effect of TDI.¹² In this study single challenge with TDI without preceding sensitization induced immediate respiratory responses in some guinea pigs, confirming the view that the immediate response induced by TDI is mediated, at least in part, by nonimmunologic mechanisms. However, airway inflammation was not induced, regardless of the presence or absence of an immediate response, by this procedure in our model, in contrast to the experiments of Gordon et al.²⁴ and Thompson et al.²⁵ This discrepancy might have derived from differences in the methods of exposure to TDI between the two studies. Late respiratory response and accompanying eosinophilic airway inflammation were induced by sensitization and subsequent multiple challenges but not by sensitization only or single challenge only in this study, suggesting that they were mediated by an immunologic mechanism.

We have demonstrated that sensitization and subsequent multiple challenges with TDI induced immediate and/or late respiratory responses at a high prevalence in guinea pigs treated with metyrapone before each challenge and that eosinophilic airway inflammation was involved in the late response. This model will be quite useful in investigating the pathogenesis of TDI-induced asthma.

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