Functional and pathological effects of prolonged hyperoxia in neonatal mice

BARBARA B. WARNER, LORIE A. STUART, RICHARD A. PAPES, AND JONATHAN R. WISPE
Division of Neonatology and Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, Ohio 45229-3039

Warner, Barbara B., Lorie A. Stuart, Richard A. Papes, and Jonathan R. Wispe. Functional and pathological effects of prolonged hyperoxia in neonatal mice. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L110–L117, 1998.—Bronchopulmonary dysplasia (BPD) commonly develops in premature infants. An improved understanding of the pathophysiology of BPD requires better models. In this study, neonatal FVB/N mice were exposed to room air or 85% oxygen for 28 days. Neonatal hyperoxia resulted in decreased alveolar septation, increased terminal air space size, and increased lung fibrosis. These changes were evident after 7 days and more pronounced by 28 days. Decreased alveolarization was preceded by decreased proliferation of lung cells. After 3 days of hyperoxia, cell proliferation was decreased compared with room air littermates. Cell proliferation continued to be decreased in the first 2 wk but normalized by 4 wk. Hyperoxia caused an increased number of inflammatory cells in lung tissue and in lung lavage fluid. Analysis of lung tissue RNA by RT-PCR showed that hyperoxia increased expression of the proinflammatory cytokines interleukin-1α and macrophage inflammatory protein-1α. Prolonged neonatal hyperoxia caused functional changes, decreasing lung volume and pulmonary compliance. We conclude that prolonged exposure of neonatal mice to hyperoxia creates a lesion that is very similar to human BPD and suggests that altered cell proliferation may be important in the pathogenesis of chronic neonatal lung disease.

INFANTS BORN PREMATURELY often survive, only to face a lifetime of debilitating diseases that are the result of their prematurity and the therapies necessary to keep them alive. Despite surfactant replacement and technological improvements, chronic lung disease, “bronchopulmonary dysplasia” (BPD), is still the major morbidity of premature birth. In fact, the incidence of BPD has not changed in the past ten years (15).

BPD is caused by acute lung injury in a vulnerable host. Acute pulmonary oxygen injury is complex and multifatorial, but reactive oxygen species are felt to be a major cause of initial injury. Subsequent to the initial injury, there is an influx of inflammatory cells that increases lung damage. Neonatal responses to hyperoxia are unique, probably because injury occurs during the period of alveolar development. One important and uniquely neonatal response to hyperoxia is diminished alveolar development. Massaro et al. (20) and Frank and Groseclose (13) demonstrated that the neonatal period is critical for normal alveolar maturation. Delays or alterations in neonatal lung growth result in permanent architectural abnormalities in alveolar development. In humans, Northway (22) initially described and named BPD and observed that pulmonary immaturity was a consistent factor in its development. The factors regulating normal lung development are incompletely understood, and the effects of hyperoxia or lung injury on them are unknown.

Research into the prevention and treatment of BPD has been hampered by a lack of suitable animal models. In 1978, participants in a National Institutes of Health conference recommended that emphasis be placed on the development of appropriate models (14). Since then, animal models of immaturity have included the rat, rabbit, lamb, and primate. Large-animal models such as the primate offer many advantages and have been extensively studied. Postnatal lung development in primates parallels human lung development and can be subject to a pattern of injury identical to that seen in preterm infants, i.e., ventilation and hyperoxia. The primate model has provided important information regarding the effect of prematurity, ventilation, and hyperoxia on postnatal lung development, surfactant protein expression, and other developmentally regulated genes (7). However, the use of primates is not practical for many investigators, and small-animal models would have several advantages, including cost, ease of maintenance, capacity for genetic manipulation, and the ability to use multiple animals in one study. There is a body of literature that supports the use and validity of rodents for studying neonatal oxygen injury. Bonikos and colleagues (4) were the first to demonstrate that neonatal mice exposed to prolonged hyperoxia developed a chronic lung disease characterized by alveolar dysplasia and fibrosis. More recently, several groups of investigators have used the newborn rat to study the effects of chronic neonatal hyperoxia (13, 16).

The newborn mouse is particularly well suited for studies of neonatal oxygen injury. Murine alveolar development begins on postnatal day 3, and saccular division is completed by the 14th postnatal day (2). This sequence, and the relative timing of alveolarization, resembles human lung development. In addition, the ability to manipulate the murine genome could prove useful for investigating the role of specific factors in lung injury or prevention. In this report, we use the “Northway protocol” (23) to characterize the morphometric and physiological consequences of prolonged neonatal oxygen exposure. We demonstrate that neonatal hyperoxia causes alterations in lung alveolarization and function. These changes are closely associated with an early and profound effect on lung cell proliferation.

METHODS

Animals and oxygen exposure protocol. The time-dated pregnant FVB/N mice were obtained from Charles River
Lung lavage. Selected mice underwent lung lavage to recover alveolar cells. Mice were given a lethal dose of pentobarbital sodium and were placed in a supine position, and trachea were cannulated as above. Room temperature normal saline was gently instilled by syringe until resistance was felt. After 1–2 min, the saline was withdrawn and replaced on ice. Each animal was lavaged five times, and lavage fluid was pooled and centrifuged at 800 rpm for 5 min. Lavage samples were not used unless 80% of instilled saline was recovered. Red blood cells present in the bronchoalveolar lavage (BAL) were lysed with a solution of 0.15 M potassium chloride, and the cell suspension was centrifuged at 800 rpm for 10 min. Cells were resuspended in 0.5 ml of PBS. Total cell counts were performed with a hemocytometer, and differential cell counts were performed on cytoospin preparations stained with Diff-Quick (Scientific Products, McGaw Park, IL). A blinded observer counting a minimum of 200 cells obtained the cell differentials.

PCR analysis of inflammatory cytokine mRNA. The expression of inflammatory cytokines in the lungs of neonatal mice exposed to oxygen or room air was analyzed by RT-PCR. Lungs were recovered, homogenized in 1 ml of 4 M guanidine-thiocyanate, 0.5% Na-lauroylsarcosine, 20 mM sodium citrate, and 0.1 M β-mercaptoethanol. Total cellular RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform method. The RNA pellet was dissolved in water, extracted with phenol and chloroform, and precipitated at −80°C with sodium acetate and absolute ethanol. Recovered RNA was quantitated spectrophotometrically, and 2 µg of RNA were used for individual RT reactions.

Reagents for the RT reactions were obtained from Gibco (Gaithersburg, MD) and used according to their protocol. Reaction mixtures were incubated at 42°C for 1 h. The reaction was stopped by boiling at 95°C for 5 min, and the product was stored at −20°C. Primers were designed using the software program Oligo (National Biosciences, Plymouth, MN). Total RNA was reverse transcribed to cDNA using the following downstream upstream primer pairs: primers mouse interleukin (IL)-1α nucleotides 125–744, 5′-CTC TAG AGC ACC ATG CTA CAG AC-3′/5′-GGC ACA TCA GGT ACG ATC CAG-3′; mouse tumor necrosis factor-α (TNF-α), 5′-GGG AGC TGG TAC TGG CAG AAG CAG-3′/5′-GGT ACA ACC CAT CGG CTG GCA-3′ resulting in a 287-bp product; mouse macrophage inflammatory protein (MIP)-1α, 5′-ACT GCC CTT GCT GGT CCT CTC T-3′/5′-AGG CAT TCA GTC CCA GGT CAG TGA-3′ resulting in a 277-bp product; mouse macrophage inflammatory protein-2 (MIP)-2α, 5′-GGC ACA TCA GGT AGC AGC TAC CAG-3′/5′-ACC CTG CCA AGG GTC TAC-3′ resulting in a 287-bp product. Reagents and protocol for PCR reactions were obtained from Stratagene (La Jolla, CA). The PCR

Lithium (Wilmington, MA). Mice were maintained on standard laboratory food and water ad libitum and kept on a 12:12-h light-dark cycle in the accredited Children's Hospital Animal Care Facilities. All portions of the experimental protocol were approved by the Children's Hospital Medical Center Animal Care and Use Committee.

Exposure to hyperoxia. Within 12 h of birth, pups from four to eight litters were pooled before being randomly redistributed to the newly delivered mothers. One-half of the newborn pups were placed in 85% oxygen, and one-half remained in room air. Nursing mothers were rotated between oxygen-exposed and room air litters every 24 h to avoid oxygen toxicity in the mothers and to eliminate maternal effects between groups. Oxygen exposures were done in 3-ti3 Plexiglas chambers into which oxygen was continuously delivered at 3.5 l/min. Continuous flow achieved a constant level of 85% oxygen and prevented CO2 accumulation. Oxygen levels were monitored with a Minio II monitor (Catalyst Research, Owings Mills, MD). Survival was recorded daily, and mice were exposed to oxygen for up to 4 wk. Body weights were recorded at the time of death.

Tissue preparation. Animals were killed by an intraperitoneal injection of pentobarbital sodium and were exsanguinated by aortic transection. Lungs were fixed in situ with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Tracheas were cannulated with a 25-gauge Silastic catheter, and paraformaldehyde was instilled at 25 cmH2O pressure. After 5 min, lungs were removed and further fixed overnight in 4% paraformaldehyde. The next day lungs were washed in PBS and serially dehydrated in increasing concentrations of ethanol before embedding in paraffin. To standardize analysis, sections were taken from the right lower lobe and upper half of the left lung. Five-micrometer tissue sections were stained with hematoxylin and eosin (H&E), Masson's trichrome for collagen, or Verhoeff-van Gieson's stain for elastin.

Morphometrical analysis. Morphometrical measurements were done using Image-1/Metamorph Imaging System, version 2 for Microsoft Windows (Universal Imaging, West Chester, PA). For measurement of the average area of individual terminal air spaces, five-micrometer H&E sections were examined with a microscope that contained a square lattice composed of 10 intersecting lines in the eyepiece. Fields for measurements of terminal air spaces were obtained from the points of intersection lines in the eyepiece. Calibrations for the images were made by acquiring standard micrometer images using the ×10 objective. These standards were then used to determine the number of pixels per micrometer. Air spaces were distinguished from tissue by image intensity and were converted to a red color, and solid tissue was converted to gray. Images were corrected for tears in air space wall extravasated debris, red blood cells, or inflammatory cells. To measure the area of individual terminal air spaces (17), the computer counted the number of pixels shaded in red per air space, and the number of pixels per air space was converted to square micrometers based on the calibrated standards. For measurement of average interstitial lengths, ×20 computer images with a superimposed square lattice composed of 10 intersecting lines was used. Measurements of terminal air spaces were obtained from the points of intersection lines. For each animal a minimum of 10 lung fields was used for the analysis. Measurement of each alveolar area and interstitial length were exported to Microsoft Excel (Microsoft, Redmond, WA).

Proliferative index. Rates of cell proliferation were determined using bromodeoxyuridine (BrdU) as a marker for dividing cells. BrdU is a thymidine analog that is incorporated into replicating DNA. Neonatal mice were injected subcutaneously if they were ≤1 wk of age and intraperitoneally if they were >1 wk of age. BrdU (ZYMED, San Francisco, CA) concentrated aqueous solution was used according to the manufacturer’s instructions (1 ml/100 g body wt). The concentrated aqueous solution was diluted 1:3 in sterile normal saline and warmed to 37°C before injection. Six hours later, mice were given a lethal injection of pentobarbital sodium, and lungs were fixed as described above. Incorporated BrdU was detected with a monoclonal anti-BrdU antibody and a streptavidin–biotin staining system. Tissue sections were counterstained with hematoxylin to identify unlabeled nuclei. Lung sections were examined using the ×40 objective, and BrdU-labeled cells and unlabeled cells were counted using the Metamorph Imaging system. A proliferative index was calculated with the equation (BrdU nuclei/total nuclei) × 100. For each animal a minimum of 10 lung fields was used for the analysis.

PCR analysis of inflammatory cytokine mRNA. The expression of inflammatory cytokines in the lungs of neonatal mice exposed to oxygen or room air was analyzed by RT-PCR. Lungs were recovered, homogenized in 1 ml of 4 M guanidine-thiocyanate, 0.5% Na-lauroylsarcosine, 20 mM sodium citrate, and 0.1 M β-mercaptoethanol. Total cellular RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform method. The RNA pellet was dissolved in water, extracted with phenol and chloroform, and precipitated at −80°C with sodium acetate and absolute ethanol. Recovered RNA was quantitated spectrophotometrically, and 2 µg of RNA were used for individual RT reactions.

Reagents for the RT reactions were obtained from Gibco (Gaithersburg, MD) and used according to their protocol. Reaction mixtures were incubated at 42°C for 1 h. The reaction was stopped by boiling at 95°C for 5 min, and the product was stored at −20°C. Primers were designed using the software program Oligo (National Biosciences, Plymouth, MN). Total RNA was reverse transcribed to cDNA using the following downstream upstream primer pairs: mouse interleukin (IL)-1α nucleotides 125–744, 5′-CTC TAG AGC ACC ATG CTA CAG AC-3′/5′-GGC ACA TCA GGT ACG ATC CAG-3′; mouse tumor necrosis factor-α (TNF-α), 5′-GGG AGC TGG TAC TGG CAG AAG CAG-3′/5′-GGT ACA ACC CAT CGG CTG GCA-3′ resulting in a 287-bp product; mouse macrophage inflammatory protein (MIP)-1α, 5′-ACT GCC CTT GCT GGT CCT CTC T-3′/5′-AGG CAT TCA GTC CCA GGT CAG TGA-3′ resulting in a 277-bp product; mouse macrophage inflammatory protein-2 (MIP)-2α, 5′-GGC ACA TCA GGT AGC AGC TAC CAG-3′/5′-ACC CTG CCA AGG GTC TAC-3′ resulting in a 287-bp product. Reagents and protocol for PCR reactions were obtained from Stratagene (La Jolla, CA). The PCR
protocol consisted of 30 cycles of denaturation, annealing, and extension at 94°C, 59°C, and 72°C, respectively, using a Perkin-Elmer Cetus DNA thermal cycler. Upon completion of the PCR reaction, the reaction products were separated by electrophoresis through 2% agarose in Tris-borate-EDTA. Product size for each cytokine was determined by comparison with DNA size standards included in the gel. Ethidium bromide-stained gels were analyzed by laser densitometry using an IS-1000 Digital Imaging System (Alpha Innotech). Densitometry results from PCR products were normalized to β-actin internal controls.

Functional analysis. The dynamics of spontaneous breathing were measured in conscious, unrestrained mice in a respiratory chamber designed specifically for mice (system model RSP2005; Kent Scientific, Litchfield, CT). Individual mice were placed in gastight chambers. Intratracheal pressure was controlled by a constant flow of air or oxygen (bias air flow). Flow changes superimposed on the bias air flow caused by mouse respirations were measured using mass air flow sensors located in the inlet and outlet ports of the chamber (model TRN3300) and analyzed using a WorkBench Respiratory Measurement System (Kent Scientific). Integration of both flow signals yields quantitative information about inspiratory and expiratory flow rates, peak inspiratory and expiratory flow, tidal volumes, and respiratory rates.

Pressure-volume analysis. To further analyze the effects of prolonged neonatal hyperoxia, lung pressure-volume relationships were measured in oxygen-exposed mice and compared with room air controls. Mice were given a lethal intraperitoneal injection of pentobarbital sodium and were placed in 100% oxygen to metabolically degas the lungs. The thorax was left undisturbed, and the tracheas were cannulated with a 22-gauge blunt-tip stainless steel needle. The abdomen was opened, and the abdominal organs were eviscerated. The tracheal cannula was connected to a three-way stopcock that was connected to a Motorola pressure transducer model MPX5010DP (Motorola, Phoenix, AZ). The reference port of the transducer was open to atmosphere. The pressure transducer was calibrated with a mercury manometer before each series of experiments. The pressure transducer output signal was direct current coupled, sampled at 20 Hz, and displayed in scrolling mode at 1/s. The pressure transducer output signal was fed into a Tektronix digital oscilloscope model 2430A (Tektronix, Wilsonville, OR). A 3-ml glass syringe was used to inject incremental volumes of air into the lungs through the stopcock. The steady-state change in the transducer output with each incremental volume change was recorded.

The lungs were slowly inflated to 30 cmH2O pressure, and the volume instilled into the trachea was recorded as total lung volume (V30; see Refs. 3, 21, and 24). In previous studies, it was determined that 30 cmH2O pressure are needed to inflate the lungs maximally without overdistending the alveoli, thus representing total lung volume. This pressure was maintained for 3 min before deflation. A drop in pressure of >5 cmH2O during this period was considered to result from an air leak, and the animal was excluded from analysis. After a period of equilibration, the lungs were incrementally inflated and deflated with 0.1–0.2 ml of air, and the pressure was recorded at each volume. Pressure-volume compliance curves during both inflation and deflation were thus generated.

Statistical analysis. Data were analyzed using Statview for Macintosh (Abacus Concepts, Berkeley, CA) using two-way ANOVA and post hoc analysis between groups by the Scheffé's F procedure. Alveolar size data followed a non-Gaussian distribution and were converted to natural log for statistical analysis.

RESULTS

Survival and growth of neonatal mice exposed to hyperoxia. Sixty percent of neonatal mice exposed to hyperoxia survive, with the majority of deaths occurring between 3 and 7 days of life. Prolonged neonatal exposure to hyperoxia adversely affected growth. Oxygen-exposed mice weighed an average of 8% less at 1 wk, 13% less at 2 wk, 15% less at 3 wk, and 26% less at 4 wk compared with room air controls. Activity level and neurological status were not obviously different between oxygen- and room-air-exposed mice.

Effects of neonatal hyperoxia on lung development. The murine lung matures postnatally, with alveolarization occurring in the first 2 wk of life (2). Continuous exposure of newborn mice to 85% oxygen over 4 wk resulted in marked changes in alveolar development and lung growth. This change was characterized by a simplification of acinar structure, with a decreased number of alveoli and greatly enlarged terminal airways (Fig. 1). Terminal air space areas after 3, 7, 14, 21, and 28 days of oxygen exposure are presented in Table 1. In room air mice, terminal air space size decreased with age, a pattern consistent with progressive alveolarization. In contrast, terminal air space size increased over time in oxygen-exposed pups. After 1 wk, terminal air spaces were significantly larger in oxygen-exposed pups compared with room air controls, and the differences persisted throughout the 4-wk experimental period.

The change in terminal air space size is accompanied by increased collagen deposition. Increased collagen deposition was evident after 2 wk of oxygen exposure, increasing through 4 wk (Fig. 1). Collagen was diffusely distributed and increased progressively with continued exposure to oxygen. Morphometrical analysis of interstitial thickness demonstrated significant increases in mean interstitial lengths by 3 and 4 wk of oxygen exposure compared with room air controls (Table 2). Lung injury is often associated with changed elastin structure or distribution (5). Light level examination of elastin in oxygen-exposed animals over time did not show any differences from room air controls.

In summary, chronic neonatal exposure to 85% oxygen resulted in markedly decreased alveolarization, increased collagen deposition, and increased interstitial thickness.

Effect of neonatal hyperoxia on lung cell proliferation. It is crucial to identify the mechanisms involved in decreased alveolarization caused by neonatal hyperoxia. Alveolarization could be decreased if proliferation of epithelial or mesenchymal progenitor cells was decreased. To determine whether the decreased alveolarization was associated with decreased cell division, lung cell proliferation was measured. BrdU was used to identify dividing cells, and proliferation indexes were calculated in mice exposed to oxygen or room air (Table 3). In room air mice, proliferative indexes begin high (30 ± 10% on third day of life) and decrease significantly over time (ANOVA P < 0.05). In oxygen-exposed mice, proliferative indexes were significantly lower at 3
and 5 days ($P < 0.05$) compared with room air littermates. After 2 wk of hyperoxia, cell proliferation was slightly increased in oxygen-exposed mice, whereas proliferation was steadily decreasing in room air littermates. By 4 wk, there was no difference in cellular proliferation between oxygen-exposed pups and room air littermates. No statistically significant change in proliferation was found over time in oxygen-exposed animals. These results demonstrate that prolonged and early neonatal hyperoxia has profound effects on lung cellular proliferation.

Oxygen-induced lung inflammation. In adult rodents, hyperoxia is associated with lung inflammation, and it is presumed that these inflammatory cells exacerbate injury. Less is known about inflammation in neonatal pulmonary oxygen injury. We studied lung inflammation by 1) examining lung sections for inflammatory cells; 2) quantitating inflammatory cells in lung lavage; and 3) measuring the expression of proinflammatory cytokines during hyperoxia.

Histology. A mild inflammatory response was seen in histological sections from oxygen-exposed mice. The inflammatory cells were located mainly in the perivascular and peribronchiolar regions. The inflammation is maximal after 2 wk of hyperoxia and diminished thereafter.
L114 EFFECTS OF PROLONGED HYPEROXIA IN NEONATAL MICE

Table 1. Median area of terminal airways

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
<th>Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOL</td>
<td>Mean, µm²</td>
<td>Range, µm²</td>
</tr>
<tr>
<td>3</td>
<td>934 ± 822</td>
<td>1,046 to 1,460</td>
</tr>
<tr>
<td>7</td>
<td>681 ± 460</td>
<td>1,554 to 1,828</td>
</tr>
<tr>
<td>14</td>
<td>412 ± 399</td>
<td>580 to 4,420</td>
</tr>
<tr>
<td>21</td>
<td>380 ± 324</td>
<td>481 to 580</td>
</tr>
<tr>
<td>28</td>
<td>470 ± 354</td>
<td>593 to 8,680</td>
</tr>
</tbody>
</table>

DOL, day of life; n, no. of animals used per group; median, median individual air space area expressed in µm² (see METHODS). *P < 0.05 in comparison between oxygen-exposed and room air littersmates.

Lung lavage. To quantitate and more clearly identify the cellular components of the inflammatory response, BAL material from oxygen-exposed and room air mice was analyzed. Results from BAL paralleled what was evident on histological sections. The total number of inflammatory cells was increased in BAL material after 14 days of hyperoxia (Table 4). In room air mice, the majority of lavaged cells were alveolar macrophages or mononuclear white cells. After exposure to oxygen, the number of polymorphonuclear white cells was increased. This neutrophilic response peaked after 2 wk of hyperoxia, with neutrophils accounting for one-third of the cells.

Analysis of cytokine expression. The expression of neutrophil chemotactic cytokines was measured by RT-PCR to begin to identify the factors responsible for the inflammatory response. Expression of TNF-α, IL-1α, MIP-2, and MIP-1α was compared in lungs of hyperoxia or room air mice (Fig. 2A). IL-1α and MIP-1α mRNA were increased after 3 days of hyperoxia and remained elevated through 14 days. Densitometric analysis of PCR products demonstrated a modest increase of 50–80% in IL-1α over 5–14 days (Fig. 2B). More significantly, the change in MIP-1α increased an average of 300–400% over the 5- to 14-day time period (Fig. 2B). TNF-α and MIP-2 expression was different between oxygen and room air mice.

In summary, histological sections and BAL from oxygen-exposed newborn pups demonstrate a polymorphonuclear inflammatory response after 2 wk of oxygen exposure. Increased expression of IL-1α and MIP-1α precedes the neutrophil influx, implicating a potential role for these neutrophil chemotactic cytokines in the inflammatory response.

Effect of neonatal hyperoxia on lung function. Lung function was tested to determine if there were functional correlates to the oxygen-induced changes in lung anatomy. Pulmonary function was studied in spontaneously breathing mice by whole body plethysmography (system model RSP 200E; Kent Scientific). For these studies, the same groups of mice were studied repeatedly to follow changes in lung function during continuous exposure to hyperoxia or room air. Noticeable changes in lung function developed by 2 wk of age, which progressed over time. In mice exposed to hyperoxia for 28 days, respiratory rates were decreased. The slowed rate of breathing was due to prolonged inspiratory and expiratory times, with decreased inspiratory and expiratory flow rates (Table 5).

To further evaluate the effects of neonatal hyperoxia, lung pressure-volume relationships and total lung volumes were compared between oxygen-exposed mice and room air controls. Figure 3 shows an inflation-deflation curve of one 4-wk-old mouse exposed to hyperoxia and one exposed to room air. Very similar pressure-volume curves were obtained from all other mice examined after 28-day exposure to hyperoxia or room air. The lungs of oxygen-exposed mice are less compliant than those of room air mice. At any inflation pressure, lung volume is less in oxygen-exposed mice. Total lung volume, measured as the volume necessary to achieve a pressure of 30 cmH₂O, was significantly decreased after prolonged exposure to hyperoxia. V₃₀ was 0.65 ± 0.06 ml (n = 8) in oxygen-exposed mice and 1.0 ± 0.1 ml (n = 6, P < 0.05) in room air mice. These

Table 3. Cellular proliferation in lungs from neonatal mice exposed to O₂ or room air

<table>
<thead>
<tr>
<th>Days</th>
<th>n</th>
<th>RA, %</th>
<th>n</th>
<th>Oxygen, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>9</td>
<td>30 ± 10</td>
<td>11</td>
<td>8 ± 4*</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>19 ± 3</td>
<td>8</td>
<td>6 ± 2*</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>13 ± 7</td>
<td>8</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>14 ± 6</td>
<td>7</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>6 ± 3</td>
<td>7</td>
<td>16 ± 5*</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>3 ± 1</td>
<td>5</td>
<td>10 ± 4*</td>
</tr>
<tr>
<td>28</td>
<td>6</td>
<td>3 ± 2</td>
<td>6</td>
<td>8 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of animals used for each group. Results are reported as the percent of cells labeled with bromodeoxyuridine (BrdU). Proliferative indexes were determined as described in METHODS, with BrdU used to label dividing cells. For each animal 10 lung fields were analyzed. *P < 0.05 in comparison between oxygen-exposed and room air (RA) littersmates.

Table 4. Bronchoalveolar lavage cell counts and differentials

<table>
<thead>
<tr>
<th>DOL</th>
<th>Group</th>
<th>n</th>
<th>Total Cells (×10⁴)</th>
<th>Differential, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PMN</td>
</tr>
<tr>
<td>7</td>
<td>RA</td>
<td>3</td>
<td>3 ± 3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>Oxygen</td>
<td>3</td>
<td>4 ± 5</td>
<td>12 ± 8*</td>
</tr>
<tr>
<td>14</td>
<td>RA</td>
<td>6</td>
<td>19 ± 18</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>Oxygen</td>
<td>6</td>
<td>21 ± 16</td>
<td>30 ± 3*</td>
</tr>
<tr>
<td>28</td>
<td>RA</td>
<td>3</td>
<td>74 ± 12</td>
<td>1 ± 2</td>
</tr>
<tr>
<td></td>
<td>Oxygen</td>
<td>3</td>
<td>75 ± 22</td>
<td>4 ± 3</td>
</tr>
</tbody>
</table>

Results are means ± SD and are expressed as the total number of cells × 10⁴/ml, with differentials expressed as the percentage of the total count; n, no. of animals/group. PMN, polymorphonuclear neutrophils; Mono, monocytes; Lymph, lymphocytes. *P < 0.05 compared with room air.
studies demonstrate that prolonged exposure of mice to hyperoxia results in decreased lung compliance. These functional changes are very similar to those seen in infants with BPD.

DISCUSSION

In this study, we demonstrate that prolonged exposure of neonatal mice to hyperoxia resulted in dysplastic lung growth with decreased alveolarization, increased collagen deposition, and increased interstitial thickness. Decreased alveolarization began early and was preceded by decreased proliferation of lung cells during the critical period of alveolar septation. With continued exposure, a mild inflammatory response became evident, which resolved over time. Architectural and histological changes were associated with changes in lung function. Oxygen-exposed animals had decreased lung volume, decreased pulmonary compliance, and decreased inspiratory and expiratory flow rates. The anatomical and functional changes in neonatal mice exposed to chronic hyperoxia are very similar to those seen in infants with BPD.

The damaging effects of hyperoxia on the mature lung have been well described (8). Less is known about neonatal pulmonary oxygen injury, but it is clear that the neonatal lung responds to hyperoxia and lung injury in a unique manner. Presumably, this is because injury occurs on the template of rapid neonatal lung growth and maturation. It appears that there is a critical period during postnatal lung development when disruptions of the temporal or anatomical patterns of lung maturation result in permanent changes (13, 20). Infants with neonatal lung injury often never recover full lung function, and pathological examination of their lungs later in life shows persistent abnormalities in lung morphology. It is critical to develop an understanding of the mechanisms by which oxygen adversely impacts lung maturation. Our results suggest that the mouse can be used for such investigations.

The hallmark features of BPD are decreased number of alveoli, increased variability in alveolar size, and interstitial fibrosis (22, 24). Northway and others (16, 23, 24) have described the effects of continuous exposure of neonatal animals to hyperoxia. In mice exposed to 80% oxygen for 2 wk or longer, lung volume and alveolar surface area were decreased, as was alveolar surface area (23). We have extended this observation, demonstrating that changes in alveolarization occur very quickly. By 1 wk of age, oxygen-exposed mice had significant changes in alveolar size and number.

The most impressive effects of neonatal hyperoxia were on lung cell proliferation. These effects were early and profound, suggesting that the effect of hyperoxia on cell division is central to dysplastic alveolar growth. Exposure to 85% oxygen for 3 days markedly inhibited cell proliferation, which remained decreased through the 1st wk of life. These effects were early and profound, suggesting that the effect of hyperoxia on cell division is central to dysplastic alveolar growth. Exposure to 85% oxygen for 3 days markedly inhibited cell proliferation, which remained decreased through the 1st wk of life. This is precisely the period of maximal alveolarization in mouse lung. There was a slight increase in proliferation after 2 wk in hyperoxia. By 4 wk of age, proliferative indexes were the same in oxygen-exposed or room air mice. In contrast to our findings, a very early effect from hyperoxia, Han et al. (16) recently reported that in neonatal rat lung the major impact of 60% oxygen on lung DNA synthesis occurred during the 2nd wk of exposure. The differences between that study and ours may be explained by the difference in oxygen exposure (85 vs. 60%) or species (mouse vs. rat). Both studies demonstrated a biphasic effect of hyperoxia on cell proliferation, with
an initial inhibition of cell division followed by an increase. Which lung cells are affected by neonatal hyperoxia has not been determined.

Oxygen could affect cell proliferation directly by decreasing DNA synthesis and cell division or indirectly by causing death of important progenitor cells. It is increasingly evident that cellular redox state and oxygen tension are important regulators of gene expression, affecting both transcription and translation (12). Both in vitro and in vivo, oxygen-induced changes in cellular proliferation have been associated with changes in growth-related proteins (6, 16). The mechanism underlying our findings remains to be elucidated.

Lung injury and dysplastic growth could also be caused by inflammatory cells and their products. Inflammatory cells can increase lung injury by producing damaging oxygen radicals and proteases. Inflammatory cells also release cytokines that can regulate the inflammatory response and growth factors that determine which cells proliferate and affect the fibroelastic structure of the lung (1, 25). In the current study, there were increased numbers of neutrophils in the lung and in BAL fluid. There was also an increase in the expression of the proinflammatory cytokines. A wide range of cytokines have been implicated in the inflammatory process. Our study was not inclusive; rather, we chose specific cytokines that are known to be chemotactic for neutrophils (10, 11, 19, 28). The results from RT-PCR analyses are not strictly quantitative; however, the magnitude of the oxygen-related increases, particularly for MIP-1α, suggests that they might be important. Additionally, expression of IL-1α and MIP-1α increased early during oxygen exposure, before any obvious tissue injury. The timing of the increases for IL-1α and MIP-1α suggests that they may be important in modulating subsequent lung inflammation.

There is abundant literature about the histological changes found with pulmonary oxygen injury, but less is known about the functional changes of hyperoxia, particularly in small animals. Recent advances in small-animal physiology allow us to compare the functional effects of neonatal hyperoxia in mice and humans. The changes in lung volume and compliance are remarkably similar in mice after neonatal hyperoxia and human infants with BPD. Despite these similarities, the respiratory rates were decreased in hyperoxia-exposed mice, whereas infants with BPD are usually tachypnic. This may be due to differential effects of hyperoxia on the small-resistance airways or reflect the additive injuries of hyperoxia and barotrauma in ventilated infants.

Recent progress has been limited in clarifying mechanisms of neonatal lung dysplasia. A major factor in this slow progress has been debate over suitable models (9). The preterm infant is biologically unique, and models must be relevant for neonatal adaptation and postnatal lung growth and development. Immature nonhuman primates are very valuable in the study of neonatal lung injury because of their developmental similarities and because they can be mechanically ventilated. Lung injury and fibrosis in the oxygen-exposed primate are similar to that found in preterm neonates. The pattern of injury found in our mouse model is homogeneous, lacking the alternating areas of compression and over-expansion seen in ventilated infants with BPD. However, large animals are expensive and require high-technology intensive care facilities. They are also not suited for studies in which genetic manipulations can be used to study injury or to test therapeutic strategies. The mouse has a long history in the study of pulmonary oxygen injury. Pappas et al. (23), after initially describing the architectural changes in humans with BPD, demonstrated that similar changes were produced in neonatal mice by prolonged exposure to oxygen. This report extends these findings. We report that the decrease in alveolarization common to many models, and a prominent feature of BPD, is associated with a decrease in cell proliferation that occurs in neonatal mice exposed to hyperoxia. The architectural changes are associated with functional changes that parallel those found in BPD. In contrast to other models of oxygen injury in which there is an intense inflammatory response, the inflammatory response in oxygen
exposed mice is mild, resolving over a period of 4 wk. This difference may arise from the level of oxygen used, developmental age, and species of animals used.

We demonstrate that prolonged exposure of neonatal mice to hyperoxia created a lesion that is anatomically and functionally similar to the human disease. We will use this model in the future to explore the altered cell division and to determine the role of cytokines and growth factors in lung injury and repair.

This work was supported by National Heart, Lung, and Blood Institute Grant K08 HL-03101–01. Address for reprint requests: B. B. Warner, Children’s Hospital Medical Center, Division of Neonatology and Pulmonary Biology, 3333 Burnet Ave., Cincinnati, OH 45229-3039.

Received 12 August 1997; accepted in final form 17 March 1998.

REFERENCES


