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Inhalation of Toluene Diisocyanate Is Associated with Increased Production of Nitric Oxide by Rat Bronchoalveolar Lavage Cells

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Isocyanates are used commercially, particularly in the manufacture of polyurethane coatings and foam. These compounds can pose an occupational health hazard since there is a risk of respiratory disease following isocyanate exposure. The purpose of the present study was to investigate whether a single, sublethal isocyanate inhalation is associated with increased production of the free radical nitric oxide (NO). Mature male Sprague–Dawley rats were exposed to air or toluene diisocyanate (TDI; 2 ppm) for 4 hr. Indices of pulmonary function were assessed before and after exposure to TDI fumes. At 20 hr postexposure, bronchoalveolar lavage cells (BALC) and fluid were harvested. NO synthase (NOS)-dependent reactive species production by alveolar macrophages was assessed by determining $N\omega$-nitro-L-arginine methyl ester-inhibitable chemiluminescence following stimulation with unopsonized zymosan. Northern blot analysis was used to index inducible NOS mRNA levels in BALC, while nitrite and nitrate (NO_x) levels were measured to determine NO_x levels in the lavage fluid and the production of NO by cultured adherent BALC was indexed by measuring nitrite levels. Exposure to aerosolized TDI was associated with an increase in the number of alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes harvested by bronchoalveolar lavage, relative to that from air-exposed rats. NO_x levels in the lavage fluid and NOS-dependent production of reactive species by alveolar macrophages were increased following TDI exposure. In addition, inducible NO production by BALC (i.e., mRNA levels and nitrite levels in BALC conditioned media) was elevated following TDI treatment. These findings indicate that pulmonary inflammatory responses induced by TDI exposure are associated with increases in inducible NO production. Therefore, the potential role of NO in the initial pulmonary response to TDI exposure warrants further investigation. © 1997 Academic Press

Isocyanates are used principally in the manufacture of polyurethane products such as foam and surface coatings. Exposure to these compounds can pose an occupational health hazard and cause respiratory toxicity (Rosenstock and Cullen, 1994). The primary disorder in symptomatic workers following isocyanate exposure is the occurrence of bronchial asthma with attendant syndromes of bronchial hyperresponsiveness and airway inflammation (Baur *et al.,* 1994; Fabbri *et al.,* 1991).

The mechanisms underlying the induction of isocyanate-induced asthmatic responses have not been fully characterized. A subgroup of exposed workers do exhibit IgE-mediated hypersensitivity; however, this population comprises 20% or less of isocyanate-sensitive individuals (Rosenstock and Cullen, 1994; Baur *et al.,* 1994). Recent studies in humans indicate that specific HLA class II genetic markers appear to be associated with susceptibility or resistance to isocyanate-induced asthma (Bignon *et al.,* 1994). Activated T lymphocytes and eosinophils are also prominent in the bronchial mucosa of symptomatic workers exposed to toluene diisocyanate (TDI) (Bentley *et al.,* 1992). Furthermore, airway inflammation and an influx of neutrophils appear to be important components of late asthmatic reactions to TDI in human subjects (Fabbri *et al.,* 1987). Collectively, these observations suggest that cell-mediated immunological mechanisms and ensuing inflammatory responses may play significant roles in the pathogenesis of isocyanate-induced asthma.

Animal models, such as the rat and guinea pig, have been used to characterize pulmonary reactions to TDI exposure. These animal models exhibit airway inflammation marked by the influx of polymorphonuclear leukocytes into extravascular airway regions following inhalation exposure to TDI (Duncan *et al.,* 1962; Gordon *et al.,* 1985; Hesbert *et al.,* 1991). In addition, TDI-induced airway hyperresponsiveness has been demonstrated in guinea pigs (Gordon *et al.,* 1985).

Nitric oxide (NO), an endogenous free radical which can be produced in the lung, has been implicated in the pathogenesis of asthma (Barnes and Liew, 1995). A pronounced increase in the concentration of exhaled NO appears to be a characteristic of asthmatic patients (Kharitonov *et al.,* 1994; Persson *et al.,* 1994). Although the cellular source of the exhaled NO has not been identified, an induction of NO

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synthesis in airway epithelial cells and some infiltrating inflammatory cells is reported to occur in established asthmatic individuals (Hamid *et al.*, 1993). It is also known that resident and recruited phagocytic cells in the lung, such as macrophages and polymorphonuclear leukocytes, are capable of producing high levels of NO upon appropriate stimulation (Blackford *et al.*, 1994; Huot and Hacker, 1990; Pendino *et al.*, 1993; Wizemann *et al.*, 1994). However, the production of NO in response to TDI exposure has not yet been documented.

The purpose of the present study was to examine early events following TDI exposure using the rat as the experimental animal model and to determine whether induction of NO synthesis is a component of this early pulmonary reaction to TDI.

MATERIALS AND METHODS

Animals and TDI exposure protocol. Mature male Sprague–Dawley rats (Hilltop, Scottdale PA) were used in experiments. The animals (350–400 g BW) were exposed to normal air or to aerosolized TDI (2 ppm) for 4 hr. The TDI exposure was conducted in a chamber constructed from a 10-gallon glass aquarium fitted with a stainless steel manifold for the delivery of TDI. Aerosolized TDI was generated by bubbling air, which had been particle- and oil-filtered and dehumidified through a 2,6 (20%)–2,4 (80%) TDI solution. The TDI concentration delivered to the chamber was monitored at 30-min intervals using spectrophotofluorometry (Rando *et al.*, 1989). The entire exposure was performed in an exhaust hood and the exhaust was first vented through hydrochloric acid to neutralize excess TDI. After the 4-hr exposure period, the animals were removed from the chamber and placed in a holding facility. This TDI dose and exposure protocol was chosen since it has been shown to produce effects limited to the trachea and lungs in guinea pigs, rabbits, and rats and results in an acute inflammatory reaction 1 day after exposure in these animal models (Duncan *et al.*, 1962).

Pulmonary function determinations. Animal breathing patterns were assessed before and after exposure to TDI with a bias flow ventilated whole body plethysmograph operated as a flow box. Box flow was measured just prior to the exposure period, just after the 4-hr exposure period, and 18 hr after the end of the exposure period for each TDI-exposed animal. Normally, flow into and out of the box is primarily due to differences in temperature and humidity between the gas that enters and leaves the lungs and to a lesser extent to the compression and rarefaction of the gas within the lungs. In this study, four indices of respiratory function based on box flow were used to evaluate the pulmonary response to TDI. The indices were (1) percentage changes in the frequency of breathing (f_B%), (2) percentage changes in tidal volume calculated from the integrated plethysmograph flow (V_T%), (3) the percentage change in the time to reach maximum expiratory flow/total expiratory time (T_{me}/T_E), and (4) the percentage change in the ratio of peak plethysmograph flow to the square root of the mean squared (RMS) plethysmograph flow (V_p/V_{RMS}).

Collection of bronchoalveolar and tissue samples. Bronchoalveolar lavage fluid and cells as well as tissue samples were harvested from the rats 20 hr after the end of the exposure period. The rats were first anesthetized with sodium pentobarbital (65 mg, ip; Butler, Columbus OH) and then exsanguinated by cutting the left renal artery. A tracheal cannula was inserted and an initial bronchoalveolar lavage was performed with 6 ml of cold Ca^{2+}/Mg^{2+}-free phosphate buffered saline (145 mM NaCl, 5 mM KCl, 9.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5.5 mM dextrose, pH 7.4). Subsequent lavages were performed with aliquots of up to 8 ml of phosphate buffered saline for a total volume of 74 ml. The initial and subsequent lavage samples were then centrifuged (500*g*, 5 min, 4°C). The supernatant from the initial lavage was processed for analyses of protein, lactate dehydrogenase (LDH), and nitrate/nitrite levels. The cell pellets from the initial and subsequent lavages were then combined and resuspended in a Hepes-buffered solution (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 1 mM $CaCl_2$, and 5.5 mM dextrose, pH 7.4). Differential cell counts were determined using an electronic cell counter equipped with a cell-sizing attachment (Coulter Model ZBI with a 256 Channelizer, Coulter Electronics, Hialeah, FL). The trachea and large bronchi were also dissected and immediately frozen in liquid nitrogen for subsequent extraction of RNA from these tissues.

Bronchoalveolar lavage fluid analyses. Protein levels in the initial acellular lavage fluid were analyzed according to the method of Bradford (1976; BioRad, Hercules CA) and values were expressed as μg/ml. LDH determinations were made using a commercially available kit (Sigma, St. Louis MO) and values were expressed as international units (IU)/ml. A fluorometric assay was used to index nitrate and nitrite (NO_x) levels in the lavage fluid (Misko *et al.*, 1993). This latter procedure was followed as described except that, for the conversion of nitrate to nitrite, the incubation period with the nitrate reductase enzyme was extended to 20 min. NO_x values in lavage fluid were expressed as micromolars.

Determination of alveolar macrophage reactive species production. The production of reactive species from alveolar macrophages was determined using luminol-enhanced chemiluminescence under basal (resting) and stimulated (unopsonized zymosan; 2 mg/ml) conditions. It should be noted that the use of unopsonized zymosan as a stimulant selectively permits evaluation of reactive species production from rat alveolar macrophages since these cells, in contrast to polymorphonuclear leukocytes, respond to this stimulant without the need for opsonization (Castranova *et al.*, 1987). The NO synthase (NOS)-dependent component was assessed by measuring the amount of the unopsonized zymosan-stimulated chemiluminescence which could be inhibited by $N\omega$-nitro-L-arginine methyl ester (L-NAME, 1 mM, an inhibitor of NO synthase), as described previously in detail (Blackford *et al.*, 1994).

Measurement of NO production by cultured bronchoalveolar lavage cells (BALC). Following the harvest of BALC and cell differential determinations, a portion of the cells was resuspended in medium (Minimum Essential Medium, BioWhittaker, Inc., Walkersville, MD) supplemented with 1 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 100 μg/ml kanamycin with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and 10 mM Hepes (Sigma Chemical Co.) before culture at 37°C in a humidified atmosphere (95% air, 5% CO_2). The cells were plated on the basis of 1×10^6 alveolar macrophages/ml/well in 24-well plates. The cultured cell population was enriched by adherence to plastic for 1 hr before nonadherent cells were removed and fresh media were replaced with or without L-NAME (1 mM), L-arginine (10 mM), or L-NAME plus L-arginine. After a further 18 hr of culture, the media were harvested for nitrite determinations using the Greiss reaction (Green *et al.*, 1982). The cultured cells were then washed with phosphate buffered saline (PBS) and lysed in 2% Triton–PBS. After transfer of the lysed cell mixture to a tube, the wells were washed with an equal volume of PBS which was combined with the initial lysate. Cellular protein content was then determined using the method of Bradford (1976; BioRad, Hercules, CA). To assess the content of the adherent cell population, a portion of cultured cells from each animal was harvested after the 1 hr adherence period using a Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (137 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.5 mM dextrose, pH 7.4). The harvested cells were then deposited on slides using a cytocentrifuge (Shandon Scientific, London, England) and stained with a Wright stain (Volu-Sol, Henderson, NV). Differential cell counts of the adherent cell population were then determined using light microscopy.

RNA analyses. Total cellular RNA was extracted using a guanidinium thiocyanate-based procedure (Chomczynski and Sacchi, 1987; Huffman *et*

TABLE 1
Indices of Pulmonary Function in Rats before and after Exposure to Aerosolized TDI

	Preexposure (%)	0 hr Postexposure (%)	18 hr Postexposure (%)
fB	100 ± 5.7	42.2 ± 5*	45.1 ± 3.3*
TV	100 ± 11.1	147 ± 13.9*	146 ± 18.5*
T_{me}/T_e	100 ± 10.2	46.4 ± 8.3*	91.5 ± 38.0
V_p/V_{rms}	100 ± 11.1	176.9 ± 16.7*	145 ± 19.4*

Note. Pulmonary function was measured just prior to (preexposure), immediately after (0 hr postexposure), or 18 hr after (18 hr postexposure) a 4-hr exposure to 2 ppm TDI. Results are presented as percentages ($X \pm$ SEM) of the mean values measured just prior to the exposure period, $N = 6$.

* $p \leq 0.05$ vs preexposure values.

TABLE 2
Differential Analysis of the BALC Population from Rats Exposed to Air or TDI

Treatment	Total cells	Alveolar macrophages	Red blood cells	PMNs and lymphocytes
Air ($N = 20$)	15.4 ± 1.5	13.4 ± 1.2	0.5 ± 0.1	0.6 ± 0.2
TDI ($N = 19$)	27.5 ± 2.8*	18.2 ± 1.6*	2.6 ± 0.6*	4.1 ± 0.9*

Note. Bronchoalveolar lavage cell (BALC) populations were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI. Results (10^{-6} cells/rat) are presented as the $X \pm$ SEM; N, number of rats.

*$p \leq 0.05$ vs air.

al., 1992) and quantified spectrophotometrically (E260 = 40 μg/ml/OD). The RNA was then size-fractioned on a 1.5% agarose gel containing 2 M formaldehyde and blotted onto a Duralose membrane (Stratagene, La Jolla, CA) using capillary-mediated bulk flow transfer. The estimated amount of total RNA analyzed for each cell or tissue sample was as follows: BALC, 5 μg; trachea, 7 μg; and large bronchi, 10 μg. Inducible (i) NOS mRNA was indexed using a ^{32}P nick-translated double-stranded cDNA probe derived from a plasmid containing a 4100-bp cDNA fragment for murine macrophage iNOS (Lowenstein *et al.,* 1992) which was a gift of Dr. S. H. Snyder and Dr. C. Lowenstein (Johns Hopkins University, Baltimore MD). The cDNA probe for use was amplified from the plasmid construct by the polymerase chain reaction technique (Gene Amp DNA Amplification Kit, Perkin Elmer Cetus, Norwalk, CT) using 20-bp synthetic DNA oligonucleotide primers (Blackford *et al.,* 1994). Northern blot hybridization for iNOS mRNA was performed using Quikhyb hybridization buffer (Stratagene) according to the manufacturer's instructions. Subsequently, the blot was boiled in RNase-free distilled water for 5 min to remove hybridized probe and the amount of 28S ribosomal RNA loaded onto the blot was assessed using the hybridization protocol of Barbu and Dautry (1989). The relative optical densities of the hybridization signals were quantified using a computerized image analysis system (Optimas, Edmonds WA). Individual iNOS mRNA sample signal levels were then divided by the corresponding 28S rRNA sample signal level to normalize values for the amount of RNA loaded.

Statistics. Differences between the groups were determined using independent *t* tests (SAS Institute, 1985). Significance was set at $p \leq 0.05$.

RESULTS

Decreases in breathing rate (f_B) and increases in tidal volume (TV) occurred following TDI inhalation (Table 1). These alterations were observed just after the 4-hr exposure period (0 hr post) and persisted for at least 18 hr (18 hr post). Indices of maximum flow/total expiratory time (Tme/Te) were decreased at 0 hr post-TDI exposure but were not significantly different from preexposure values at 18 hr postexposure (Table 1). However, the peak expiratory plethysmograph flow/RMS of plethysmograph flow (V_p/R_{RMS}) remained elevated at both postexposure times studied as the flow pattern became more pulsatile in nature (Table 1).

Differential analysis of the BALC population is consistent with the occurrence of an acute inflammatory response following the inhalation of TDI since elevated numbers of alveolar macrophages, red blood cells, and the combined polymorphonuclear leukocyte (PMN) and lymphocyte cell population were observed 20 hr postexposure, i.e., after the end of the exposure period (Table 2). In the air-exposed (control) rats, approximately equal percentages of lymphocytes and PMNs were observed, whereas PMNs predominated in this cell population after TDI exposure (71 ± 2%). In the initial acellular lavage fluid sample, NO_x levels were elevated in TDI-exposed rats (Table 3). However, protein levels and LDH activity in these same samples were not significantly elevated in TDI-exposed rats compared to air-exposed rats.

The basal production of free radicals by BALC, indexed as resting chemiluminescence, was increased following TDI exposure (Fig. 1). In addition, free radical production by alveolar macrophages in response to unopsonized zymosan was increased following TDI by 184% above the levels measured with cells from air-control rats (Fig. 1). Furthermore, a significant amount of free radical production by alveolar macrophages was inhibited by L-NAME and was thus dependent upon NOS activity (Fig. 2). TDI exposure was also associated with a significant increase in BALC steady-state iNOS mRNA levels (Fig. 3). However, no detectable signal

TABLE 3
NO_x, Protein Levels, and LDH Activity in the Initial Acellular Bronchoalveolar Lavage Fluid from Air or TDI-Exposed Rats

	NO_x (μM)	Protein (μg/ml)	LDH (IU/ml)
Air ($N = 5$)	4.7 ± 0.2	333 ± 41	41.0 ± 3.9
TDI ($N = 5$)	7.7 ± 1.2*	426 ± 80	50.2 ± 10.8

Note. Acellular lavage fluid samples were collected 20 hr after the end of a 4-hr exposure to 2 ppm TDI. Results are presented as the $X \pm$ SEM; N, number of rats.

* $p \leq 0.05$ vs air.

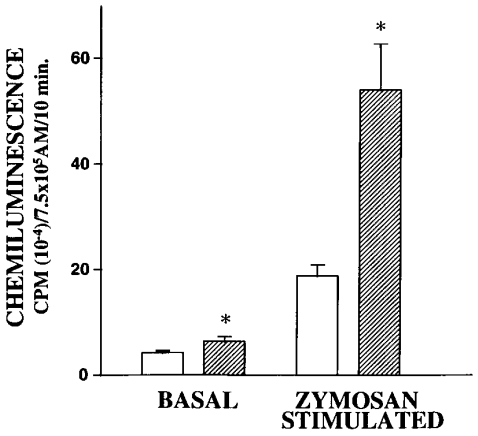

FIG. 1. Basal and unopsonized zymosan-stimulated chemiluminescence in BALC from air (open bars) or TDI-treated (hatched bars) rats. Cells were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI. Results are presented as the $\bar{X} \pm$ SEM; $N = 9$ for the air and 8 for the TDI group. *$p \leq 0.05$ vs air.

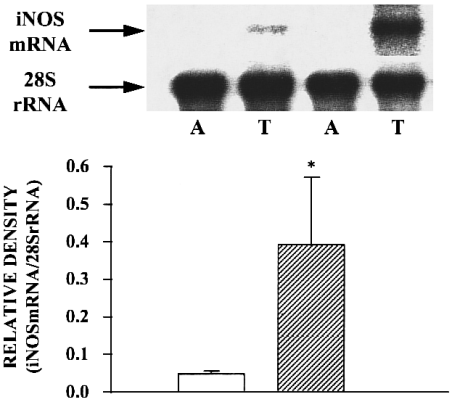

FIG. 3. (Top) Northern blot analysis of iNOS mRNA and 28S rRNA in BALC obtained from representative air (A) or TDI (T)-treated rats. Cells were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI. (Bottom) Relative amounts of iNOS mRNA in BALC harvested from air (open bar) or TDI-exposed rats (hatched bar). Results are presented as the $\bar{X} \pm$ SEM; $N = 6$ per group. *$p \leq 0.05$ vs air.

for iNOS mRNA was observed in tracheal or large bronchiolar tissue extracts (data not presented).

Following TDI inhalation, harvested BALC produced substantially more NO in culture (measured as media nitrite levels) than controls (Fig. 4). Furthermore, a significant inhibition of NO production by BALC from TDI-treated animals was observed in the presence of a NOS inhibitor (L-NAME), and this inhibition was partially reversed by the addition of excess L-arginine (Fig. 5). The adherent BALC from air and TDI-treated rats contained similar percentages of alveolar macrophages, PMNs, eosinophils, and red blood cells. However, an increased number of lymphocytes was observed in the adherent BALC population from TDI-treated rats (Table 4). Interestingly, the amount of nitrite detected in the media was linearly related to the number of lymphocytes in culture (Fig. 6), whereas no significant correlations were noted for other cell types (data not shown).

DISCUSSION

In the past, it has been shown that mice and guinea pigs have a decreased breathing rate following exposure to TDI (Karol *et al.*, 1980; Sangha and Alarie, 1979). The rat responded similarly in this study as breathing rates fell immediately postexposure to 42.2% of their preexposure values and remained at 45% of their preexposure values until 18

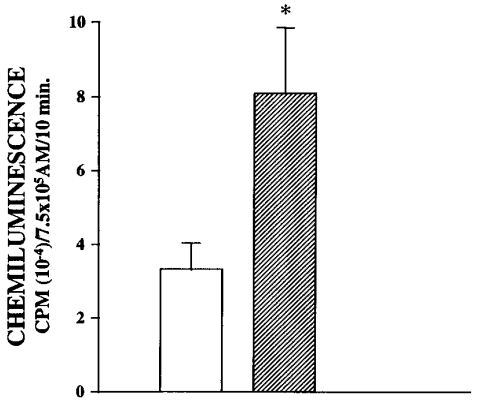

FIG. 2. NOS-dependent chemiluminescence from alveolar macrophages after *in vivo* exposure to air (open bar) or aerosolized TDI (hatched bar). Cells were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI. NOS-dependent chemiluminescence was calculated as the amount of the response inhibited by L-NAME, an inhibitor of the NOS enzyme. Results are presented as the $\bar{X} \pm$ SEM; $N = 6$ for the air and 4 for the TDI group. *$p \leq 0.05$ vs air.

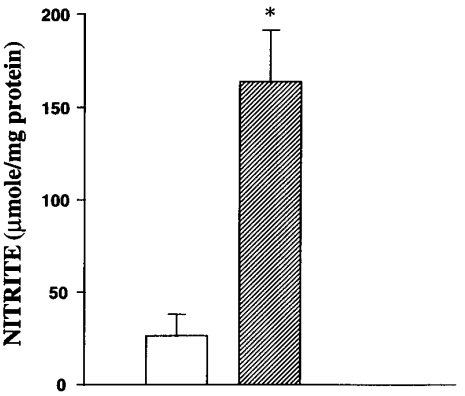

FIG. 4. *In vitro* NO production (measured as nitrite) by adherent BALC following *in vivo* exposure to air (open bar) or TDI (hatched bar). Cells were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI and cultured *in vitro* as described under Materials and Methods. Results are presented as the $\bar{X} \pm SEM$; $N = 5$ per group. *$p \leq 0.05$ vs air.

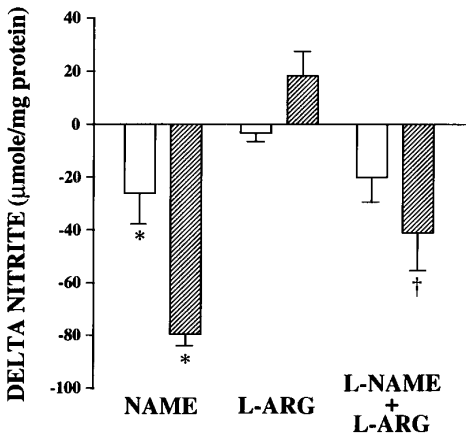

FIG. 5. Effect of L-NAME and/or L-arginine (Arg) on *in vitro* NO production (measured as nitrite) by adherent BALC following *in vivo* exposure to air (open bars) or TDI (hatched bars). Cells were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI and cultured *in vitro* as described under Materials and Methods. Results are expressed as the difference (delta nitrite; $\bar{X} \pm$ SEM) in nitrite levels from those in adherent BALC conditioned media alone for each treatment; $N = 5$ per group. *$p \leq 0.05$ vs media alone. †$p \leq 0.05$ vs L-NAME alone.

hr postexposure. The percentage change in tidal volume, $V_{T\%}$, increased in exposed animals to 140% of their preexposure values as breathing rate decreased but the increase was not sufficient to prevent a decrease in minute ventilation. $V_{T\%}$ remained elevated at 18 hr postexposure. It has been shown in humans (Morris *et al.,* 1995) that the time to reach maximum flow/total expiratory time (T_{me}/T_E) correlates with specific airway conductance. In this study, (T_{me}/T_E) decreased immediately following exposure to TDI which is consistent with an increase in specific airway resistance. Interestingly, (T_{me}/T_E) appeared to recover to control values at 18 hr postexposure, although the variability of the measurement at this time was high, while TV and f_B did not recover. Finally, peak plethysmograph flow divided by the RMS value of plethysmograph flow ($\dot{V}_p/V_{RMS}$), defined as the crest factor, was used as an index of the shape of the plethysmograph flow signal with respect to time. It was found that the crest factor increased significantly following TDI exposure and remained elevated at 18 hr postexposure. The increase in the crest factor reflected changes in the airflow pattern which became more pulsatile as peak flow increased to a much greater extent than the effective or RMS flow.

In addition to alterations in pulmonary function, exposure of this rat animal model to TDI was associated with an inflammatory response. This was evidenced by increased numbers of red blood cells, lymphocytes, and PMNs harvested by bronchoalveolar lavage from the lungs of exposed animals. Although protein levels and LDH activity in the lavage fluid from TDI-exposed rats were not significantly different from those in control animals in the present study, values from the TDI-exposed rats were more variable than those from control animals. In fact, the protein and LDH values within the TDI group were positively correlated ($r = 0.98$, $n = 5$; $p < 0.05$), suggesting that, at the time studied, overt disruption of the alveolar air–blood barrier and cell damage was occurring in some TDI animals. In addition to the apparent occurrence of airway inflammation, stimulated free radical production from pulmonary phagocytes, as indexed using luminol-enhanced chemiluminescence, was increased following TDI exposure. Collectively, these findings are similar to those found during lung oxidant damage states which can occur following exposure to high oxygen tension (Dedhia *et al.,* 1993), ozone (Esterline *et al.,* 1989), or silica (Blackford *et al.,* 1994).

As is the case for oxidant lung damage following ozone (Pendino *et al.,* 1993) or silica exposure (Blackford *et al.,* 1994), acute exposure to TDI was also associated with increased NO production. In the present study, a generalized increase in lung NO production following acute TDI inhalation was suggested by the observed elevation in NO_x levels in initial bronchoalveolar lavage fluid samples. The increased NO production appeared to derive primarily from an upregulation of the inducible NOS isoform in bronchoalveolar lavage cells. This is supported by the observation of increases in steady-state iNOS mRNA levels in and *ex vivo* production of nitrite by bronchoalveolar lavage cells from TDI-exposed rats. Alveolar macrophages appear to be one source of NO under these conditions. This is supported by our finding that a NOS inhibitor attenuated free radical production from alveolar macrophages. However, we cannot

TABLE 4
Differential Analysis of the Cultured BALC Population from RATS Exposed to Air or TDI (% of Cells)

Treatment	AM	LYM	PMN	EOS	RBC
Air ($N = 5$)	80.2 ± 6.9	2.0 ± 0.1	1.6 ± 0.8	0.2 ± 0.2	16.8 ± 7.0
TDI ($N = 5$)	71.8 ± 6.7	8.8 ± 2.6*	3.8 ± 2.1	2.0 ± 1.3	13.6 ± 5.5

Note. Results are presented as the $X \pm$ SEM; *N*, number of rats. AM, alveolar macrophages; LYM, lymphocytes; PMN, polymorphonuclear leukocytes; EOS, eosinophils; RBC, red blood cells.

* $p \leq 0.05$ vs air.

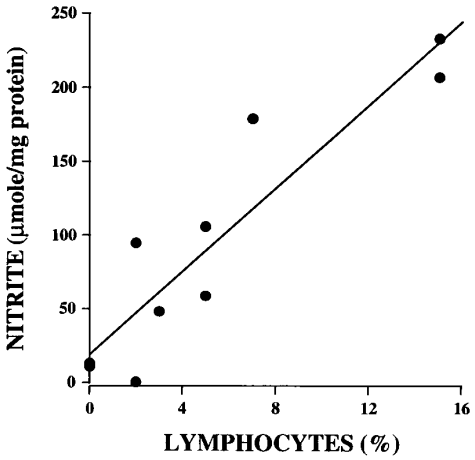

FIG. 6. Linear regression plot of the number of lymphocytes in the cultured cell populations vs *in vitro* NO production (measured as nitrite) for BALC from both air and TDI-exposed rats. Cells were harvested from the rats 20 hr after the end of a 4-hr exposure to 2 ppm TDI and cultured *in vitro* as described under Materials and Methods. The correlation coefficient was 0.918.

exclude the possibility that PMNs are also a source of the increased NO generated following acute TDI exposure. In the case of acute exposure to silica, iNOS mRNA levels are increased in fractions of bronchoalveolar lavage cells enriched for either the alveolar macrophage or PMN cell population (Blackford *et al.,* 1994).

Evidence is accumulating to suggest an important involvement of cell-mediated immune mechanisms following TDI exposure. It has been speculated that low-molecular-weight chemical agents, such as TDI, can interact cognitively with T lymphocytes and results in cell activation and the release of cytokines (Bernstein and Bernstein, 1994). We found that nitrite levels produced by adherent BALC correlated significantly with the number of lymphocytes in culture. Interferon gamma, which can be released from activated lymphocytes, is a potent stimulator of NO production by alveolar macrophages (Jorens *et al.,* 1991). Thus, an initial exposure to TDI may result in a pulmonary inflammatory response, an increased number of activated lymphocytes in the airways, the release of interferon gamma, and production of high levels of NO from alveolar macrophages.

The role(s) that NO may play in the body responses to TDI exposure have not been fully defined in the present study. Our findings suggest that the NO produced by alveolar macrophages contributes to the pool of free radicals generated by these cells following TDI exposure. This is supported by our observation that a NOS inhibitor significantly attenuated alveolar macrophage-dependent chemiluminescence responses. NO could also possibly modulate inflammatory responses induced by acute exposure to TDI. Following an acute exposure to TDI, an influx of eosinophils into the airway mucosa has been observed (Gordon *et al.,* 1985). Recently, NO has been shown to prolong eosinophil survival (Beauvais *et al.,* 1995). This could be one mechanism whereby an increase in NO synthesis could contribute to an amplification of the inflammatory reaction following TDI exposure. In addition, NO has the potential to affect important aspects of pulmonary function. For instance, NO has been implicated in the modulation of bronchial reactivity. Low levels of NO, produced by constitutive isoforms of the NOS enzyme, appear to counteract airway constriction but there is a distinct possibility that high levels of NO may enhance bronchial reactivity via a downregulation of constitutive NO production in airway cells (Nijkamp and Folkerts, 1994). However, whether the NO which is released from lung phagocytes following acute exposure to TDI participates in any of the observed changes in pulmonary function measurements observed in the present study is not known at the present time.

In summary, we have found that acute exposure to TDI fumes in a rat animal model is associated with pulmonary function changes and evidence of airway inflammation. Such exposure was also associated with an induction of iNOS activity in bronchoalveolar lavage cells. The specific contribution of elevated levels of NO to the pulmonary function and inflammatory changes which were observed following TDI inhalation and the possible role of NO in the development of isocyanate-induced asthma will require further time-course, dose–response, and *in vivo* studies using NOS inhibitors.

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REFERENCES

Barbu, V., and Dautry, F. (1989). Northern blot normalization with a 28S rRNA oligonucleotide probe. *Nucleic Acids Res.* **17,** 7115.

Barnes, P. J., and Liew, F. Y. (1995). Nitric oxide and asthmatic inflammation. *Immunol. Today* **16,** 128–130.

Baur, X., Marek, W., Ammon, J., Czuppon, A. B., Marczynski, B., Raulf-Heimsoth, M., Roemmelt, H., and Fruhmann, G. (1994). Respiratory and other hazards of isocyanates. *Int. Arch. Occup. Environ. Health* **66,** 141–152.

Beauvais, F., Michel, L., and Dubertret, L. (1995). The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. *FEBS Lett.* **361,** 229–232.

Bentley, A. M., Maestrelli, P., Saetta, M., Fabbri, L. M., Robinson, D. S., Bradley, B. L., Jeffery, P. K., Durham, S. R., and Kay, A. B. (1992). Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. *J. Allergy Clin. Immunol.* **89,** 821–829.

Bernstein, J. A., and Bernstein, I. L. (1994). Clinical aspects of respiratory hypersensitivity to chemicals. In *Immunotoxicology and Immunopharmacology* (J. H. Dean, M. I. Luster, A. E. Munson, and I. Kimber, Eds.), pp. 617–642. Raven Press, New York.

Bignon, J. S., Aron, Y., Ju, L. Y., Kopferschmitt, M. C., Garnier, R., Mapp, C., Fabbri, L. M., Pauli, G., Lockhart, A., Charron, D., and Swierczewski, E. (1994). HLA Class II alleles in isocyanate-induced asthma. *Am. J. Respir. Crit. Care Med.* **149,** 71–75.

Blackford, J. A., Antonini, J. M., Castranova, V., and Dey, R. D. (1994). Intratracheal instillation of silica up-regulates inducible nitric oxide synthase gene expression and increases nitric oxide production in alveolar macrophages and neutrophils. *Am. J. Respir. Cell Mol. Biol.* **11,** 426–431.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72,** 248–254.

Castranova, V., Lee, P., Ma, J. Y. C., Weber, K. C., Pailes, W. H., and Miles, P. R. (1987). Chemiluminescence from macrophages and monocytes. In *Cellular Chemiluminescence* (K. Van Dyke and V. Castranova, Eds.), pp. 3–19. CRC Press, Boca Raton, FL.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Dedhia, H. V., Ma, J. Y. C., Vallyathan, V., Dalal, N. S., Banks, D., Flink, E. B., Billie, M., Barger, M. W., and Castranova, V. (1993). Exposure of rats to hyperoxia: Alteration of lavagate parameters and macrophage function. *J. Toxicol. Environ. Health* **40,** 1–13.

Duncan, B., Scheel, L. D., Fairchild, E. J., Killens, R., and Graham, S. (1962). Toluene diisocyanate inhalation toxicity: Pathology and mortality. *Am. Ind. Hyg. Assoc. J.* **23,** 447–456.

Esterline, R. L., Bassett, D. J. P., and Trush, M. A. (1989). Characterization of the oxidant generation by inflammatory cells lavaged from rat lungs following acute exposure to ozone. *Toxicol. Appl. Pharmacol.* **99,** 229–239.

Fabbri, L. M., Boschetto, P., Zocca, E., Milani, G., Pivirotto, F., Plebani, M., Burlina, A., Licata, B., and Mapp, C. E. (1987). Bronchoalveolar neutrophilia during late asthmatic reactions induced by toluene diisocyanate. *Am. Rev. Respir. Dis.* **136,** 36–42.

Fabbri, L. M., Picotti, G., and Mapp, C. E. (1991). Late asthmatic reactions, airway inflammation and chronic asthma in TDI sensitized subjects. *Eur. Respir. J.* **4**(Suppl. 13), 136s–138s.

Gordon, T., Sheppard, D., McDonald, D. M., Distefano, S., and Scypinski, L. (1985). Airway hyperresponsiveness and inflammation induced by toluene diisocyanate in guinea pigs. *Am. Rev. Respir. Dis.* **132,** 1106–1112.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [^{15}N]-nitrate in biological fluids. *Anal. Biochem.* **126,** 131–138.

Hamid, Q., Springall, D. R., Riveros-Moreno, V., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S., and Polak, J. M. (1993). Induction of nitric oxide synthase in asthma. *Lancet* **342,** 1510–1513.

Hesbert, A., Ban, M., Bonnet, P., Simon, P., Bottin, M. C., Lemonnier, M., and de Ceaurriz, J. (1991). Interdependence of polymorphonuclear neutrophils and macrophages stained for N-acetyl-β-glucosaminidase in lavage effluents from toluene diisocyanate-exposed rat lungs. *Tox. Lett.* **56,** 53–59.

Huffman, L., Michalkiewicz, M., Vrana, K. E., and Hedge, G. A. (1992). Expression of preproNPY and precursor VIP mRNAs in rats under hypo- or hyperthyroid conditions. *Mol. Cell Neurosci.* **3,** 418–424.

Huot, A. E., and Hacker, M. P. (1990). Role of reactive nitrogen intermediate production in alveolar macrophage-mediated cytostatic activity induced by bleomycin lung damage in rats. *Cancer Res.* **50,** 7863–7866.

Jorens, P. G., Van Overveld, F. J., Bult, H., Vermeire, P. A., and Herman, A. G. (1991). L-arginine-dependent production of nitrogen oxides by rat pulmonary macrophages. *Eur. J. Pharmacol.* **200,** 205–209.

Karol, M. H., Dixon, C., Brady, M., and Alarie, Y. (1980). Immunologic sensitization and pulmonary hypersensitivity by repeated inhalation of aromatic isocyanates. *Toxicol. Appl. Pharmacol.* **53,** 260–270.

Kharitonov, S. A., Yates, D., Robbins, R. A., Logan-Sinclair, R., Shinebourne, E. A., and Barnes, P. J. (1994). Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* **343,** 133–135.

Lowenstein, C. J., Glatt, C. S., Bredt, D. S., and Snyder, S. H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. USA* **89,** 6711–6715.

Misko, T. P., Schilling, R. J., Salvemini, D., Moore, W. M., and Currie, M. G. (1993). A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* **214,** 11–16.

Morris, M. J., Madqwick, R. G., and Lane, D. J. (1995). Analysis of tidal expiratory flow pattern in the assessment of histamine-induced bronchoconstriction. *Thorax* **50,** 346–352.

Nijkamp, F. P., and Folkerts, G. (1994). Nitric oxide and bronchial reactivity. *Clin. Exp. Allergy* **24,** 905–914.

Pendino, K. J., Laskin, J. D., Shuler, R. L., Punjabi, C. J., and Laskin, D. L. (1993). Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. *J. Immunol.* **151,** 7196–7205.

Persson, M. G., Zetterström, O., Agrenius, V., Ihre, E., and Gustafsson, L. E. (1994). Single-breath nitric oxide measurements in asthmatic patients and smokers. *Lancet* **343,** 146–147.

Rando, R. J., Hammad, Y. Y., and Chang, S. (1989). A diffusive sampler for personal monitoring of toluene diisocyanate (TDI) exposure. I. Design of the dosimeter. *Am. Ind. Hyg. Assoc. J.* **50,** 1–7.

Rosenstock, L., and Cullen, M. R. (1994). *Textbook of Clinical Occupational and Environmental Medicine.* Saunders, Philadelphia.

Sangha, G. K., and Alarie, Y. (1979). Sensory irritation by toluene diisocyanate in single and repeated exposures. *Toxicol. Appl. Pharmacol.* **50,** 533–547.

SAS Institute (1985). *SAS User's Guide: Basics.* SAS Institute, Cary, NC.

Wizemann, T. M., Gardner, C. R., Laskin, J. D., Quinones, S., Durham, S. K., Goller, N. L., Ohnishi, S. T., and Laskin, D. L. (1994). Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J. Leukocyte Biol.* **56,** 759–768.