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The Alveolar Type II Epithelial Cell: A Multifunctional Pneumocyte

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The Alveolar Type II Epithelial Cell: A Multifunctional Pneumocyte. CASTRANOVA, V., RABOVSKY, J., TUCKER, J. H., AND MILES, P. R. (1988). *Toxicol. Appl. Pharmacol.* **93**, 472–483. The epithelial surface of the alveoli is composed of alveolar type I and type II cells. Alveolar type I cells comprise 96% of the alveolar surface area. These cells are extremely thin, thus, minimizing diffusion distance between the alveolar air space and pulmonary capillary blood. Type II cells are spherical pneumocytes which comprise only 4% of the alveolar surface area, yet they constitute 60% of alveolar epithelial cells and 10–15% of all lung cells. Four major functions have been attributed to alveolar type II cells: (1) synthesis and secretion of surfactant; (2) xenobiotic metabolism; (3) transepithelial movement of water; and (4) regeneration of the alveolar epithelium following lung injury. Therefore, alveolar type II cells play important roles in normal pulmonary function and in the response of the lung to toxic compounds which may cause lung damage. Techniques have now been developed to isolate and purify alveolar type II epithelial cells from lung tissue. Such cellular preparations afford bioassay systems to monitor the effects of occupational or environmental pollutants on alveolar pneumocytes and should yield important information concerning the etiology of pulmonary disease in the alveolar region of the lung.

A classic approach to pulmonary toxicology is to monitor the effects of *in vivo* or *in vitro* exposure to various agents on the function of isolated lung tissue or purified pneumocytes. For technical reasons, the most common pulmonary bioassays have involved tracheal smooth muscle and alveolar macrophages. Tracheal smooth muscle preparations are useful in the study of agents which cause chest tightness, decreased dynamic lung volumes, or occupational asthma. Alveolar macrophages serve as a system to test the effects of agents on susceptibility to pulmonary infection, general cellular viability, and hypersecretion of reactive substances and enzymes which may lead to lung damage. However, several lung diseases involve cells located in the alveolar septa. Such diseases include emphysema, edema, fibrosis, and oxidant injury. For this reason, purified preparations of cells from this region of the lung would be valuable in investigating several types of toxic lung damage.

The morphology of the alveolar region of the lung is shown in Fig. 1. The alveolar space is surrounded by type I and type II epithelial cells. Alveolar type I cells are long, thin cells which comprise 96% of the epithelial surface area. These thin cells serve to minimize the distance between the alveolar air space and pulmonary capillaries, thus, maximizing gas exchange. In contrast, type II cells are spherical pneumocytes which are approximately 9 μm in diameter (Jones *et al.*, 1982; Haies *et al.*, 1981). Although these pneumocytes constitute only 4% of the alveolar epithelial surface area, they represent 60% of alveolar epithelial cells by number (Crapo *et al.*, 1983).

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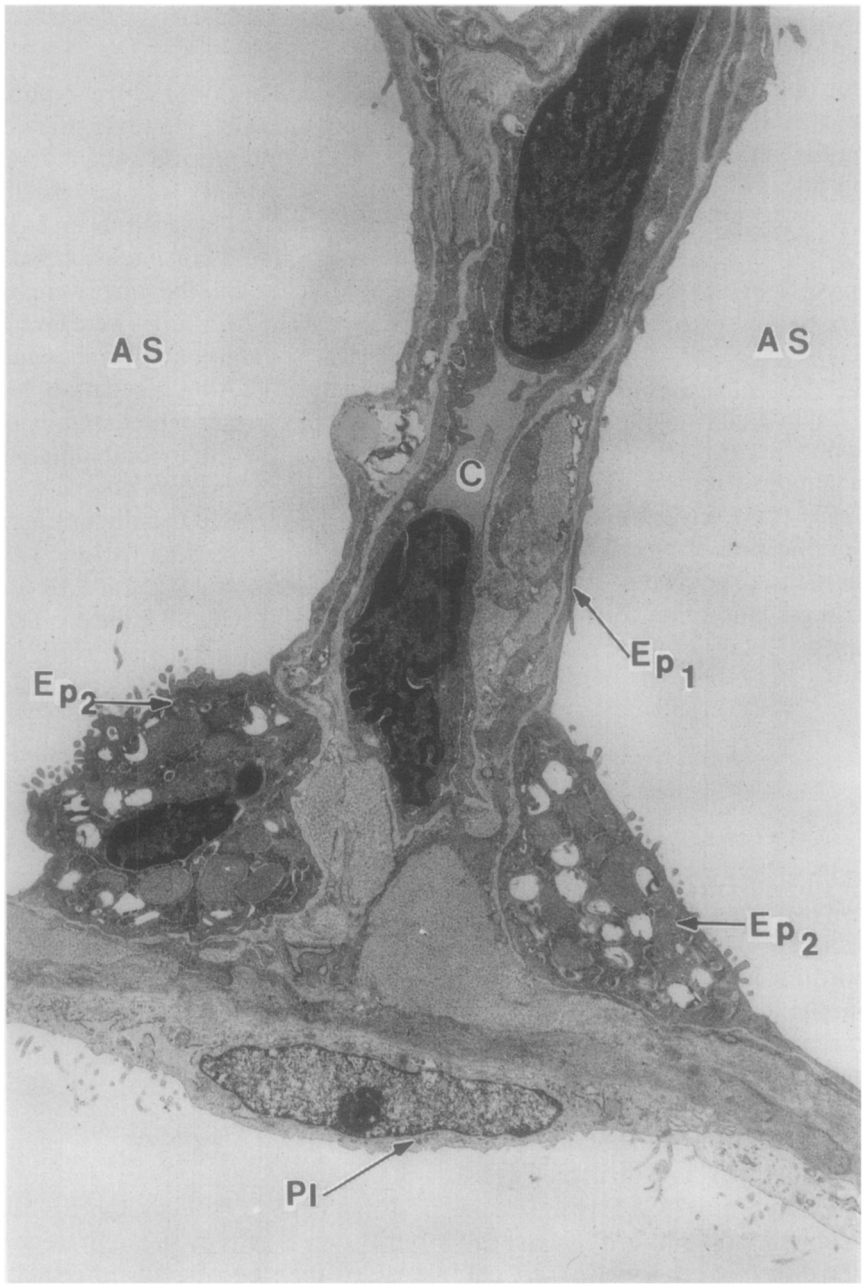

FIG. 1. A transmission electron micrograph of the alveolar septal region of a rat lung (×3200). The alveolar air space (AS), pulmonary capillary (C), type I epithelial cell (Ep1), type II epithelial cell (Ep2), and visceral pleura (PI) are labeled.

Several techniques are now available to isolate and purify alveolar type II cells and a number of functions have been attributed to this pneumocyte. Therefore, alveolar type II cells should serve as a useful preparation to evaluate the potential toxicity of environ-

mental and occupational pollutants. The purpose of this manuscript is to describe methods for obtaining purified preparations of alveolar type II cells and to review data concerning the various properties and functions of this pneumocyte.

ISOLATION AND PURIFICATION OF ALVEOLAR TYPE II CELLS

Several methods have been employed to isolate and purify type II cells. These methods include enzymatic digestion of lung tissue to free lung cells from the pulmonary epithelium and purification of type II cells by discontinuous density gradients, primary culture, centrifugal elutriation, or unit gravity sedimentation. Details of these methods are given below.

A. Density Gradient Centrifugation

Purification of alveolar type II cells according to density was first reported by Kikkawa and Yoneda (1974). Briefly, rat lungs were perfused with medium to remove blood elements from the lung and lavaged to remove alveolar macrophages. Lungs were excised and the bronchial tree was removed. The lungs were then sectioned into 0.5mm^3 blocks. Tissue blocks were mixed with colloid barium sulfate (1% w/v) and incubated in 2% trypsin for 20 min at 37°C. Trypsinization was stopped by the addition of 15% fetal calf serum to the dispersed cells, which were collected by filtration through nylon cloth. The cell suspension was then layered on a discontinuous Ficoll gradient (densities 1.047, 1.058, and 1.100) and centrifuged for 1 hr at 1700*g*. Type II cells were collected in a zone above 1.047. This preparation yielded 0.6 million cells/rat, 90% of which were type II cells.

B. Plating

Dobbs and Mason (1979) also purified type II cells by density. However, they extended the purification process using differential adherence in primary culture. Briefly, lungs were perfused and lavaged. Then 8–10 ml of a fluorocarbon-albumin emulsion was instilled via the trachea and the lungs were incubated for 20 min at 37°C. Lungs were lavaged, filled with elastase (40 units/ml), and incubated for 20 min at 37°C. After incubation, lungs were minced into 1-mm^3 pieces, and incubated in 20% fetal calf serum to inhibit digestion. The cell suspension was then filtered through nylon mesh and added to a discontinuous albumin gradient (densities 1.040 and 1.085). The gradients were centrifuged at 270*g* for 20 min at 4°C. Type II cells (28 million cells/rat, 80% pure) were collected at the 1.040–1.085 interface. Cells were suspended (2 million cells/ml) in culture medium, added to 75-cm^2 tissue culture flasks, and incubated at 37°C in a 10% CO_2:90% air incubator. After 1 hr of incubation, the nonadherent cells were removed and incubated (1.0 million cells/ml) in 35-mm plastic petri dishes for 22 hr at 37°C. During this time, type II cells adhered to the dish and contaminating cells were decanted. This procedure resulted in type II cells which were 92% pure with a seeding efficiency of 68%.

C. Centrifugal Elutriation

Jones *et al.* (1982) developed a method to purify type II cells by size, a procedure which avoided the step of gradient centrifugation. Briefly, lungs were perfused and lavaged. Elastase (40 units/ml) was instilled via the trachea and the lungs were incubated for 20 min at 37°C. The lungs were minced and digestion was arrested with 25% fetal calf serum. After 10 min of incubation at 37°C, this suspension was strained through nylon mesh. These cells were loaded into an elutriation ro-

tor (Model JE-6, Beckman Instrument, Fullerton, CA) at a flow rate of 10 ml/min and a rotor speed of 2000 rpm. At this flow rate, small cells were removed. After 200 ml of fluid had been collected, the flow rate was increased to 19 ml/min. Type II cells (10 million cells/rat, 85% pure) were collected at this flow rate.

D. Unit Gravity Sedimentation

A combination of density gradient centrifugation and unit gravity sedimentation has been employed by Brown *et al.* (1984). Briefly, lungs were perfused and lavaged. Lungs were then filled with a fluorocarbon-albumin emulsion and incubated at 37°C for 20 min. After incubation, lungs were lavaged, filled with elastase (7 units/ml), and incubated for 20 min at 37°C. After digestion, lungs were minced and digestion was arrested with fetal calf serum. The cells were layered on a metrizamide gradient (densities 1.040 and 1.090) and centrifuged at 160*g* for 20 min at 4°C. Type II cells were collected from the 1.04–1.09 interface, washed, resuspended in 75 ml of 1% Ficoll medium, loaded into a unit gravity separator containing a continuous 2–4% Ficoll gradient (density 1.009 to 1.015), and allowed to sediment at unit gravity for 2–5 hr at 25°C. Fractions 15–19 contained 9 million cells/rat which were 90% type II cells.

IDENTIFICATION OF TYPE II CELLS

Isolated alveolar type II epithelial cells can be identified by the presence of characteristic lamellar inclusion bodies. These lamellar bodies are apparent under an electron microscope as darkly stained spiral structures (Fig. 2). These lamellar bodies can be visualized using light microscopy with the Papanicolaou stain. Using this stain inclusion bodies are identified as deep blue granules surrounded by a clear halo (Kikkawa and Yoneda, 1974). Mason and Williams (1976) have shown that a lipophilic fluorescent dye, phosphine 3R (Roboz Surgical Instrument, Washington, DC), is concentrated in the lamellar bodies of type II cells. Therefore, type II pneumocytes can be identified under a fluorescent microscope by the presence of yellow inclusion bodies (Fig. 3).

FUNCTIONS OF ALVEOLAR TYPE II CELLS

A. Synthesis and Secretion of Surfactant

Macklin (1954) was the first to propose that the alveolar epithelium was covered by a mucoid film which was vital to normal lung function. Morphological evidence for this thin polysaccharide-rich alveolar lining material soon followed (Chase, 1959; Goniowsky and Biczyskowa, 1964). The alveolar lining material was found to be a lipoprotein complex (Pattle and Thomas, 1961; Klaus *et al.*, 1961) which contains nonserum proteins, phospholipids, and neutral lipids (Reifenrath, 1973). The composition of alveolar lining material, i.e., surfactant, is given in Table 1 (Sanders and Longmore, 1975). Surfactant is composed mainly of lipid. Approximately $\frac{2}{3}$ of this lipid is phosphatidylcholine with $\frac{2}{3}$ of the phosphatidylcholine being saturated, i.e., disaturated phosphatidylcholine (DSPC).

Macklin (1954) proposed that surfactant was synthesized and secreted by granular pneumocytes, i.e., type II cells, located in the alveolar epithelium and that lamellar bodies were storage vesicles for this surfactant (Fig. 2). This hypothesis was confirmed by Askin and Kuhn (1971) who published autoradiographs of lungs from rats treated intravenously with tritiated palmitate. They found that this fatty acid was incorporated into DSPC and was indeed concentrated into the lamellar bodies of alveolar type II cells. Therefore, a major function of type II pneu-

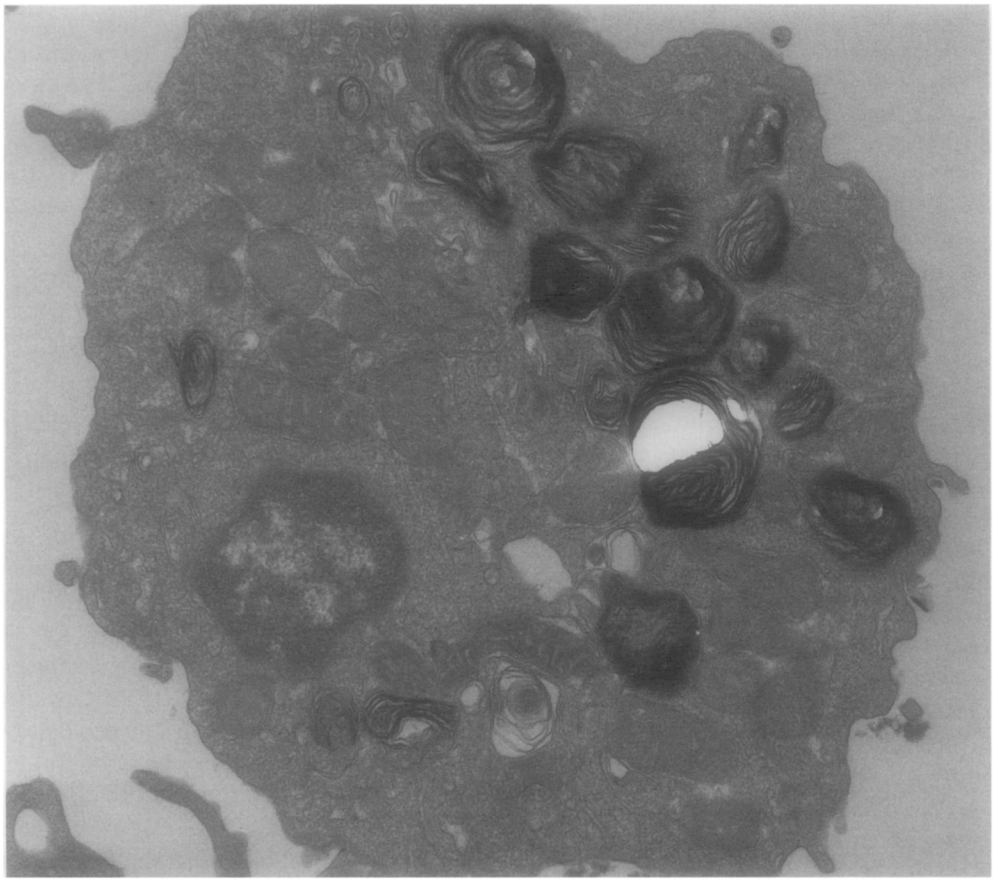

FIG. 2. A transmission electron micrograph of an alveolar type II epithelial cell isolated from rat lung (×13,500). Samples were postfixed with osmium tetroxide and stained with uranyl acetate and Reynolds lead citrate. Characteristic lipid inclusions (lamellar bodies) are apparent as darkly stained spiral structures.

mocytes is the synthesis and secretion of surfactant.

It is clear that pulmonary surfactant is critical to normal lung function. Its major function is to decrease the surface tension at the air–liquid interface of the lung. This property of alveolar fluid was first discovered by Pattle (1955). Clements (1957) further investigated this phenomenon. He reported that surfactant not only lowers the surface tension of lung fluid but that surface tension is also dependent on surface area, i.e., surface tension decreases with a decrease in surface area. The ability of surfactant to decrease a surface tension results in decreased work required to inflate the lung, alveolar stability, and prevention of edema (Clements *et al.*, 1958; Staub, 1966). The surface tension properties of surfactant have been attributed to DSPC while the ability of surfactant to rapidly form a surface film seems to be a property of the surfactant apoprotein components (Clements, 1977). A second function of surfactant is its role in the lung's response to infection. Pretreatment of *Staphylococcus aures* with alveolar lining material dramatically increases the ability of alveolar macrophages to kill the bacteria *in vitro* (LaForce *et al.*, 1973; Juers *et al.*, 1976). Indeed, surfactant not only enhances bacterial killing but also significantly

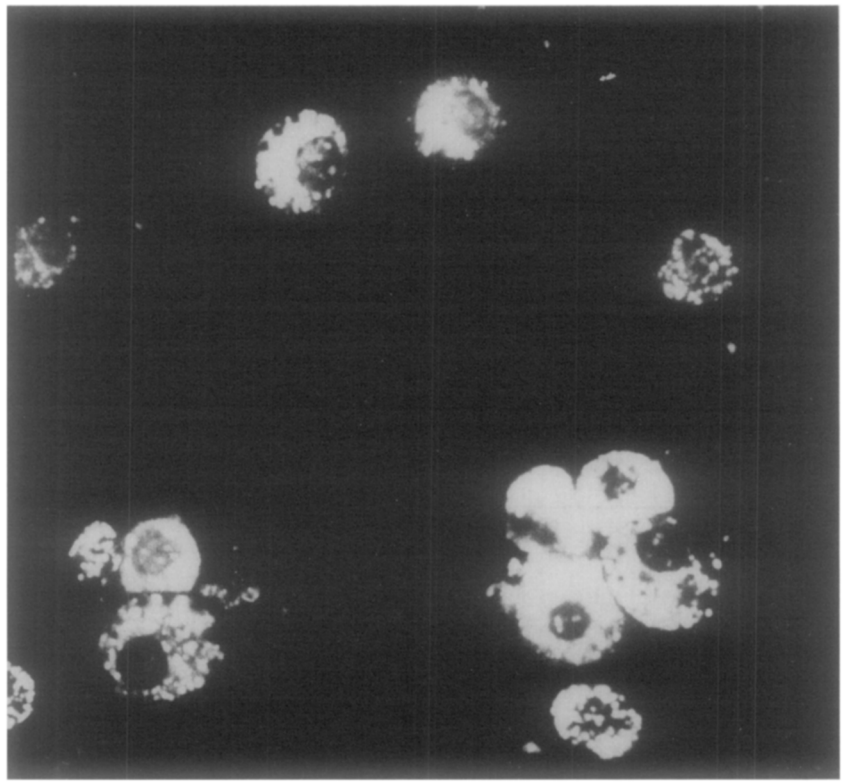

FIG. 3. A fluorescence micrograph of a purified preparation of alveolar type II epithelial cells (×1200). Cells were equilibrated for 2 min with phosphine 3R dye (0.002%) and observed under a fluorescence microscope set at an absorption peak of 477 nm and an emission peak of 512 nm. Type II cells are identified by the presence of yellow lamellar bodies.

increases phagocytosis of bacteria by alveolar macrophages (O'Neill *et al.*, 1984). A third function of surfactant may be to protect the lung from potentially toxic inhaled particles. Indeed, Wallace *et al.* (1985) have shown that pretreatment of silica or kaolin with DSPC dramatically decreases the cytotoxicity of these particles to alveolar macrophages. Therefore, the lipidotic response to inhalation of various dusts may be a pulmonary defense mechanism.

As discussed above, surfactant serves to lower surface tension, aids in bacterial killing, and protects the lung from inhaled particles. Exposures which would alter the ability of type II cells to synthesize or secrete surfactant could greatly affect lung function. Therefore, assays of surfactant synthesis and secretion should be of great interest to pulmonary toxicologists. Surfactant synthesis can be monitored by determining the *in vitro* incorporation of radiolabeled palmitate or choline into DSPC by isolated type II cells (Batenburg *et al.*, 1978; Mason and Dobbs, 1980. An example of the time course for DSPC synthesis is given in Fig. 4 (Miles *et al.*, 1983). Surfactant release can be monitored *in vitro* by measuring release of labeled DSPC from type II cells (Mason *et al.*, 1977; Dobbs and Mason 1979; Brown and Longmore, 1981). Briefly, radiolabeled palmitate is added to type II cells in culture and allowed to incorporate into DSPC for 22 hr at 37°C. After this time, the medium is replaced with isotope-free me-

TABLE 1

COMPOSITION OF PULMONARY SURFACTANT

	Percentage		
Lipid	91		
Phospholipids		81	
Phosphatidylcholine			72
Saturated			45
Unsaturated			27
Phosphatidylglycerol			5
Phosphatidylethanolamine			2
Others			2
Neutral Lipids		10	
Triglyceride			3
Cholesterol			7
Protein	7		
Carbohydrate	2		

dium and release of labeled DSPC monitored over time as shown in Fig. 5. Surfactant release from type II cells in culture is increased by approximately threefold by β-adrenergic agonists (L-epinephrine, L-isoproterenol, or DL-terbutaline at 1×10^{-5} M) and by approximately fivefold by tetadecandyl phorbol acetate (1×10^{-5} M). In contrast, cholinergic agonists fail to enhance surfactant synthesis (Dobbs and Mason, 1979; Brown and Longmore, 1981).

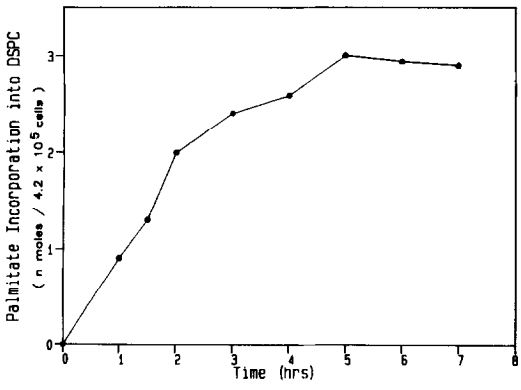

FIG. 4. Time course for incorporation of ^{3}[H]palmitate into DSPC by isolated rat alveolar type II cells suspended in phosphate-buffered medium (8.3×10^5 cells/ml) containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 1.0 mM palmitate.

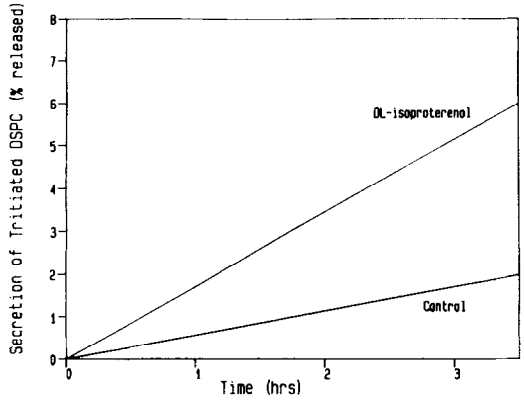

FIG. 5. Secretion of DSPC from a primary culture of type II cells with and without β adrenergic stimulation, i.e., L-isoproterenol (10^{-5} M) added at time zero. Type II cells (1×10^6 cells/ml) were incubated in culture with ^{3}[H]palmitate for 22 hr at 37°C. After this time, nonadherent cells and labeled medium were removed and the adherent cells rinsed three times before fresh medium was added (zero time). Tritiated DSPC was measured in the medium and cells at various times thereafter.

B. Xenobiotic Metabolism

The lung is a site for metabolism of foreign substances (xenobiotics), which include drugs and environmental pollutants (Minchin and Boyd, 1983; Bend *et al.*, 1985). Metabolism results in the formation of water soluble compounds which may be either less toxic or more reactive depending on the nature of the intermediates, the relative amounts of activating and nonactivating enzymes, and the availability of substrates and cofactors. A major mechanism for xenobiotic metabolism in lung is the cytochrome *P*-450-dependent monooxygenase system (EC 1.14.14.1). This enzyme system is a class of heme proteins that catalyzes the monooxygenation of a wide variety of lipophilic substances in the presence of O_2 and NADPH (Gillette *et al.*, 1972).

Early studies established that pulmonary cytochrome *P*-450-dependent monooxygenase activities are localized in the microsomal fraction of lung cells (Hook *et al.*, 1972) and are associated with no more than three dis-

tinct forms (Ueng and Alvarez, 1982; Serabjit-Singh *et al.*, 1983). Two types of pneumocytes exhibit substantial *P*-450-dependent monooxygenase activity, i.e., the alveolar type II epithelial cell and the bronchiolar, nonciliated Clara cell (Devereux *et al.*, 1979; Jones *et al.*, 1983; Baron and Kawabata, 1983; Rabovsky *et al.*, 1986). Each cell type is rich in endoplasmic reticulum and is sensitive to the presence of toxic agents (Baron and Kawabata, 1983). The contribution of alveolar macrophages to *P*-450-dependent metabolism is uncertain. Reports on the presence or absence of cytochrome *P*-450 in alveolar macrophages have been conflicting and indicate that many factors including substrate may be operative (Baron and Kawabata, 1983; Minchin *et al.*, 1985).

A useful *P*-450-dependent monooxygenase activity for toxicological studies is ethoxyresorufin deethylase (ERase). ERase activity is measured in a direct fluorometric assay (Burke *et al.*, 1977) and is thought to be representative of *P*-450-dependent forms that metabolize polycyclic aromatic hydrocarbons (Burke *et al.*, 1977; Phillipson *et al.*, 1984). Alveolar type II cells from untreated rats exhibit minimal activity ($\leq$0.04 pmol resorufin/min·10^6 cells). However, substantial ERase activity (0.54 pmol resorufin/min·10^6 cells) is obtained following induction, i.e., exposure to β-naphthoflavone (βNF) for 48 hr (Fig. 6). ERase activity of rat type II cells is 1000-fold more sensitive to inhibition by α-naphtoflavone (ID50 approximately 10^{-8} M) than by metyrapone (Rabovsky *et al.*, 1986). Hence this activity is probably associated with the form(s) that are induced by polycyclic aromatic hydrocarbons (Ullrich and Kremers, 1977). The ability to measure cytochrome *P*-450-dependent monooxygenase activity (ERase and activities associated with other *P*-450 forms) in type II cells will enable researchers to study the relationship between this cell type and the detoxication of xenobiotics in pulmonary tissue. Alveolar type II cells are not only alveolar epithelial cells.

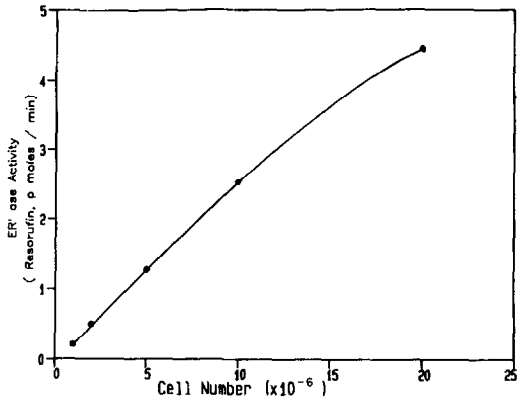

FIG. 6. Ethoxyresorufin deethylase activity as a function of number of induced type II cells in 2 ml of medium. Activity was determined by monitoring the production of fluorescent product (resorufin) at 36°C with an excitation wavelength of 530 nm and an emission wavelength of 585 nm in the presence of 2.5 mM substrate (resorufin ethyl ether). Induction of *P*-450 activity was accomplished by treating rats with β-naphthoflavone for 48 hr (80 mg/kg injected intraperitoneally).

They are also in close contact with the capillary blood supply. Hence xenobiotic metabolism in alveolar type II cells will apply to circulating organic compounds as well as inhaled substances.

C. Transepithelial Water Movement

It is essential for normal lung function that the alveoli be dry, since edema would increase the distance between the alveolar air space and pulmonary capillary blood, thus, effectively decreasing gas exchange. Evidence is mounting that alveolar type II cells may play an important role in alveolar transepithelial water movement. Jones *et al.* (1982) were the first to report that type II cells possess the ability to extrude large amounts of sodium and water across the plasma membrane. Their data showed that type II cells precooled at 2°C were swollen and contained a large amount of intracellular sodium. Upon warming, these pneumocytes rapidly decreased their intracellular sodium concentra-

TABLE 2

WATER AND IONIC CONTENT OF TYPE II CELLS[a]

	37°C	2°C
Cell H_2O ($\mu l/10^7$ cells)	2.1	3.2
% H_2O (by wt)	63	70
Mean cell volume (μm^3)	325	451
Cell Na^+ (mmol/liter cell H_2O)	51	72
Cell K^+ (mmol/liter cell H_2O)	107	80
Total cations (mmol/liter cell H_2O)	158	152
Cell Cl^- (mmol/liter cell H_2O)	70	69

[a] Cellular water and ionic content was measured at 2°C and after a 30 min incubation at 37°C. Cell H_2O and percentage H_2O were measured gravimetrically, Na^+ and K^+ were measured by flame photometry, and Cl^- was measured using a chloridometer. Mean cell volume was calculated from cell H_2O divided by percentage H_2O. Cytoplasmic Na^+ was corrected for bound Na^+. Total cation concentration was calculated as K^+ plus corrected Na^+ content.

tion, increased intracellular potassium concentration, and decreased cellular volume (Table 2). This water movement was rapid and relatively large and was inhibited by low temperature or ouabain, i.e., inhibition of Na^+–K^+ pump which can extrude large quantities of sodium resulting in net water movement (Fig. 7). Recently, methods have been developed which allow the formation of type II cell monolayers in culture. These type II cell monolayers exhibit dome formation, i.e., discrete areas of the monolayer raise off of the surface of the tissue culture plate (Goodman and Crandall, 1982; Mason *et al.*, 1982). Domes are thought to result from active transport of sodium transepithelially from the medium to the substratum. Indeed, dome formation was completely blocked by inhibitors of oxidative phosphorylation (KCN, low temperature, or 2,4-dinitrophenol), inhibitors of the Na^+–K^+ pump (ouabain or vanidate), or inhibitors of Na^+–H^+ exchange (amiloride) (Goodman *et al.*, 1983). Evidence indicates that the Na^+–H^+ exchange pathway moves sodium passively into type II cells from the alveolar side while the Na^+–K^+ pump actively extrudes sodium to the basal lateral side (Mason *et al.*, 1982). Water follows this transepithelial movement of sodium, thus, keeping the alveoli dry. This transepithelial water movement is dependent upon cAMP and β-adrenergic stimulation, since dome formation and ionic current were increased approximately twofold by 8-bromo-cAMP, theophylline, or β-adrenergic agonists (Goodman *et al.*, 1984; Cott *et al.*, 1983). Since type II cells may function to keep alveoli dry, assays such as dome formation by type II cell monolayers may prove useful for screening compounds which may cause pulmonary edema.

D. Recovery from Oxidant Injury

Upon exposure to high oxygen tensions, the alveolar epithelium becomes damaged (Mustafa and Tierney, 1978). Although alveolar type I cells are susceptible to oxidant injury, alveolar type II cells seem resistant to oxidant damage (Cross, 1974; Crapo *et al.*, 1980). Indeed, Adamson and Bowden (1979) have shown that after exposure to 90% O_2 mitotic figures were found in type II cells. This activity was associated with an increase in

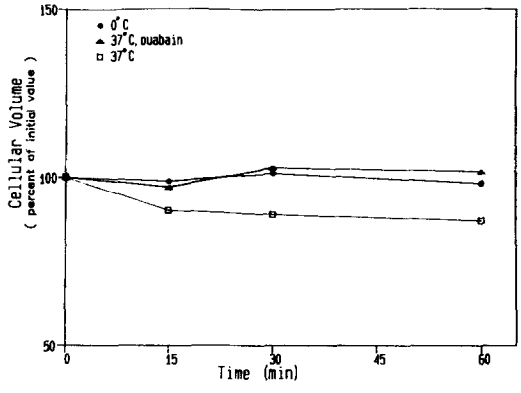

FIG. 7. Time courses of volume changes of isolated type II cells at different temperatures and in the presence of ouabain (10^{-4} M), i.e., an inhibitor of Na^+–K^+ ATPase. Mean cell volumes were determined by electronic cell sizing.

DNA synthesis, i.e., thymidine incorporation, and proliferation of type II cells. Pulse label studies suggest that type II cells proliferate and de-differentiate into type I cells, thus, repairing the alveolar epithelium. Transformation of type II cells to type I cells has also been described following NO_2 exposure (Evans *et al.*, 1975). It has been suggested that the resistance of alveolar type II cells to oxidant injury may be due to the existence of a specialized pathway for transport of the antioxidant, vitamin C, into these pneumocytes. Indeed, Castranova *et al.* (1983) have shown that alveolar type II cells possess a Na^+-ascorbate cotransport system which can accumulate ascorbate from a plasma level of 0.1 mM to an intracellular level of 3.2 mM. This transport system was dependent on extracellular sodium and was depressed by inhibitors of mitochondrial electron transport (KCN) and glycolysis (iodoacetic acid). It exhibited a V_{max} of 14.2 nmol/10^7 cells·hr and a $K_{1/2}$ of 5 mM for ascorbate. If agents alter this transport system, the lung's ability to resist and recover from oxidant exposure could be compromised.

SUMMARY

Alveolar type II epithelial cells serve at least four vital functions. They synthesize and secrete surfactant, metabolize foreign compounds, transport water out of alveoli, and repair the alveolar epithelium after oxidant injury. With the development of techniques to isolate and purify type II cells, the effects of various occupational and environmental pollutants on this multifunctional pneumocyte can be evaluated.

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