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MEASUREMENT OF THE RELEASE OF INFLAMMATORY MEDIATORS FROM RAT ALVEOLAR MACROPHAGES AND ALVEOLAR TYPE II CELLS FOLLOWING LIPOPOLYSACCHARIDE OR SILICA EXPOSURE: A COMPARATIVE STUDY

R. S. Kanj,[1,2] J. L. Kang,[3] V. Castranova[1,2]

[1]National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, West Virginia, USA,
[2]West Virginia University School of Medicine, Department of Physiology and Pharmacology, Morgantown, West Virginia, USA, and
[3]Department of Physiology, Division of Cell Biology, Ewha Womans University, College of Medicine, Seoul, South Korea

Evidence suggests that hyperproduction of reactive oxidants and inflammatory mediators plays a critical role in adverse pulmonary responses to silica or lipopolysaccharide (LPS). The objective of this study was to evaluate the role of alveolar macrophages (AM) and alveolar epithelial type II cells (TII) in the induction of pulmonary inflammation and injury in response to these pulmonary toxicants. To support this objective, the release of several inflammatory mediators from primary rat AMs and TII cells was compared under similar culture and exposure conditions. The responsiveness of RLE-6TN, a rat type II cell line, was also compared to primary rat TII cells under the same culture conditions, following exposure to LPS or silica. The following findings were made. (1) Although AMs were generally found to release more inflammatory mediators than TII cells following LPS or silica exposure, primary TII cells clearly produced significant levels of mediators that could be capable of contributing considerably to lung inflammation and injury. (2) Since the responses of the RLE-6TN cell line to LPS or silica exposure were generally considerably less intense and required higher concentrations of stimulant than those measured in primary rat TII cells, RLE-6TN cells may not be an ideal substitute for primary TII cells in studying pulmonary inflammation. (3) LPS was more potent than silica in inducing inflammatory cytokine release from the three cell types. However, compared to LPS, silica exhibited equal or greater potency as an inducer of cellular oxidant generation, especially from primary TII cells.

The lung can be susceptible to disease because of its direct contact with the outside environment. Alveolar macrophages (AMs) phagocytize and clear inhaled microbes and particles, thus playing an important role in lung defense. However, when exposed to high dust burdens, AMs secrete reactive products and cytokines, which can produce lung inflammation and damage. This excessive inflammation is believed to play a critical role in occupational lung

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Address correspondence to Vincent Castranova, PhD, NIOSH, 1095 Willowdale Road, M/S 2015, Morgantown, WV 26505, USA, E-mail: vicl@cdc.gov

diseases (Zhang et al., 2000). Recent evidence suggests that alveolar type II epithelial cells (TII) may also contribute to lung inflammation (Blau et al., 1994; Crippen et al., 1995; Stringer & Kobzik, 1998; Finkelstein et al., 1997).

Stimulated AMs can produce reactive oxygen species through a respiratory burst, which directly contributes to killing microorganisms (Johnston, 1978). When incapable of killing foreign organisms by themselves, AMs recruit more phagocytic cells to the area by releasing chemokines, such as macrophage inflammatory protein-2 (MIP-2), which act to attract polymorphonuclear leukocytes (PMNs), monocytes, and other cells from the pulmonary capillaries to the alveolar airspaces (Nielson et al., 1995). TII cells comprise about 4% of the alveolar epithelial surface but constitute approximately 60% of the alveolar epithelial cell population in number (Crapo et al., 1983). Like AMs, TII cells play a major antiviral role by producing interferon upon viral exposure (Hahon & Castranova, 1989). They can also produce $NO^{\bullet}$, ROS, and cytokines in response to different stimulants (Punjabi et al., 1994; Blau et al., 1994) and thus contribute to cell recruitment in response to an inflammatory agent (Crippen et al., 1995). Like primary TII cells, rat lung epithelial-T-antigen negative cells (RLE-6TN), derived from rat alveolar TII cells by Driscoll et al. (1995a), contain lamellar bodies for surfactant storage and exhibit the ability to produce inflammatory mediators when stimulated (Tao & Kobzik, 2003; Driscoll et al., 2001). However, unlike primary TII cells, RLE-6TN cells maintain their morphology and the ability to produce surfactant for several days in culture.

Of major importance to lung pathology are two main airborne contaminants: silica and lipopolysaccharide (LPS). Silica or silicon dioxide (SiO_2), mostly in it quartz form, is a common mineral found in the earth's crust (Peters, 1987). Crystalline silica can directly produce cellular toxicity. It can also initiate an inflammatory response that could include increased oxidant production and proinflammatory mediator secretion as a result of the respiratory burst and nuclear transcription factor-κB (NF-κB) activation in target and recruited cells (Kang et al., 2000; Shi et al., 1998; Vallyathan & Shi, 1997). LPS is a lipid-carbohydrate component of endotoxin, which is found in the outer cell membrane complex of gram-negative bacteria (Mayeux, 1997). Endotoxin, including the active LPS component, can be associated with indoor or outdoor organic dust particles if humidity and temperature conditions are favorable to microbial growth. Although recognition and intracellular signalling can differ with the target cell and LPS receptor type, the result of this binding with most target cells is usually the stimulation of an inflammatory response (Blau et al., 1994; Mayeux, 1997).

Among the inflammatory mediators of interest in this study are oxygen species, $NO^{\bullet}$, and various chemokines and cytokines. Oxygen metabolites can be very toxic to cells if produced excessively. Such oxygen metabolites are termed reactive oxygen species (ROS) and include the hydroxyl radical ($OH^{\bullet}$), superoxide radical (O_2^{-}), and hydrogen peroxide (H_2O_2) (Castranova,

1998; Shi et al., 1998). When AMs are exposed to silica, high levels of ROS can produce direct cell damage by oxidizing lipids (Dalal et al., 1990). Silica or LPS can also produce direct cell and/or tissue damage by inducing cellular production of other proinflammatory mediators (Castranova et al., 1996; Castranova, 2000). Similar to ROS, reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$), nitrite (NO_2), and peroxynitrite ($ONOO^{\bullet}$), are vital for normal cell functioning but can be toxic if produced excessively by stimulated cells (Rao, 2000). Similar to ROS, RNS are generally very unstable and highly reactive. They can induce direct or indirect cell and/or tissue damage by producing oxidizing and/or nitrating cellular damage (Jorens et al., 1993). RNS may also induce production of other proinflammatory mediators regulated by activator protein-1 (AP-1) and nuclear transcription factor-κB (NF-κB) (Comhair & Erzurum, 2002; Chen & Castranova, 2004). As for tumor necrosis factor-alpha (TNF-α), this cytokine has been proposed to be very important during inflammatory processes, especially in the recruitment and activation of inflammatory cells, cell proliferation, and extracellular matrix protein synthesis (Driscoll et al., 1995b; Driscoll, 1996). It can stimulate the production of other proinflammatory cytokines, such as MIP-2 (Driscoll et al., 1996; Barrett et al., 1999a). IL-1β is an early-response proinflammatory cytokine with many similar effects as TNF-α, while IL-6 is a proinflammatory cytokine associated with fibrogenic activity (Driscoll, 1996; Lohmann-Matthes et al., 1994).

Hyperproduction of reactive oxidants and inflammatory mediators has been shown to play a role in adverse pulmonary responses to silica or LPS (Huffman et al., 1998; Ning et al., 2000). It has been proposed that AMs and TII cells are involved in the production of these reactive and inflammatory products. However, no data were found that compared the release of several inflammatory mediators from AMs and TII cells under the same culture conditions, same exposures, and equivalent cell concentrations. Further, no literature was found that compared the responsiveness of a rat type II cell line to primary rat TII cells under similar culture conditions and following exposure to LPS and silica. Therefore, the main objective of this study was to clarify the roles of AMs and TII cells in lung pathology resulting from LPS or silica exposures. This objective would address the following unresolved questions. (1) What is the relative contribution of AMs and TII cells to the production of oxidants, chemokines, and cytokines in response to LPS and silica? The answer to this question would help in revealing which cell type contributes more inflammatory mediators following lung injury produced by LPS or silica. It would also help reveal which agent is more potent in stimulating inflammatory mediator release and hence inducing more severe lung injury. (2) Is the responsiveness of RLE-6TN cell lines and primary TII cells similar? The answer to this question would determine if the RLE-6TN cell line could be used in research in place of primary TII cells, since the TII isolation technique consumes animals, material, and time, and is thus costly.

METHODS

Animal Model

All primary cells were collected from naïve male Sprague-Dawley rats [Hla: (SD) CVF] weighing 250–300 g (Hilltop Lap Animals, Scottdale, PA). The animals were housed in a AAALAC-accredited, specific-pathogen-free, environmentally controlled facility. The rats were monitored to be free of endogenous viral pathogens, parasites, mycoplasms, *Helicobacter*, and CAR *Bacillus.* Rats were acclimated for at least 5 d before use, and were housed in ventilated cages that were provided with HEPA-filtered air, with Alpha-Dri virgin cellulose chips and hardwood Beta-chips used as bedding. The rats were maintained on ProLaB 3500 diet and tap water, both of which were provided ad libitum.

AM Isolation by BAL

Rats were anesthetized intraperitoneally with sodium pentobarbital (65 mg/kg body weight), and the renal artery was cut to exsanguinate the animals. The trachea was cannulated with an 18-gauge needle. While massaging the lungs, aliquots of a solution of ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS: 150 m*M* NaCl, 1.9 m*M* KH_2PO_4, 9.35 m*M* Na_2PO_4, pH 7.3–7.5) were repetitively introduced and withdrawn from the lungs through a 10-ml syringe attached to the tracheal cannula. The first lavage used 2 ml/100g body, while subsequent lavage volumes were 8 ml each. The total volume of bronchialveolar lavage (BAL) fluid collected during this procedure was approximately 80 ml. This fluid was then centrifuged at $1000 \times g$ for 10 min, and the supernatant discarded and the pellet resuspended in 1 ml Ham's F-12 medium (pH 7.07–7.40) supplemented with 1 m*M* glutamine, 100 U/ml of penicillin–streptomycin, and 10% fetal bovine serum (FBS). AMs were counted using an electronic cell counter (Coulter Multisizer II, Coulter Electronics, Hialeah, FL) equipped with a 256-channel cell sizer. AMs were identified as having a diameter of 9–18 μm (Castranova et al., 1990). The AM yield was $12 \pm 4 \times 10^6$ cells/rat. The purity obtained by BAL was 90–98% as verified microscopically (Porter et al., 2002a). Cell viability measured by LDH activity release, trypan blue exclusion and total protein (TPRO) was 89 ± 5% (see methods that follow).

TII Isolation and Purification by Panning

TII isolation was performed according to Jones et al. (1982). Rats were anesthetized intraperitoneally with sodium pentobarbital (65 mg/kg body weight), and the renal artery was cut to exsanguinate the animals. The diaphragm was carefully pierced to deflate the lungs, the chest cavity cut open along the midrib line, and the heart and lungs were removed *en bloc*. The lungs were perfused by instilling approximately 20 ml calcium- and magnesium-free PBS through the right ventricle into the pulmonary artery. Perfusion was completed by instilling approximately 10 ml of the PBS solution through

the left ventricle into each of the pulmonary veins until the lungs were white. The trachea was cannulated and the lungs lavaged (7 times with a total 50 ml) with Ca^{2+}, Mg^{2+}-free PBS. This BAL fluid, which consisted mainly of AMs, was then discarded. The lungs were subsequently lavaged twice with 8 ml of a digestion solution consisting of 40 U/ml type I porcine elastase, twice crystallized (ICN Biomedicals, Inc., Aurora, OH), and 0.018% DNase from bovine pancreas (USB Corporation, Cleveland, OH) in PBS. The rinsed lung was then filled with digestion solution and left to digest for a total of 30 min in a 37°C water bath. Following digestion, the trachea and the main bronchi were dissected away and discarded. The lungs were chopped with a tissue chopper set at a cut thickness of 0.5 mm. Digestion was then stopped by suspending the minced lung tissue in an elastase inhibitor solution (25% FBS and 0.018% DNase in PBS), and the resultant suspension was successively filtered through 3 nylon mesh sheets of smaller pore sizes (150, 330, and 440 mesh/cm^2, New York Stencil, New York). The filtrate was centrifuged at 1000 × g for 10 min. The pellet containing unpurified TII cells was then resuspended in 1 ml PBS. The cells were then counted and added to the panning plate for TII purification.

The panning plate was prepared according to Dobbs et al. (1986) whereby a 100-mm tissue culture dish was coated with approximately 2 mg immunoglobuloin G rat (IgG) (Sigma, St. Louis, MO) in 4 ml Tris-base (50 m*M*, pH 9.5) for 3 h at room temperature. After this coating period the IgG-treated plate was washed 5 times with 10 ml PBS, and once with 10 ml culture medium (Ham's F-12 medium supplemented with 1m*M* glutamine, 100 U/ml of penicillin–streptomycin, and 10% FBS). The cells were added at a concentration of 20–30 × 10^6 cells/10 ml culture medium/IgG-coated culture dish. After incubation for 1 h at 37°C, the plate was gently rocked. The supernatant, which contained the purified TII cells, was collected and centrifuged. The pellet was resuspended in 1 ml culture medium. The cells were counted using an electronic cell counter (Coulter Multisizer II, Coulter Electronics, Hialeah, FL) equipped with a 256-channel cell sizer that counts cells according to their size. TII cells were identified as having a diameter of 7–12 μm (Jones et al., 1982). The yield was 25 ± 8 × 10^6 cells/rat, and the viability was 89.6 ± 2.4%. TII cells were incubated overnight before using them in any exposure in order to let them recover from the isolation procedure.

TII Identification and Purity Measurement by Fluorescence Microscopy

TII cells were identified by fluorescence microscopy using phosphine 3R (Roboz Surgical Instrument, Washington, DC) staining, whereby the lipophilic dye concentrates in the lamellar bodies, which store the surfactant to give a yellow-green fluorescence (Mason & Williams, 1976). Phosphine 3R solution in PBS was added to the TII cells suspension at a final concentration of 0.002%. Two minutes later, the stained cells were viewed under a fluorescence microscope (Olympus AX70 photomicroscope equipped with a Sony 3CCD color video camera DXC 9000) at an absorption peak of 477 nm and an

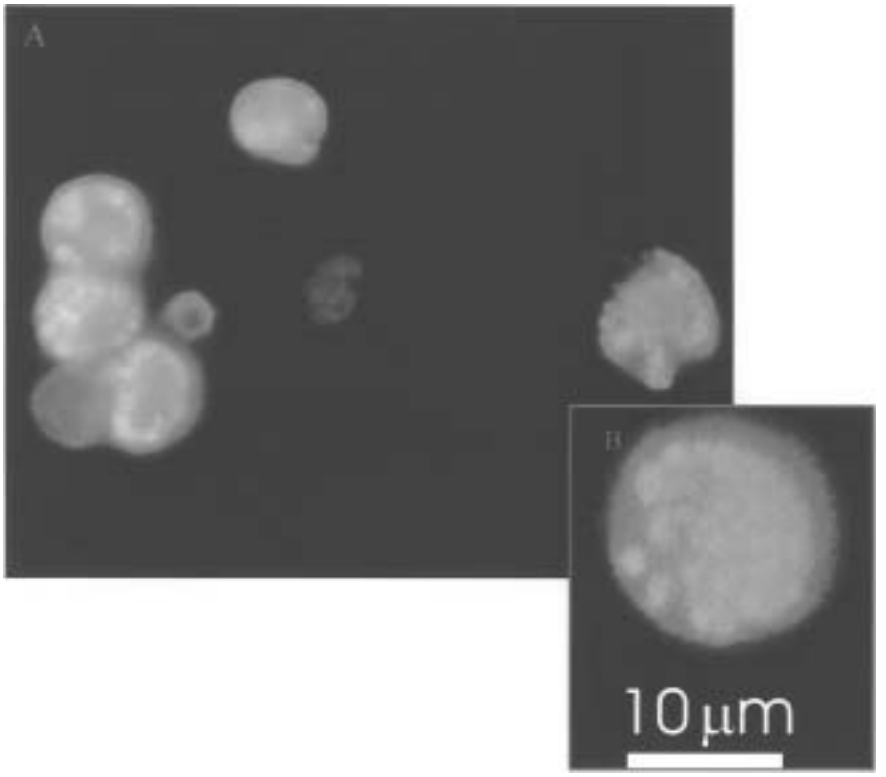

FIGURE 1. (A) Fluorescence microscopy of TII cells stained with phosphine 3R. The globular inclusions emitting fluorescence are the lamellar bodies, which contain surfactant and concentrate the lipophilic dye. (B) Focusing on a single TII cell staining positively with phosphine 3R.

emission peak of 512 nm (Jones et al., 1982). Phosphine 3R staining of the isolated TII cell preparation is shown in Figure 1.

Rat Lung Epithelial-T-Antigen Negative (RLE-6TN) Cells

RLE-6TN cells were derived from alveolar TII cells by Driscoll et al. (1995a). Like primary TII cells, RLE-6TN cells contain lamellar bodies for surfactant storage. They have the ability to produce cytokines, $NO^{\bullet}$, and other ROS when stimulated (Tao & Kobzik, 2003; Driscoll et al., 2001). Unlike primary TII cells, RLE-6TN cells maintain their morphology and the ability to produce surfactant even after several days in culture. RLE-6TN cells were obtained commercially (American Type Culture Collection, Manassas, VA).

Cell Viability Measurements

Cell viability and number were monitored during cell culture to ensure that cell treatments did not produce excessive cellular toxicity or decrease the number of cells surviving in culture.

Lactate dehydrogenase (LDH) activity LDH activity release was used as an indicator of cell cytotoxicity. To measure LDH activity, the exposure plates were spun at 1000 × g for 10 min, after which the supernatant was collected, and part of it was used for LDH measurement. A Cobas Mira Plus analyzer (Roche Diagnostics Systems, Branchburg, NJ) was used to measure LDH activity release based on the increase in absorbance at 340 nm during the oxidation of lactate to pyruvate, a reaction catalyzed by LDH. Viability was determined as LDH activity released into the supernatant under control and test conditions compared to LDH activity released by the same concentration of lysed cells.

Total cellular protein (TPRO) TPRO was used as an indicator of the number of cells attached to the culture plate. To measure TPRO, cells were sonicated using a Branson sonifier 450 (VWR Scientific, Columbus, OH) for 30 s at a duty cycle of 30% and an output control of 3, and then centrifuged for 10 min at 1000 × g. TPRO was measured from the supernatant using a Cobas Mira Plus analyzer (Roche Diagnostics Systems, Branchburg, NJ) based on a colorimetric reaction (absorbance maximum at 540 nm).

Trypan blue exclusion Trypan blue (Sigma, St. Louis, MO) dye exclusion technique was used as an indicator of cell cytotoxicity (Miles et al., 1988). Trypan blue solution was added to the cell culture wells at a final concentration of 0.04%. After 4 min on ice, the cells were fixed with 10% formalin and observed under a light microscope. The percentage of cells excluding dye was taken as the percent viability.

O_2 consumption Mitochondrial function measured by O_2 consumption was also an indicator of cell viability and cellular function. To measure oxygen consumption, a Gilson 5/6 oxygraph (Gilson Medical Electronics, Middleton, WI), equilibrated with a Clark electrode, was used. A baseline was established before adding 3×10^6 cells in 1.5 ml of calcium- and magnesium-supplemented (1 m*M* each) PBS into a preheated 37°C chamber equipped with a stirrer. O_2 consumed by the cells was measured over 10 min using a standard curve constructed by equilibrating the Clark electrode with solutions bubbled with gases of various O_2 concentrations.

Cell Culture

Cell culture medium used for all cell types was Ham's F-12 medium (Biowhittaker, Walkersville, MD) supplemented with 1 m*M* glutamine, 100 U/ml of penicillin–streptomycin, and 10% FBS. For RLE-6TN cells, the culture medium was supplemented with pituitary extract (bovine, 10 μg/ml) (Sigma, St. Louis, MO), insulin (bovine, 5 μg/ml) (Sigma, St. Louis, MO), IGF-1 (human, recombinant, 2.5 ng/ml) (Boehringer Mannheim, Indiannapolis, IN), and holotransferrin (bovine, 25 μg/ml) (Sigma, St. Louis, MO). All exposures were done in vitro using 12-well culture plates (Fisher, Pittsburgh, PA). Silica exposures used Min-U-Sil 5 (99% of silica particles < 5 μm in diameter; purity = 98.5% crystalline silica) obtained from U.S. Silica Corporation (Berkeley Springs, WV) at concentrations of 0–100 μg/ml for 18-h exposures or 0–600 μg/ml for

30-min exposures. LPS (Sigma, St. Louis, MO) exposures were at concentrations of 0–10 μg/ml for 18-h exposures of AMs and TII cells or 0–100 μg/ml for 18-h exposures of RLE-6TN cells. LPS exposure concentrations for the 3 cell types for all 30-min exposures were 0–600 μg/ml. The tissue culture plates were centrifuged following exposures at 1000 × g for 10 min before collecting the medium. While some of the postexposure medium was used right away for LDH activity and TPRO measurement, the rest of it was stored at −70°C for future TNF-α, MIP-2, IL-1β, IL-6, and $NO^{\bullet}$ measurement. The cells attached to the bottom of the wells were used to measure trypan blue exclusion. In all experiments, exposure time for $NO^{\bullet}$, TNF-α, MIP-2, IL-1β, and IL-6 studies were 18 h. Exposure time for ROS and O_2^- studies was 30 min. These in vitro exposure times were taken from previous time-course studies from our laboratory (Zeidler et al., 2003). All exposures were performed in a 37°C, 10% CO_2 incubator.

Superoxide Anion (O_2^-) Measurement by Cytochrome *c* Reduction

Measurement of O_2^- release using cytochrome *c* spectrophotometry relies on the change in absorbance of cytochrome *c* (550 nm) following its reduction by O_2^- (Miles et al., 1978). Cytochrome *c* (Sigma, St. Louis, MO) dissolved in HEPES-buffered medium (10 m*M* HEPES, 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, and 5.5 m*M* D-glucose; pH 7.4) was added to all wells at a final concentration of 0.12 m*M*. Cells (2×10^6 cells/ml in 96-well microplates) were exposed to 0–600 μg/ml LPS or 0–600 μg/ml silica for 30 min at 37°C. Cytochrome *c* reduction was measured at 0 and 30 min of exposure using a SpectraMax 250 spectrophotometer. Conversion from ΔOD units to O_2^- concentration was performed using the following formula: $CL = \Delta OD/e$ where C is the O_2^- concentration (m*M*), L is the path length (cm), ΔOD is the change in cytochrome *c* absorbance, and e is the O_2^- extinction coefficient of 18.7 $mM^{-1}\ cm^{-1}$.

ROS Measurement by Luminol-Dependent Chemiluminescence (CL)

Measurement of ROS using luminol (5-amino-2,3-dihydro-1,4-phthalazinedion)-amplified chemiluminescence relies on the oxidation of luminol to form a luminol radical that produces chemiluminescence (Miles et al., 1978). A 22 m*M* luminol stock solution was made by dissolving 4 mg luminol (Sigma, St. Louis, MO) in 100 μl dimethyl sulfoxide (DMSO) and then adding 900 μl of HEPES-buffered medium to the DMSO-dissolved luminol. The 96-well plate with a cell concentration of 2×10^6 cells/ml in HEPES-buffered medium was placed in the 37°C preheated measurement chamber where 1 m*M* luminol (final concentration) was added to each well at time 0. PBS, LPS (0–600 μg/ml), or silica (0–600 μg/ml) was then injected into specified wells, and ROS were measured for 30 min with emission at 460 nm on a LB96P microplate luminometer. Chemiluminescence (CL) was expressed as relative light units (RLU) per 2×10^6 cells per 30 min.

$NO^{\bullet}$ Measurement

Frozen postexposure supernatants were thawed and used for NO determinations by measuring its oxidation products, nitrate (NO_3) and nitrite (NO_2), collectively referred to as total $NO^{\bullet}$. A total $NO^{\bullet}$ assay kit, obtained from Assay Designs, Inc. (Ann Arbor, MI), was used to measure $NO^{\bullet}$ in 96-well microplates. The kit included a nitrate reductase to convert NO_3 to NO_2. It also included Greiss reagents that interact with NO_2 in a colorimetric reaction, the products of which absorb light at 540 nm. A series of NO_2 standards was run in parallel with the samples, creating a standard curve used to convert light absorbance units into $NO^{\bullet}$ concentrations. A SpectraMax 250 spectrophotometer was used to read the microplate absorbances and convert them to total $NO^{\bullet}$ concentrations.

Cytokine and Chemokine Measurement

Frozen postexposure supernatants were thawed and used for cytokine and chemokine measurements. TNF-α, MIP-2, IL-1β, and IL-6 concentrations were measured using specific enzyme-linked immunosorbent assay (ELISA) kits obtained from Biosource International (Camarillo, CA). The kits included 96-well microtiter plates with cytokine-specific antibody-coated wells. A series of cytokine standards was run in parallel with the samples, and this standard curve was used to convert light absorbances into cytokine concentrations. A SpectraMax 250 spectrophotometer was used to read the microplate absorbances and convert them to cytokine concentrations.

Statistics

All data are means ± SEM of at least three experiments. Data were analyzed by analysis of variance (ANOVA) followed by Tukey's test, with $p < .05$ considered statistically significant.

RESULTS

Cell Cytotoxicity Following LPS or Silica Exposures

Cell cytotoxicity was measured to check for any cell death that might have been produced by LPS or silica exposure, and the number of cells was measured to check if the cell concentration remained constant throughout the 18-h exposure period. LPS did not produce substantial cytotoxicity or cell disintegration (no difference from control) at any of the concentrations used in these cultures over the 18-h exposure period in AMs, TII cells, or RLE-6TN cells (data not shown). Furthermore, silica did not produce substantial cytotoxicity or cell disintegration at 50 μg/ml in AMs, TII cells, or RLE-6TN cells. However, some cytotoxicity was noted at the 100 μg/ml silica exposure for some assays; that is, 100 mg/ml silica had no effect on total cellular protein in the culture, decreased trypan blue exclusion by as much as 8%, and increased LDH activity leakage by 8, 16, and 45% in TII cells, RLE-6TN cells, and AMs, respectively.

NO• Release Upon LPS or Silica Exposure

LPS concentration-responsively increased NO• production from the three cell types (Figure 2). Primary AMs produced considerably more NO• in response to LPS than primary TII cells, which in turn were more sensitive (producing NO• at lower concentrations of LPS) than RLE-6TN cells in response to LPS exposure. Silica exposure in vitro did not result in any detectable NO• production from the three cell types (data not shown). In contrast, silica is a potent inducer of NO• production from BAL cells after in vivo exposure, and a potent inducer of iNOS in AMs and TII cells in vivo (Porter et al, 2002b). Huffman and colleagues (1998) have shown that silica induction of iNOS in AMs requires communication between alveolar macrophages and neutrophils. Such cross-talk was absent in this in vitro study.

TNF-α Release Upon LPS or Silica Exposure

Both LPS and silica concentration-responsively increased TNF-α production from primary AMs and primary TII cells, but not from RLE-6TN cells (Figure 3). In addition, primary AMs were more responsive to both LPS and silica in TNF-α release than primary TII cells, which in turn were more active

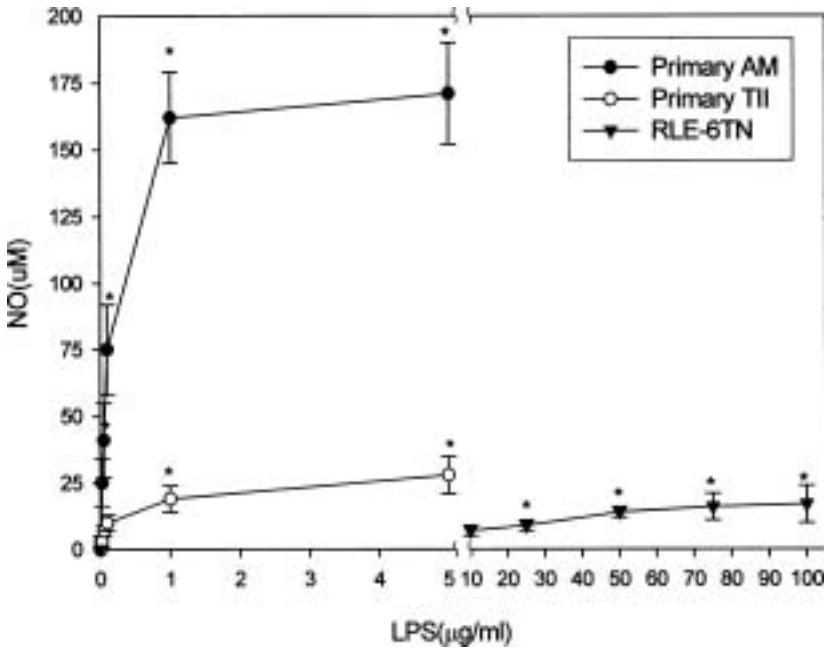

FIGURE 2. Exposure-induced NO• release from the three cell types upon LPS exposure: 1×10^6cells/ml were exposed to increasing concentrations of LPS for 18 h. Note the break in the *x* axis. Asterisk indicates a significant difference ($p < .05$) from control (0 LPS) for each cell type.

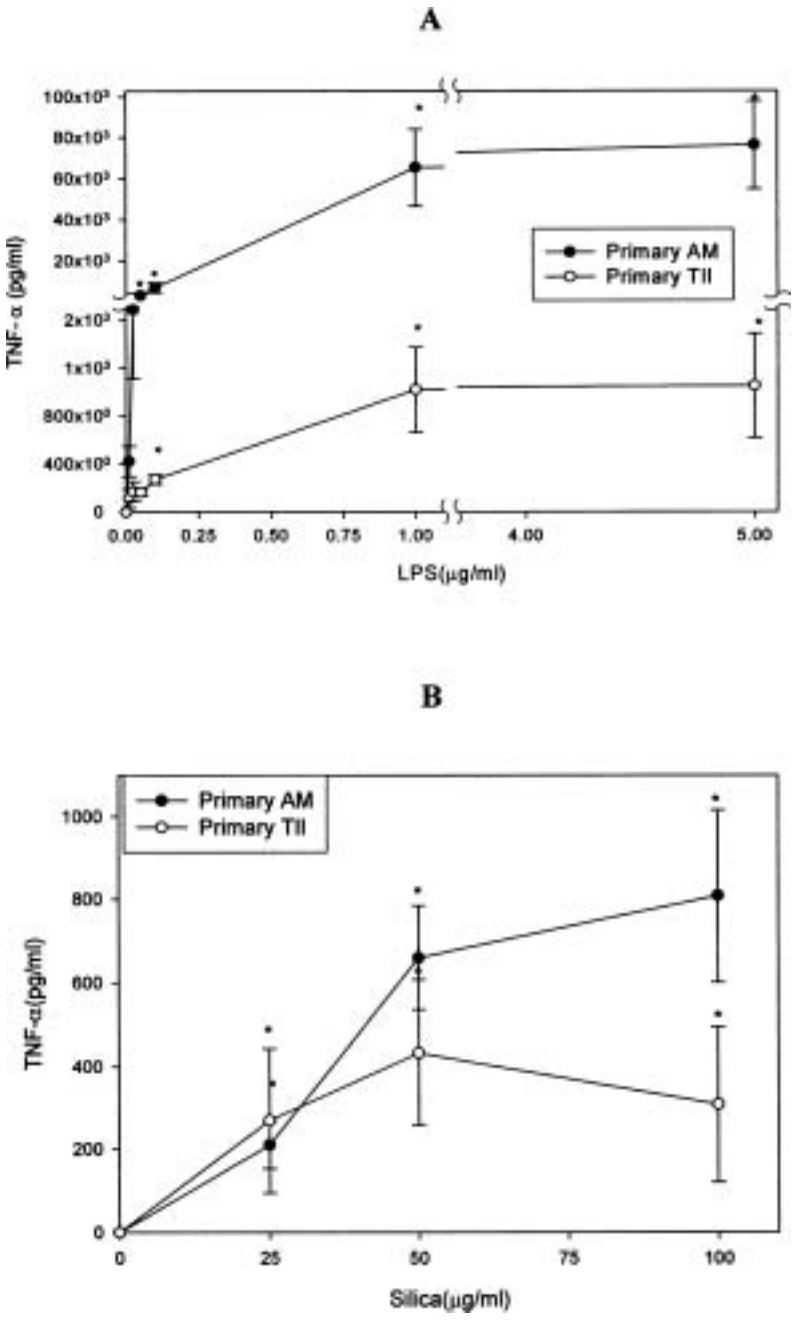

FIGURE 3. Exposure-induced TNF-α release from three rat alveolar cell types upon (A) LPS or (B) silica exposure: 1×10^6 cells/ml were exposed to increasing concentrations of stimulant for 18 h. Note the breaks in the axes. Asterisk indicates a significant difference ($p < .05$) from control (0 stimulant) for AMs and TII cells.

than RLE-6TN cells. LPS induced far greater production of TNF-α in AMS and primary TII cells than silica.

MIP-2 Release Upon LPs or Silica Exposure

Both LPs and silica concentration-responsively increased MIP-2 production from primary AMs, primary TII cells, and RLE-6TN cells (Figure 4). Primary AMs were more active in stimulant-induced MIP-2 release than primary TII cells, which in turn were more active than RLE-6TN cells. LPS induced greater production of MIP-2 in the three cell types than silica.

IL-1β Release Upon LPS or Silica Exposure

LPS and silica concentration-responsively increased IL-1β production from primary AMs and primary TII cells, but not from RLE-6TN cells (Figure 5). Primary AMs were more active in stimulant-induced IL-1β release than primary TII cells, which in turn were more active than RLE-6TN cells. Unlike the production of $NO^{\bullet}$, TNF-α, and MIP-2, where LPS was more potent than silica, both agents induced comparable production of IL-1β in the cells types investigated.

IL-6 Release Upon LPS or Silica Exposure

LPS and silica concentration-responsively increased IL-6 production from primary AMs, primary TII cells, and RLE-6TN cells (Figure 6). The 3 cell types had somewhat comparable abilities to produce IL-6 at 5 mg/ml LPS. However, RLE-6TN cells were not responsive to low (< 0.1 μg/ml) concentrations of LPS, while AMs and TII cells exhibited similar responsiveness. Upon silica exposure, primary AMs were less active in stimulant-induced IL-6 release than primary TII cells, which in turn were less active than RLE-6TN cells. LPS induced a greater production of IL-6 in the 3 cell types than silica.

O_2^- Release With LPS or Silica Exposure

Silica concentration-responsively increased O_2^- production from primary AMs, primary TII cells, and RLE-6TN cells (Figure 7). Upon silica exposure, primary AMs and TII cells had comparable ability to produce O_2^- at levels somewhat greater than RLE-6TN cells. Silica induced a greater production of O_2^- in the three cell types than did LPS, since no significant O_2^- release from the three cell types was noted with LPS exposure (data not shown).

ROS Release With LPS or Silica Exposure

LPS concentration-responsively increased ROS production in primary AMs measured externally as CL (Figure 8). However, LPS failed to stimulate ROS in primary TII cells or RLE-6TN cells (data not shown). Silica concentration-responsively increased ROS production in primary AMs and TII cells, but not in RLE-6TN (Figure 9). Upon silica exposure, AMs and TII cells had comparable abilities to produce ROS. Silica and LPS stimulated comparable ROS production in AMs. However, silica was a more potent stimulant of ROS production in TII cells than LPs (Figures 8 and 9).

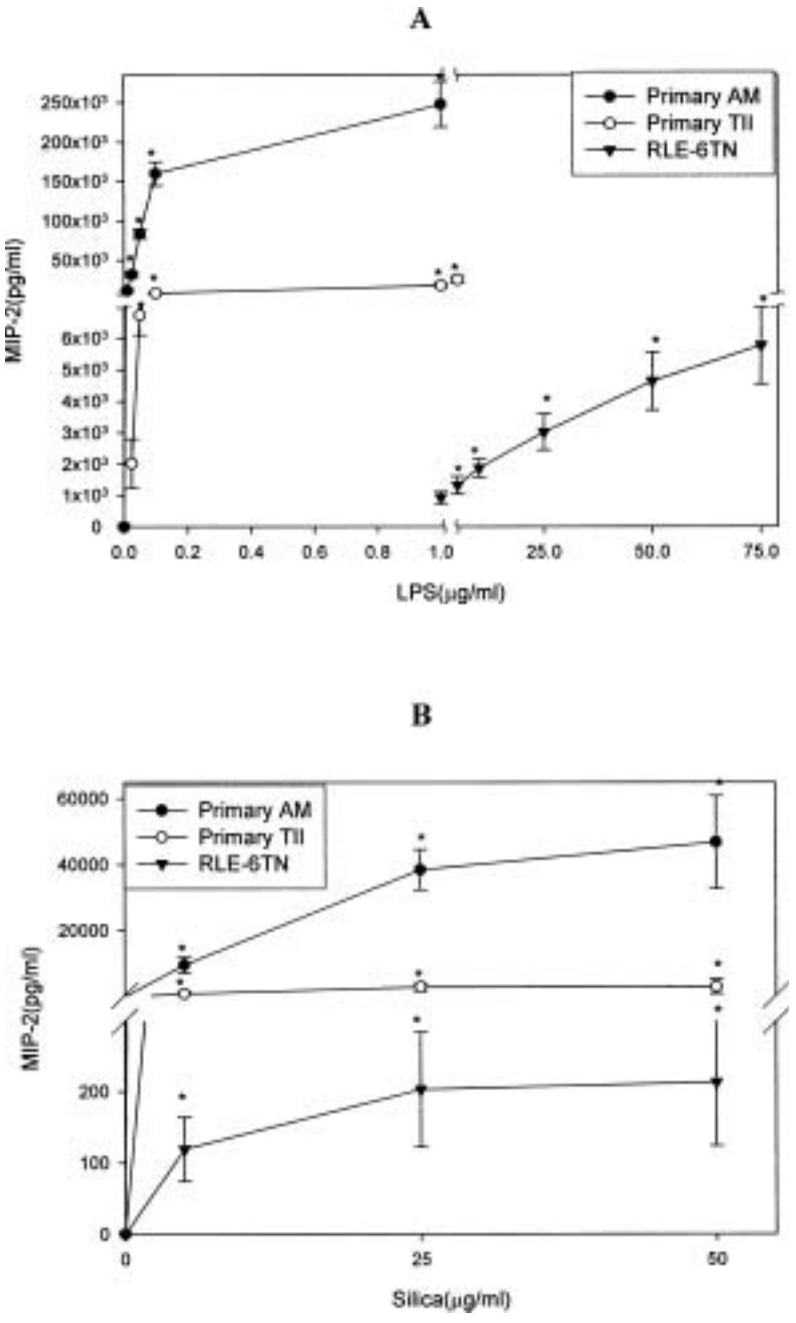

FIGURE 4. Exposure-induced MIP-2 release from the 3 rat alveolar cell types upon (A) LPS or (B) silica exposure: 1×10^6 cells/ml were exposed to increasing concentrations of stimulant for 18 h. Note the breaks in the axes. Asterisk indicates a significant difference ($p < .05$) from control (0 stimulant) for each cell type.

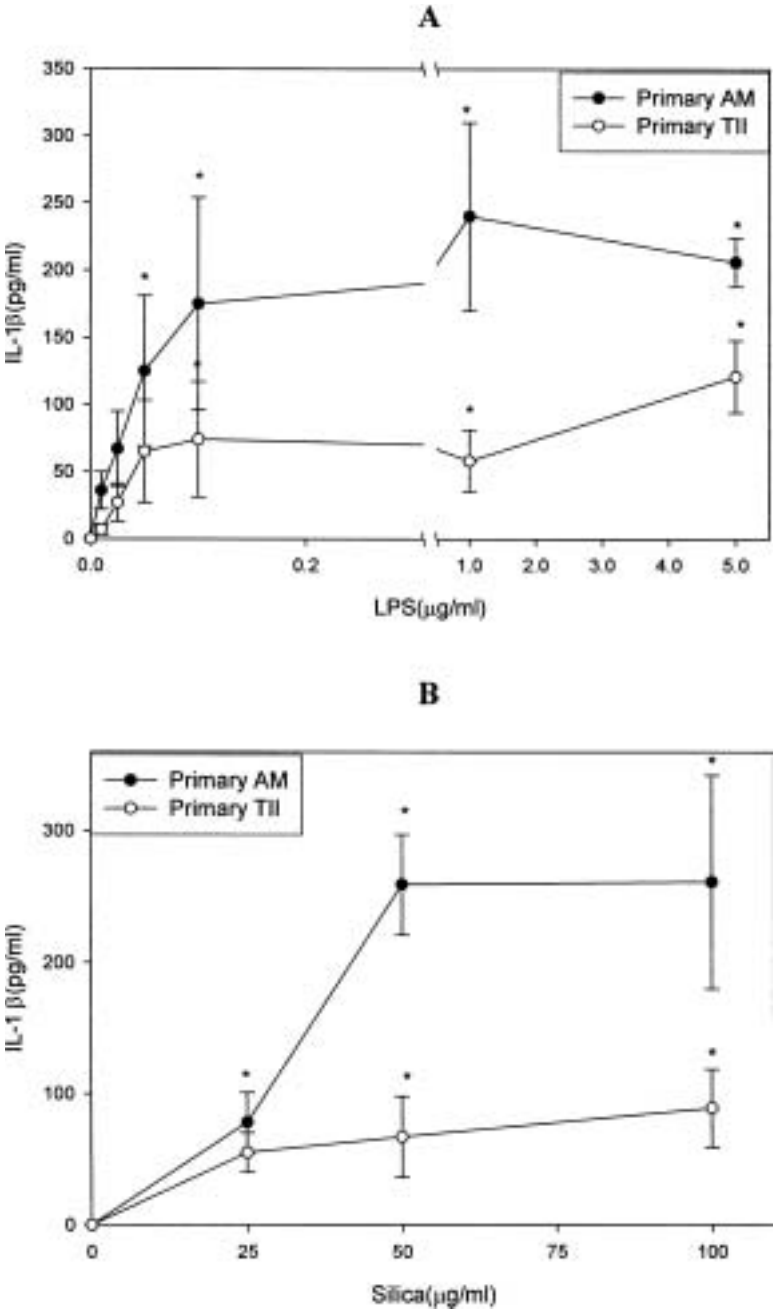

FIGURE 5. Exposure-induced IL-1β release from the three rat alveolar cell types upon (A) LPS or (B) silica exposure: 1×10^6 cells/ml were exposed to increasing concentrations of stimulant for 18 h. Note the breaks in the axes. Asterisk indicates a significant difference ($p < .05$) from control (0 stimulant) for each cell type.

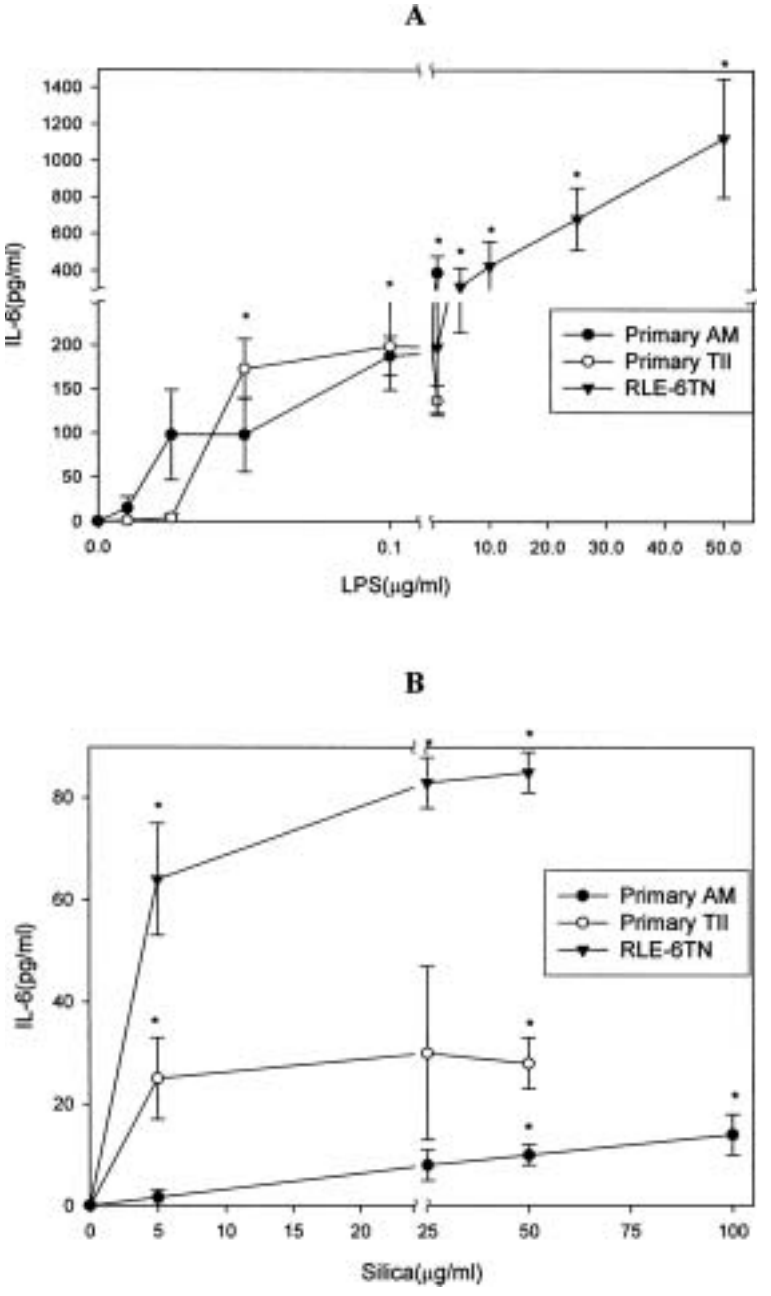

FIGURE 6. Exposure-induced IL-6 release from the 3 rat alveolar cell types upon (A) LPS or (B) silica exposure: 1×10^6 cells/ml were exposed to increasing concentrations of stimulant for 18 h. Notes the breaks in the axes. Asterisk indicates a significant difference ($p < .05$) from control (0 stimulant) for each cell type.

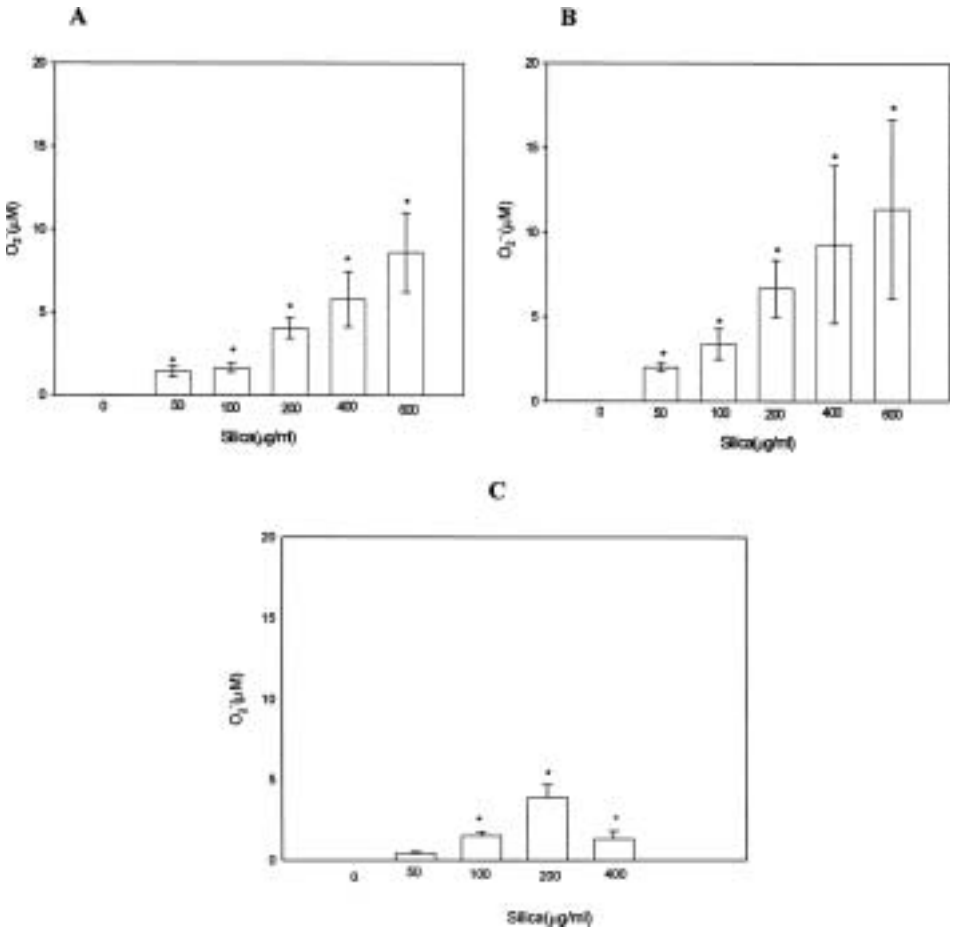

FIGURE 7. Exposure-induced O_2^- release (measured as cytochrome *c* reduction) from (A) AMs, (B) TII cells, and (C) RLE-6TN cells upon silica exposure: 2×10^6 cells/ml were exposed to increasing concentrations of silica for 30 min. Asterisk indicates a significant difference ($p < .05$) from control (0 silica).

DISCUSSION

Comparing Primary AMs and Primary TII Cells

AMs are professional phagocytes that clear inhaled microbes and particles from the lungs. Therefore, it has been proposed that AMs are a major source of inflammatory cytokines and reactive products, which can produce lung damage and inflammation and lead to occupational lung diseases (Castranova, 2000). The data in the present study support this hypothesis. However, the results suggest that, although AMs are more active than TII cells in producing many inflammatory mediators upon in vitro LPS and silica exposure, TII cells

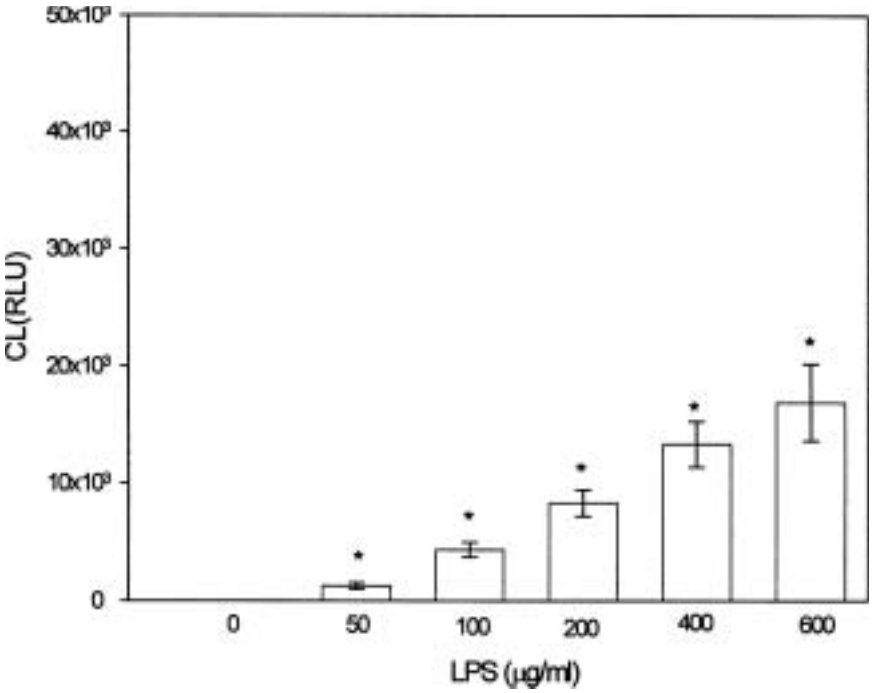

FIGURE 8. Exposure-induced ROS (measured as luminol-dependent chemiluminescence) release from AMs upon LPS exposure: 2×10^6 cells/ml were exposed to increasing concentrations of LPS for 30 min. Asterisk indicates a significant difference ($p < .05$) from control (0 LPS). Chemiluminescences, CL; relative light units, RLU.

are also capable of producing highly significant levels of inflammatory products. Indeed, data from the present study support evidence suggesting that alveolar type II epithelial cells (TII) may significantly contribute to lung inflammation by a direct interaction with a stimulant (Blau et al., 1994; Crippen et al., 1995; Finkelstein et al., 1997). However, data from the present study also extend previous findings by measuring mediator release from primary TII cell cultures instead of TII-like cell lines used in most previous studies. In addition, few studies have compared the secretory activity of AMs and TII cells under similar exposure and culture conditions. Among the very few studies comparing AMs and TII cells, data from Crestani et al. (1994), support our findings that TII cells produce more IL-6 than AMs basally in vitro.

Data presented in the present study indicate that TII cells could produce comparable amounts of ROS as AMs upon silica exposure. It is of interest that TII O_2 consumption measured basally in preliminary studies characterizing the viability of TII cells was approximately 193 ± 82 nmol/10^6 cells/h. This value is comparable to values reported previous by our laboratory (Jones et al., 1982) and is significantly greater than AM oxygen consumption (~46.2 nmol/10^6 cells/h) as reported by Castranova et al. (1980). TII cells are more dependent

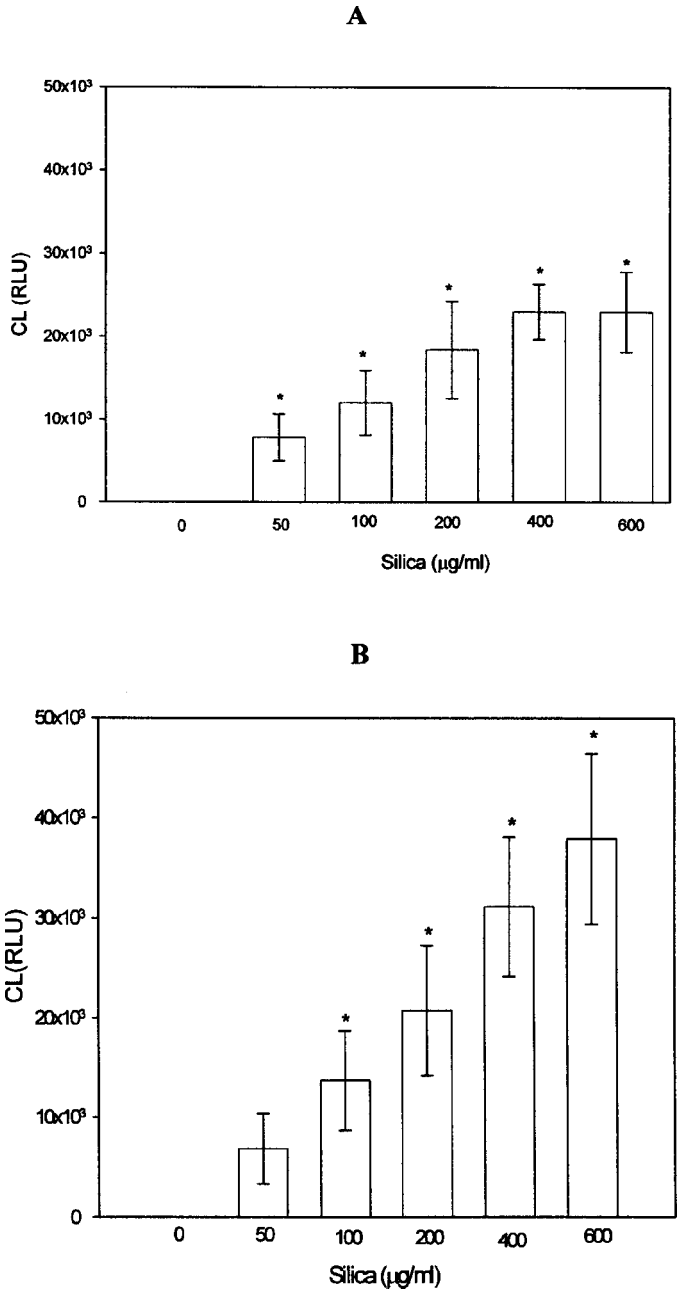

FIGURE 9. Exposure-induced ROS (measured as luminol-dependent chemiluminescence) release from (A) AMs and (B) TII cells upon silica exposure: 2×10^6 cells/ml were exposed to increasing concentrations of silica for 30 min. Asterisk indicates a significant difference ($p < .05$) from control (0 silica). Chemiluminescence, CL; relative light units, RLU.

on mitochondrial respiration, whereas AMs are more dependent on glycolysis as an energy source (Fisher et al., 1980). This is manifested basally and upon exposure. Driscoll et al. (2000) reported a significant increase in mitochondrial-derived H_2O_2 in RLE-6TN cells following silica exposure. In addition, their results link these mitochandrial-derived ROS to silica-induced transcription of mRNA for inflammatory cytokines and chemokines.

Comparing Primary TII and RLE-6TN Cells

The RLE-6TN cell line was established as a less costly and time-consuming alternative to TII cell isolation in studies involving pulmonary epithelial cells (Driscoll et al., 1995a). Indeed, since then, the RLE-6TN cell line has been used in place of primary TII cells in many experiments studying the pulmonary epithelium. However, the sensitivity and level of responsiveness of this cell line compared to primary TII cells have not been fully investigated. For this reason, the present study had, among other aims, the goal to compare primary rat TII cells to RLE-6TN cells, a rat TII cell line. For this purpose, the same mediators released from the two cell types were measured under comparable conditions. Primary TII cells were significantly more active in producing $NO^{\bullet}$, TNF-α, MIP-2, IL-1β, and ROS than RLE-6TN cells upon LPS exposure. Upon silica exposure, primary TII cells were significantly more active in producing TNF-α, MIP-2, IL-1β, and ROS than RLE-6TN cells. In addition, primary TII cells showed a higher sensitivity to LPS and silica than RLE-6TN. Indeed, a much higher LPS or silica concentration was required to initiate significant mediator release from RLE-6TN cells. For some mediators, namely, TNF-α and IL-1β, even very high stimulant concentrations, which produced some cell injury, were incapable of inducing significant mediator release from RLE-6TN cells. In agreement with this conclusion, Driscoll et al. (1996) reported that induction of MIP-2 mRNA from primary TII cells was greater than from RLE cells following silica exposure. Primary TII cells also responded at a lower silica concentration and were thus more sensitive than the cell line. In contrast, Driscoll et al. (1996) reported that LPS induced similar levels of MIP-2 mRNA in both cell types. However, primary TII cell were cultured for 3 d after isolation prior to exposure in that study. Reports indicate that morphological and biochemical changes in primary TII cells are more probable with time in culture (Dobbs et al., 1986). This could explain the reason for the lower responsiveness of primary TII cells to LPS in the Driscoll et al. (1996) study. Another study reporting lower primary TII cell responsiveness was presented by Punjabi et al. (1994), where no significant induction of $NO^{\bullet}$ production was detected in primary TII cells in response to 1 μg/ml LPS. A close examination of the Punjabi et al. (1994) study reveals that a low cell density (3×10^5 cells/well) was used. It is possible that $NO^{\bullet}$ production from the lower cell number could not be detected by the Greiss reaction technique.

A significant conclusion from the present investigation is that although using the RLE-6TN cell line is a less costly and less time-consuming method to study pulmonary epithelial TII cells, the activity and/or sensitivity of this cell

line does not necessarily reflect the responses of primary TII cells. Hence, RLE-6TN cells may not be a good substitute for primary TII cells in mechanistic studies of toxicology.

Comparing the Potency of LPS to Crystalline Silica

As mentioned earlier, silica and LPS are two airborne contaminants of major importance to lung pathology. Crystalline silica is highly fibrogenic and biologically toxic (Castranova, 2000). While silica can directly produce cellular toxicity, it can also initiate an inflammatory response that includes increased oxidant production and proinflammatory mediator secretion as a result of nuclear transcription factor-κB (NF-κB) activation in target and recruited cells (Kang et al., 2000; Vallyathan & Shi, 1997). Likewise, LPS binding to target cells also induces the synthesis and release of many inflammatory mediators, such as the proinflammatory cytokines TNF-α, IL-β, and IL-6 (Chen et al., 1995; Christman et al., 1998). In the present study, LPS was found to be more potent than silica in inducing $NO^{\bullet}$, TNF-α, MIP-2, and IL-6 release from the three cell types. In contrast, LPS and silica had a comparable ability to induce IL-1β production from the three cell types, whereas silica was more potent than LPS in activating O_2^- production.

Although Chen et al. (1995) reported a greater activation of NF-κB after stimulation of a macrophage cell line with silica than with LPS, this activation was measured at a single time (6 h postexposure), which may have missed the peak LPS response. Indeed, considerable literature supports the hypothesis that LPS is more potent than silica in inducing transcription and cytokine release from AMs. Blackford et al. (1994) have shown that iNOS mRNA and $NO^{\bullet}$ production increase much more in AM's following in vivo exposure of rats to LPS (sevenfold for the mRNA and ninefold for $NO^{\bullet}$ production, respectively) than following silica exposure (threefold and fourfold, respectively). Dubois et al. (1989) also reported that TNF and leukotriene (LT) B_4 release from primary AMs, following in vitro LPS exposure, was higher than the release from AMs following silica exposure. Much less data exist that compare LPS and silica exposure in primary TII or RLE-6TN cells. Some data from Driscoll et al. (1996) suggest that MIP-2 mRNA from primary TII and RLE-6TN cells following in vitro LPS exposure is greater than that induced following silica exposure. As for ROS, although silica and LPS have been both reported to induce ROS release in some TII cell lines and AMs (Barrett et al., 1999a, 1999b; Shi et al., 1999), the relative potency of these two stimuli was not directly compared. Chen et al. (1998) reported that the antioxidants ascorbate or formate decreased silica-induced NF-κB activation in the macrophage cell line RAW 264.7 to a greater extent that LPS-induced NF-κB activation. This suggests that ROS production was greater after silica exposure than after LPS exposure. Zeidler et al. (2003) also reported slightly lower H_2O_2 levels in murine AMs exposed in vitro to LPS than silica.

As mentioned in the Results section, in vitro exposure to silica did not stimulate release of $NO^{\bullet}$ from any of three cell types tested. Indeed, Huffman

et al. (1998) have shown that although in vivo exposure to silica can stimulate $NO^{\bullet}$ production from bronchoalveolar lavage cells (BALC), it cannot do so in vitro. However, conditioned medium from BAL (harvested from rats) following in vivo silica exposure was effective in making naive AM's produce $NO^{\bullet}$ in response to silica exposure in vitro. Therefore, cross-talk between pneumocytes appears to be important for $NO^{\bullet}$ production in response to silica.

Conclusion

After having compared the release of several inflammatory mediators from AMs, TII cells, and RLE-6TN cells under similar culture conditions, exposures (basally, or following LPS or silica exposures), and cell concentrations, the following conclusions can be made: (1) Although AMs were generally found to release more inflammatory mediators than TII cells following LPS or silica exposures. TII cells clearly produced significant levels of mediators, which could be capable of contributing to lung inflammation and injury following occupational exposure. (2) Since the RLE-6TN TII cell line response to LPS and silica exposures were generally considerably less intense and required higher concentrations of stimulant than those measured in primary TII cells, RLE-6TN cells may not be an ideal substitute for primary TII cells in mechanistic and toxicological studies. (3) LPS was found to be a more potent inducer of inflammatory cytokines from AMs, TII cells and RLE-6TN cells than silica. However, silica was found to be as potent as LPS or even more potent with respect to stimulation of O_2^- and ROS generation, especially from TII cells.

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