Cytokines and oxygen radicals after hyperoxia in preterm and term alveolar macrophages

HENRY J. ROZYCKI, PAUL G. COMBER, AND THOMAS F. HUFF

Departments of Pediatrics and Microbiology and Immunology, School of Medicine of Virginia Commonwealth University, Richmond, Virginia 23298-0276

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Rozyczki, Henry J., Paul G. Comber, and Thomas F. Huff. Cytokines and oxygen radicals after hyperoxia in preterm and term alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 282: L1222–L1228, 2002. First published January 18, 2002; 10.1152/ajplung.00230.2001.—To determine if the alveolar macrophage inflammatory cytokine response to oxygen differs in premature cells, macrophages were obtained from litters of premature (27 days) and term (31 days) rabbits. The majority of these cells were nonspecific esterase positive and actively phagocytosed latex particles. The cells that expressed cytokines also reacted with a monoclonal antibody against rabbit macrophages. After incubation overnight in 5 or 95% oxygen, the amount of interleukin (IL)-1β and IL-8 mRNA was assessed by RT-PCR and the amount of cytokine protein by quantitative immunofluorescence microscopy. The preterm macrophage showed a significant increase in cytokine mRNA and protein after overnight incubation in 95% oxygen. This response was not seen in the term cells. Only premature macrophages had a significant increase in intracellular oxygen radical content, measured by 2’,7’-dichlorofluorescin analysis, after incubation in 95% oxygen. This enhanced inflammatory cytokine response to oxygen may be one mechanism involved in the early development of chronic lung disease in premature infants.

chronic lung disease; prematurity; rabbits

CHRONIC LUNG DISEASE (CLD) can occur in newborn infants who require supplemental oxygen and positive pressure ventilation after birth. The incidence of CLD is directly proportional to the degree of prematurity (38). This is true for the classic form of this condition, originally called bronchopulmonary dysplasia, as well as the newer type of CLD, which occurs after less intense exposure to hyperoxia and volutrauma. This latter condition is associated with the younger and smaller infants who are surviving in greater numbers compared with 30 years ago (25).

It has been known for almost 20 years that infants who would go on to develop CLD have a stronger and more prolonged acute inflammatory response in their lungs compared with those who recover from respiratory distress syndrome (RDS) without long-term sequelae (34). More recently, several investigators reported that inflammatory mediators such as interleukin (IL)-1β (37), IL-6 (5), tumor necrosis factor (TNF)-α (27), and IL-8 (29) are present in higher concentrations in lung lavage from babies who develop CLD. These mediators are present in animal models of CLD (9), and there is evidence that they play an active role in the early pathophysiology of CLD (33).

Although several different environmental and infectious stimuli have been implicated in the initiation of the lung injury that culminates in CLD, the major ones are mechanical ventilation and oxygen toxicity (1). In some animal models, the latter is felt to be the stronger risk factor for CLD (10). Oxygen, through formation of oxygen radicals, can damage tissue, and radicals can act as secondary messengers to promote elaboration of mediators (42). Because the premature lung is deficient in its antioxidant capacity (3), exposure to oxygen is likely to lead to an increase in oxygen radical formation (17). After injury from oxygen, an inflammatory reaction develops in the lungs, orchestrated through the local elaboration of chemical mediators, including the cytokines listed above. Many different cells in the lung can express inflammatory mediators (28), but the alveolar macrophage is the most likely initial source of cytokines after exposure to hyperoxia (7).

The relationships between prematurity and CLD and between early inflammation and CLD are well established. However, the relationship between prematurity and the pulmonary inflammatory response to oxygen has not been extensively investigated. Specifically, it is not known whether the premature alveolar macrophage reaction to hyperoxia is different from that seen in macrophages from term lungs. In part, this may have been due to limitations in techniques available to study cells that are present in relatively fewer numbers in the premature lungs. By pooling cells obtained by lung lavage from litters of rabbits and by employing amplification techniques to measure the amount of cytokine mRNA and protein expressed after in vitro hyperoxia exposure, we have been able to examine how prematurity affects the alveolar macrophage cytokine response to oxygen.

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METHODS

Female New Zealand White rabbits with timed pregnancies were obtained from commercial breeders and kept for at least 4 days to allow for acclimatization. Animals were given food ad libitum, provided with nesting boxes, and given 12-h light/dark cycles.

Litters of preterm (27-day gestation) and term (31-day gestation) fetuses were delivered by hysterotomy immediately after pentobarbital sodium euthanasia of the doe, and each pup was given a lethal dose of pentobarbital intraperitoneally within seconds of delivery. This prevented significant exposure to room air oxygen. All protocols were approved by the Institutional Animal Care and Use Committee.

Lung lavage was performed through a 23- or 25-gauge (for the term and preterm pups, respectively) butterfly needle placed in the trachea under direct visualization. Each pup was lavaged three times with 1 ml of warmed 150 mM NaCl-1 mM EDTA solution. The aspirates from each pup in the litter were pooled and kept on ice. For adult rabbits, lavage was performed with five aliquots of 10 ml each of the NaCl-EDTA solution.

The pooled samples were centrifuged at 1,200 g × 10 min at 4°C, the red cells were lysed in 1 ml of endotoxin-free water, and the cells were were centrifuged at 1,200 g × 3 min at 4°C before being suspended in complete medium (RPMI 1640 with 10% fetal calf serum, 2 mM l-glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin). Fetal calf serum was from Sigma (St. Louis, MO), and medium and additives were from Bio-Whittaker (Walkersville, MD). Manual cell counts and viability by 0.2% trypan blue exclusion were performed, and the volume was adjusted to a final concentration of 25 × 10^6 live cells/ml. In sterile 96-well plates, 0.2 ml of cell suspension (5 × 10^5 cells) was placed in a well, and the plates were incubated in 5% O_2-5% CO_2-95% humidity (model 165 Ciba-Corning blood gas analyzer; Bayer Diagnostics, Tarrytown, NY). In some experiments, adult or term cells were incubated in low or high oxygen with or without 1 mg/ml lipopolysaccharide, serotype 026:B6; Sigma).

Cell characterization. Cells from preterm and term litters were incubated on chamber slides and then processed for May-Grunwald/Giemsa staining for morphology, for nonspecific esterase staining (Sigma), or for phagocytosis, based on an overnight incubation in 5% oxygen with 9 × 10^7 1 µm sterile latex beads (Sigma) in 0.2 ml of complete medium. Alveolar macrophages were identified by immunofluorescent staining with RAM11, a monoclonal antibody specific for an intracellular antigen in rabbit macrophages (41) as described below.

Reverse transcription-polymerase chain reaction. Total RNA was isolated using a single step guanidine-urea-phenol system (Ultraspec II; Biotex, Houston, TX), extracted in chloroform, precipitated in isopropanol and then 75% ethanol, and stored at −80°C in diethyl pyrocarbonate-treated water. It was uncommon to have sufficient RNA from the newborn animals to perform any direct quantitation, so a housekeeping gene, β-actin, was used in a semiquantitative RT-PCR assay to correct for differing yields of RNA.

An aliquot of the total RNA was reverse transcribed to cDNA using the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT) at 20°C × 10 min, 42°C × 30 min, and 99°C × 5 min, total volume 20 µl. An aliquot of the cDNA was then used for the PCR reaction. The PCR mixture was first heated to 95°C for 5 min, during which the primer pairs for the IL-1β or IL-8 gene (at 1.2 µM) and for β-actin (0.6 µM) were added. The PCR reaction then proceeded through 36 cycles at 95°C × 1 s, 60°C × 1 s, and 72°C × 2 s, followed by a 10-min extension at 72°C. For IL-1β, the primers were 5'-GACCTGCTTGAAGGCTTTTATAG and 5'-TCCCTGTTGTTGCTCGACGTATGA, which nets a 437-bp product between positions 890 and 453 of the rabbit gene (32). The IL-8 primers were 5'-GAGTGGACCTCACTGTGCAA and 5'-GCCAGTGGCAACAAACA and defined a 594-bp portion.
of the IL-8 gene between position 197 and 791 (43). The other primer pair was 3'-CTCCGTGAGGATCTCTGAGGGTGTAG and 5'-GGGAGAAGATGCCCCAGATGTTGTT, giving a 221-bp product from position 660 to 421 of the rabbit β-actin gene (42). PCR products were resolved on a 2% agarose gel, transferred to a Nytran membrane (Schleicher & Schuell, Keene NH) overnight in 2× SSC, and then ultraviolet cross-linked to the membrane (Stratolinker UV, 1,200 Ci n).

For the Southern blot, the IL-1β or IL-8 and the β-actin probes (100 ng each) were end-labeled with 750 and 150 μCi 32P, respectively (DNA 5'-End Labeling Kit; Boehringer-Mannheim, Indianapolis, IN). The IL-1β probe CCGTGCCT-GGTTATTGAAAGACGATAAACC was complementary to position 679–859 of the rabbit gene (32). The IL-8 probe, CCGTGCCTAGGCTGCTGAAATGTTAG, was complementary to positions 430–559 of the rabbit gene (43), and the β-actin probe, CATCGTATGACCTCTGGACGAGGTCGATCAC, was complementary to positions 525–554 for rabbit β-actin (20). The blot was incubated for 1 h at 37°C in hybridization solution (0.1% BSA (wt/vol), 0.1% polyvinylpyrrolidone (wt/vol), 0.1% Ficoll, 2× SSC, 0.1% SDS (wt/vol), 0.025% yeast RNA (wt/vol) in 50 mM sodium phosphate, pH 6.5) and then hybridized overnight at 37°C in the same solution containing both probes. The blot was then washed with 2× SSC-0.1% SDS × 10 min at 37°C twice and 0.2× SSC-0.1% SDS × 1 h at 37°C twice. The labeled blot was exposed to X-ray film at room temperature. At least three different experiments were performed with each age group, and Figs. 2 and 3 are representative of the same results obtained within each age group.

**Intracellular immunofluorescent staining.** Lung lavage cells were incubated on glass chamber slides (LabTek 8 chamber slides; Nalge, Napierville, IL) using 0.4 ml (~1× 10^4 cells/chamber) in medium containing 0.133 μl of monensin solution/well (Cytostain kit; PharMingen, San Diego, CA). After aspirating off the medium with monensin, we fixed and stained the cells according to the manufacturer’s instructions accompanying the Cytostain kit. In brief, the cells were first fixed for 20 min in fixation/permeabilization buffer. All subsequent dilutions and washes were performed with the wash solution supplied, which contains saponin and 0.1% sodium azide in phosphate-buffered saline. Wells were washed twice between each incubation. Blocking antibodies (100 μg/ml) were incubated for 10 min, and first and second antibodies for 30 min. All incubations were performed at 4°C, and the final incubation was in the dark.

Antibodies utilized for IL-1β staining were, in sequence, goat IgG (Sigma), polyclonal goat anti-human IL-1β (5 μg/ml; R & D Systems, Minneapolis, MN), pig IgG (Sigma), and fluorescein isothiocyanate (FITC)-labeled pig anti-goat IgG (1:30; Boehringer-Mannheim). Antibodies utilized for IL-8 were mouse IgG (Sigma), mouse anti-rabbit IL-8 (5 μg/ml, clone 2g3.2; PharMingen), rat IgG (Sigma), and FITC-labeled rat anti-mouse IgG (1:50; PharMingen). Rabbit macrophage staining was performed utilizing mouse IgG (Sigma), mouse anti-rabbit macrophage (1.2 μg/ml, clone RAM11; Dako, Carpinteria, CA), donkey IgG (Sigma), and Texas red-labeled donkey anti-mouse IgG (1:100; Jackson Immunoresearch, West Grove, PA).

After being stained, the cells were sequentially photographed during exposure to light at 450–480 nm for FITC and then at 550–590 nm for Texas red, if necessary. All slides were either visualized immediately or stored in the dark at 4°C for no more than 3 days. Multiple ×200 images were captured from an Olympus Provis system microscope through the attached high-speed charge-coupled device camera (SenSys camera; Photometrics, Tucson, AZ).

Fluorescence was quantitated using IPLab Spectrum version 3.1 scientific image analysis software (Signal Analytics, Vienna, VA). Background fluorescence was subtracted from each image. To control for nonspecific fluorescence, the average fluorescence pixel density from cells not incubated with the primary antibody was subtracted from the fluorescence pixel density/cell from cells exposed to both the primary and FITC-labeled secondary antibody. Mean fluorescence levels were calculated from at least 100 cells per well. Mean cellular fluorescence levels for each oxygen exposure were analyzed for significant differences by two-way ANOVA and Tukey’s post hoc t-test.

**Fig. 2.** Southern blot of RT-PCR products from preterm and term rabbit alveolar macrophages incubated overnight in 5 or 95% O2. In each lane the top band is IL-8 (594 bp) and the bottom band is β-actin (221 bp). The preterm cells show a small amount of IL-8 DNA in 5% O2 and a much larger amount after overnight incubation in 95% O2. The term cells also have a small amount of IL-8 after 5% O2 but no increase after exposure to 95% O2. Representative blot from at least 3 experiments/age group.

**Fig. 3.** Southern blots of RT-PCR products from adult (A), term (B), and preterm (C) rabbit alveolar macrophages incubated overnight in 5 or 95% O2. The adult and term cells were also exposed to endotoxin. In each lane the top band is IL-1β (437 bp) and the bottom band is β-actin (221 bp). Neither the adult nor term macrophages demonstrate the same cytokine response to hyperoxia that the premature macrophages show, but the adult and term cells can respond to endotoxin. Representative blots from at least 3 experiments/age group.
Intracellular oxygen radical content. After overnight incubation in 5 or 95% oxygen, the cells from the premature and term rabbits were incubated for 15 min in fresh medium supplemented with 10 μM 2',7'-dichlorofluorescein (DCF). DCF has been used to measure intracellular oxygen species in a large variety of cell types, including macrophages in many different species (22). The unoxidized dye is nonfluorescent and freely permeable through the cell membrane, but once exposed to reactive oxygen species (ROS), the DCF produced is both fluorescent and more polar. The fluorescent dye remains within the cell for up to 1 h (36). Overall, the relative amount of fluorescent dye present measures general oxidative stress and the steady-state concentration of ROS. The cells were then exposed to fluorescent light, and microscopic images of dye-exposed and dye-unexposed cells were digitally captured. The amount of auto fluorescence for each oxygen concentration and gestational age was calculated by image analysis from the non-DCF-exposed cells, and this value was subtracted from the fluorescence intensity values of the DCF-exposed cells. At least 100 cells per sample were used for the calculation. To determine if there were any significant differences between premature and term alveolar macrophages in the fluorescent density after hyperoxia, we analyzed the data by two-way ANOVA and then by Tukey’s test.

RESULTS

Cell characteristics. Each litter consisted of between 5 and 13 pups with a median of 8 pups per litter. The average total cell yields were 18,500 ± 8,900 (mean ± SE) cells/pup in premature rabbit litters and 102,900 ± 36,600 cells/pup for the term litters. The Mann-Whitney’s test showed that the term litters had significantly more cells (P < 0.05), but this difference was not significant when cell yield was corrected for weight (619 ± 299 cells/g for the premature rabbits, 2,058 ± 732 cells/g for the term, P = 0.1). Term cells were >95% macrophage by morphology, nonspecific esterase positivity, and latex bead phagocytosis observation. In the preterm wells, 73–86% of the cells were macrophages by the three criteria, whereas the rest appeared to be epithelial cells.

In Fig. 1 are representative sequential photomicrographs of cells lavaged from a litter of preterm rabbits incubated overnight in 95% oxygen and stained with anti-IL-1β and anti-rabbit macrophage antibodies. The bright cells, which are positive for IL-1β, are also positively identified as macrophages.

Induction of cytokine message. As seen in the representative example in Fig. 2, macrophages from the preterm rabbit expressed a small amount of IL-8 mRNA in 5% oxygen, but after overnight exposure to 95% oxygen there was an increase in the cytokine message. The term macrophages did not demonstrate a similar IL-8 mRNA change after hyperoxic exposure. Similarly, only preterm macrophages showed a vigorous IL-1β message induction in 95% oxygen. The term and adult macrophages expressed IL-1β mRNA when incubated with endotoxin but not when exposed to hyperoxia (Fig. 3).

Induction of IL-8 protein. The preterm macrophages also expressed more IL-8 protein after hyperoxic exposure compared with term cells. Figure 4 includes representative photomicrographs of fluorescently labeled anti-IL-8 from the two age groups after 5 and 95% oxygen exposure. The mean fluorescence per cell in each group is shown on the graph in Fig. 5 and is

Fig. 4. Photomicrographs of preterm and term macrophages fluorescently stained for intracellular IL-8. Cells from each gestational age were incubated in 5 or 95% O₂. Magnification ×100.

Fig. 5. Bar graph of mean pixel density/cell of alveolar macrophages incubated in 5% or 95% O₂ and fluorescently stained for intracellular IL-8 after subtracting background and autofluorescence. #P < 0.05 vs. preterm 5%.
significantly elevated in the preterm 95% oxygen-exposed cells compared with the 5% oxygen exposed.

**Intracellular oxygen radical accumulation.** When cells were incubated overnight in 5 or 95% oxygen and then incubated for 15 min in medium with DCF, there was a significant rise in the mean fluorescent density in the premature macrophages, which was not seen in the term cells. Figure 6 includes representative photomicrographs of cells from each age in each oxygen concentration, and Fig. 7 is a graph of the mean values for each group. Only the preterm cells had a significant rise in cellular oxygen radical content after hyperoxia.

**DISCUSSION**

When exposed to concentrations of oxygen far in excess of what is available in utero, the antioxidant-deficient premature lung produces high amounts of reactive oxygen intermediates (ROI) (15, 17). In many, although not all, cell types, ROI activate transcription promoters such as nuclear factor (NF)-κB (31). Most of the inflammatory cytokines that have been found in the lungs of premature infants who develop CLD, including IL-1β, TNF-α, IL-6, and IL-8, have NF-κB target sequences in their promoter region (4). This would explain how exposure to oxygen or to oxidant stress can promote expression of these cytokines in vitro (11–13, 35). It would also explain how, in the very premature infant with the least developed antioxidant defenses, exposure to even small amounts of direct oxygen toxicity could provoke the initial inflammatory signs that lead to CLD.

Evidence to support this pathophysiological model for CLD has all been indirect to date, due, in some measure, to the technical difficulties inherent in studying preterm animals or cells. The data presented in this study are, as far as we know, the first that directly demonstrate that cells from the premature lung behave differently when exposed to hyperoxia from those from term or adult lungs.

First, when incubated overnight, the premature macrophages had a significant increase in their intracellular oxygen radical content as measured by DCF fluorescence. DCF may be activated by a variety of radicals (21). Furthermore, activation of DCF may depend not only on the production rate of one or more radicals but also on the complex system of enzymatic and nonenzymatic antioxidants. Higher DCF fluorescence, therefore, may reflect the overall steady-state oxygen radical status within the cell. Thus these results do not identify a specific antioxidant deficiency or deficiencies in the preterm vs. the term rabbit macrophage.

When incubated overnight in 95% oxygen, preterm alveolar macrophages increased their mRNA expression of the proinflammatory cytokines IL-1β and IL-8. The amount of intracellular IL-8 protein was also significantly elevated. There were too few macrophages to measure secreted IL-8 in the medium. The macrophages lavaged from the lungs of term rabbits did not respond. Term rabbits are more resistant to hyperoxia-induced lung injury compared with premature rabbits, just as premature human infants are more vulnerable to lung injury and CLD (16). One reason may be that the term lung can upregulate its antioxidant capabilities, whereas the preterm lung cannot (16). Our findings now add another difference between the premature and the term lung after exposure to hyperoxia.
The macrophage is a major source of inflammatory cytokines in the lung. Macrophages from patients with acute RDS produce increased amounts of IL-8 (14). When rats were rendered macrophage deficient, they not only tolerated hyperoxia (7) but they also did not demonstrate a rise in their intrapulmonary NF-κB activation (30). Macrophages have been seen in the human fetal lung as early as 20 wk of gestation (2), but the number of macrophages recovered by lung lavage is low until a few hours to a day before delivery (23). By 3–4 days of life, macrophage numbers begin to approach adult levels when corrected for lung or body weight (39). Although Jacobs et al. (24) showed that, in preterm monkeys, the presence of RDS depressed the number of macrophages, more recent work has shown that the number of macrophages increases after birth with or without surfactant treatment in preterm infants with RDS (19). During the course of CLD, the macrophage is the predominant leukocyte obtained by lung lavage (8). It is likely that, after preterm labor and birth, macrophages are present and active. So, although in the unactivated lung used in our experiments the low macrophage number in the premature lung may reduce the significance of an increase in cytokine expression, this may not be true in the preterm infant in whom the lung macrophage population increases quickly after birth.

Because there are fewer macrophages in fetal animals delivered operatively, it is more difficult to study their function, but no alternative currently exists for obtaining unstimulated alveolar macrophages from premature animals. Pooling material from the lungs of complete litters of fetal rabbits that have not breathed allowed us to obtain a significant number of unstimulated preterm alveolar macrophages. In spite of this, we had to use techniques such as RT-PCR and quantitative immunohistochemistry to generate a detectable amount of DNA and protein for analysis. These techniques are extremely sensitive and so may provide false positive results. One source of error may be the relatively more heterogeneous cell population obtained from the preterm lungs compared with the term and adult lungs. This may especially affect the RT-PCR. However, the IL-1β-producing cells in our study reacted strongly with a monoclonal antibody that is specific for rabbit macrophages.

There is some controversy regarding the source(s) of alveolar macrophages in the fetus. Evidence supports the model that the macrophage is derived from circulating monocyctic cells that undergo final differentiation in the tissues (18). More recently, data from lung explant cultures have given rise to the hypothesis that fetal lung macrophages may originate from mesenchymal cells present in the pulmonary tissue (40). It is possible that the cells taken from the preterm rabbits were progeny of this mesenchymal source and those from the term animals came from hematopoietic sources. This possible difference in ontogeny might explain their difference in responses to oxygen, but, since it is not possible, as of yet, to differentiate these populations of macrophages (if they exist), this explanation must remain speculative. Another possible explanation for our findings is a higher cell death rate in the macrophages from the older animals; i.e., these cells died before they were able to increase cytokine levels. Both hyperoxia and excess ROI can induce cell death, both by apoptosis and necrosis (6). This does not seem likely in this study, however, since the preterm and term rabbit macrophages cultured overnight in 95% oxygen were still capable of expressing equal amounts of β-actin message. Another possibility is that the cytokine reaction of term macrophages, while similar to that of the preterm cells, is delayed beyond the overnight oxygen exposure that can stimulate the premature macrophages. The term macrophage did produce IL-1β mRNA in response to endotoxin during the same incubation period. Even if there was some delay in the term cell, the earlier response of the premature cells is consistent with the pathophysiological model described above.

It is not clear if the overproduction of proinflammatory cytokines or the underproduction of counterregulatory cytokines, or a combination of both, is a predominant cause of lung damage in the premature infant. Jones et al. (26) noted that not only persistence of IL-8 but absence of IL-10 were seen in premature infants with RDS. Only inflammation promoters were examined in this study. The effect of prematurity on macrophage expression of anti-inflammatory cytokines has not been studied to date.

This study examined only one concentration of oxygen and one period of exposure. The relative paucity of preterm macrophages makes examination of a wide range of treatment conditions difficult. It is also difficult to test a series of antioxidants to determine whether oxygen radical blockade will reduce cytokine expression in premature macrophages, so our finding of a relationship among oxygen exposure, proinflammatory cytokine expression, and prematurity cannot yet be causally linked with a significant degree of certainty.

Because CLD is a disease of prematurity, determining how the preterm lung responds to relevant stimuli is critical. The data presented here outline two such differences, concentrating on one important risk factor and one cell response. Future studies will be needed to examine whether there are differences in the premature response to other agents, such as volutrauma or infection, and to other cells and the lung as a whole. Identifying the difference in the premature response may provide targets for therapies aimed at preventing CLD in premature infants.

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REFERENCES
5. Bagchi A, Viscardi RM, Taciak V, Ensr0 JE, McCrea KA, and Frank L and Sosenko IR. 16. 44x324