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Oxygen-Induced Alterations in Lung Vascular Development in the Newborn Rat

ROBERT J. ROBERTS,[25] KENNETH M. WEESNER,[22] AND JOHN R. BUCHER,[23]

Division of Neonatology and Clinical Pharmacology, Department of Pediatrics and Toxicology Center, Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, Iowa, USA

Summary

Newborn rats were exposed to air or hyperoxic conditions for the first 6 days of life. Resulting effects on the pulmonary vascular bed were determined by analysis of barium angiograms, scanning electron microscopy of methylmethacrylate corrosion casts and whole lung, morphometric estimations of pulmonary arteries/area and capillary number/area, and arterial blood gas measurements. Similar studies were also performed on the lungs of animals allowed to recover in air for 1 and 2 wk. Although the general pattern of the pulmonary arterial bed by barium angiograms appeared similar, diminished branching or underfilling of the distal arterial segments was more frequently encountered in hyperoxic-exposed animals. Morphometric examination and corrosion casts revealed differences in vascular pattern and density between hyperoxia and air-exposed animals. The number of capillaries/mm^2 of lung tissue was less in hyperoxic-exposed pups than controls after 6 days of exposure to hyperoxia but markedly increased to slightly above control levels by 2 wk of air recovery. The number of 20–50 μm size vessels/mm^2 followed a similar pattern of change. Corrosion casts of lungs exposed to 6 days of hyperoxia revealed less microvascular density compared to air controls, but after 1 wk recovery in air, hyperoxic-exposed animals had a more extensive network of microvessels. Maximum Pa_{O_2} attained by animals in the various groups closely resembled the patterns of change in microvessel density. These findings support the thesis that a major alteration of lung vascular growth and development occurs subsequent to exposure of the newborn to hyperoxia.

Retarded lung growth and maturation have been demonstrated to result from early neonatal exposure of rats to concentrations of normobaric hyperoxia as low as $Fi_{O_2} = 0.4$ (3). This earlier work from our laboratory demonstrated the potential that exposure to hyperoxia may have in contributing to many of the lung changes previously reported in infants who have developed manifestations of chronic lung disease. A serious functional abnormality which is common to most of these infants is an altered ventilation-perfusion distribution. This has been documented in infants with bronchopulmonary dysplasia (19), Mikity-Wilson disease (18) and chronic pulmonary insufficiency of prematurity (12), all diseases which require, or stem directly from periods of hyperoxic therapy. Swyer *et al.* (18) have reported fewer and smaller capillaries in the lungs of infants who died from Mikity-Wilson disease than were found in the lungs of infants who died from non-respiratory causes. On the other hand, Pratt (16) has shown that pulmonary capillary proliferation occurred in a number of adult humans who succumbed after receiving prolonged periods of hyperoxic therapy for respiratory distress.

The objective of this study was to determine if alterations in pulmonary vascular development occur during, or after exposure of newborn rats to hyperoxia. We therefore examined the effect of exposure to hyperoxia on the pulmonary vascular bed by the injection of barium contrast medium into the pulmonary artery, by morphometric analyses of light level histologic sections of lung tissue, and by infusion of a methylmethacrylate casting medium into the pulmonary vascular bed with examination of the resulting cast by scanning electron microscopy. The functional significance of any structural defects was assessed by examining changes in the maximum arterial oxygen tension attained by neonatal rats breathing 99% oxygen, 1% halothane. The association between hyperoxia-induced changes in arterial blood gases and changes in vascular development has been examined in neonatal rats immediately after 6 days of hyperoxic exposures, and also in animals allowed to recover from such exposures for periods of 1 and 2 wk in air.

MATERIALS AND METHODS

Pregnant rats were obtained from BioLab Corp. St. Paul, MN. Pups born within the 24 h period before the start of a hyperoxic exposure were pooled and randomly grouped into litters each consisting of 11 pups. Hyperoxic exposures were carried out in modified infant incubators as previously described (3). Dams were rotated between hyperoxic and air litters every 24 h during the exposures. Exposures were terminated on the sixth day. A random selection of pups from each exposure group was taken for the analyses outlined below. All the remaining animals were placed in air for 7 or 14 days, at which time they were subject to the same procedures performed on the 6-day-old animals. Litter size was, therefore, reduced to six to eight during days 7–13, and to three to four for days 14–20. During these "air recovery" periods dams were rotated between the litters every 48 h.

The lungs were prepared for light microscopic studies by tracheal infusion, using a solution of cacodylate buffered glutaraldehyde (3%) at a pressure of 25 cmH_2O. Tracheas were then ligated and the lungs immersed in fixative overnight at 4°C. Two specimens were collected from the middle portion of the left lobe and one specimen each from the base of the lower lobe and apex of the upper right lobe. The tissues were diced into 1-mm cubes, postfixed in phosphate buffered osmium tetroxide (2%) for 1–2 h, washed in distilled water, *en bloc* stained with uranyl acetate, dehydrated in graded methanols, and embedded in Epon 812. Sections of 1–2 μm in thickness were stained with toluidine blue.

Light level morphometric studies were performed at a magnification of ×1000 with an AO microscope (American Optical, Buffalo, NY) equipped with a 25-square eyepiece grid. The number of capillaries was determined within each 0.1 mm^2 grid. Capillaries were defined as irregularly shaped endothelial lined vessels contained within the architecture of saccule and alveolar walls. Morphometric counts of vessels 20–50 μm, 50–100 μm, and 100–250 μm in diameter were also performed (9). The number of animals utilized in these studies is indicated in Tables 1 and 2. A total of 60–90 grids per slide were counted and averaged.

The heart and lungs of selected animals from each exposure group were removed *en bloc* and frozen for angiogram analysis of the vascular bed as per Rendas *et al.* (17). Each lung was allowed to thoroughly thaw at 37°C for 1 h before cannulation of the

Table 1. *Effects of hyperoxic exposures on lung capillary development and on the arterial P_{O_2} attained by animals while breathing 99% oxygen, 1% halothane*

F_{IO_2} group	n	Capillary[1] number per mm^2	n	Arterial P_{O_2}[2]
6 days of age (entire time spent in oxygen concentration indicated)				
0.21	8	62 + 9.0	21	296 + 7
0.4	8	58 + 11.0	9	284 + 13
0.8	8	44 + 4.0	6	268 + 7
>0.95	8	39 + 2.0[3]	12	225 + 12[3]
13 days of age (first 6 days in respective oxygen concentrations, next 7 days in air)				
0.21	6	73 + 4.0	9	333 + 29
0.4	7	84 + 5.0	6	326 + 21
0.8	6	75 + 6.0	7	375 + 21
>0.95	6	69 + 2.0	7	409 + 21
20 days of age (first 6 days in respective oxygen concentrations, next 14 days in air)				
0.21	6	74 + 6.0	6	380 + 20
0.4	8	91 + 6.0	6	420 + 11
0.8	6	89 + 11.0	5	427 + 16
>0.95	7	86 + 7.0	6	398 + 16

[1] Capillary counts were performed as described in "Materials and Methods" on tissue sections from lungs of animals in the various exposure groups, values represent mean ± S.E. expressed as capillaries/mm^2.

[2] Arterial P_{O_2} was determined as described in "Materials and Methods" on animals in the various groups. Animals were removed from hyperoxic or air exposures a minimum of 2 h before preparation for sampling.

[3] Significantly different from air control $P < 0.05$ Student's t test.

Table 2. *Number of small pulmonary vessels in hyperoxic-exposed and control animals at 6, 13, and 20 days of age*[1]

F_{IO_2} group	n	20–50 μm vessels/mm^2	50–100 μm vessels/mm^2	20–250 μm total vessels/mm^2
6 days of age (entire time spent in oxygen concentration indicated)				
0.21	7	5.7 + 1.0	0.6 + 0.1	6.4 + 0.6
>0.95	8	4.6 + 0.7	0.5 + 0.0	4.5 + 0.8
13 days of age (first 6 days in respective oxygen concentration, next 7 days in air)				
0.21	6	6.2 + 0.8	0.3 + 0.1	6.6 + 0.8
>0.95	6	4.2 + 0.3[2]	0.3 + 0.1	4.6 + 0.2
20 days age (first 6 days spent in respective oxygen concentration, next 14 days in air)				
0.21	3	6.5 + 0.4	0.3 + 0.1	7.0 + 0.4
>0.95	3	7.3 + 0.6	0.3 + 0.1	7.7 + 0.6

[1] Vessel counts were performed as described in "Materials and Methods" on tissue sections from lungs of animals in the various exposure groups. Values represent means ± S.E. expressed as vessel numbers/mm^2. n = number of animals studied.

[2] Significantly different from control $P < 0.025$ Student's t test.

pulmonary artery and trachea. A heated (60°C) suspension of barium contrast medium suspended in gelatin (Micropaque Nicholas Labs Ltd., Slough, England; Fisher 275 Bloom gelatin) and water (525 g: 50 g: 450 ml) was infused into the vascular bed at a reservoir pressure of 70 mmHg. After 5–8 min of infusion the glutaraldehyde solution was instilled through the tracheal cannula. After inflation of the lung was complete the infusion was stopped, the trachea tied off, and the lung and heart placed on ice to solidify the barium gelatin mixture. Lungs were then immersed overnight in glutaraldehyde. Radiographs were taken using Kodak X-Omat TL film (Eastman Kodak Co., Rochester, NY) and a Hewlett Packard 43805 N x-ray system (Hewlett Packard, McMinnville, OR). Cross sections were then obtained from the right and left lobes, and these were postfixed, embedded, and stained as indicated previously. With this procedure barium was found in arteries as small as 30–50 μm in diameter.

Methylmethacrylate corrosion casts were obtained by cannulating the pulmonary artery with polyethylene 50 tubing, excising the left ventricular apex, and inflating the lungs with saline (0.9% NaCl) at 25 cmH_2O pressure. Blood was cleared from the pulmonary vascular bed before casting by careful flushing with saline. The polymer mixture was prepared from methylmethacrylate monomer (Aldrich) Baston's compound A, Baston's compound B catalyst (Polyscience Inc., Warrington, PA) in a ratio of 1:1:0.5 (5, 11). Immediately after one drop of promotor (Baston's C) was added to 1 ml of this mixture, the polymer was drawn into a tuberculin syringe and infused through the polyethylene tubing into the pulmonary vascular bed. The infusion was controlled by a constant infusion pump (Harvard Model 600-000 Harvard Inc., Dover, MA) at a rate of 0.2 ml/min maintaining a pressure of 40–50 mmHg. The infusion was discontinued after the appearance of a liberal amount of polymer through the left ventricular apex and complete blanching of the lungs. The preparation was allowed to harden and then extirpated from the chest. Residual lung tissue was removed by corrosion in a KOH solution (34 g/100 ml) at 60°C for 3–5 days. The solution was changed and the casts were rinsed with distilled water daily.

After complete tissue removal the casts were dehydrated in graded ethanol, sectioned, critical point dried, mounted, sputter-coated and scanned using a JEOL JSM 35-C scanning electron microscope. Scanning electron micrographs were obtained at ×20, ×40, ×100, ×300, and ×1000 magnifications on all lobes using the same working distance. Representative whole lung samples (0.5-mm thick cross sections) from all treatment groups were also examined by scanning electron microscopy after tracheal fixation (25 cmH_2O pressure) with glutaraldehyde. Photographs were examined to detect qualitative differences between the treatment groups. In addition, the number of arteries <100 μm in diameter/photograph (at ×100) were counted and compared between groups.

The maximum attainable arterial oxygen tension was determined in animals immediately after the 6-day hyperoxic exposures and also in animals allowed to recover for 1 and 2 wk in air. Animals were removed from the hyperoxic exposures at least 2 hr before determination of arterial P_{O_2}. Animals were lightly anesthetized with ether and immobilized. A mixture of 99% O_2, 1% halothane was then delivered through a hood covering the head for at least 5 min before obtaining an arterial blood sample via the descending aorta. Arterial blood was collected in a heparinized syringe and its oxygen tension determined with an Instrumentation Laboratory Blood Gas Analyzer 712 (Instrumentation Laboratory, Lexington, MA).

To minimize investigator bias, animals were identified only by a number throughout these procedures. The morphometric analyses were done in a blind fashion. The animal experiments were performed in accordance with the guidelines of the University of Iowa Animal Care Committee. Statistical differences between hyperoxic and control animals were determined by ANOVA for capillary counts, Student's t test for vessel counts and chi square analysis for counts of vessels on scanning electron photomicrographs.

RESULTS

The effect of exposure of the newborn rat to hyperoxia on the growth and development of the pulmonary vasculature was investigated in animals exposed to F_{IO_2} = 0.21, = 0.4, = 0.8, or > 0.95 from birth to 6 days of age, and in animals allowed to recover in air for 1 or 2 wk after this 6-day hyperoxic exposure. Pulmonary arterial angiograms were accomplished by injection of barium-gelatin contrast medium into the pulmonary artery. Figure 1 is a comparison of representative angiograms of 6-, 13-, and 20-day-old animals exposed to F_{IO_2} = 0.21 or > 0.95 for the first 6 days of life. The general pattern of arterial branching was similar in air

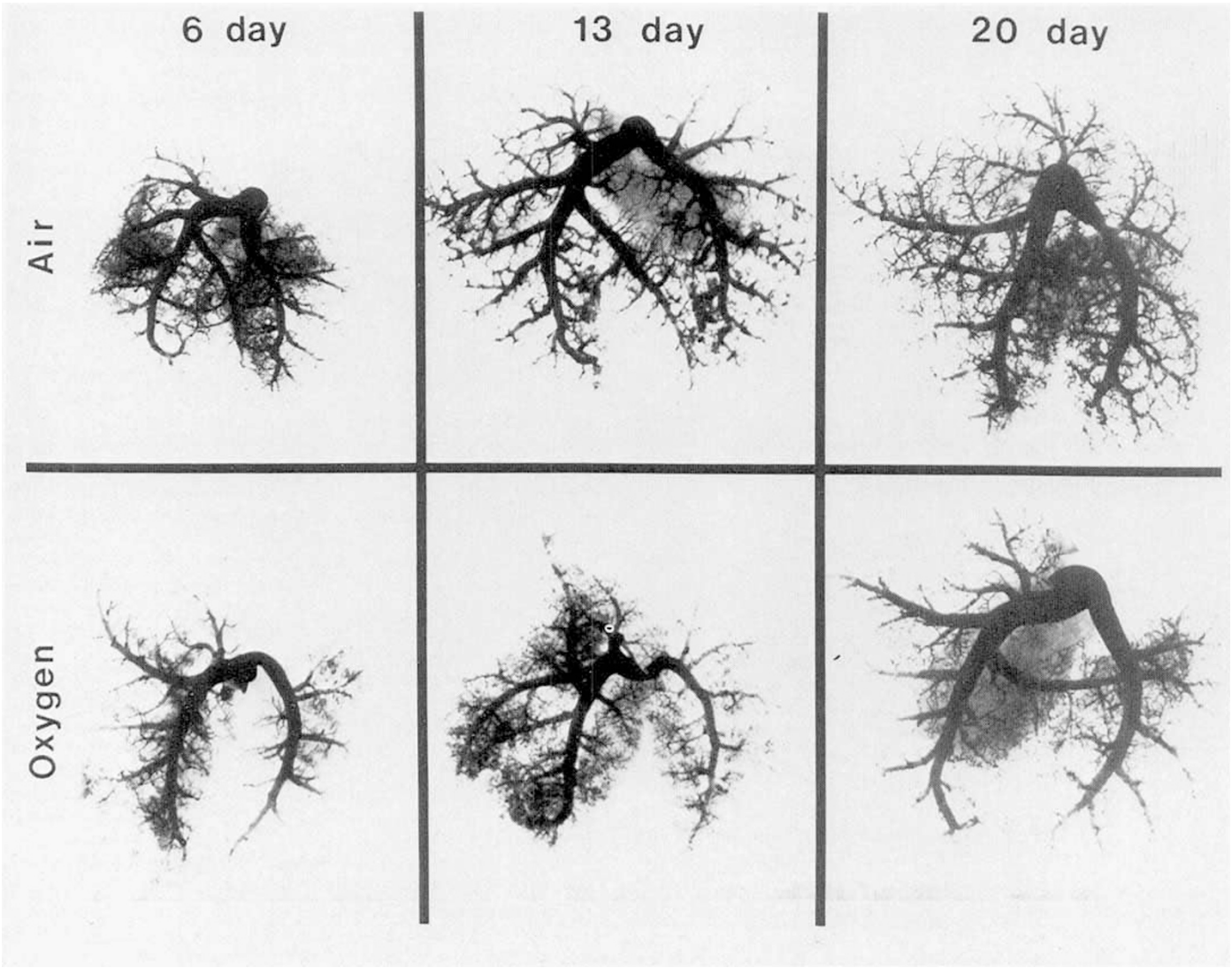

Fig. 1. Barium pulmonary artery angiograms performed in animals exposed to F_{IO_2} 0.21 (*top portion*) or > 0.95 (*bottom portion*) for 6 days and after recovery in room air for 7 (13 day) and 14 days (20 day).

versus hyperoxic-exposed lungs at all ages. The maturation of the pulmonary vascular bed is manifest by increase in size of the major arteries and by branching and extension of the secondary and tertiary arteries. There appeared to be areas of diminished arterial filling in the secondary and tertiary vessels of the hyperoxic-exposed lungs (Fig. 1 bottom panels).

Because the barium angiogram technique only defines arterial vessels greater than 50 μm in diameter, corrosion casts of the pulmonary vascular bed were employed to allow resolution of the microvascular bed. The casting technique has the advantage of penetration of the polymer through the pulmonary microvascular network and has been used by Hijiya to study bleomycin-induced pulmonary vascular injury in adult rats (8).

The scanning electron micrographs of casts from newborn animals exposed to hyperoxia for 6 days reveal major differences in the pulmonary vasculature when compared to air-exposed controls (Fig. 2). The pulmonary vasculature in control animals has a uniform appearing pattern at 6 days with small arteries giving rise to a large number of microvessels. In contrast, the hyperoxic-exposed lungs have a different appearing vascular organization. There are fewer visible small arteries ($\chi^2 = 12.68$, $P < 0.005$) which give rise to microvessels, and the spacing between microvessels appears greater than in control animals. Scanning electron photomicrographs of vascular casts of 13-day-old animals (exposed to >95% oxygen or room air for 6 days then allowed to recover for 7 days in room air) reveal a persistance of fewer numbers of small (20–50 μm) vessels in the hyperoxic-exposed lung (Fig. 3). But the microvascular network, which was present at 6 days, appears to be much more extensive and grossly different in organizational pattern from 13-day control animals (panel D *versus* B).

Morphometric estimation of capillary density showed fewer capillaries per/mm^2 area in the lungs of hyperoxic-exposed animals at the conclusion of the 6-day exposures (Table 1). The capillary numbers were decreased in hyperoxia in a dose-related manner. Capillary number/mm^2 area caught up to control values during the first wk of recovery in air, and showed a tendency towards an increase over control in the lungs of the hyperoxic-exposed animals examined at the end of the second wk of recovery in air.

Maximum arterial oxygen tensions, also given in Table 1, showed that the reduction in capillary density (capillary number/mm^2) might be of importance in limiting gas exchange in the neonatal rat. A dose-related reduction in the maximum arterial oxygen tension attained while breathing 99% O_2, 1% halothane was demonstrated by rats which were exposed to F_{IO_2} = 0.21, =

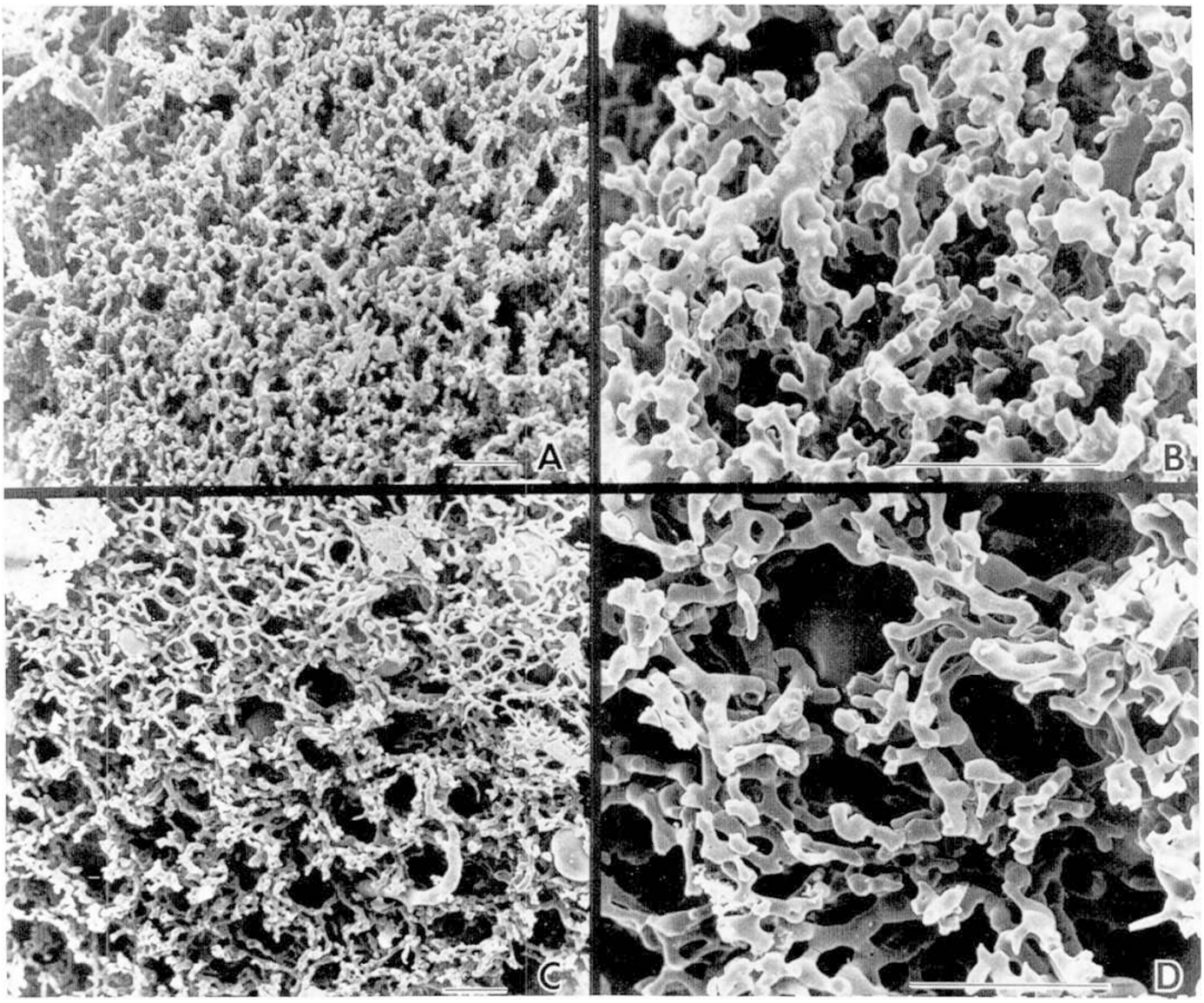

Fig. 2. Scanning electron micrographs of methylmethacrylate corrosion casts of the pulmonary vascular bed in 6-day-old rats exposed to F_{IO_2} 0.21 (*A and B,*) or $F_{IO_2} > 0.95$ (*C and D,*). The magnifications from left to right are ×40 and ×300. Reference line at lower right corner in each frame is 100 μm in length.

0.4, = 0.8, or > 0.95 during days 1–6 of life. After a 1-wk recovery period in air the dose response relationship reversed, with those animals initially exhibiting the lowest gas exchange capabilities, showing the highest ($F_{IO_2} > 0.95$ group). After the second wk of recovery in air, the dose response relationship was less obvious. Nonetheless, these data are suggestive of an adaptive response of the gas exchange capability of the lung following the return to a normoxic environment.

Morphometric estimation of small vessels (20–250 μm) showed no statistical differences between $F_{IO_2} > 0.95$ exposed and control rat pups at 6 days (Table 2). There were significantly fewer of the 20–50 μm size vessels at 13 days in hyperoxic-exposed lungs. By 2 wk of recovery there was a catch-up to controls. The small vessel morphometric estimations in control animals are comparable with those reported by Meyrick and Reid (13).

Scanning electron micrographs of cross sections of fixed inflated lungs show striking differences between control and hyperoxic-exposed rats. In the 6-day-old controls the alveolarization process can be demonstrated with subdivision of the air spaces with secondary septae (Fig. 4, panels A and B). In contrast, the alveolarization process does not appear to be occurring in the 6-day-old pup exposed to $F_{IO_2} > 0.95$. Instead, there is a generalized dilation of the airspaces present (Fig. 4, panels C and D). At 13 days of age the alveolarization process has continued in the control animals (Fig. 5, panels A and B). The 13-day-old animals exposed to oxygen for 6 days and allowed to recover in air for 7 days show a persistence of large air spaces scattered throughout the lung fields, but some secondary septal formation has appeared (Fig. 5, panels C and D).

DISCUSSION

The sensitivity of the developing vessels of the retina to elevated arteriolar oxygen tension has been well documented (1, 2) and is now generally accepted as the causative factor in the clinical condition retrolental fibroplasia (14). The sensitivity of the endothelial lining of the pulmonary vascular bed of the adult rodent to the toxic actions of hyperoxia is also well documented (20) and is

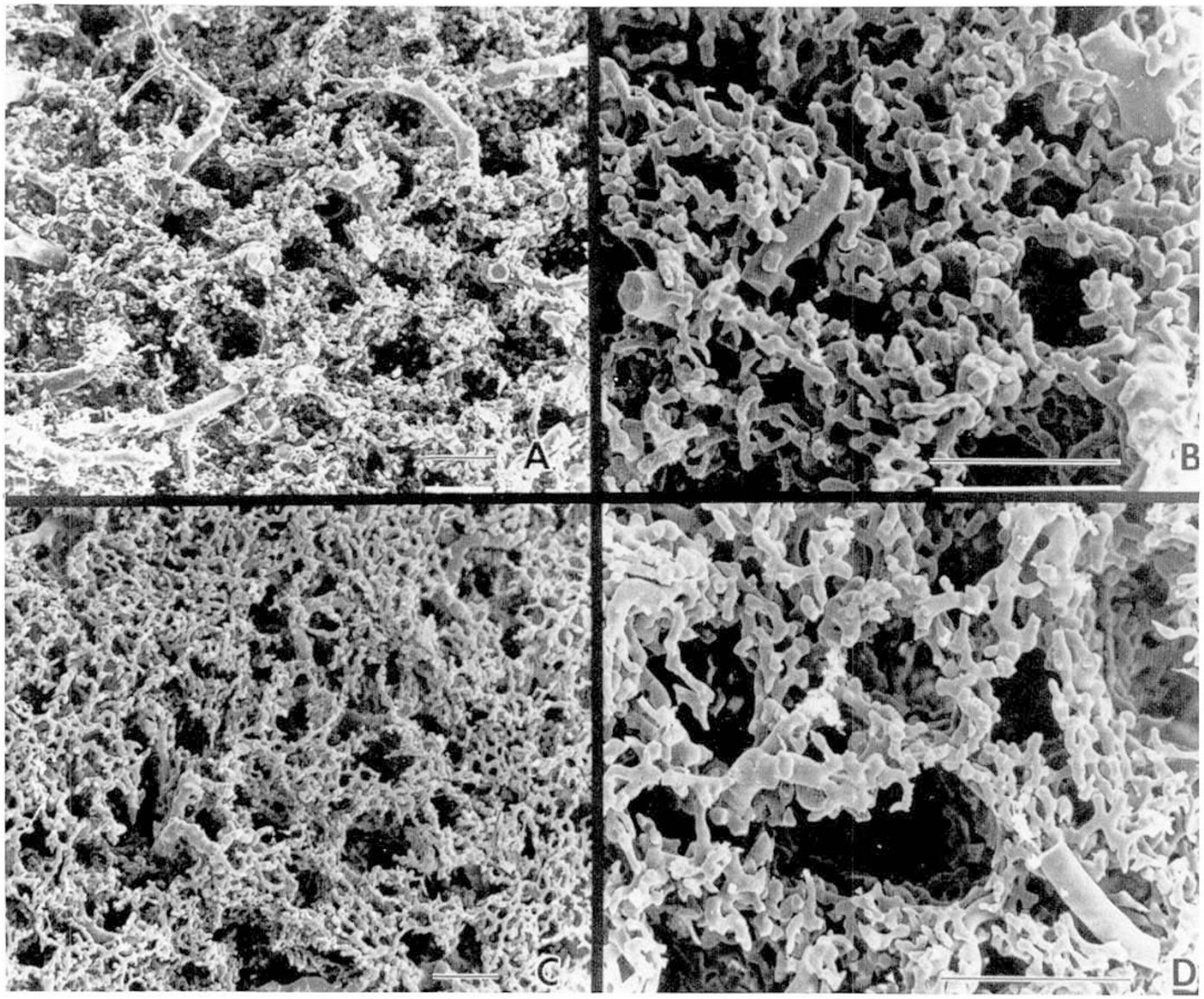

Fig. 3. Scanning electron micrographs of methylmethacrylate corrosion casts of the pulmonary vascular bed in animals exposed to F_{IO_2} 0.21 (*A and B,*) or $F_{IO_2} > 0.95$ (*C and D*) for 6 days, then allowed to recover in room air for 7 days. The magnifications from left to right are ×40 and ×300. Reference line at lower right corner in each frame is 100 μm in length.

proposed to be, at least in part, responsible for the formation of pulmonary edema and the development of respiratory distress in animals exposed to elevated oxygen concentrations. Inasmuch as hyperoxic exposure of the newborn of certain species, including the rat, has not been associated with similar acute lethal consequences (7), hyperoxia does produce a spectrum of lung changes in newborn rats. A recent article from this laboratory detailed the effects of exposure to various concentrations of normobaric hyperoxia on retarding lung growth and the development of alveoli in newborn animals (3).

Pulmonary angiograms and morphometric measurements have been used to study normal pulmonary artery development in pigs, humans, and rats (6, 9, 17). Our pulmonary angiograms, vascular casts and small vessel and capillary morphometry studies reveal a marked disruption of qualitative and quantitative aspects of pulmonary vascular maturation in hyperoxic-exposed animals compared to controls. Capillary counts have demonstrated a reduction in the number capillaries per mm^2 of lung parenchyma in newborn animals exposed to hyperoxia. Small vessel counts and vascular cast studies show a reduced number of 20–50 μm vessels in hyperoxic-exposed animals. The reduction in capillary number/mm^2 correlates well with the reduction in maximal arterial P_{O_2} at 6 days in hyperoxic-exposed rat pups. Although the corrosion casts of 6-day hyperoxic-exposed lungs show the presence of a microvascular (5–15 μm) network (Fig. 2) the vascular pattern and density appear different from controls and consistent with the morphometry studies, which indicated a reduced number of capillaries per unit area. The scanning electron micrographs of whole lung tissue provides visual confirmation of decreased septal density that might be anticipated from the microvascular density and pattern seen with the vascular casting technique (Fig. 2 *versus* Fig. 4). The reduced alveolarization process with hyperoxic exposure is thus associated with a diminished number and density of microvessels in the existing septal walls. This dysmorphic vascular network also might be expected to provide less gas exchange in the hyperoxic-exposed animals compared to controls. The apparent effect of hyperoxia on the alveolarization process (diminished) and microvascular development suggests that a marked disruption of ventilation to perfusion relationships are to be expected.

At 13 days of age (end of the first recovery period) the pattern

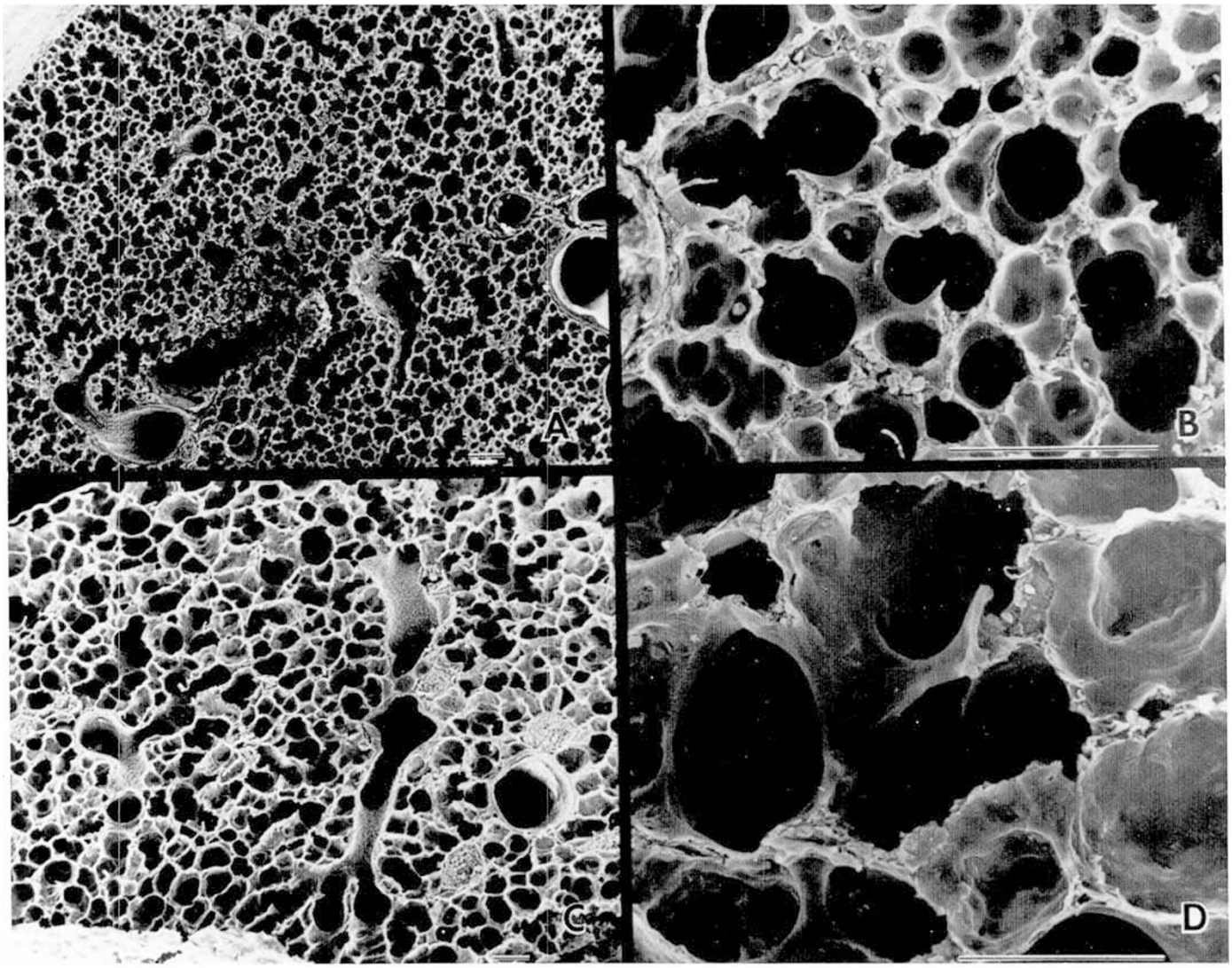

Fig. 4. Scanning electron micrographs of fixed, inflated lungs from 6-day-old rats exposed to F_{IO_2} 0.21 (*top portion A and B*) or $F_{IO_2} > 0.95$ (*bottom portion C and D*). The magnifications from left to right are ×40 and ×300. Reference line at lower right corner in each frame is 100 μm in length.

of reduced maximal P_{O_2} and capillary number/mm^2 in hyperoxic-exposed animals was reversed, with those animals showing the least efficient gas exchange after 6 days of hyperoxia (>0.95 group) exhibiting the highest exchange capability. The casts of hyperoxic-exposed lung now have a very extensive microvascular network (Fig. 3) compared to controls of the same age or hyperoxic-exposed lung at 6 days (Fig. 2). This suggests that either pulmonary changes other than those related to capillary density may be called upon to provide an improved gas exchange, or that the new capillary growth occurring during the recovery period is oriented in such a manner as to maximize contact with the alveolar gas exchange surface. Of note in this regard is the appearance of new septal growth by scanning electron microscope studies (Fig. 5), which may involve normal microvascular/septal development.

Although we have not determined the arterial oxygen tensions under conditions of air breathing in the 6-day hyperoxic-exposed rats, upon return to air it is likely those animals experience difficulty maintaining a normal P_{aO_2}. The increase in lung capillary density occurring during the 2-wk air recovery period and the more extensive microvascular network seen in casts at 13 days may reflect a response to arterial hypoxemia. A remarkably similar observation has been recently reported in retinal vessels of kittens where arterial hypoxemia produced a more severe vasoproliferative retinopathy following hyperoxic injury (15). An alternative explanation for our findings is that they reflect the presence of more immature septa in the lungs of the hyperoxic-exposed animals, because immature secondary septa contain a dual rather than single capillary (4). Similar changes in pulmonary capillary volume were noted by Kapanci *et al.*, (10) in studies in which adult monkeys were allowed to recover in air after a 1- or 2-wk exposure to $F_{IO_2} = 1.0$.

The clinical correlates of these experimental observations in newborn animals may be found in the various forms of chronic respiratory disease in neonates, including bronchopulmonary dysplasia, who require prolonged oxygen therapy (12, 18, 19). Disassociation of the normal maturation process of the ventilation and perfusion components of the lung, resulting in part from hyperoxic treatment, would obviously preclude normal oxygenation. The degree of the adverse effects on lung development would be expected to coincide with the quantitative aspects of the hyperoxic treatment, the intrinsic vulnerability of the individual lung to hyperoxia, and the state of lung maturity at the time of hyperoxic exposure (3). Additional work is currently underway in our laboratory to further characterize the effects of hyperoxia on pulmonary vascular development and to determine a mechanism by which hyperoxia could effect these changes.

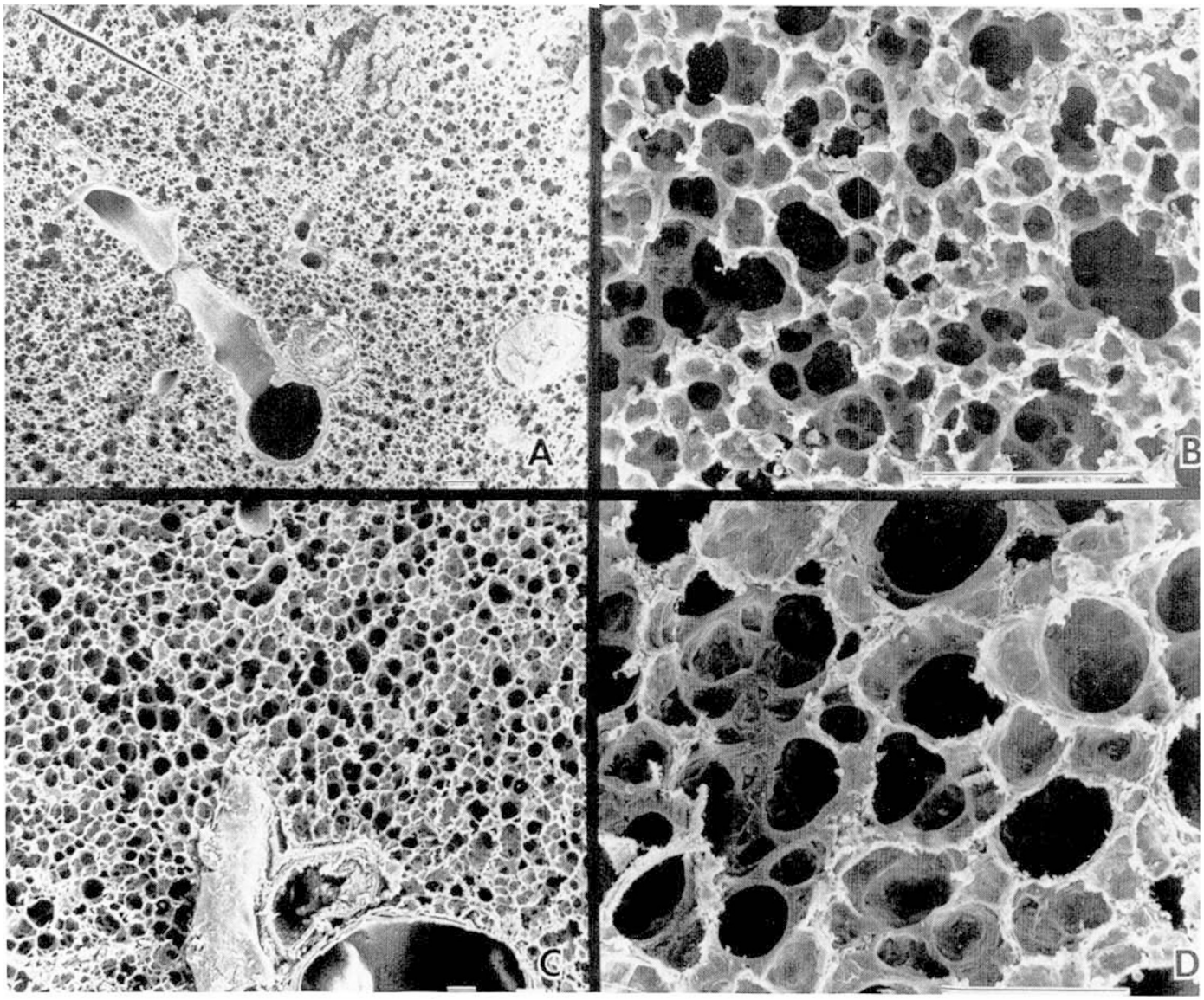

Fig. 5. Scanning electron micrographs of fixed, inflated lungs from 13-day-old rats exposed to F_{IO_2} 0.21 (*top portion A and B*) or $F_{IO_2} > 0.95$ (*bottom portion C and D*) for 6 days, then allowed to recover in room air for 7 days. The magnifications from left to right are ×40 and ×300. Reference line at lower right corner in each frame is 100 μ in length.

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22. Current address, Department of Pediatrics, Bowman Gray School of Medicine, Winston-Salem, North Carolina.
23. Current address, Department of Biochemistry, Michigan State University, East Lansing, MI.
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25. Requests for reprints should be addressed to: Dr. R. J. Roberts, Pediatric Clinical Pharmacology, Depts. Pediatrics and Pharmacology, Bowen Science Bldg. 2-521, University of Iowa, College of Medicine, Iowa City, IA 52242
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