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Research Article

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Time Course of Changes in Surface Tension and Morphology of Alveolar Epithelial Cells in CO_2-induced Hyaline Membrane Disease *

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Carbon dioxide (CO_2) exposure was found to result in production of pulmonary hyaline membranes in rodents by a number of investigators (1–3). In previous studies with Niemoeller (4), we were able to show that incidence of hyaline membrane formation progressively decreases in guinea pigs during prolonged exposure to CO_2 after compensation of the respiratory acidosis, suggesting a nonspecific acidosis effect.

A lack of surface-active material (surfactant) has been observed in lungs of newborn infants with atelectasis and hyaline membranes (5). The question arose whether the transient CO_2-induced hyaline membrane formation is associated with a loss in lung surfactant and changes in alveolar lining cells, since previous studies show a relationship between lung surfactant and transformation of lamellar bodies of alveolar lining cells. Buckingham and Avery (6) were able to detect a surfactant in the lung of the fetal mouse on the same day of gestation on which Woodside and Dalton (7) noted the first appearance of lamellar bodies in the alveolar lining cells. Klaus and co-workers (8) demonstrated that *1*) after vagotomy of guinea pigs, the lung surfactant and the number of alveolar bodies are reduced, *2*) the surfactant is found in the mitochondrial fraction of ultracentrifuged lungs and not in the mitochondrial fractions of liver and spleen, and *3*) toads and pigeons lack both surfactant and lamellar bodies.

This report extends the observations of Klaus and co-workers and brings further evidence for the relationship between changes in lung surfactant and transformation of lamellar bodies in alveolar cells during the uncompensated phase of CO_2-induced acidosis.

Methods

Male guinea pigs of the Hartley strain weighing between 400 and 600 g were exposed to 15% CO_2 and 3% CO_2 in air (21% O_2) to follow up previous studies (4). The gas mixtures were prepared in the laboratory by mixing pure CO_2 with compressed air in high pressure cylinders. These were analyzed with the Scholander apparatus. A plastic chamber [1] was employed for the experiments. In each set of exposures, guinea pigs were placed into individual metabolic cages within the chamber. The animals were carefully selected. After arrival at the laboratory, the guinea pigs were housed in individual cages, and measurements of body weight were made for 3 to 4 days. Only animals that gained weight and had a leukocyte count below 11,000 were used in the experiments. The carbon dioxide concentration in the chamber was continuously monitored with a Beckman infrared CO_2 analyzer and the oxygen content intermittently with a Beckman O_2 analyzer. The CO_2 concentrations were kept at 15% and 3% within limits of $\pm 0.5\%$ and the oxygen concentration at 21% $\pm 1\%$. The exposure chamber was installed in an air-conditioned room. A closed circuit system within the chamber circulated air continuously through silica gel containers. With these means the environmental temperature was kept at 78° ± 2° F and the humidity at 65 to 75%. Ammonia vapor was absorbed by boric acid placed in a second closed circuit within the chamber. The exposure chamber was opened every morning for a period of about 3 to 5 minutes to fill the water and food containers and take out the urine and feces.

Blood samples were drawn from the abdominal aorta under pentobarbital anesthesia while the animals breathed through a mask the same CO_2 gas mixture to which they had been exposed. Blood pH and P_{CO_2} were determined first with an Epsco blood parameter ana-

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[1] American Sterilizer Co., Erie, Pa.

lyzer and in the later part of the study with an Instrumentation Laboratory blood gas and pH analyzing system. Blood corticosteroids were determined with the technique of Silber, Busch, and Oslapas (9). The lungs were removed within seconds after exsanguination. One lung was frozen for later measurements of surface tension. The major part of the other lung was used for light and electron microscopic studies.

Electron microscopic studies. Cold (4° C) 4.5% glutaraldehyde solution adjusted with 0.1 M cacodylate to pH 7.3 was injected into the main bronchi and the lung immersed into the fixative (10). Fixation was allowed to proceed at 4° C for 24 hours, after which the specimen was transferred for several days into an isomolal wash solution of sucrose-cacodylate, pH 7.3; the latter procedure was repeated once. Representative sections of the fixed tissues were then used for frozen sections, paraffin embedding, usually followed by hematoxylin-eosin or special staining, and for electron microscopy. The tissues, for the latter studies, were cut into 1-mm blocks and then osmicated by Palade's method (11), dehydrated, and embedded in epoxy resin as described by Luft (12) or Freeman and Spurlock (13). Sections 1 to 2 μ thick were stained with toluidine blue (14) and examined with the light or phase microscope. Ultrathin sections were examined with an Elmiskop 1, usually after staining with lead by the method of either Karnovsky (15) or Reynolds (16).

From each experimental animal, 12 blocks were prepared for electron microscopic studies. Of these, on the average, four blocks were sectioned and examined. Ten sections per block were studied and lamellar bodies in the individual granular pneumocytes counted. The data on lamellar bodies, presented in Table I, are based on 40 sections per experimental animal. Because of the two-dimensional properties of sections, the number of lamellar bodies observed represents only a fraction of the total number per cell.

Surface tension measurements. The specimens of lung were kept frozen until immediately before each study of surface properties. Samples of 0.7 to 3.0 g (the majority between 1 and 2 g) were minced into 1- to 2- mm pieces and stirred about 3 minutes in a solution of 30 ml of 0.85% saline at room temperature, 23° C. The mixture was then filtered through gauze into a Teflon trough, 12.8 × 5 cm, and allowed to "age" for 20 minutes. The surface area was changed over a 10-minute period from 55 cm^2 to 8.5 cm^2 and back to 55 cm^2. Surface tension was measured as the downward pull of the surface film on a partially submerged frosted platinum strip. The displacement of the platinum in the solution was minimal and resulted in less than 1 dyne per cm change in weight from the buoyancy effect.

The effect of sample size on the result was checked by the observation that 0.7 g of normal lung in 30 ml saline achieved a very low tension of 3 dynes per cm on one occasion, and 3.0 g of abnormal lung in 30 ml saline did not go below 26 dynes per cm on another.

The number of cycles of the film was not the same for each sample. The film was compressed and ex-

TABLE I

*Effect of exposure to 15% CO_2 in 21% O_2 on surface characteristics of lungs and morphology of alveolar epithelial cells in guinea pigs**

Condition	No. of animals	pH	P_{CO_2}	Corticosteroids†	Lung wt	Maximal surface tension	Minimal surface tension†	Film compressibility‡	Electron microscopy; no. of lamellar bodies per alveolar lining cell§ Normal‖	Abnormal¶
			mm Hg	*μg/L*	*% body wt*	*dynes/cm*	*dynes/cm*			
Control	5	7.405 0.025	44 4.0	24 (7) 3	0.68 (16) 0.02	44 3.9	8.2 5.2	1.36 0.35	14.9 [200] 1.3	0 [200] 0
15% CO_2 in 21% O_2										
1 hour	6	7.010** 0.035	119.0** 4.5	52 (6)** 8.6	0.90** 0.10	39 1	3 (2) 0	1.72 0.01	7.5** [240] 2.9	6.3** [240] 2.6
6 hours	6	7.09** 0.05	114.1** 8.2	47 (6)** 7.2	1.15** 0.18	25 0	2.7 (3) .7	1.72 0.11	3.4** [240] 3.3	9.5** [240] 5.1
1 day	5	7.106** 0.028	110.3** 8.0	61 (11)** 38	1.0** 0.20	39 5.6	21.6** 3.0	0.57** 0.05	0** [240] 0	13.9** [240] 4.8
2 days	5	7.200** 0.030	97.2** 10.0	52 (6)** 21	0.91** 0.13	39.8 3.3	14.0 4.2	0.98 0.17	4.0** [200] 1.7	7.9** [200] 2.5
7 days	4	7.340 0.035	89.5** 7.5	32 (5) 15	0.80 0.07	41.3 4.7	14.9 4.6	0.95 0.25	15.7 [160] 2.1	3.0 [160] 0.7
14 days	5	7.371 0.036	84.4** 11.0	24 (5) 10	0.78 0.06	42.0 3.0	11.0 4.0	1.14 0.3	13.9 [160] 0.9	0.9 [160] 0.6

* Based on examination with the light and electron microscope, the over-all number of granular pneumocytes did not appear to change throughout the experiment, although it is not possible to estimate the number of these cells per gram lung tissue.
Data are means and standard deviations.
† The number in parentheses represents the number of animals when different from the number listed in the first column.
‡ Film compressibility is [2 (γ maximum $-$ γ minimum)]/[γ maximum $+$ γ minimum].
§ The number in brackets represents the number of sections counted.
‖ Normal lamellar bodies contain very electron opaque material arranged in bars, clumps, or a myeloid pattern.
¶ Abnormal lamellar bodies were without clearly defined electron opaque structures but may contain lipid droplets.
** Statistically different from control at the 5% level and better.

TABLE II

Surface characteristics of lungs and morphology of alveolar epithelial cells in guines pigs after exposure to 3% CO_2

Condition	No. of animals	pH	P_{CO_2}	Corticosteroids	Lung wt	Maximal surface tension	Minimal surface tension	Film compressibility	Electron microscopy; lamellar bodies per alveolar lining cell: Normal	Abnormal
			mm Hg	*μg/L*	*% body wt*	*dynes/cm*	*dynes/cm*			
3% CO_2 in 21% O_2										
4 days	4	7.320*	57.5*	38.3*	0.87*	37.5	7.3	1.36	11.6	4.5* (160)†
		0.028	12.3	7.1	0.26	3.8	2.9	0.19	4.6	2.5
8 days	3	7.360	48.0*	36.4*	0.70	43.7	6.0	1.52	11.8	1.4 (120)†
		0.020	6.1	3.7	0.07	1.4	1.2	0.24	2.9	1.8

* Statistically different from control at the 5% level and better.
† No. of sections counted.

tended until no further reduction in surface tension took place, which was usually from 3 to 15 cycles, or over periods of 30 to 150 minutes.

The highest and lowest tensions noted on the last cycle were recorded as γ maximum and γ minimum.

Results

Table I shows the changes in blood pH, P_{CO_2}, corticosteroids, lung surface characteristics, and lamellar bodies per alveolar lining cell produced by prolonged exposure to 15% CO_2. Short exposure for 1 and 6 hours to 15% produced a severe uncompensated respiratory acidosis, in which the pH fell to 7.1 and the P_{CO_2} increased to 114 mm Hg. During this period alveolar edema was marked, particularly after 6 hours of exposure, although no hyaline membranes were detected. Lung weight expressed in percentage of body weight nearly doubled during this period. The surface tension of lung extracts did not change. However, the number of normal lamellar bodies decreased significantly. After 1 day of exposure to 15% CO_2, hyaline membranes were found in all of the animals, and average minimal surface tensions rose from 8 to 21 dynes per cm, film compressibility correspondingly decreased, and nor-

TABLE III

Summary of histopathological changes observed with the light microscope

Condition	No. of animals	Atelectasis* Incidence	Atelectasis* Grade	Congestion† Incidence	Congestion† Grade	Edema‡ Incidence	Edema‡ Grade	Hemorrhage§ Incidence	Hemorrhage§ Grade	Hyaline membranes Incidence	Hyaline membranes Grade	Phagocytic‖ pneumocytes Incidence	Phagocytic‖ pneumocytes Grade
		%		%		%		%		%		%	
Controls	13	23	0.5	15	0.5–1	0		23	0.5–1	0		0	
15% CO_2/21% O_2													
1 hour	4	100	0.5–2	100	1.0	100	0.5–1	50	1–2	0		0	
6 hours	7	100	0.5–1	100	1–3	100	1–2	100	2–3	0		57	0.5–1
1 day	7	100	1–4	100	0.5–3	100	0.5–4	100	0.5–3	100	0.5–3	71	0.5–2
2 days	14	79	1–3	36	0.5–2	64	0.5–3	93	0.5–3	71	0.5–1	79	0.4–3
7 days	5	80	0.5–2	40	1–2	0		20	0.5	0		60	1–2
14 days	7	14	1	43	1–2	0		14	0.5	0		57	0.5–1
3% CO_2/21% O_2													
4 days	4	100	1–2	30	0.5	66	0.5			66	0.5	66	0.5–1
8 days	3	100	1–2	50	5–2	25	0.5	25	0.5			50	0.5

* Atelectasis present predominantly in subpleural zone.
† Of capillaries in alveolar wall.
‡ Presence of capillary transudate within alveolar air spaces.
§ Intracellular.
‖ Increase of phagocytic pneumocytes (alveolar macrophages within the alveolar lumen).

mal lamellar bodies had disappeared in the large alveolar lining cells (granular pneumocytes). During the period from the second to the fourteenth days of exposure, compensation of the respiratory acidosis developed, the incidence of pulmonary edema and hyaline membranes decreased progressively, and surface tension of lung extracts as well as lamellar bodies returned to normal.

The blood corticosteroid levels paralleled the changes in hydrogen ion concentrations with a marked increase during the initial period of uncompensated respiratory acidosis and a return to control values during the compensatory phase of respiratory acidosis.

Exposure to 3% CO_2 for 4 and 8 days produced a slight respiratory acidosis, small increases in lung weight/body weight ratio, and a number of abnormal lamellar bodies (Table II). However, there were no changes in surface tension, although a few hyaline membranes were observed in animals exposed for 4 days to 3% CO_2. Corticosteroid levels in the blood were slightly but significantly increased, indicating presence of a stress response.

Macroscopic findings. The gross examination of the lungs of animals exposed for 1 hour to 15% CO_2 revealed the same findings as that of controls. After 6 hours of exposure, however, stippling of the pleural aspects of most lungs was observed. This stippling consisted of minute foci of red dots measuring up to 2 mm in diameter. The lungs appeared much larger than those of the control animals because of the presence of frothy edema fluid. After 24 hours of exposure the lungs were still enlarged and edematous and exhibited wedge-shaped, depressed, segmental areas of dark red discoloration, up to half a lobe in extent. Essentially the same gross findings were obtained on the second day of exposure with the exception that the areas of atelectasis were smaller than on day 1. After 7 and 14 days of exposure to 15% CO_2 the gross appearance of the lungs was identical to that of control animals.

Light microscopy. Glutaraldehyde- and formalin-fixed sections from at least two different parts

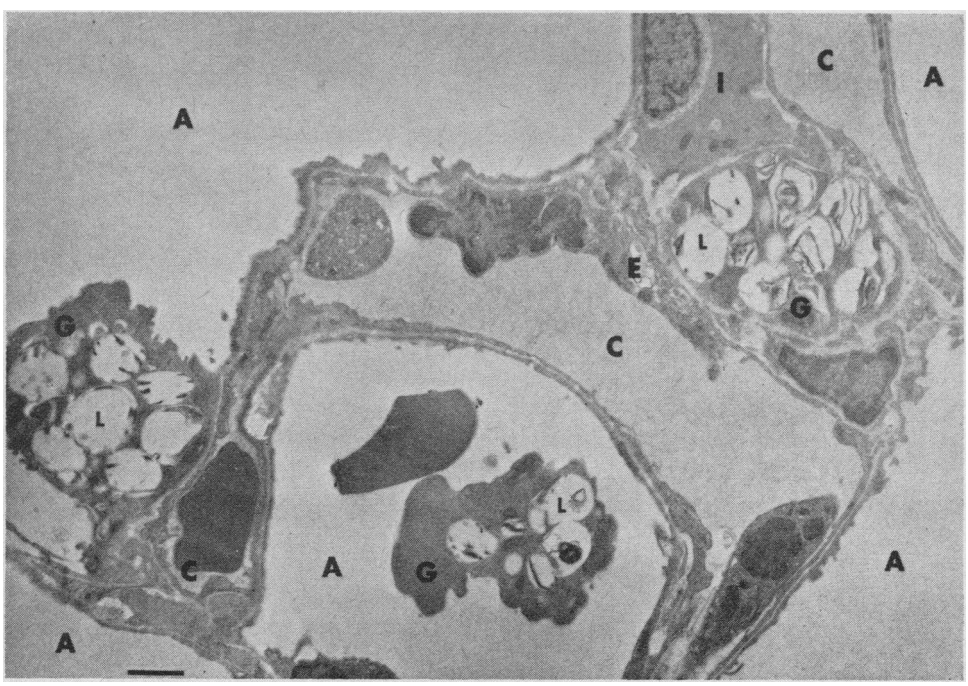

FIG. 1. LOW MAGNIFICATION MICROGRAPH OF NORMAL LUNG SHOWING PARTS OF FIVE ALVEOLAR SPACES (A), THREE CAPILLARIES (C) IN ALVEOLAR SEPTA, AND THREE GRANULAR PNEUMOCYTES (G). The capillary endothelial cells are marked E and septal interstitial cells I. Numerous lamellar bodies (L) are present in the granular pneumocytes. × 7,200.

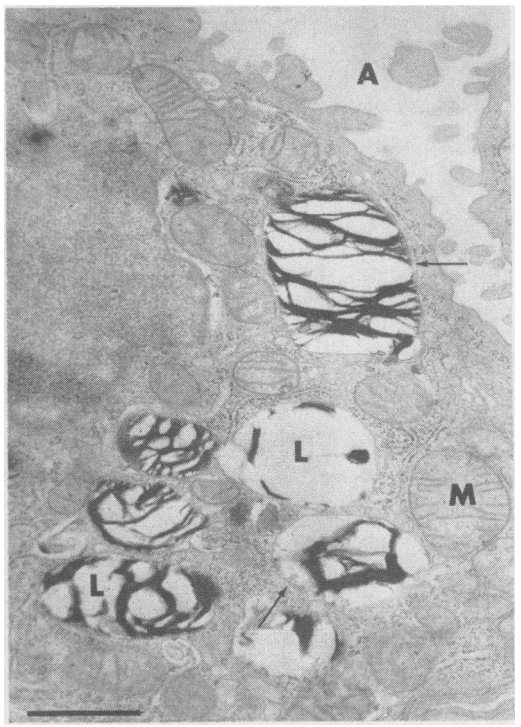

FIG. 2. HIGHER MAGNIFICATION OF PART OF A GRANULAR PNEUMOCYTE OF A CONTROL ANIMAL SHOWING SEVEN ELECTRON OPAQUE LAMELLAR BODIES (L) ON CROSS AND LONGITUDINAL SECTIONS. Note the persistent double membrane on some of the lamellar bodies (arrows). Mitochondria are designated M. × 16,500.

of the lungs were used in most cases for the evaluation of histopathological changes produced by exposure to CO_2. A summary of the light microscopic findings is presented in Table III.

The histological alterations were found to be essentially identical to those described by Niemoeller and Schaefer (4). Since the experimental animals were sacrificed at shorter time intervals of exposure to CO_2 than in the previous study (4), it was possible to delineate the time course of the development of the lung lesions, particularly of subpleural atelectasis and hyaline membranes, more accurately. After 1 and 6 hours of exposure to 15% CO_2 no hyaline membranes were observed, although atelectasis, congestion, edema, and hemorrhages were found in all cases. Pulmonary edema and hemorrhages reached a peak at 6 hours and 24 hours and began to subside on the second day of exposure. All animals exhibited hyaline membranes after 1 day of exposure; the membranes disappeared completely by the seventh day of CO_2 exposure.

The number of phagocytic pneumocytes increased sharply with the appearance of more severe pulmonary lesions (Table III). There were occasional polymorphonuclear leukocytes among the cell debris and hyaline membranes on days 1 and 2. The number of leukocytes in the blood did not change throughout the whole exposure period to 15% CO_2.

Attention was given to the so-called perivascular lymphoid nodules described by Thompson, Hunt, Fox, and Davis (17) as regular occurrences in the lungs of normal guinea pigs from a variety of strains used for research. These lymphoid nodules were also frequently observed in normal guinea pigs of the Hartley strain used in our study. Exposure to 15% CO_2 for prolonged periods did not change the number of lymphoid nodules nor did it affect their histological appearance.

Histochemical studies. Light microscopic examination of tissue stained for neutral fats with sudan IV showed lipid accumulations in the granular pneumocytes on day 2 and particularly day 4 of the 15% CO_2 experiments. The same findings were observed with flaming red and sudan black B staining. Luxol fast blue B, a stain specific for phospholipids, stained the lamellar bodies in controls and was taken up again by the altered lamellar bodies after 2 or more days of exposure to 15% CO_2.

Electron microscopic studies were carried out on lung tissues after incubation in a cytochemical medium for acid phosphatase. No reaction products were observed in the lamellar bodies.

Electron microscopic findings (15% CO_2 experiments). The terms for the cellular epithelial elements of the alveolus proposed by Macklin (18) and Businco and Giunti (19) will be used in this report: phagocytic, membranous, and granular pneumocyte. The latter two cells, although distinctly different, are often interchangeably referred to as niche cell, epicyte, (large) alveolar epithelial cell, nucleated squame, or vacuolated and nonvacuolated alveolar cell. The phagocytic pneumocyte, which is also called alveolar macrophage or free histiocyte, is referred to only briefly in the light microscopic studies. This cell has

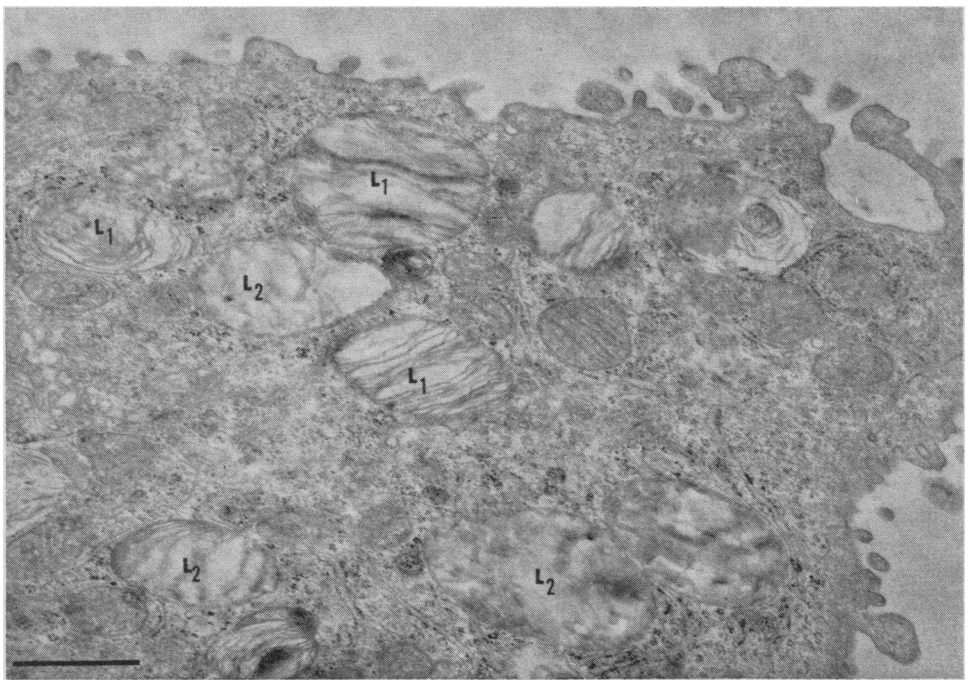

FIG. 3. MICROGRAPH OF LUNG EXPOSED FOR 7 DAYS TO CO_2 SHOWING LAMELLAR BODIES WITH MULTIPLE FINE LAMELLAE (L_1) OR LIPID (L_2). × 19,600.

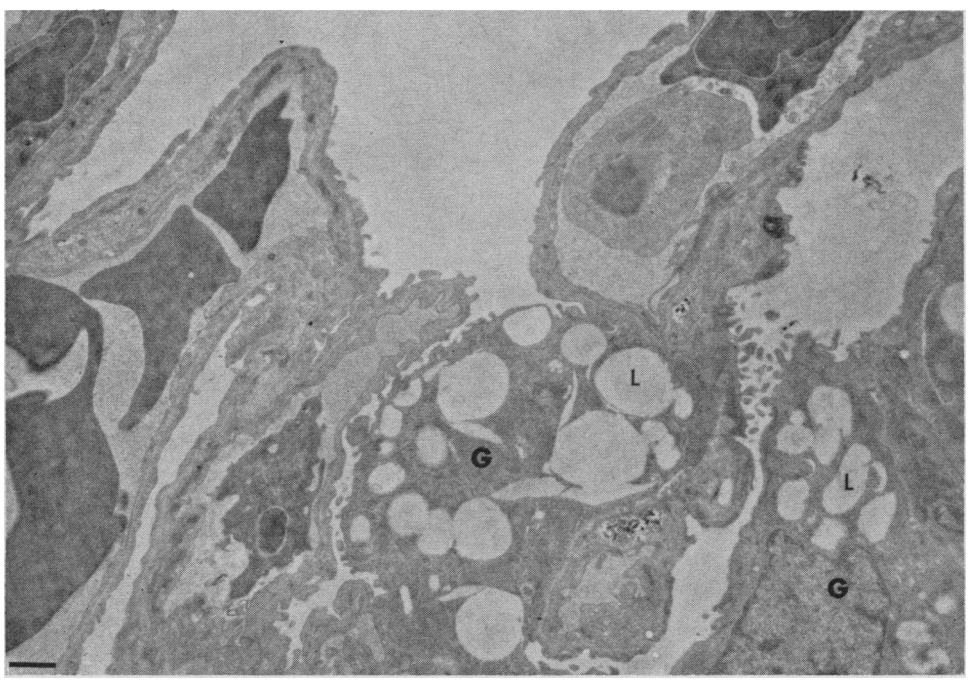

FIG. 4. LOW MAGNIFICATION PHOTOGRAPH OF LUNG EXPOSED TO CO_2 FOR 24 HOURS SHOWING TWO GRANULAR PNEUMOCYTES (G) WITH ENLARGED "EMPTY" LAMELLAR BODIES (L). × 6,700.

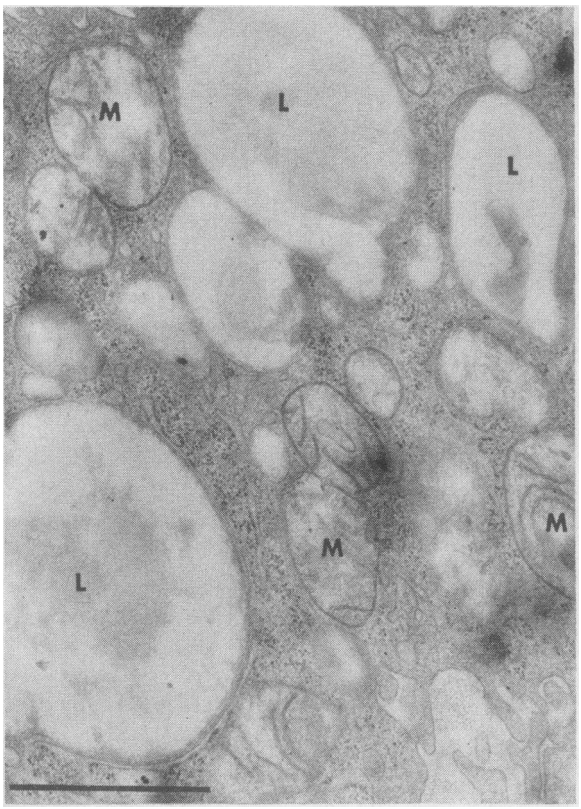

FIG. 5. THE "EMPTY" LAMELLAR BODIES (L) SEEN ON FIGURE 4 CONTAINING VERY ELECTRON TRANSLUCENT FLUID WITH SLIGHTLY MORE ELECTRON OPAQUE MATERIAL IN THEIR CENTERS. M designates mitochondria, some of which are swollen. × 24,800.

been extensively investigated (20) and its fine structure described, among others by Karrer (21). The membranous pneumocyte is the cell that lines with its attenuated cytoplasm about 95% of the alveolus (22, 23). The third cell, the granular pneumocyte, is almost the size of the phagocytic pneumocyte (alveolar macrophage) and is at least partially attached to the basement membrane, usually in the niche formed by protruding septal capillaries or by the contact of the walls of more than two adjacent alveoli [Figures 1 (G), 2, and 3]. These plump cells line only a small part of the alveoli into which they project a few short pseudopods; they are often partially capped by a thin layer of cytoplasm of the adjacent membranous pneumocytes. The granular pneumocyte has a moderate amount of rough and smooth endoplasmic reticulum, a distinct Gilgi zone, and abundant mitochondria (23). These cells also contain characteristic structures usually called lamellar bodies (lamellar forms, mitochondrial transformations, osmophilic bodies, or plasmosomes), which some investigators consider to be altered mitochondria (24–28). These structures are usually lined by a single unit membrane and contain in an electron transparent matrix coarse bars of very electron opaque material usually arranged in a lamellated (Figure 2), sometimes circular or irregular, fashion (Figure 1). However, the ratio of electron opaque over electron transparent material may vary within the individual being and among normal animals (Figures 1 and 2).

Evidence for a secretory function of lamellar forms. Light microscopic studies reported by Macklin in 1938 were suggestive of the discharge of "osmophilic bodies" from granular pneumocytes into the alveolar space (18, 29, 30). Simi-

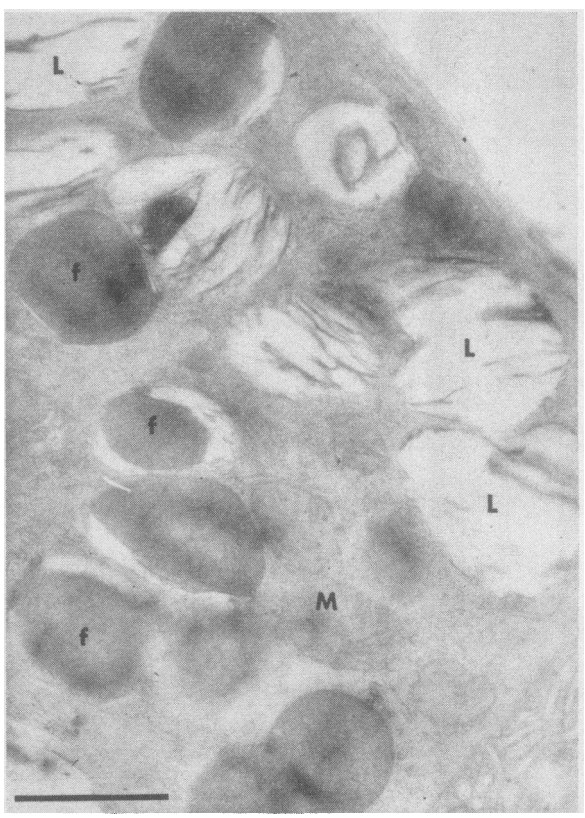

FIG. 6. LAMELLAR BODIES PARTIALLY OR COMPLETELY FILLED WITH FAT DROPLETS (f) ADJACENT TO NEAR NORMAL LAMELLAR BODIES (L) AFTER 4 DAYS OF CO_2 EXPOSURE. × 20,400.

lar findings were reported by von Hayek (31). The proximity to the cell surface of lamellar bodies as occasionally observed with the electron microscope, and the occasional intra-alveolar presence of electron opaque material resembling lamellar bodies, prompted previous investigators to postulate that these structures are normally discharged from the granular pneumocytes (27). We found in our studies on numerous occasions in control as well as experimental animals lamellar bodies in different stages of emergence from the granular pneumocyte (32). Moreover, during CO_2 exposure, fluid of varying electron opacity accumulates within the lamellar bodies instead of the lamellar bars. A bleb of this electron transparent fluid is sometimes observed to accumulate in front of a ruptured lamellar body before it spreads out along the cell surface to form a thin film identifying the granular pneumocyte conclusively as a cell with an exocrine function (32).

Changes in granular pneumocytes. If one assumes that the lamellar bodies are of mitochondrial origin, then there must be at least two types of mitochondria in granular pneumocytes, or the mitochondria present must be of different susceptibility to CO_2 intoxication, e.g., at different stages of their life cycles. Of two mitochondria in the same cell, one will show the characteristic changes caused by CO_2 while its neighbor exhibits a perfectly normal ultrastructure (Figures 5 to 7). After 1 hour of CO_2 exposure the number of forms with multiple fine lamellations increases. A considerable percentage of these forms shows an apparent arrest of the fusion of cristae at 6 hours after onset of exposure to the gas (Figure 7). However, the predominant appearance of the lamellar body is by now that of an "empty" vesicle filled with very electron transparent fluid that may contain an irregular network of very thin moderately electron opaque filaments (Figure 7). An occasional normal or near nor-

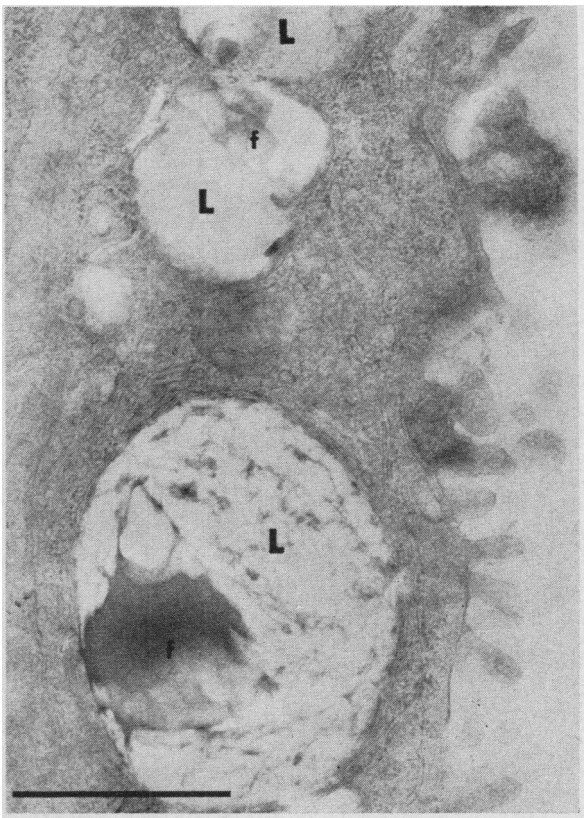

FIG. 8. AFTER 48 HOURS OF CO_2 EXPOSURE, THE NETWORK OF ELECTRON OPAQUE MATERIAL IN THE LAMELLAR BODIES (L), WHICH MAY ALSO CONTAIN FAT DROPLETS (f). × 27,000.

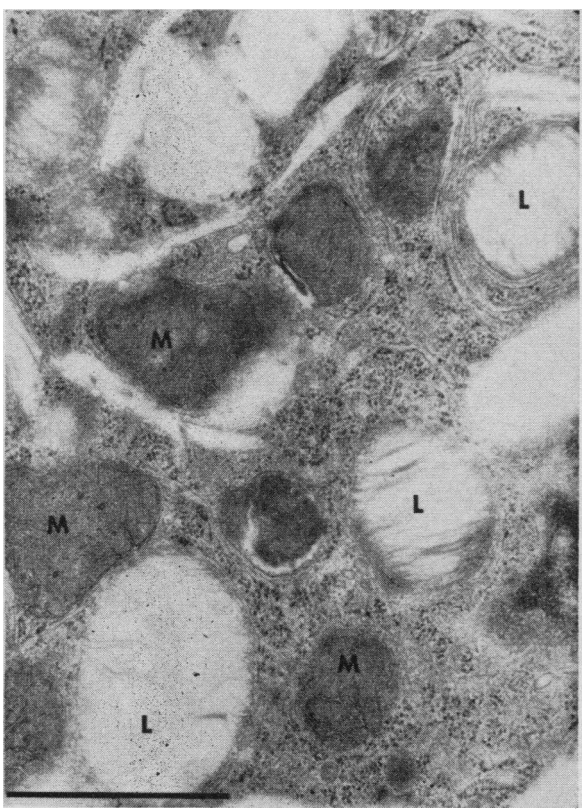

FIG. 7. FINE LAMELLATIONS IN THE LAMELLAR BODIES (L) PRODUCED BY 6-HOUR EXPOSURE TO CO_2. Note adjacent normal mitochondria (M). × 28,000.

mal lamellar body may still be present. The same "empty" lamellar body can be seen as long as 24 hours after the start of the experiment, but now many of these structures contain a dense network of moderately coarse, irregular, interconnecting filaments of medium electron opacity (Figures 4, 5). Material of the same degree of electron density then appears in globular, triangular, or irregular masses; it occupies smaller parts of the vesicle 24 hours after onset of exposure (Figure 5) and the major portion on the second day (Figure 8) when there is a pronounced increase in electron opacity of the material filling the atypical lamellar bodies. On the second day, as well as occasionally on the first day, a few of these vesicles are noted to coalesce, a fact that would account for the occasionally observed abnormal giant lamellar bodies.

The granular pneumocytes have a near normal cytologic pattern after 7 days of exposure to CO_2 (Figure 3). Some cells will still contain fat-filled lamellar bodies among the normal lamellar bodies; other cells will show increased numbers of lamellar bodies with multiple fine lamellations and thus resemble, in this respect, the early changes of about 1 hour of exposure to CO_2. Their appearance is virtually normal after 14 days of exposure to 15% CO_2 (Figure 9).

Hyaline membranes. The appearance of the hyaline membranes produced by CO_2 is strikingly similar to those in infants noted by van Breeman, Neustein, and Bruns (33) and particularly by Campiche and associates (34, 35).

Animals exposed 1 to 6 hours to CO_2 have definite fine structural changes in the membranous pneumocytes (small alveolar lining cells). These cells show initially increased pinocytotic activity as manifested by the large number of pinocytosis vesicles (1 hour) followed by a definite decrease and later on absence of these vesicles (6 hours) (Figure 10). Concomitant swelling with pronounced loss of electron opacity of the membranous part of the cytoplasm lining the alveolar air space occurs as well as the accumulation of a layer of edema fluid of varying thickness (apparently a capillary transudate) (1 to 6 hours) (Figures 10 and 11). This is followed by disintegration of some of the damaged membranous pneumocytes

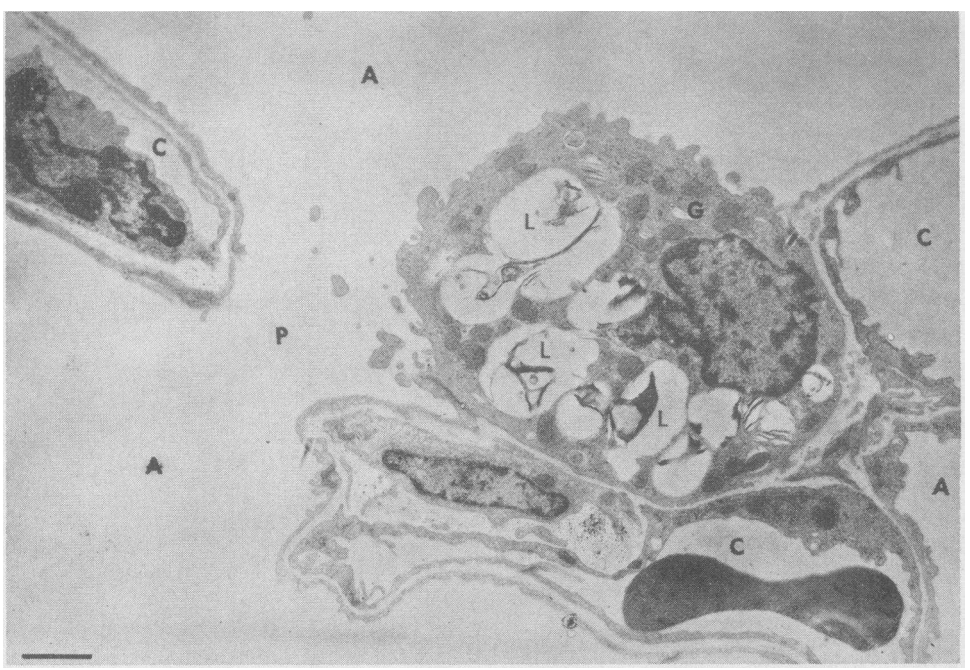

FIG. 9. A GRANULAR PNEUMOCYTE (G) IN ONE OF ITS TYPICAL LOCATIONS NEAR A PORE OF KOHN (P). Its lamellar bodies (L) are filled with elongated, irregular bars of very electron opaque material. This animal was exposed to CO_2 for 14 days. Alveolar lumen, A; capillary, C. × 11,000.

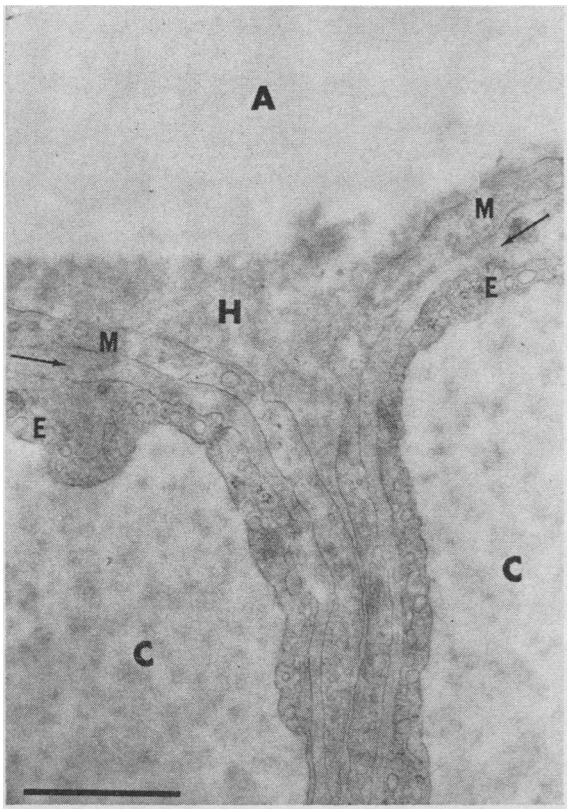

FIG. 10. SIX HOURS OF CO_2 EXPOSURE: A THIN LAYER OF EDEMA FLUID (H), WHICH IS ALREADY MORE ELECTRON OPAQUE THAN THE INTRACAPILLARY FLUID (C), COVERS SWOLLEN MEMBRANOUS PNEUMOCYTES (M). The arrows point to the basement membrane sandwiched between alveolar lining cell and capillary endothelial cell (E). × 20,000.

and cells lining the alveolar ducts and respiratory bronchioles, the fragments of which mingle with the layer of edema fluid, eventually forming a "hyaline" membrane (days 1 and 2) (Figures 11 to 14). The electron opacity of the layer of edema fluid, whether mixed or not with cell debris, increases steadily until it far exceeds that of the plasma in the adjacent capillaries (Figures 10 and 11). Very few of these membranes contain a meshwork of fibrin (Figure 11), but occasional fibrin fibrils are always present. These hyaline membranes are at least focally adherent to the denuded basement membranes or surviving alveolar and bronchiolar lining cells (Figures 13 and 14).

Effects on other types of cells. Only during 1 to 6 hours of exposure to CO_2 can one observe a rare damaged endothelial or interstitial cell. This damage usually consists of moderate swelling of a few of a cell's mitochondria. The membranous part of the endothelial cells of the capillaries appears, in addition, slightly increased in thickness (up to 2 days of exposure). An increased number of polymorphonuclear leukocytes is also present within the capillary lumina during this time period. These leukocytes are, however, almost never seen outside the vessels. On occasion a few extravasated erythrocytes can be observed during the first and second days of an experiment.

Results of 3% CO_2 experiments. Most of the lamellar bodies of the granular pneumocytes are of normal appearance after exposure to a 3% CO_2 atmosphere for 4 days. The abnormal forms show only a moderate deviation from normal and resemble the lamellar bodies of pneumocytes exposed to 15% CO_2 for 2 days, i.e., the contents

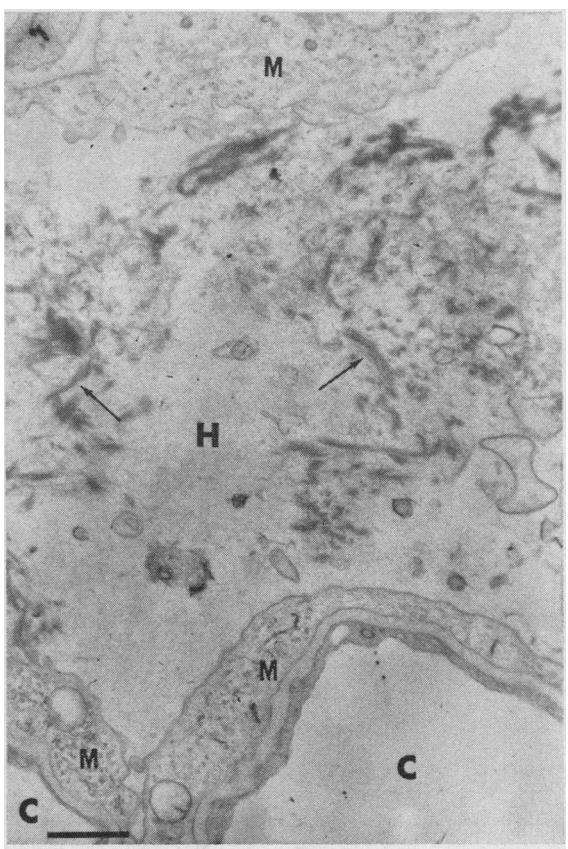

FIG. 11. AN ALVEOLAR SPACE PARTLY FILLED WITH EDEMA FLUID (H) CONTAINING REMNANTS OF CELLS AND FIBRIN (ARROWS) 24 HOURS AFTER CO_2 EXPOSURE. M designates membranous pneumocytes; C, capillary lumina. × 11,500.

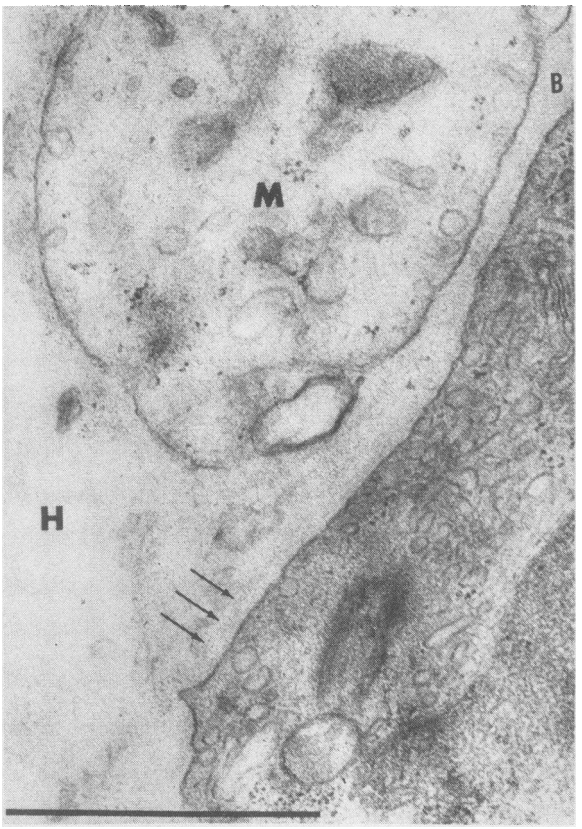

FIG. 12. AFTER 24 HOURS OF CO_2 EXPOSURE: A NORMAL ENDOTHELIAL CELL SEPARATED FROM THE EDEMA-FLUID FILLED ALVEOLAR LUMEN (H) BY A PARTLY DENUDED BASEMENT MEMBRANE (ARROWS). Attached to the latter (B) in the right upper half of the micrograph is a degenerating swollen membranous pneumocyte (M). × 39,400.

of these lamellar bodies consist of a coarse network of electron opaque material that may contain lipid droplets of varying size. Lamellar bodies with much thinner lamellae are also present in increased numbers.

A near normal cytologic pattern is present after 8 days of exposure to 3% CO_2. We observed a few atypical lamellar bodies with multiple thin lamellae.

Discussion

There appear to be four phases in the pulmonary pathology caused by exposure to 15% CO_2. During the first phase, lasting up to 6 hours exposure to 15% CO_2, a severe respiratory acidosis exists associated with pulmonary effusion and simultaneous changes in most of the lamellar bodies of the granular pneumocytes. The surface tension is not affected by these early changes, nor is there any evidence of hyaline membranes during this period. However, the presence of hyaline membranes is the outstanding feature of the second phase—between 6 and 24 hours of exposure. At this time the surface tension has reached its peak. All normal lamellar bodies in the granular pneumocyte have disappeared; only abnormal forms are present. The hyaline membranes consist mainly of inspissated proteinaceous material (edema fluid) that contains cell debris of disintegrated membranous pneumocytes and, very rarely, occasional strands of fibrin.

During the third (repair) phase, from the second to the seventh days, the respiratory acidosis gradually becomes compensated, the surface tension returns to normal levels, and the lamellar bodies gradually take on normal appearance. The pulmonary edema is absorbed, and the hya-

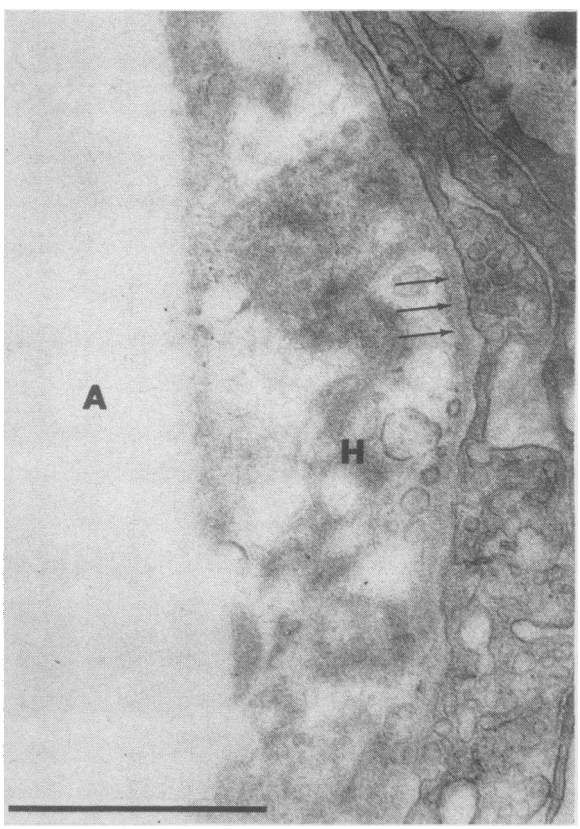

FIG. 13. A COMPLETELY NAKED BASEMENT MEMBRANE (ARROWS) LINED ON ITS ALVEOLAR ASPECT (A) BY A THIN LAYER OF INSPISSATED EDEMA FLUID (H) THAT CONTAINS CELL DEBRIS (24 HOURS OF CO_2 EXPOSURE). × 33,500.

line membranes disappear. The final phase is one of recovery in which the pH is compensated, although the P_{CO_2} remains elevated. A normal cytological pattern is re-established.

This study on CO_2-induced hyaline membrane disease permits elucidation of two simultaneously occurring processes: *1*) The pulmonary edema develops rapidly during the first 6 hours. The normally present pinocytotic activity of the small alveolar lining cells (membranous pneumocytes) appears to increase during the first hour, followed by a near complete cessation at 6 hours, which indicates a reduced removal of edema fluid from the alveolar spaces. The striking finding at 1 day of exposure is the disintegration of the alveolar lining cells, the debris of which mingles with the edema fluid. This mixture becomes more and more inspissated and represents the hyaline membranes. *2*) Another process involving changes in lamellar bodies parallels the pulmonary effusion even in the early stages. This suggests that the respiratory acidosis is associated with both processes, leading to an increased extravasation of capillary fluid with resulting edema and affecting simultaneously the lamellar bodies in the granular pneumocytes. Since the maximal amount of edema fluid is present after 6 hours of exposure without affecting the surface tension, it is more likely that the changes in lamellar bodies are related to the maximal reduction in surface-active material found after 24 hours, at which time the maximal alterations of lamellar bodies are present.

The electron microscopic appearance of the lamellar bodies indicates that the bars making up the lamellae consist mainly of a phospholipid or lipoprotein (which was also demonstrated with special stains with the light microscope) and are suspended in a material of very low electron opacity, possibly a mucopolysaccharide (36). The lamellae are apparently quite soluble because after excretion into the alveolar space one finds only occasionally minute remnants of these structures, if any at all. The susceptibility of the granular pneumocytes to 15% CO_2, judging from their cytological appearance, seems to be limited to certain changes in lamellar bodies. After being exposed for 6 hours to the gas, these organelles begin to exhibit a complete structural disarrangement and "emptiness" indicative of a metabolic breakdown. The appearance of neutral fat, particularly

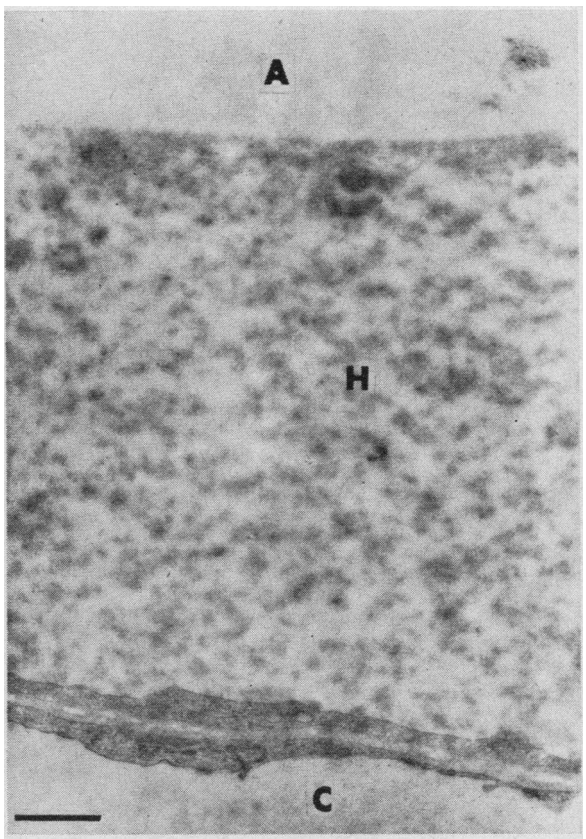

FIG. 14. MICROGRAPH OF TYPICAL HYALINE MEMBRANE (H) AFTER 24 HOURS OF CO_2 EXPOSURE. The membrane contains occasional foci of cell debris. A denotes the alveolar lumen and C the capillary lumen. × 10,400.

on the second day of an experiment, can be considered the first sign of an adjustment to the new environment and recovery; synthetic pathways for triglycerides are known to be very resistant to adverse conditions (37, 38).

In our experiments the effects of exposure to 3% CO_2 were similar but quantitatively less than those observed under 15% CO_2 and did not produce changes of surface tension and marked hyaline membranes.

Normal lamellar bodies were seen in different stages of movement from the granular pneumocytes into the alveolar space, and in CO_2-exposed animals pathological lamellar bodies discharged their contents of varying electron opacity into the alveolar space, forming a bleb at the borderline of the edema fluid. Furthermore, the time course of changes in lamellar body transformation and the associated alterations in surface tension, observed during CO_2 exposure, support the identi-

fication of lamellar bodies in the granular pneumocytes as the cellular elements responsible for the secretion of the surface-active material.

The recent excellent reports by Campiche and his colleagues (34, 35) on electron microscopic studies of lungs from infants who had succumbed to hyaline membrane disease leave no doubt about the histo- and cytopathology, particularly in regard to the composition of hyaline membranes. The similarity between hyaline membranes in humans and those produced by CO_2 intoxication in guinea pigs is striking. This may indicate that CO_2 effects are a common factor in the development of the disease, known to have multiple causes. The present investigations have demonstrated the time course of formation and disappearance of CO_2-induced hyaline membranes. Under these conditions, the various states in the formation of hyaline membranes are delineated, such as prolonged extravasation of plasma, cessation of removal of this exudate by pinocytosis, necrosis and disintegration of cells, intermingling, and inspissation of these elements leading to hyaline membranes. Further studies of other factors, e.g., fat metabolism, might reveal a correlation with the outlined time sequence and should give further insight into the mechanism of development of hyaline membranes.

Summary

Atelectasis and hyaline membranes produced by exposure of guinea pigs to 15% CO_2 were found to be associated with disappearance of lamellar bodies in the large alveolar lining cells (granular pneumocytes) and an associated decrease in surfactant as indicated in the rise of minimal surface tension of the lungs. This process is limited to the uncompensated phase of respiratory acidosis and is reversed during the compensatory phase. The parallel time course in changes of surface tension and alterations of lamellar bodies in the granular pneumocytes provides additional evidence for the identification of the latter as the cells responsible for the secretion of surfactant.

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