Nitric oxide production by rat bronchoalveolar macrophages or polymorphonuclear leukocytes following intratracheal instillation of lipopolysaccharide or silica

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Exposure of the lung to lipopolysaccharide (LPS) or silica results in an activation of alveolar macrophages (AMs), recruitment of polymorphonuclear leukocytes (PMNs) into bronchoalveolar spaces, and the production of free radicals. Nitric oxide (NO) is one of the free radicals generated by bronchoalveolar lavage (BAL) cell populations following either LPS or silica exposure. The purpose of the present study was to assess the relative contributions of AMs and PMNs to the amounts of NO produced by BAL cells following intratracheal (IT) instillation of either LPS or silica. Male Sprague Dawley rats (265–340 g body wt.) were given LPS (10 μg/100 g body wt.) or silica (5 mg/100 g body wt.). BAL cells were harvested 18–24 h post-IT and enriched for AMs or PMNs using density gradient centrifugation. Media levels of nitrate and nitrite (NOx; the stable decomposition products of NO) were then measured 18 h after *ex vivo* culture of these cells. Following IT exposure to either LPS or silica, BAL cell populations were ~ 20% AMs and ~ 80% PMNs. After density gradient centrifugation of BAL cells from LPS- or silica-treated rats, cell fractions were obtained which were relatively enriched for AMs (~ 60%) or PMNs (~ 90%). The amounts of NOx produced by the AM-enriched fractions from LPS- or silica-treated rats were ~ 2–4-fold greater than that produced by the PMN-enriched fractions. Estimations of the relative contribution of AMs or PMNs to the NOx produced indicated that: (i) following LPS treatment, 75%–89% of the NOx was derived from AMs and 11%–25% from PMNs; and (ii) following silica treatment, 76%–100% of the NOx was derived from AMs and 0–24% from PMNs. Immunohistochemistry for inducible NO synthase on lung tissue sections supported these findings. We conclude that AMs are the major source of the NO produced by BAL cells during acute pulmonary inflammatory responses to LPS or silica.

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1. Introduction

Exposure of the lungs of humans and experimental animals to a number of different substances can result in pulmonary inflammation. For instance, it has been well documented that inhalation or intratracheal instillation of microbial products, such as endotoxin-derived lipopolysaccharide (LPS), or particulates, such as silica, can initiate a pulmonary inflammatory response (Lapp and Castranova 1993; Burrell 1994). A key component of

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Abbreviations used: AMs, Alveolar macrophages; BAL, bronchoalveolar lavage; iNOS, inducible nitric oxide synthase; IT, intratracheal; LPS, lipopolysaccharide; NO, nitric oxide; PMNs, polymorphonuclear leukocytes.

lung inflammatory responses to either LPS or silica is an activation of alveolar macrophages (AMs) and recruitment of polymorphonuclear leukocytes (PMNs) into bronchoalveolar spaces (Lapp and Castranova 1993; Sharar *et al* 1994). This bronchoalveolar population of cells, consisting of AMs and PMNs, has then been shown to release a number of inflammatory mediators.

Nitric oxide (NO) is one of the inflammatory mediators which is generated following exposure of lungs to either LPS or silica (Knowles *et al* 1990; Blackford *et al* 1994). NO is a free radical and appears to play an important role in the defense against pathogens, and appears to be involved in the tissue damage associated with inflammatory processes (Ialenti *et al* 1992; Gaston *et al* 1994; Moilanen and Vapaatalo 1995). The marked increase in NO production during pulmonary inflammatory responses to either LPS or silica is primarily due to an upregulation of inducible NO synthase (iNOS) activity. One important source of the NO generated during these pulmonary inflammatory responses is the population of bronchoalveolar cells. Evidence for this includes the finding that iNOS mRNA levels increase in cells harvested by bronchoalveolar lavage (BAL) from either LPS- or silica-treated rats (Blackford *et al* 1994; Huffman *et al* 1997). Furthermore, free radical production by these cells is significantly attenuated in the presence of iNOS inhibitors (Antonini *et al* 1994; Blackford *et al* 1994; Huffman *et al* 1997). In addition, increases in media levels of nitrate and nitrite, the stable decomposition products of NO, are observed following *ex vivo* culture of BAL cells from either LPS-exposed or silica-exposed rats (Huffman *et al* 1997, 1998). Although this evidence demonstrates that NO is produced by BAL cell populations following either LPS or silica treatment, the specific cellular source(s) of NO is not clear, i.e. whether AMs, PMNs, or both cell types are involved.

There is some evidence to suggest that AMs are an important source of the NO produced during pulmonary inflammatory responses to either LPS or silica. For instance, the capacity of AMs to respond directly to LPS with increases in NO production has been well documented (Jorens *et al* 1991; Warner *et al* 1995). AMs also are a likely source of the NO produced following silica exposure (Huffman *et al* 1998; Porter *et al* 2002). However, much less information exists concerning NO production by BAL PMNs. While it is known that PMNs have the capacity to express iNOS mRNA and produce NO, much of the work in this area has involved the study of NO production by peritoneal or circulating blood PMNs (McCall *et al* 1989; Wright *et al* 1989; Yui *et al* 1991; Kolls *et al* 1994; Evans *et al* 1996). To our knowledge, no study has addressed the specific contribution of lung-recruited PMNs to the amounts of NO produced by BAL cell populations following LPS or silica exposure. This is of mechanistic importance because it is possible that the PMNs that are recruited into the lung during acute inflammatory responses may produce a spectrum of inflammatory mediators differing from those which can be produced by PMNs at other body sites.

Therefore, the objective of the present study was to determine the relative contributions made by AMs and PMNs to the amounts of NO produced by BAL cell populations following the intratracheal instillation of LPS or silica. BAL cells were harvested from either LPS-treated or silica-treated rats. Density gradient centrifugation was used to obtain cell fractions which were enriched in either AMs or PMNs. NO production was then assessed by measuring media levels of nitrate and nitrite following *ex vivo* culture of these cell fractions.

2. Materials and methods

2.1 *Animals*

The animals used in these experiments were male Sprague-Dawley [Hla:(SD) CVF] rats weighing 265 g–340 g at the time of study. The rats were obtained from Hilltop Lab Animals (Scottdale, PA,USA). The animals were housed in an AAALAC-accredited, specific pathogen-free facility. The rats were monitored to be free of endogenous viral pathogens, parasites, mycoplasms, helicobacter and CAR bacillus. The rats were acclimated for at least 5 days before use and were housed in ventilated cages which were provided with HEPA-filtered air. Alpha-Dri virgin cellulose chips and hardwood Beta-chips were used as bedding. The rats were provided Prolab RMH rodent diet and tap water *ad libitum*, and maintained under controlled light (12 h of light, 12 h of darkness) and temperature (22–24°C) conditions.

2.2 *Intratracheal instillation*

Rats were lightly anesthetized with an intraperitoneal injection of 2 mg/100 g BW sodium methohexital (Eli Lilly Co., Indianapolis, IN, USA). The rats then received an intratracheal (IT) instillation of LPS (*Escherichia coli* 055:B5; Difco Laboratories, Inc., Detroit, MI, USA) at a dose of 10 μg/100 g body wt. or crystalline silica (Min-U-Sil, < 5 μm diameter, US Silica Corp., Berkeley Springs, WV, USA) at a dose of 5 mg/100 g body wt. Control animals were instilled with endotoxin-free saline (0·9% NaCl; Baxter HealthCare Corp., Deerfield, IL, USA). Each rat received 0·1 ml/100 g body wt. of solution. The animals were then allowed to recover for 18 h following control or LPS treatment, or for 24 h following control or silica treatment. These times were chosen because acute pulmonary

inflammatory responses to LPS or silica occur during this period (Blackford *et al* 1994; Huffman *et al* 1997).

2.3 *Collection of BAL cell samples*

The rats were euthanized with sodium pentobarbital (> 100 mg/kg, ip; Sleepaway, Fort Dodge, IA, USA) and the left renal artery was cut. A tracheal cannula was inserted and the lung was lavaged with ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; 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) at a volume of 6 ml for the first lavage and 8 ml for subsequent lavages. A total of 80 ml of BAL fluid was collected from each rat. The samples were centrifuged (500 *g*, 5 min, 4°C) to pellet the cells and the supernatants were aspirated to waste. BAL cells from similarly treated rats were then combined. In the case of saline treatment, BAL cells from 2–6 rats were combined. Following LPS treatment, BAL cells from 1–2 rats were combined. For silica-treated rats, BAL cells from 4 animals were combined. The cells were then washed a total of three times with 5 ml of PBS. The cells were resuspended in PBS and aliquots of the initial BAL cell samples were removed for determinations of cell number, cell populations, viability, and for *ex vivo* cell culture. The remainder of the BAL cell samples were enriched for either AM-containing or PMN-containing fractions using density gradient centrifugation.

2.4 *Density gradient centrifugation*

In this study, we used two density gradient centrifugation methods to enrich rat BAL cell populations for either AMs or PMNs following IT treatments. We found that when a Percoll-based medium was used, consistent banding patterns for cells from control as well as LPS-treated rats were obtained. However, this was not the case for cell populations from silica-treated rats. In this case, diffuse, poorly defined cell banding patterns were noted. A Histopaque method was then used to separate different populations of BAL cells from either control or silica-treated rats. This method resulted in a clear pattern of cell separation for samples from either saline or silica-treated rats. Therefore, in this investigation, a Percoll-based medium was used to enrich BAL cell populations for AMs or PMNs from LPS-treated rats and the Histopaque method was used to enrich BAL cell populations from silica-treated animals.

2.5 *Percoll method for density gradient centrifugation*

BAL cells were layered onto a continuous gradient of 70% Percoll (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The gradient was then centrifuged (345 *g*, 30 min, 4°C). For BAL cells from control rats, a single band of cells localized between densities of 1·060 and 1·073. In the case of BAL cells from LPS-treated rats, two bands of cells were noted. An upper band localized between densities of 1·053 and 1·069 (AM-enriched fraction) while a lower band localized between densities of 1·072 to 1·108 (PMN-enriched fraction). These bands of cells were transferred to separate tubes, washed, and resuspended in PBS. Aliquots of the cell fractions were then removed for determinations of cell number, cell populations, viability, and for *ex vivo* cell culture.

2.6 *Histopaque method for density gradient centrifugation*

BAL cell populations obtained from either control or silica-treated rats were initially subjected to hypotonic lysis to remove red blood cells. Cells were then layered onto a Histopaque double density gradient composed of equal amounts of Histopaque 1083 and Histopaque 1119 (Sigma, St. Louis, MO, USA). The gradients were then centrifuged (400 *g*, 30 min, RT). For BAL cells from control rats, a single opaque band of cells localized at the interface between the PBS diluent and Histopaque 1083. In the case of BAL cells from silica-treated rats, an upper opaque band of cells localized at the interface between the PBS diluent and Histopaque 1083 (AM-enriched fraction). In addition, there was a cell-containing pellet (PMN-enriched fraction). These cell fractions were transferred to another tube, washed, and resuspended in PBS. Aliquots of the cell fractions were then removed for determinations of cell number, cell populations, viability, and for *ex vivo* cell culture.

2.7 *Determination of BAL cell number, cell populations, and viability*

Total counts of cells greater than 5 μm in the BAL cell suspensions were determined using an electronic cell counter equipped with a cell-sizing attachment (Coulter Multisizer II, Coulter Electronics, Hialeah, FL, USA). Portions of the harvested cells were then deposited on slides using a cytocentrifuge (Shandon Scientific, London, England) and stained with a modified Wright-Giemsa stain (Hema-Tek 2000, Bayer Corp., Elkhart, IN, USA). The percentages of AMs and leukocytes present on the slides were determined by counting 100 cells using light microscopy. Greater than 99% of the counted cells were either AMs or PMNs for all the treatment groups. Following LPS treatment, the PMNs were almost exclusively neutrophils and contained less than 1% eosinophils. In the case of silica treatment, the majority of PMNs

were neutrophils, but this cell population also contained 5%–25% eosinophils.

Viability of the cell samples was assessed using a trypan blue exclusion test. The percentages of viable cells were then determined by placing aliquots of the treated cells in a hemocytometer and scoring 100 cells for either the absence (viable cells) or the presence (dead cells) of blue staining. Before density gradient centrifugation, viabilities of BAL cells from control rats ranged from 79%–91%. Viabilities of BAL cells from LPS-treated rats and silica-treated rats ranged from 90%–99% and 70%–88%, respectively. Following density gradient centrifugation of BAL cells from either LPS-treated or silica-treated rats, viabilities of the AM-enriched fractions were 94%–95% (LPS treatment) or 75%–83% (silica treatment). The viabilities of the PMN-enriched fractions were 95%–98% (LPS treatment) or 80%–93% (silica treatment).

2.8 *Measurement of NO production by BAL cells*

The production of NO by BAL cells was assessed by measuring media nitrate and nitrite levels following *ex vivo* culture of cells. Nitrate and nitrite are stable decomposition products of NO and measurement of these products is widely used to index the amount of NO produced by cultured cells (Tracey 1992). BAL cells were suspended in culture medium (Earle's Minimum Essential Medium; BioWhittaker, Walkersville, MD, USA) supplemented with 1 mM glutamine, 100 U/ml each penicillin, streptomycin, and kanamycin, with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 10 mM HEPES. The cells were then placed into wells of 24-well tissue culture plates (Costar Corp., Cambridge, MA, USA). In the case of BAL cells from control and LPS-treated rats, cells were plated at a density of $0{\cdot}25 \times 10^6$ viable cells/0·5 ml/well. In experiments with BAL cells from control and silica-treated rats, cells were plated at a density of 1×10^6 viable cells/0·5 ml/well. These cell densities were chosen based upon results from preliminary experiments. The cells were then incubated for 18 h at 37°C in an incubator with an atmosphere of 95% air-5% CO_2 and a relative humidity of 90%. After the incubation period, the tissue culture plates were centrifuged (500 *g*, 10 min, 4°C) and the cell-free supernatants were stored at – 80°C prior to analysis. NOx was determined as previously described (Porter *et al* 2002). Briefly, nitrate in the media samples was reduced to nitrite, and then nitrite was determined by flow injection analysis colorimetry at 540 nm using the Griess reaction (QuickChem 8000, Lachat Instruments, Milwaukee, WI, USA). The results are expressed as μM.

2.9 *Estimation of the contribution of AMs or PMNs to NOx production*

To estimate the amount of NOx produced by AMs or PMNs, algebraic equations were used. One equation used data obtained from the AM-enriched fractions and the other equation used data obtained from the PMN-enriched fractions. In each of these equations, the number of AMs (per 100 cells) and the number of PMNs (per 100 cells) in the cell fraction were set equal to the amount of NOx produced (μM) by the cell fraction. For example, the following set of algebraic equations were obtained in one experiment.

$$56\ (\text{AMs}) + 44\ (\text{PMNs}) = 18{\cdot}4\ \mu\text{M NOx}$$

$$8\ (\text{AMs}) + 92\ (\text{PMNs}) = 5{\cdot}8\ \mu\text{M NOx}.$$

We then mathematically solved for the relative amounts of NOx produced by either AMs or PMNs and expressed the results as the percentage of NOx which was derived from each cell type.

2.10 *Immunohistochemistry for iNOS*

After euthanasia, lungs were removed from control, or LPS-treated, or silica-treated animals (lungs were not lavaged) and inflated by instillation of 10% neutral buffered formalin. The left lobes were processed within 24 h and 5 μm paraffin sections were used for immunohistochemistry as outlined previously (Porter *et al* 2002). Briefly, after rehydration, microwave antigen retrieval with citrate buffer was performed, followed by peroxidase blocking with methanol and H_2O_2. Slides were incubated overnight at 4ºC with iNOS monoclonal antibody (N32020; BD Biosciences Pharmingen, San Diego, CA, USA). Localization was achieved using a streptavidin-biotin-peroxidase system for use on rat specimens (K0609; DAKO, Carpinteria, CA, USA) with diaminobenzidene (Zymed Laboratories, South San Francisco, CA, USA) as the chromogen. Slides were examined with an Olympus AX70 microscope and photographs were taken using a Quantix digital camera (Photometrics, Tucson, AZ, USA) with QED acquisition software (QED Imaging Inc., Pittsburgh, PA, USA).

2.11 *Statistical analyses*

Statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA). A *t*-test was used to determine differences between groups. Significance was set at $P < 0{\cdot}05$.

3. Results

NOx production by BAL cell populations from control, or LPS-treated, or silica-treated rats are shown in figure 1. These values represent the amounts of NOx produced by cell populations initially harvested following these treatments, i.e. before density gradient centrifugation. As expected, both LPS and silica treatment resulted in increases in NOx production by BAL cells. Following LPS treatment, there was approximately a 10-fold increase in NOx production. Following silica treatment, approximately 11 μM of NOx was produced by BAL cells, whereas NOx levels were non-detectable in control samples.

BAL cell populations from control rats were composed almost entirely of AMs (97%–100%). Treatment with either LPS or silica resulted in a significant influx of PMNs into bronchoalveolar spaces. The relative percentages of AMs and PMNs in BAL cell populations following either LPS or silica treatment are shown in figure 2. Before density gradient centrifugation, BAL cell populations from either LPS-treated or silica-treated rats were composed of approximately 20% AMs and 80% PMNs. After density gradient centrifugation, these cell populations separated into two factions which were relatively enriched for AMs (~ 60%) or PMNs (~ 90%; figure 2).

The amounts of NOx produced by the enriched BAL cell populations from either LPS-treated or silica-treated rats are shown in figure 3. The amounts produced by the AM-enriched fractions were approximately 2-4-fold greater than those produced by the PMN-enriched fractions. Estimations of the percent contribution of AMs or PMNs to the amounts of NOx produced are presented in table 1. Following LPS treatment, approximately 80% of the NOx produced appeared to derive from AMs and 20% from PMNs. The range across three separate experiments was 75%–89% from AMs and 11%–25% from PMNs. Following silica treatment, approximately 90% of the NOx produced appeared to derive from AMs and 10% from PMNs. In this case, the range across three separate experiments was 76%–100% from AMs and 0%–24% from PMNs.

Immunohistochemical results for iNOS staining are presented in figure 4. AMs were strongly positive for iNOS in the lungs of both the LPS-treated and silica-treated rats (figure 4); controls had very little positive staining (data not shown). In LPS-treated animals, there were many PMNs in alveolar areas and around blood vessels. Although some of the PMNs in alveolar areas displayed positive staining for iNOS, most did not (figure 4A). In silica-exposed rats, AMs in the alveoli were associated with iNOS positive, amorphous material along with a number of cells with condensed or fragmented nuclei. Although few PMNs were observed in bronchoalveolar areas following silica treatment, those that were found were negative for iNOS staining (figure 4B).

4. Discussion

The results from our study demonstrate that AMs are the primary source of the NO produced by rat BAL cells dur-

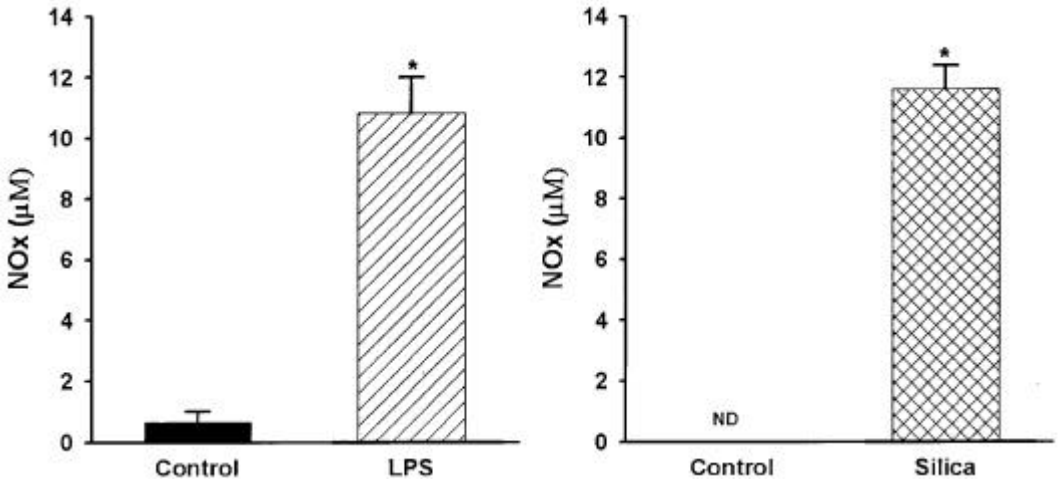

Figure 1. NOx production by BAL cell populations from control, LPS, or silica-treated rats before density gradient centrifugation. BAL cells were cultured for 18 h at $0{\cdot}25 \times 10^6$ viable cells/0·5 ml (LPS experiment) or 1×10^6 viable cells/0·5 ml (silica experiment). Media were then collected and analysed for nitrate and nitrite (NOx). Each value is the mean ± SE of three separate samples from one experiment. These results are representative of three separate experiments for each treatment. ND, Non-detectable.*$P \leq 0{\cdot}05$ vs control.

ing acute pulmonary inflammatory responses to LPS or silica. Our findings also indicate that lung-recruited PMNs contribute to a much lesser extent to the NO produced following these treatments. To our knowledge, this is the first report which has specifically assessed the respective contributions made by AMs and PMNs to the amounts of NO produced by BAL cell populations following exposure to LPS or silica. In previous investigations, evidence has been presented to suggest that AMs are a source of the NO produced by rat BAL cell populations after *in vivo* LPS or silica treatment (Antonini *et al* 1994; Blackford *et al* 1994; Huffman *et al* 1997; Porter *et al* 2002). However, in those studies, the specific amounts of NO derived from AMs were not evaluated. In addition, the potential contribution of PMNs to NO production by BAL cell populations was not determined. Therefore, our study provides new information concerning NO production by AMs and PMNs during acute pulmonary inflammatory responses to either LPS or silica.

Regarding NO production by PMNs, it is known that PMNs are capable of expressing iNOS mRNA and producing NO (Evans *et al* 1996; Kolls *et al* 1994; McCall *et al* 1989; Wright *et al* 1989; Yui *et al* 1991). However, much of the work in this area has involved the study of peritoneal or circulating blood PMNs. It is possible that PMNs recruited into bronchoalveolar spaces may produce a spectrum of inflammatory mediators which differs from that produced by PMNs at other body sites. Therefore, assessment of the contribution that lung-recruited PMNs make to the production of specific inflammatory mediators, such as NO, is of importance. Previously, it was shown that iNOS mRNA levels were increased in rat BAL cell populations which had been enriched for PMNs after the intratracheal instillation of silica (Blackford *et al* 1994). However, the actual production of NO was not evaluated in that study. Furthermore, it is known that both transcriptional and post-transcriptional mechanisms can be important in the control of iNOS expression (Weisz *et al* 1994; Rao 2000). In this regard, our evaluation of the actual amount of NO produced by BAL PMNs provides new information.

Immunohistochemical evidence from the present study also supports our *ex vivo* culture data concerning NO production by BAL PMNs and AMs. Following LPS treatment, we found that PMNs in bronchoalveolar areas were generally unstained for iNOS, whereas AMs were positive for iNOS protein. In regards to iNOS staining following silica treatment, the PMNs observed in bronchoalveolar areas were negative for iNOS staining. However, the AMs were positive for iNOS protein. In addition, Porter *et al* (2002) found that, following the long-term inhalation of silica, rat lung AMs and alveolar epithelial cells stained positively for iNOS protein, whereas PMNs were unstained or only weakly positive.

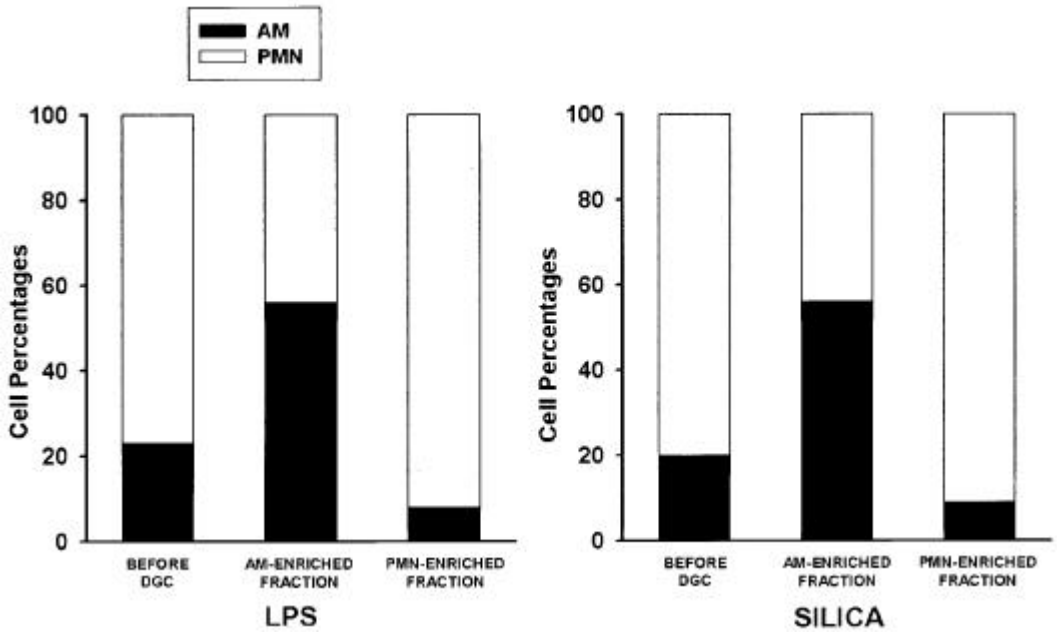

Figure 2. Percentages of AMs and PMNs in BAL cell populations from either LPS-treated or silica-treated rats before density gradient centrifugation (DGC) and after density gradient centrifugation. The AM-enriched and PMN-enriched fractions were the cell fractions obtained after density gradient centrifugation. These results are representative of three separate experiments for each treatment.

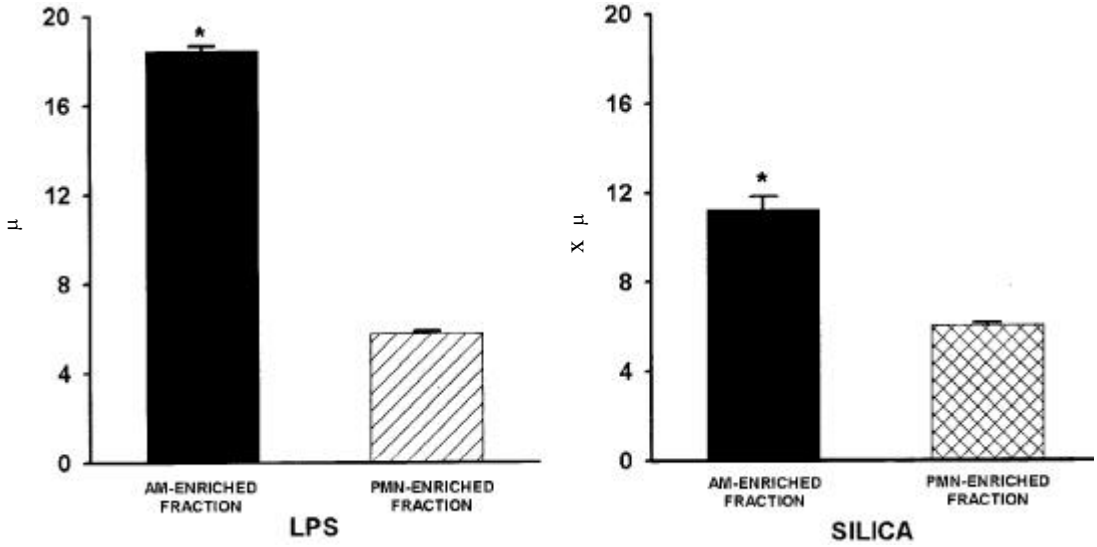

Figure 3. NOx production by AM- and PMN-enriched BAL cell fractions from LPS or silica-treated rats. BAL cell fractions were cultured for 18 h at $0{\cdot}25 \times 10^6$ viable cells/0·5 ml (LPS experiment) or 1×10^6 viable cells/0·5 ml (silica experiment). Media were then collected and analysed for nitrate and nitrite (NOx). Each value is the mean ± SE of three separate samples from one experiment. These results are representative of three separate experiments for each treatment. *$P \leq 0{\cdot}05$ vs PMN-enriched fraction.

Table 1. Estimation of the percent contribution of AMs and PMNs to NOx production following IT instillation of LPS or silica.

	Treatment group	
Cell type	LPS	Silica
AMs	81 ± 4%	92 ± 8%
PMNs	19 ± 4%	8 ± 8%

Values are the means ± SE for three separate experiments.

This finding supports our conclusion that AMs are the primary source of NO following silica exposure.

It should be noted that we only evaluated NO production by AMs and PMNs at one time-point after exposure to LPS or silica. It is possible that the cellular source of NO could change as a function of time. For example, it has been shown that there is a kinetic expression of various cytokines, including tumour necrosis factor-α and interleukin 1-β by rat BAL cells during LPS-induced acute lung inflammation (Xing *et al* 1994). Results from that study indicate that initially AMs are the primary source of cytokines. However, at later time points, infiltrating PMNs become the predominant source of cytokine production by BAL cell populations. This may also be the case for NO production by BAL cells following exposure of the lung to LPS or, perhaps, silica. It would be of interest to explore this possibility in future investigations. Nevertheless, our results do indicate that AMs are the major source of NO production by BAL cell populations at 18 or 24 h following LPS or silica treatment, i.e. times when acute pulmonary inflammatory responses to LPS or silica occur.

In these experiments, we used two types of density gradient centrifugation methods to enrich BAL cell populations for AMs or PMNs. It is interesting to note that the Percoll-based method worked well for BAL cells from LPS-treated rats, but not for BAL cells harvested following silica treatment. In the latter case, we observed diffuse, poorly defined cell banding patterns when the continuous Percoll-based density gradient was used. It is unclear why BAL cells from silica-treated rats could not be separated using the Percoll method. Since Percoll is composed of colloidal silica coated with polyvinylpyrrolidone, perhaps the silica-based nature of this medium is a contributing factor. Alternatively, it is known that AMs phagocytize silica particles *in vivo*. Since each AM is capable of ingesting a different number of silica particles, and thus would have different bouyant densities, separation of AMs and PMNs on the continuous Percoll-based density gradient used in the present study may not be possible. Although our attempts to use a Percoll-based method to separate BAL cell populations from silica-treated rats were unsuccessful, we found that a discontinuous Histopaque density gradient method could be used. Thus, another result from our study is the finding

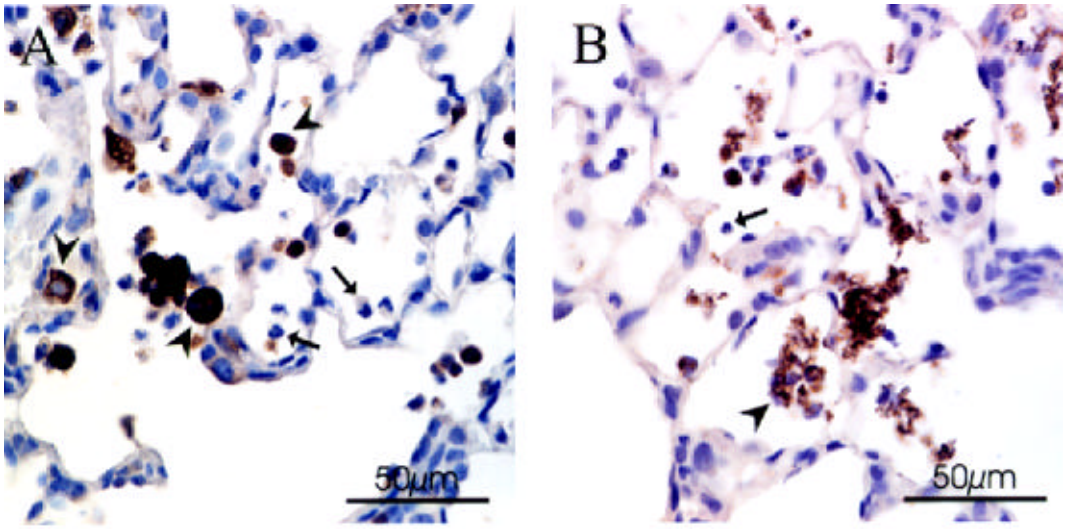

Figure 4. Immunohistochemical localization of iNOS in paraffin sections of rat lungs. **(A)** Rat instilled with LPS. AMs are highly positive for iNOS, while the PMNs are negative. **(B)** Rat instilled with silica. Within the alveoli, AMs are associated with iNOS positive, amorphous material; PMNs, when seen, are negative. (Arrows, PMNs; arrowheads, AMs).

that different density gradient centrifugation methods may have to be used to enrich BAL cell populations depending upon the treatment.

In summary, it is important to define the contributing roles of AMs and lung-recruited PMNs to the cascade of inflammatory mediators produced following exposure of the lung to different stimuli. The results from the present study provide new information concerning the relative contribution of AMs and PMNs to the amounts of NO produced following exposure of the lung to LPS or silica. Our findings indicate that the majority of the NO produced by BAL cells during acute pulmonary inflammatory responses to LPS or silica derives from AMs. However, PMNs appear to contribute to some extent to the generation of this free radical.

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