NORMAL RABBIT ALVEOLAR MACROPHAGES

I. The Phagocytosis of Tubular Myelin*

BY BARBARA A. NICHOLS

(From the Francis I. Proctor Foundation and Department of Microbiology, University of California, San Francisco, California 94143)

Abundant alveolar macrophages inhabit the normal lung, where they have a vital role in host defense, removing foreign particles from their vicinity (1-3). Possibly, as recently suggested, (4) they are also involved in the turnover of surfactant, the phospholipid material which lines the surface of the alveoli. Surfactant lowers the surface tension between air and tissues, thereby stabilizing the alveoli (5-7). The substance is generated in lamellar bodies of type II epithelial cells of the alveolar wall (8, 9) and is elaborated onto air-sac surfaces, where it forms a complex lining. Believed to consist of two parts, the lining layer presumably has (a) a 5-nm surface film containing the hydrophobic regions of the phospholipids oriented toward the air-phase (5), and (b) a partially aqueous subphase ranging from 30 nm to several micrometers in depth (10, 11) which harbors reserve phospholipids. In addition to a lining layer in the air spaces, surfactant appears, too, in the form of aggregates called "tubular myelin" (12, 13) easily identified by their distinctive substructure. The present studies were undertaken to determine whether the phagocytic activities of normal rabbit alveolar macrophages include the ingestion of recognizable forms of surfactant, such as tubular myelin.

Materials and Methods

Materials. Alveolar macrophages were obtained from the lungs of 10 normal New Zealand albino rabbits. Lung tissue from several of these animals was also studied. For comparison, resident macrophages were obtained from the peritoneal cavities of three normal New Zealand albino rabbits. Pentobarbital (Beuthanasia Special) was procured from H. C. Burns Pharmaceuticals, Oakland, Calif.

Methods, Collection of Tissues. Rabbits were killed immediately before experiments by means of an intravenous injection of approximately 200 mg of sodium pentobarbital in 0.5 ml. To ensure a minimal lapse of time before fixation, no attempt was made to excise the lungs before specimen retrieval; the trachea was merely exposed rapidly and clamped below the larynx. With a 16-gauge needle and a 50-ml syringe, the fixative was gently introduced via the trachea into the lungs, and then the fluid was withdrawn. Several washes of 30-40 cm³ each from one animal yielded frothy fluid. On centrifugation, the foam rose to the top, and a small pellet of cells separated to the bottom. When lung tissue was studied, the initial fixative was introduced by the same route. Small pieces were then dissected out and minced in fixative. This procedure resulted in fixation of cells and tissues within moments after the lethal injection of barbiturate.

For comparison with alveolar macrophages, resident peritoneal macrophages (from unstimu-
lated animals) were collected immediately after sacrifice by washing out the peritoneal cavity with fixative. In all cases, fixation of the cells or tissue occurred within 1-3 min after sacrifice.

**Fixation.** Tissues were fixed in 1% formaldehyde-3% glutaraldehyde with CaCl₂ (14) at room temperature for 2-4 h and postfixed overnight at 4°C in 1% OsO₄ in acetate-Veronal buffer, pH 7.4, and 5% sucrose. The specimens were stained in block for 1 h at room temperature in 0.5% uranium acetate in acetate-Veronal buffer (final pH of the stain 5.4) with 4% sucrose (15). Additional lung specimens (primarily for examination of surfactant) were fixed with a mixture of glutaraldehyde and osmium (16) for 1 h at 4°C and stained in block overnight in uranyl acetate.

**Subsequent Processing.** All specimens were dehydrated in ethanol and propylene oxide, and embedded in Epon. Thin sections were cut with a diamond knife on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.), stained with aqueous uranyl acetate and Reynolds' lead citrate. They were examined in a Siemens Elmiskop 1A electron microscope (Siemens Corp., Iselin, N. J.) operating at 80 kV with a 50 μm objective aperture.

**Results**

**The Inclusions of the Alveolar Macrophage.** The typical alveolar macrophage (Fig. 1), 15-25 μm in diameter, has a voluminous cytoplasm in which the most conspicuous features are its many distinctive dense inclusions. In a medial thin section, as many as 100 of these bodies per cell may be counted, ranging from 100 nm to 2 μm in diameter and clustered in greatest profusion at the outer limits of the Golgi region. Although these inclusions are of almost infinite structural variety (Figs. 1 and 2), they share in common a characteristic content of myelin figures (so named because they resemble the membranes of myelin sheaths around nerve axons). In section, the figures are often circular, comprised of alternate dense and lucent thin layers which form a concentric pattern of evenly spaced rings against a matrix of moderate density. The number of myelin figures within a single inclusion varies considerably; many small circular profiles or a sole large, membranous whorl may occupy the entire content. The diversity of these bodies is particularly well-illustrated in Figs. 2 and 6. Their heterogenous appearance suggests that they may be secondary lysosomes such as dense bodies or residual bodies, the residues of incomplete lysosomal digestion.

Other inclusions are seen which are fewer in number and differ from the typical dense bodies in two respects. First, they lack the dense matrices characteristic of the dense bodies, and second, the myelin figures that fill them are comprised of irregularly spaced rather than evenly spaced membranes (Fig. 8). The multitude of inclusions with their remarkable content of myelin figures intimates that these phagocytes ingest large quantities of polar lipids.

**The Inclusions of the Peritoneal Macrophage.** For comparison, the structure of a macrophage resident in the peritoneal cavity was analyzed. It is clear that the macrophages that reside in the peritoneal cavity (Fig. 3) are strikingly different in morphology from alveolar macrophages. They completely lack the dense inclusions typical of the alveolar macrophages, although there is ample evidence of endocytic activity in their pinocytic vesicles and the irregularity of their surface membranes. The inclusions present in peritoneal macrophages are smaller, less dense, and considerably fewer in number than those in alveolar macrophages. Above all, they lack the myelin figures which are the hallmark of the alveolar macrophage inclusions. The inset (Fig. 3), illustrates at high magnification the characteristic structure of an inclusion from a peritoneal macrophage.
Fig. 1. Fig. 1 is an electron micrograph of a typical alveolar macrophage. These large phagocytes are unusual because of the distinctive morphology of their many inclusions (i) which almost universally contain a number of myelin figures (seen to better advantage in Fig. 2). The nucleus (n) with its centrally located nucleolus (nu) and dispersed chromatin occupies a characteristic location at one side of the cell. The Golgi complex (G) contains numerous stacks of cisternae near the centriolar region at (c). The moderate content of mitochondria (m) and RER (er) is for the most part confined to the outer regions of the cytoplasm. The highly irregular surface of the cell represents sections through lamellipodia. Fixed in formaldehyde-glutaraldehyde 4 h, postfixed in 1% osmium overnight, and stained in block in buffered uranyl acetate. × 9,800.
FIG. 2. Higher magnification views of inclusions (i) in alveolar macrophages. These organelles, limited by a unit membrane, typically have a matrix of moderate density in which myelin figures of greater density are situated (seen to best advantage in inset). The latter are multilayered, membranous in appearance, and commonly circular in section. Specimen preparation as in Fig. 1 × 24,000. Inset × 74,000.
Fig. 3. Portion of a typical resident macrophage from the peritoneal cavity of a normal rabbit. The morphology differs from that of the alveolar macrophage in that the inclusions (i) are markedly fewer. Those present are of low density and reveal little internal organization (seen to better advantage in inset). The irregular outlines of the cellular periphery attest to its endocytic activity. The Golgi complex (G) is central, and the moderately abundant RER (er) is situated toward the exterior. Clusters of filaments 10–15 nm in diameter are prominent throughout the cytoplasm. Mitochondria are indicated at (m). Specimen prepared as in Fig. 1. × 14,000. Inset × 64,000.
Tubular Myelin. The fixative, after its use to wash the cells from the lungs, is frothy and more turbid than one would expect from the relatively small pellet of cells it contains. Apparently much of the cloudiness of the fixative wash solution is due to the presence of surfactant, which is washed out with the macrophages. Tubular myelin (Figs. 4 and 5), the form of surfactant most thoroughly examined by morphologic techniques (see 17), was commonly encountered along with the alveolar macrophages in the specimens.

Tubular myelin consists of an elaborate complex of membrane-like bilayers forming twisted masses of "tubules" which are square in cross-section, as described previously by Weibel and Gil (10, 11). Each side measures approximately 60 nm. Although the internal squares of the latticework share their walls with those of the four adjacent tubules, the membrane-like walls of all are approximately the same width (15 nm) (Fig. 5). Internally, a dense central dot appears against a matrix of low density, and a thin indistinct connection forms a link between this dot and two opposite corners of the tubules (Fig. 5). This was best demonstrated in specimens fixed in a mixture of glutaraldehyde and osmium (16) and stained overnight in block in uranyl acetate. Because the tubules are convoluted, their total length cannot be determined in thin sections. However, segments nearly 2-μm long are common (Fig. 4). Aggregates of tubular myelin often seem to be limited by a single continuous, membrane-like bilayer (arrows, Fig. 4); the significance of this arrangement is undetermined.

Myelin figures are also ubiquitous in the fixative wash fluid (Fig. 4) and in the alveoli of lung specimens. They, too, have been observed in the alveolar spaces by previous investigators (17-19). Comprised of concentric membranous whorls, they are somewhat irregularly organized (differing in this respect from the myelin figures of the inclusions which usually manifest a constant spacing between lamina). Membranes of the extracellular myelin figures are often continuous with those of the tubular myelin (Fig. 4), as if one originated from the other (20). Whether the myelin figures are a true component of the extracellular spaces or an artifact produced during the introduction of fixative into the lung cannot be stated with certainty. However, the presence of identical structures within macrophage vacuoles (see following section) strongly suggests that they were present before fixation.

The Phagocytosis of Tubular Myelin by Alveolar Macrophages. Images illustrating the phagocytosis of tubular myelin by macrophages (Fig. 6) were occasionally observed. Tubular myelin and extracellular myelin figures were situated at the cellular surface, surrounded by cytoplasmic projections which appear to be enveloping and ingesting them. Moreover, phagocytic vacuoles containing recognizable residues of these structures are found in the cytoplasm (Figs. 7 and 8). The structure of the tubular myelin may be retained intact for at least a transient period after internalization, as depicted in Fig. 7. These findings indicate that alveolar macrophages phagocytize tubular myelin. However, the engulfment of other particles such as dust or bacteria was not observed during the course of this study.

Discussion

The Phagocytosis of Tubular Myelin. In the course of this investigation, we discovered that alveolar macrophages phagocytize tubular myelin. Macro-
Fig. 4. Tubular myelin embedded with macrophages from lung washings. This micrograph illustrates an extensive array of tubular myelin (tm). Areas where the tubules are seen in cross-section reveal that they measure approximately 60 nm per side, and a central density is apparent in many tubules. Longitudinal sections of tubules indicate the lengths that they may attain. Myelin figures (mf) consist of circular whorls of membranes, some of which may be in continuity with the tubular myelin (tm). Although the overall configuration consists of a highly tortuous mass of tubules, note that there is one limiting side (arrows), suggesting that formation may be initiated in a linear fashion along a single lamina. Specimen prepared as in Fig. 1. × 21,000.
Fig. 5. Higher magnification cross-sectioned view of tubular myelin (tm) from a section of the lung. This resolves the internal structure of the tubules more clearly. Note the central density from which fine projections extend into the corners of the tubules. Points of continuity between the tubular myelin and the surrounding membranes are seen at arrows. The alveolar epithelium (ep) is seen at upper left. Specimen fixed in a mixture of glutaraldehyde and osmium at 4°C and stained overnight in block in uranyl acetate. × 79,000.

Phages were observed in the actual process of ingesting tubular myelin and the extracellular myelin figures that are often in continuity with it. In addition, cytoplasmic vacuoles were found that contained these structures.

Tubular myelin was first observed in the alveoli almost 20 yr ago (18), but much still remains to be learned concerning its source and function. It has been the subject of intensive investigation in the laboratory of Weibel (17) who named it after three dimensional reconstruction analyses which revealed it is a tightly packed "tubular" system (12). It was in his laboratory that attempts were made to fix the surfactant lining layers by perfusion of the lungs through the vasculature. These and other studies revealed that tubular myelin appears to be a constant component of the extracellular lining layers of the alveoli. The recent studies of Gil and Reiss (13) are significant since for the first time, they showed conclusively that tubular myelin is a form of surfactant. In homogenates of lungs, they found by the use of various techniques (electron microscopy, biochemistry, surface tension measurements) that tubular myelin has surface-tension-lowering properties like that of surfactant. In chemical composition it consists primarily of polar lipids, one of which, dipalmitoyl lecithin is unusual since it is found only in the lung.
FIG. 6. Portion of an alveolar macrophage depicting the phagocytosis of tubular myelin (tm). Lamellipodia (l) extend from the surface of the macrophage and surround an aggregation of tubular myelin before its internalization. Note also the typical inclusions (i), RER (er) and mitochondria (m). Specimen prepared as in Fig. 1. × 37,500.
The origin of tubular myelin has yet to be established. It has been suggested that tubular myelin is a breakdown product of surfactant, since it is increased in amount in oxygen-poisoned lungs (21). But it has also been proposed that it may be a storage form of surfactant (22).

**Extracellular Myelin Figures.** The origin of myelin figures found in washes from the lung is impossible to state unequivocally. Clearly, phospholipid-containing materials such as those comprising the surfactant lining layer, may undergo considerable rearrangement during the wash-out procedure, despite the use of fixative as the wash fluid. Several facts, however, indicate that myelin figures represent true extracellular constituents of the alveolus: (a) Similar membrane configurations have been illustrated in virtually all micrographs of lungs in earlier publications, regardless of the preparative procedures employed. (b) The myelin figures and their continuities with the tubular myelin are similar to those described by Williams (20) in fetal rat alveoli. (c) Phagosomes, apparently newly formed, containing similar structures can be observed in the macrophage cytoplasm. This last point constitutes perhaps the most convincing evidence that such myelin figures inhabited the alveolar spaces.
ALVEOLAR MACROPHAGES PHAGOCYTIZE TUBULAR MYELIN

before fixation. The morphology of the extracellular myelin figures resembles that of isolated lamellar bodies (13). In vivo, lamellar bodies secrete their contents into the alveoli; thus, it is possible that the myelin figures originate directly from the lamellar bodies.

The Turnover of Surfactant. Currently, although there is general agreement that type II epithelial cells produce surfactant, little is known about its removal after it is denatured. Several routes have been considered: (a) removal up the airways (7); (b) transport across the alveolar epithelium (23); or (c) phagocytosis and digestion by alveolar macrophages (4, 6, 22, 23).

A recent study (23) analyzed the clearance from the lung of aerosolized dipalmitoyl lecithin (DPL), a major component of surfactant. The cellular distribution of the tritium-labeled DPL was followed with frozen-section autoradiography at intervals of 1 min, 2, and 12 h. The label was immediately taken up by the lung tissues, but was lost progressively from the type I alveolar epithelial cells and appeared in the liver, spleen, kidney, blood, and urine. At the same time, it was retained in the type II alveolar epithelial cells and increased in amount in macrophages. Geiger et al. (23) concluded that DPL is rapidly absorbed into the lung by the type I cells and transferred into the blood or lymph, although other interpretations of these results might be considered.

The work of Naimark (4) provided strong impetus to the proposal that alveolar macrophages function in the uptake of used surfactant. In his recent exploration of lipid metabolism in the rat lung, rats were given intravenous injections of labeled palmitate, a precursor of surfactant, and three aspects of the lung were then analyzed for subsequent activity – the total lung homogenate, extracellular material in the lavage fluid, and the cells in the washings (presumably macrophages). As could be anticipated, the label first became localized in the lung, consistent with its uptake during surfactant synthesis in the type II cells. Later (3 h), the label emerged in the extracellular compartment, the site of secreted surfactant; and significantly later, the label finally appeared in the macrophages, supporting the view that these phagocytes engulf material previously elaborated onto the alveolar surfaces. The results of our present study provide support for Naimark’s findings.

In view of the primarily (90%) lipid nature of surfactant, it is not surprising that the inclusions of alveolar macrophages contain residues of lipid degradation. The many inclusions replete with swirls of myelin figures, hint of an abundant ingesta of polar lipids. That such polar lipids (phospholipids and fatty acids) form myelin figures when in an aqueous medium is now well-established (24). It should be noted also that there is evidence in other tissues that lipids are degraded more slowly than other materials (25–27) which may result in accumulations of partially degraded lipids. Comparing the normal alveolar macrophage with the resident peritoneal macrophage dramatized the striking diversity in the quantity and quality of their respective inclusions. The relatively few inclusions in peritoneal macrophages are plain and rather featureless. Their matrices are finely granular and heterogeneous, with only an occasional membranous profile, despite the considerable amounts of membrane phospholipids.

Abbreviation used in this paper: DPL, dipalmitoyl lecithin.
which are constantly being internalized during the endocytic activities of these peritoneal phagocytes.

These disparities in appearance between the two macrophage populations lend firm support to the theory that the polar lipids in the inclusions of alveolar macrophages have some extracellular source. Hence, it would be reasonable that surfactant subphase material, which normally envelops the macrophages of the alveoli, is likewise the source of the polar lipids observed in their inclusions. Since the "subphase" is an aqueous environment containing surfactant precursors as well as tubular myelin and extracellular myelin figures, large amounts of phospholipid substances may also be internalized by pinocytosis.

The Phagocytic Activities of Alveolar Macrophages. Investigators attempting to examine the normal flora of the alveoli have discovered that in healthy individuals, the alveoli are sterile (28, 29). Apparently two different mechanisms cooperate to produce this result. First, it is well-documented that alveolar macrophages are effective in removing bacteria from the alveoli (1-3). In animals exposed over a 4-h period to bacteria-laden aerosols (less than 3 µm in diameter), the phagocytes rapidly engulfed bacteria deposited in the alveoli (1). And it is generally accepted that alveolar macrophages form the first line of defense against microorganisms during the onset of a respiratory infection (1, 3). Second, it is also well-established that only droplets or particles of very small size (0.5-2 µm) gain access to the alveoli; larger ones are removed in the upper airways (30, 31). Hence, it is probable that under most conditions, the majority of airborne microorganisms are trapped in the upper respiratory tract (31). Therefore, it is not surprising that in the present investigation, macrophages ingesting bacteria were not seen, nor were recognizable structures such as bacterial remnants seen within macrophage phagosomes. From the foregoing considerations, it appears that the sterility of the alveoli results from a relatively low incidence of bacterial contamination in normal animals under normal circumstances, as well as from the efficiency of the macrophages as phagocytes. Thus, assisting in the turnover of surfactant may prove to be one of the major endocytic functions of alveolar macrophages, in addition to their other functions in the clearance of foreign particles which reach the alveoli.

Summary

Normal rabbit alveolar macrophages are engorged with large, dense inclusions which contain whorls of myelin figures, suggesting an exogenous source of polar lipids in their diet. One contributory source of such lipids is surfactant, since macrophages were seen ingesting tubular myelin and vacuoles containing remnants of it were found in the cytoplasm. Thus, as indicated previously in kinetic studies, it appears that alveolar macrophages participate in the turnover of surfactant. However, the relative importance of the macrophage in comparison to other pathways of surfactant removal remains to be determined. It is also noteworthy that although tubular myelin and myelin figures were abundant in the fixative used to wash out the lungs, bacteria were not found in it or in the macrophages. Thus, removal of obsolete surfactant may prove to be one of the major endocytic functions of alveolar macrophages.
I would like to thank Friederike Boost for her excellent technical aid, Steve Parente for the photographic reproductions, Rosamund Michael for editorial assistance, and Doctors Mary Bentfeld and Mary Williams for their critical reading of the manuscript.

Received for publication 15 June 1976.

References

1. Green, G. M., and E. H. Kass. 1964. The role of the alveolar macrophage in the clearance of bacteria from the lung. *J. Exp. Med.* 119:167.
2. Langmuir, A. D. 1961. Epidemiology of airborne infection. *Bacteriol. Rev.* 25:173.
3. Green, G. M. 1970. The J. Burns Amberson lecture—In defense of the lung. *Am. Rev. Resp. Dis.* 102:891.
4. Naimark, A. 1973. Cellular dynamics and lipid metabolism in the lung. *Fed. Proc.* 32:1967.
5. Pattle, R. E. 1965. Surface lining of lung alveoli. *Physiol. Rev.* 45:48.
6. Clements, J. A. 1970. Pulmonary surfactant. *Am. Rev. Resp. Dis.* 101:984.
7. Goerke, J. 1974. Lung surfactant. *Biochim. Biophys. Acta.* 344:241.
8. Bensch, K., K. Schaefer, and M. E. Avery. 1964. Granular pneumocytes: electron microscopic evidence of their exocrinic function. *Science (Wash. D. C.)*. 145:1318.
9. Sorokin, S. P. 1966. A morphologic and cytochemical study on the great alveolar cell. *J. Histochem. Cytochem.* 14:884.
10. Weibel, E. R., and J. Gil. 1968. Electron microscopic demonstration of an extracellular duplex lining layer of alveoli. *Respir. Physiol.* 4:42.
11. Gil, J., and E. R. Weibel. 1969. Improvements in demonstration of lining layer of lung alveoli by electron microscopy. *Respir. Physiol.* 8:13.
12. Weibel, E. R., G. S. Kistler, and G. Tondury. 1966. A stereologic electron microscope study of "tubular myelin figures" in alveolar fluids of rat lungs. *Z. Zellforsch. Mikrosch. Anat.* 69:418.
13. Gil, J., and O. K. Reiss. 1973. Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. *J. Cell Biol.* 58:152.
14. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27:137A.
15. Farquhar, M. G., and G. E. Palade. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* 26:263.
16. Hirsch, J. G., and M. E. Fedorko. 1968. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate. *J. Cell Biol.* 38:615.
17. Weibel, E. R. 1973. Morphological basis of alveolar-capillary gas exchange. *Physiol. Rev.* 53:419.
18. Campiche, M. 1960. Les inclusions lamellaires des cellules alvéolaires dans le poumon du Raton. *J. Ultrastruct. Res.* 3:302.
19. Leeson, T. S., and C. R. Leeson. 1966. Osmophilic lamellated bodies and associated material in lung alveolar spaces. *J. Cell Biol.* 28:577.
20. Williams, M. C. 1975. Conversion of lamellar body membranes into tubular myelin in the alveolar spaces of the late fetal rat lung: TEM and HVEM observations. *J. Cell Biol.* 67:456A.
21. Kistler, G. S., P. R. B. Caldwell, and E. R. Weibel. 1967. Development of fine structural damage to alveolar and capillary lining cells in oxygen-poisoned rat lungs. *J. Cell Biol.* 32:605.
22. Mason, R. J. 1976. Metabolism of alveolar macrophages. In *Defense Mechanisms of the Lung*. J. Brain, D. Proctor, and A. Reid, editors. Marcel Dekker Inc., New York. In press.
23. Geiger, K., M. L. Gallagher, and J. Hedley-Whyte. 1975. Cellular distribution and clearance of aerosolized dipalmitoyl lecithin. *J. Appl. Physiol.* 39:759.
24. Stoeckenius, W. 1959. An electron microscope study of myelin figures. *J. Biophys. Biochem. Cytol.* 5:491.
26. Fowler, S., and C. de Duve. 1969. Digestive activity of lysosomes. III. The digestion of lipids by extracts of rat liver lysosomes. *J. Biol. Chem.* 244:471.
26. Elsbach, P. 1973. On the interaction between phagocytes and micro-organisms. *N. Engl. J. Med.* 289:846.
27. Cohn, Z. A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leucocytes and macrophages. *J. Exp. Med.* 117:27.
28. Laurenzi, G. A., R. T. Potter, and E. H. Kaas. 1961. Bacteriologic flora of the lower respiratory tract. *N. Engl. J. Med.* 265:1273.
29. Lees, A. W., and W. McNaught. 1959. Bacteriology of lower-respiratory-tract secretions, sputum, and upper-respiratory-tract secretions in "normals" and chronic bronchitics. *Lancet.* 2:1112.
30. Hatch, T. F. 1961. Distribution and deposition of inhaled particles in respiratory tract. *Bacteriol. Rev.* 25:237.
31. Wright, G. W. 1961. Structure and function of respiratory tract in relation to infection. *Bacteriol. Rev.* 25:219.