Lung Development in the Fetal Primate *Macaca Nemestrina*. III. HMD

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Summary

Delivery of *M. nemestrina* at 80% of normal gestation provides a population of neonates at high risk for hyaline membrane disease (HMD). The diagnosis of HMD was made by the presence of reticulogranular densities and air bronchograms on chest radiographs. Patchy atelectasis was seen in the lungs of animals assigned by clinical and radiographic criteria to the HMD group and not in the normal lungs of animals matched for gestational and postnatal age. Total phospholipid and phosphatidylcholine in whole lung, airway lavage fluid, and surface-active materials were lower in animals with HMD. Amniotic fluid L/S ratios were lower in the group that developed HMD. Pressure-volume measurements indicated decreased distensibility and unstable terminal air spaces in the HMD group. Alveolar-arterial oxygen pressure differences

were greater in animals with HMD. There were no differences between HMD and normal groups in body weight, lung weight, percent dry lung weight, gestational age, and postnatal age at death. This primate species, subjected to premature delivery, is a suitable animal model of HMD in human neonates.

Speculation

An animal model of hyaline membrane disease (HMD) in a primate species is of interest to investigators of developmentally related pulmonary disorders. More complete knowledge of abnormalities in pulmonary function in HMD will contribute to development of both preventive and therapeutic measures and affords the opportunity to determine risks and benefits of such treatments to the fetus and newborn.

Introduction

Hyaline membrane disease (HMD) is the major cause of neonatal mortality in the United States (10). Rational prevention and therapy of HMD may be derived from more complete knowledge of abnormalities in pulmonary function that occur in this disorder. Definition of altered pulmonary mechanics, surfactant, and structure, particularly the sequential changes during onset and subsequent recovery, is not possible in human neonates with HMD. However, an animal model allows control of variables that affect these measurements. Evaluation of methods of prevention and treatment of HMD that may involve maternal and fetal risk is also an advantage afforded by the study of HMD in an animal model. We propose that the primate *Macaca nemestrina* is a suitable model for HMD when delivered prematurely.

Development of the model for HMD followed studies of lung development in fetal *M. nemestrina* from 108 to 168 (term) days gestation (15,22). After 130 days phospholipid (PL) and phosphatidylcholine (PC) in whole lung and surface active materials (SAM) markedly increased (22). This was associated with an increase in total lung volume and alveolar stability. After 150 days pressure-volume relationships were similar to those described at term (22). We reasoned that premature delivery of fetal animals at an intermediate stage of development, between 130 and 145 days, would provide a population of neonates at high risk for HMD that would be sufficiently mature to survive ex utero. The production of the model and description of abnormalities in chest radiographs, gas exchange, pressure-volume measurements, structure, and PL in lung, SAM, and amniotic fluid are the subjects of this report. Preliminary results of these studies have been reported in abstract (20).

Materials and Methods

Pregnant *M. nemestrina* were obtained from the University of Washington Regional Primate Center Field Station, Medical Lake, Washington. Duration of gestation was known within one day as a result of matings of individually caged females. Pregnancies were terminated by cesarean section as previously described (15). Samples of amniotic fluid were obtained by needle aspiration of the amniotic cavity prior to uterine incision. At delivery the animals were placed under a radiant heater and dried thoroughly with towels. The oropharynx was cleared of fluid by bulb syringe. When the animals did not breathe spontaneously, intermittent positive pressure with oxygen was administered with an anesthesia bag and endotracheal tube until spontaneous ventilation was present. A 3.5 Fr. ga. catheter was inserted through an umbilical artery and into the aorta. The tip of the catheter was positioned at a level between the sixth and eighth thoracic vertebrae. A tracheostomy was performed using a modified blunt 13 ga. needle as the tracheostomy tube. This tube was suctioned approximately hourly to maintain a patent airway. Chest radiographs were obtained within one hour following delivery to determine aortic catheter and tracheostomy tube positions and to evaluate the lung fields. They were repeated between four and six hours of age.

Animals were maintained in incubators during the experiments. The environmental temperature was adjusted in an attempt to maintain core temperatures of 37°C. Dextrose (5%) in water was infused through the arterial catheter (Holter Pump No. 903, Extracorporeal Medical Specialties, King of Prussia, PA) at a constant rate (1 ml/hour). Animals breathed humidified gas which was delivered at a flow rate approximately 5 times the minute ventilation. Fractional inspired oxygen concentration (F_{iO_2}) was adjusted to maintain PaO_2 50-80 mm Hg. Animals who developed persistent apnea or hypercarbia ($PaCO_2 > 70$ mm Hg) were mechanically ventilated with a respiration pump (Harvard Apparatus, Millis, MA). Carbon dioxide tension in arterial blood was maintained in the range 30-45 mm Hg in mechanically ventilated animals. Arterial blood gas tensions and pH were measured hourly or 15 minutes after a change in F_{iO_2} or ventilator settings (pH/Blood Gas Analyzer 713, Instrumentation Laboratories, Lexington, MA). Heparinated placental blood obtained at delivery from the umbilical cord was used to replace sample volumes. Alveolar-arterial oxygen tension differences were calculated from a modification of the alveolar air equation and blood gas tensions measured after the animals breathed oxygen for at least 20 minutes.

Animals were sacrificed between 6 and 8 hours of age by injection of pentobarbital, 150 mg/kg, through the aortic catheter. The lungs were removed immediately and weighed. The right upper lobe was cannulated and removed for microscopic studies as described below. Samples of the remainder of the right lung were excised and weighed for dry weight determinations and homogenization (15). The left lung was weighed and pressure-volume measurements were performed (22). The lung was degassed in a vacuum bell-jar using 15 cm H_2O negative pressure and airlessness determined by visual inspection. All lungs were distended with a maximum pressure of 35 cm H_2O to ensure maximum inflation without causing lung rupture. Lung volume was calculated by dividing the maximum volume of the left lung by the wet weight of the left lung. The left lung was then lavaged with 3 ml of homogenization medium. The fluid was gently withdrawn with a syringe. The procedure was performed five times in each lung. The fluid was centrifuged at 150 x g for 5 minutes to remove cells and debris, and the supernatant was extracted for lipids as described below.

The methods used to isolate SAM, extract and purify lipids, measure lipid phosphorus, separate phospholipids, and calculate lecithin/sphingomyelin (L/S) ratios have been previously described (22). Disaturated phosphatidylcholines (DPC) were measured by the method of Mason et al (19). An internal standard of dipalmitoyl- $1-^{14}C$ phosphatidylcholine (Applied Science Laboratories, Inc., State College, PA) was used to determine experimental losses. Recovery was $95.5 \pm 5.5\%$ (mean ± 1 SD), $n = 24$. Ninety-nine percent of fatty acids recovered in disaturated fractions were saturated, and palmitic acid was 92% of total saturated fatty acids.

The right upper lobe was studied by light and electron microscopy. Glutaraldehyde (1.5%) in 0.1 M α -collidine buffer, pH 7.4, was instilled into the lobar bronchus at 20 cm H_2O pressure. This pressure was maintained for four hours while the lobe was suspended in a bath of fixative. The lobe was stored overnight in glutaraldehyde at 4°C and was then sliced with razor blades (1-2 mm thick slices). The slices were rinsed in 0.1 M α -collidine, post-fixed in 1% osmium tetroxide in 0.1 M α -collidine buffer, and stained en bloc in 1.5% uranyl acetate. The tissue was dehydrated in ethanol and embedded in Epon 812. One μ m sections were cut with steel knives, and further sampling procedures for thin sectioning were done according to the methods of Boatman and Lowe (3). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100-S electron microscope. Microscopic examinations were performed without knowledge of the clinical and radiographic evaluations and results of other analyses.

Animals were grouped for data analysis on the basis of clinical evidence of chest wall retractions and tachypnea and chest radiographs. The diagnosis of HMD was made by the presence of reticulogranular densities and an air bronchogram on chest radiographs. Radiographs were evaluated by seven examiners independently and without knowledge of the clinical conditions of the animals and the results of pressure-volume and biochemical measurements. When the diagnosis of HMD or normal was made by at least 4 of the 7 examiners the animal was as-

signed to the HMD or normal groups, respectively. When assignment on that basis was not possible those animals were not included in the analyses.

Differences between means were tested for significance by student's t test.

Results

Eleven monkeys were delivered by cesarean section between 135 and 142 days gestation. A diagnosis of HMD was made in five animals; three animals were normal; and in three animals a diagnosis could not be made by the stated criteria. The five animals with HMD had clinical evidence of respiratory distress with chest wall retractions and tachypnea; cyanosis was present in ambient atmosphere. Four of five animals in the HMD group were ventilated mechanically because ventilatory failure and/or apnea occurred between the second and fourth hours of the experiments. The peak F_{iO_2} requirement was 1.0 in all HMD animals. The three normal animals breathed spontaneously during the entire course of the experiments. Two normal animals received oxygen in low concentration (F_{iO_2} 0.21 - 0.40). The third normal animal had satisfactory PaO_2 in air for the entire experiment. Environmental temperatures fluctuated due to performance of procedures, and core temperatures were maintained in the range 35-38°C.

Chest radiographs of HMD and normal animals of the same gestational and postnatal age are shown in Figure 1.

The normal and HMD groups are compared in Table 1. Both groups were similar in gestational age and postnatal age at death. There were no differences between the groups in body weight, lung weight, and percent dry lung weight. Alveolar-arterial oxygen pressure difference was greater in animals with HMD. Lung volume at 35 cm H_2O pressure was lower in animals with HMD.

The results of pressure-volume measurements during deflation are shown in Figure 2. The percentage of maximum lung volume retained at 15, 10, 5, and 2.5 cm H_2O pressure was lower in the HMD group. The large variance in the HMD group resulted from one animal in which air trapping occurred. In this animal 36% of the total lung volume was retained at 0 cm H_2O pressure.

The results of phospholipid measurements are shown in Table 2. Total PL and PC in whole lung, airway lavage fluid, and SAM were lower in animals with HMD. Total DPC in lung was lower in the HMD group. Determinations of DPC in SAM were made in only one normal and two HMD animals, and statistical comparison is not possible. The finding of an approximately five-fold greater amount in the normal animal suggests that the recovery of SAM DPC is also lower in HMD. Amniotic fluid L/S ratios were lower in the group of animals that developed HMD.

The results of microscopic examinations were consistent with the radiographic diagnoses (Figures 3 and 4). Patchy atelectasis, characteristic of HMD (16), was seen in the lungs of animals in the HMD group and not in the normal lungs. The extent of the atelectasis varied from animal to animal within the HMD group. The walls of the airspaces were thicker in the HMD group compared to normals (Figures 3a and 4a). Extensive damage to the epithelial cells lining the sacculles, including cell detachment from the basement membrane and the presence of necrotic cells in the airspaces (Figures 3b and 4b), was seen in HMD animals.

Discussion

These experiments indicate that *M. nemestrina* fetuses delivered by cesarean section at about 80% of term gestation may manifest respiratory distress and radiographic signs of HMD. This primate species, subjected to premature birth, is a suitable animal model of HMD in human neonates.

The assignment to HMD and normal groups was made in 45 percent and 27 percent, respectively, of the animals delivered between 135 and 142 days gestation. In the remaining 27 percent of animals a definite assignment to one or the other groups could not be made by the stated criteria. Selection of this gestational age range for premature delivery provided the opportunity to study both normal and HMD animals. Differences in SAM measurements and pressure-volume relationships between the two groups probably reflect variations in rates of lung maturation that influence the incidence of HMD.

Animals with HMD had lower amniotic fluid L/S ratios, airway PL, and homogenate SAM PL compared to normals. Also, total lung volume and the percentage of total lung volume retained at low airway pressure was lower in the HMD group, indicating decreased distensibility and unstable terminal air spaces, respectively. These findings indicate a deficiency in effective surfactant and are consistent with data from studies of human lungs that indicate that surfactant is deficient in HMD (1,4,14). Also consistent with human data is the finding that L/S ratios in amniotic fluid differentiated normal animals and those who developed clinical and radiographic signs of HMD (5,11,24).

These investigations do not delineate the mechanism of reduced SAM recovery and abnormal pressure-volume relationships measured at six to eight hours postnatal age. However, the finding of lower amniotic fluid L/S ratios in HMD suggests that deficiency of surfactant at delivery is the major factor in the genesis of HMD in *M. nemestrina*. Other factors such as insufficient surfactant synthesis (9,12) or surfactant inactivation due to alveolar wall injury and transudation of plasma proteins into air spaces (6,26) may contribute to progressive atelectasis and the increase in clinical severity of HMD after its onset. Studies of synthesis and secretion of SAM, detailed analyses of SAM composition in both health and disease, and careful surface tension measurements to evaluate function-structure relationships of SAM complexes are needed to assess the roles of these factors.

The clinical diagnosis of HMD was substantiated by histological and ultrastructural examination of the lung tissue. Patchy atelectasis, increased thickness of the saccular septa, and epithelial cell necrosis were characteristic of the HMD animals. The extent of atelectasis may have been diminished by the fixation procedure (airway instillation of fixative at 20 cm H_2O pressure). The increased thickness of the saccular walls may be due to a number of factors: interstitial edema formation, intracellular edema and cellular necrosis, and inflammatory response (cellular infiltration). Endothelial cell swelling and capillary occlusion was noted in both the normal and HMD animals. Part of the collapse of the vasculature may be attributable to the method of fixation; that is, much of the blood may have drained from the vasculature during dissection of the right upper lobe and subsequent fixation by airway instillation. Cellular debris in the airspaces (due to epithelial cell necrosis) was characteristic of the HMD animals.

Ventilation was assisted in four of five HMD animals and has been reported to increase PL recovered in airways lavage (21). However, this effect of ventilation is much too small to account for the differences described here in PL in whole lung and SAM between normal and HMD lungs. The value for airway PL of 2.2 g/g dry lung in the non-ventilated animal compared to the group mean of 2.2 $\pm$ 0.7 (SD) and lower airway PL in the HMD group compared to the normal group

indicates that assisted ventilation was not responsible for the difference between groups.

This homologue of HMD should be of interest to investigators of developmentally related pulmonary disorders. The model provides opportunity to measure gas exchange, lung mechanics, structure, and surfactant metabolism in order to define initial abnormalities and subsequent changes that occur during the course of HMD. The mode of action of therapeutic techniques such as positive airway pressure (2,8,13,23) may also be assessed. This model is also suitable for the study of pharmacologic acceleration of maturation of the fetal lung (7,17,18, 25). Such investigations are necessary to develop additional rational methods of prevention and treatment of HMD.

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Table 1. Individual and mean values in normal and HMD animals.

	Gestational age (days)	Postnatal age (hours)	Body Wt. (g)	Lung Wt. (g)	Lung Wt. (% dry)	P(A-a)O ₂ (mm Hg)	Lung Vol. (cc/g)
Normal	137	6.0	379	5.31	14.2	497	2.9
	137	6.9	320	4.85	13.2	357	3.3
	142	8.0	414	6.39	13.2		2.3
mean	139	7.0	368	5.52	13.5	427	2.8
SD	3	1.0	47	0.79	0.6	99	0.5
HMD	135	6.9	257	5.44	14.0	637	1.2
	136	6.9	340	4.90	15.2	595	1.4
	136	6.7	349	6.53	11.6	597	
	137	6.3	315	5.59	15.9	611	1.3
	139	6.4	372	6.28	12.8	611	1.0
mean	137	6.6	327	5.75	13.9	610	1.2
SD	2	0.3	44	0.66	1.8	17	0.2
P	NS	NS	NS	NS	NS	<0.01	<0.005

Table 2. Individual and mean phospholipid measurements in normal and HMD animals.¹

	Whole lung			Airways lavage		SAM			AF
	PL	PC	DPC	PL	PC	PL	PC	DPC	L/S
Normal	108.6 ²	56.7	28.7	25.0	19.8	27.8	22.3	10.8	3.8
	108.0	55.1	25.4	19.4	15.8	25.8	21.7		3.6
	90.9	48.5	21.8	9.9		12.7			
Mean	102.5	53.4	25.3	18.1	17.8	22.1	22.0		3.7
SD	10.0	4.4	3.4	7.6	2.8	8.2	0.4		0.1
HMD	76.8	32.6	10.7	3.2		4.7		1.4	1.2
	73.5	32.7	12.9	1.9	1.0	8.7	4.5	2.4	1.5
	91.5	37.4	14.5			5.9			1.5
	75.0	34.0	12.6	1.6	1.1	3.8	2.6		1.0
	82.5	34.5	13.5	2.2		6.3	3.5		
$\bar{x}$	79.8	34.3	12.8	2.2	1.1	5.9	3.5	1.9	1.3
SD	7.4	2.0	1.4	0.7	0.1	1.9	0.9	0.7	0.3
P	<0.01	<0.001	<0.001	<0.01	<0.025	<0.005	<0.001		<0.001

¹PL, phospholipid; PC, phosphatidylcholine; DPC, disaturated phosphatidylcholine; SAM, surface active materials; AF, amniotic fluid; L/S, lecithin/sphingomyelin.

²Units for whole lung, airway lavage, and SAM values are mg/g dry lung.

Figure 1. — Chest radiographs of *M. nemestrina*. (a) Normal. The catheter in the pleural space was used to measure pleural pressure in other studies. (b) Hyaline membrane disease. Gestational age was 137 days in both animals. Body weights were (a) 320 g and (b) 315 g.

Figure 2. - Pressure-volume measurements during deflation. The upper curve (■) represents 3 normal animals. The lower curve (●) represents 5 animals in the HMD group. The bars indicate 1 SD.

Figure 3. - The typical appearance of lung tissue in a normal animal. Gestational age was 137 days. (a) Light microscopic appearance of the parenchyma. The sacculi are well expanded and have relatively thin walls. 150x. (b) An electron micrograph of a saccular septum. It is lined by thin type I epithelial cells; a cuboidal type II cell is also shown on the right side of the septum. In the interstitium, fibroblasts (F) show intracellular edema and the endothelial cells lining the capillaries are swollen. The lumens of capillaries tend to be collapsed and/or occluded (arrows). 3600x.

Figure 4. - The typical appearance of lung tissue in a HMD animal. Gestational age was 137 days. (a) Light microscopic appearance of an expanded, non-atelectatic region of the parenchyma. The saccular walls are increased in thickness compared to the normal group. 150x. (b) An electron micrograph of one of the thinner saccular septa, as shown in (a). Epithelial cells are swollen and, in some cases, detached, leaving an exposed basement membrane (arrowheads). In the interstitium, fibroblasts (F) show intracellular edema. The endothelial cells are also swollen and the capillary lumens are collapsed and/or occluded (arrows). 3600x.