STUDIES ON THE PULMONARY AIR-TISSUE BARRIER. PART IV: CYTOCHEMICAL TRACING OF MACROMOLECULES DURING ABSORPTION†

In previous experiments it was shown that albumin introduced into the lungs of dogs and guinea pigs was almost completely absorbed within 48 hours, while absorption of synthetic nonglobular compounds of similar molecular weight was remarkably slow. A comparison of the absorption rate of this protein with other molecules and electrolytes of molecular weight up to 160,000 is made in Figure 1. Quantitative immunochemical studies on the serum of animals, the lungs of which had been instilled with isotope-labeled albumin or globulin, indicated that the majority of these molecules was absorbed into the circulatory system antigenically intact; in several instances more than two thirds of the absorbed proteins could be accounted for intact in the blood of an animal. Comparison of the role of the pulmonary blood with lymph from the right lymphatic and thoracic duct in the absorption of protein introduced into alveoli provided evidence for the relatively minor contribution of the lymphatic vessels in this process. Presented here are efforts at visualization by cytochemical means at the ultrastructural level of this transfer process of protein molecules; our findings indicate that macromolecules may cross the air-blood barrier by being transported directly into the pulmonary capillary blood in the pinocytotic vesicles of the membranous pneumocyte (alveolar lining cell) and endothelial cell.

MATERIALS AND METHODS

Graham and Karnovsky's adaptation for the cytochemical demonstration of peroxidase was used throughout all experiments. A 500 mg. % solution of the tracer, horse-radish peroxidase (M. W. 40,000) in isosmolal Essential Basal Medium Eagle enriched with 10% horse or guinea pig serum (pH 7.3) was instilled into the lungs of guinea pigs. The volume of this instillation mixture was kept to less than 5% of the total lung volume; the instillate was introduced into one lobe of the lung by bronchial catheter, as described previously, and the animals were sacrificed in 15-minute intervals up to one hour and then at 30-minute intervals up to four hours after instillation. The lung was then inflated with 2% buffered glutaraldehyde solution under 20 cm. of hydrostatic pressure and the specimens were kept immersed in the fixative for four hours. Sixty micron-thick sections and minute tissue blocks of less than 1 mm. were then rinsed in a buffered wash solution followed by incubation for 30-60 minutes in the cytochemical
Horseradish peroxidase as tracer in lung | BENSCH, DOMINGUEZ

![Graph]

**Fig. 1.** Comparison of percentages of various compounds absorbed from the lung after intrapulmonary instillation.

reaction mixture. Following this, the tissue was post-fixed in OsO₄ and embedded in Maraglas. Ultrathin sections, unstained or stained with lead salts, were studied with an Elmiskop I.

**RESULTS**

Normally, there are three types of cells that are exposed to the alveolar air: the alveolar lining cell or membranous pneumocyte (which lines more than 90% of the alveolar wall), the granular pneumocyte (niche cell, type II cell containing the osmophilic inclusion bodies) and the roving alveolar macrophage or phagocytic pneumocyte. The minimal air-blood pathway, about $0.5 \times 10^{-4}$ cm. in the human, consists of the attenuated cytoplasm of the membranous pneumocyte and the cytoplasm of the juxtapositional capillary endothelial cell and the basement membrane of each of these two cell types. Both of these cells contain normally large populations of pinocytotic vesicles of about 700 A diameter (Fig. 2).

After intrapulmonary instillation, the tracer protein, demonstrable as aggregates of the cytochemical reaction product consisting of moderately electron opaque, relatively uniform-sized granules (of about 150-200 Angstroms diameter) with a fuzzy periphery, appeared in close apposition to the alveolar surface of the membranous pneumocytes (attenuated alveolar lining cell). (Fig. 3). Soon thereafter (after 15-30 min.), the tracer
protein was visible in pinocytotic vesicles with lumina which were still in direct continuity with the air space, or in pinocytotic vesicles located in the cytoplasm subjacent to the alveolar surface of this cell type (Figure 4). Lung tissue, exposed for longer periods of time (one to two hours) to the peroxidase, showed the tracer protein in pinocytotic vesicles in transit between the alveolar and stromal surface of the membranous pneumocytes, in the interstitial tissue bordering on an alveolar lining cell or the space between the alveolar lining cell and the subjacent endothelial cell of a capillary; eventually (after 60-120 minutes) peroxidase was demonstrable in pinocytotic vesicles of these endothelial cells, first in the abluminal parts of these cells, later on near the vascular lumen (Figs. 5 and 6). Occasionally, the marker protein could be found within the plasma of the capillaries.

Peroxidase was not taken up, at least during the time period of these experiments, by the second type of alveolar lining cell, the granular pneumocyte. In contrast, alveolar macrophages (phagocytic pneumocytes) ingested rapidly and avidly the protein present in the instillate. In this cell, the material was taken up into small vesicles which eventually fused to form digestion vacuoles (lysosomes). (Fig. 7).

DISCUSSION

The experiments reported here were intended to elucidate, at the fine structural level, the path taken by macromolecules absorbed intact by the lung. Previously-provided evidence for the rapid absorption of a large proportion of protein molecules from fluid instilled into alveoli suggested the existence of a mechanism by which large molecules are transported rapidly and in large quantities into the pulmonary capillary blood. The use of a protein, horseradish peroxidase, that can be recognized readily under the electron microscope (at the site where the enzyme is located, an electron opaque, poorly soluble oxidation product of the benzidine substrate accumulates) permitted the tracing of the absorptive pathway: pinocytotic vesicles appear to transport the tracer protein (MW 40,000) across the attenuated alveolar lining cells, then discharge their contents into the interstitial space of the alveolar septum where it is pinocytosed by the capillary endothelial cells; the contents of these pinocytotic vesicles are eventually discharged into the vascular lumen.

Capillary permeability for fluid and crystalloids, according to Starling’s hypothesis, is a passive process regulated by the interplay between osmotic and hydraulic forces.11-18 This assumption was extended by Pappenheimer’s postulate that this diffusion of small lipid insoluble molecules occurs through hypothetical pores in the capillary wall and that molecules up to the size of hemoglobin could thus move across an endothelial cell.14-18 Electron micro-
FIG. 2. Normal alveolar air-blood barrier. There are numerous pinocytotic vesicles (short arrows) in the alveolar lining cell (membranous pneumocyte) (M) as well as the capillary endothelial cell (E), many of which are either in the process of forming on the cell surface or discharging their contents (long arrows). "A" designates the alveolar lumen, "I" the alveolar interstitium, "L" the capillary lumen, which contains parts of two erythrocytes. Magnification: 48,600 ×.
FIG. 3. Survey view of a lung fixed 15 minutes after instillation with a solution containing horseradish peroxidase; demonstration of the latter protein by a moderately electron opaque cytochemical reaction product (see text) localizes the peroxidase as a black film overlying the surface of the membranous pneumocytes (arrows), as well as coarsely-granular black deposits in the alveolar lumen (double arrows). "A" designates the alveolar lumen, "L" the capillary lumen which contains three erythrocytes and a leukocyte, and "G" the granular pneumocyte. The alveolar wall, at the center of the bottom of the photograph, borders on an alveolar duct. Magnification: 5,200 ×.
Fig. 4. Peroxidase, 30 minutes after intra-alveolar instillation, is present, not only as a dark, finely granular layer upon the surface of the membranous pneumocyte, but also in pinocytotic vesicles deep in the cytoplasm of this cell (arrows). “M” designates the membranous pneumocyte, “E” the endothelial cell, and the dark bar, the interstitial space between these two cell types; “L” designates the lumen of an alveolar capillary which also contains parts of two erythrocytes and, “A” the alveolar lumen. Magnification: 45,600 X.
Fig. 5. The lungs of animals that had received intrapulmonary instillations of horseradish peroxidase solution one hour prior to death show the dark-staining horseradish peroxidase (i.e., its reaction product), not only on the surface of the membranous pneumocytes and in their pinocytotic vesicles (short arrow), but also in the alveolar interstitial space subjacent to this cell type, as borne out by the black line marked by the white arrow. Numerous of the pinocytotic vesicles of the capillary endothelial cell (to the left of the black line) also contain the peroxidase (long arrow). "A" designates alveolar lumen, and "L" the capillary lumen. Magnification: 44,000 ×.
Fig. 6. Large quantities of peroxidase were present in the interstitial space between membranous pneumocytes and endothelial cells in specimens obtained 1 to 2 hours after intrapulmonary instillation of peroxidase solution; this illustration shows the peroxidase as a dense, dark line extending from the top to the bottom of the photograph (thus nicely delineating the entire interstitial space). Numerous of the pinocytotic vesicles of the endothelial cell of the capillary contain peroxidase (arrow). "A" designates the alveolar lumen, "M" a membranous pneumocyte, "E" an endothelial cell, and "L" the capillary lumen. Magnification: 36,000 x.
Fig. 7. The upper two thirds of the illustration are occupied by a phagocytic pneumocyte (alveolar macrophage). Within 15 to 30 minutes after exposure to the peroxidase solution, macrophages were found to contain peroxidase in membrane-bound structures ranging from pinocytotic vesicles to phagocytic vacuoles of more than one micron in diameter (arrows). "P" designates the phagocytic pneumocyte, "A" the alveolar lumen; present in the left lower corner of the illustration is part of an alveolar wall. Magnification: 10,400 ×.
scopic studies of endothelial cells, however, failed to confirm this theory, which demanded the existence of pores up to 65 A in diameter. But the presence of small vesicles in endothelial cells and the uptake of electron opaque colloids by these structures prompted microscopists, notably Palade, to identify these pinocytotic vesicles as a carrier for macromolecules and colloidal substances across the capillary lining cells.\(^{18-28}\) Re-examination of this problem during the mid-sixties, by means of cytochemical techniques and the use of tracer proteins, led, in contrast, to the postulate that the binding material holding together neighboring endothelial cells and sealing the intercellular gap (originally described as intercellular cement), actually contains the rigid smooth-walled channels of Pappenheimer's hypothesis; the pinocytotic vesicles were assigned, among others, a secretory function providing the mucopolysaccharide lining of the endothelial surface.\(^{18-20}\) Lately, however, more evidence for the transport function of the pinocytotic vesicles in endothelial cells was provided by further studies by Palade and other investigators,\(^{21}\) and a change of mind about the importance of pinocytotic vesicles as vehicles of transendothelial vesicular transport took place in other quarters.\(^{22}\)

The pinocytotic vesicles of the alveolar lining cells and the capillary endothelium appeared to be the main mode of transport of the protein into the blood in our experiments. Several of the various factors that must play a role in pinocytosis are still poorly understood; among these are the control of the rate, energy required\(^ {23}\) and direction of movement of these vesicles. For instance, our findings show that the absorbed material was moved from the (alveolar) lumen towards the basement membrane in the membranous pneumocytes, while, in contrast, in the alveolar capillary endothelial cells, the vesicular movement proceeded toward the capillary lumen. The latter is of interest in view of the numerous studies that demonstrated transcellular transport toward the abluminal surface in endothelial cells. Although our finding lends strength to the hypothesis that a rotatory microcirculation with an assumed axis parallel to the endothelial cell surface may bring about the streaming of neighboring vesicles in opposite directions,\(^ {24}\) it gives, in the final analysis, rise to more questions than answers.

Pinocytosis, a property of the majority of the cells of the alveolar wall, must play a major role in the removal of the intra-alveolar fluid that probably escapes in minute quantities from the pulmonary vascular system under normal conditions, and in large amounts in a variety of diseases. This mechanism keeps us from drowning in a transudate that is returned into the circulation. The same transcellular transport system undoubtedly also plays a significant part in the turnover of the surfactant layer that normally covers the alveolar surface. On the other hand, this ability of the lung, the
body's largest area of contact with the environment, to act as a port of entry for macromolecules including extraneous protein, is of importance medically. The absorption of large quantities of antigen, present either in the air in the form of dust or in an accidental aspirate, may help explain a variety of hypersensitivity reactions; these may either be generalized or limited to (parts of) the lung.

SUMMARY

Proteins instilled into the lungs of dogs and guinea pigs are, as was shown in preceding experiments, absorbed rapidly and antigenically intact; comparison of the role of the pulmonary blood and lymph in the removal of proteinaceous fluid from the alveoli provided evidence for the minor role of the lymphatics in this process. Described here is the visualization by cytochemical means at the ultrastructural level of this transfer process; the study indicates that macromolecules can be transported directly into the pulmonary capillary blood by the pinocytotic vesicles of the membranous pneumocyte (type I alveolar cell) and endothelial cell.

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