

Alveolar Type II and Clara Cells: Isolation and Xenobiotic Metabolism

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This paper describes one isolation procedure for two pulmonary cell types and discusses how these cells are being used for toxicological studies. Alveolar Type II cells and Clara cells have been isolated from rabbits and rats and separated into highly enriched fractions. The lungs were digested with protease, and the pulmonary cell digests were separated into discrete fractions on the basis of cellular size and density differences. Several studies have been conducted to compare the metabolism of xenobiotics in these cell types and three examples are discussed. The metabolic activation and covalent binding of the pulmonary toxin, 4-ipomeanol was found to occur in both Clara and Type II cells *in vitro*, although to a much greater extent in the Clara cells. Also, the metabolism of several substrates, including 7-ethoxycoumarin, coumarin and benzo(a)pyrene, was compared in the isolated cell fractions and found to be much greater in the Clara cells than in the Type II cells. Immunohistochemical analysis and gel electrophoresis have also been used to demonstrate the presence of the two major rabbit pulmonary cytochrome P-450 isozymes in both the isolated Clara cells and alveolar Type II cells.

Introduction

Until the mid 1970s, very little research involving isolated pulmonary cells was reported. Separation of pulmonary cells has been a difficult problem because of the complex and heterogeneous nature of the lung. The lung contains some 40 varied cell types (1). However, with the development of advanced biochemical tools and equipment, new techniques have been adapted for the isolation of several pulmonary cell types, including the alveolar Type II cell and the nonciliated bronchiolar epithelial (Clara) cell. The purpose of this chapter is not to review all literature on pulmonary cell isolation, but to describe and comment on one isolation procedure for two pulmonary cell types, the alveolar Type II cell and the Clara cell. The applications of these techniques in toxicological and pharmacological research will also be discussed.

The alveolar Type II cell, a cell unique to lung, has been isolated to near homogeneity in several laboratories (2-5). These cells, recognized by their characteristic lamellar inclusions, have been shown to synthesize and secrete the surface active material which lines the alveolar sac (6,7). Another major function of the Type II cell is that of a progenitor for the alveolar Type I cell during normal development (8) and following Type I cell injury (9,10). The presence of cytochrome P-450 and

xenobiotic metabolism has also been demonstrated in Type II cells isolated from rabbit (2) and rat (11).

Another secretory cell of the lung which seems to possess important nonrespiratory functions is the Clara cell of the bronchiolar epithelium. The role of the Clara cell in surfactant production is unclear, although some evidence indicates this as a function (12-14). The presence of extensive endoplasmic reticulum, abundant mitochondria and numerous osmophilic granules distinguishes the Clara cell from other epithelial cells and gives ultrastructural evidence for high metabolic activity (15). Enzyme histochemical studies also show high metabolic activity in the Clara cell (12,13,16). High concentrations of cytochrome P-450 in the Clara cell have been demonstrated using autoradiographic (17,18) and immunohistochemical (19) techniques. Also, the presence of cytochrome P-450 and high foreign compound metabolizing activity with several substrates have been demonstrated in Clara cells isolated from rabbit lung (20-23).

Advantages of Isolated Cell Systems in Toxicological Research

Isolated cells provide a number of advantages not found in other models for toxicological research. Cell populations can be manipulated and monitored easily whereas many factors often complicate other model systems. Many properties of the intact tissue including permeability are retained and can be investigated

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readily. Drug uptake and metabolism without the use of extraneous cofactors can be measured.

Because of the complexity of the lung, isolated pulmonary cells are especially suited to certain types of toxicological investigations on lung. An important use of isolated pulmonary cells is for study of localization of mixed-function oxidase components and xenobiotic biotransformation in lung. Localization of pulmonary carcinogenesis and chemical toxicity in lung may be related to the presence or absence of toxication-detoxication pathways in particular cell types. Observations of other xenobiotic metabolism research models such as pulmonary microsomal preparations and the isolated perfused lung do not indicate the cellular location of these enzymes. Even histochemical studies of tissue sections do not always reveal the true localization of these enzymes (22). Cross-contamination, differential effects of fixation on cells and the heterogeneous nature of the lung make histochemical determinations of enzyme localization inaccurate in lung.

Another use of isolated pulmonary cells in toxicological studies where other models might be limited is in the investigation of certain mechanisms of action of cell specific toxicity. For example, the mechanism of cytotoxicity of the pulmonary specific toxin, 4-ipomeanol, has been shown to be associated with metabolic activation by cytochrome P-450 and covalent binding in Clara cells (17,18). However, *in vitro*, both Clara and alveolar Type II cells covalently bind ipomeanol (23). Glutathione conjugation to activated ipomeanol metabolites has been demonstrated to be important in moderation of ipomeanol toxicity (24). Examination of the presence of glutathione in the isolated cell preparations is significant to this problem and may differ from the *in vivo* situation. In the isolated cells, we can study metabolic activation by cytochrome P-450 enzymes in the endoplasmic reticulum simultaneously with glutathione conjugation in the cytoplasm as well as measuring cellular glutathione levels.

Animal Models

Because of species variation in lung size and differences in pulmonary xenobiotic metabolism, choice of animal model is of prime consideration when isolating cells for toxicological research on the lung. Since the rabbit lung is large relative to other laboratory animals and has high levels of mixed function oxidase activity, it is a good model for developing appropriate techniques for pulmonary cell isolation. However, rabbit pulmonary mixed-function oxidases are relatively unresponsive to the classic phenobarbital- or 3-methylcholanthrene-type inducers (25). To investigate inducible systems of xenobiotic metabolism in pulmonary cells, another animal model such as the rat must be used (11).

Cell Isolation Technique

One method of obtaining nearly homogeneous populations of alveolar Type II cells and Clara cells from rabbit

is outlined in Figure 1. Since detailed methods of these cell isolation procedures are described elsewhere (21,26), only important comments and considerations about the methods will be discussed here. As many red blood cells are removed by perfusion as possible, because the best digestions are consistently obtained from the most thoroughly perfused lungs. Since alveolar macrophages are similar in size and density to Clara cells, as many macrophages are removed as possible by lavage. The proteolytic enzyme is instilled into the trachea to expose the epithelial cells to the enzyme. Many digestive enzymes have been tested, but the Protease I appears to release the highest percentage of Type II and Clara cells from rabbit lung.

Several methods can be used to separate Type II and Clara cells from the cell digest. The main advantages of the elutriator centrifuge are speed and separation of large numbers of cells without overloading. The elutriator

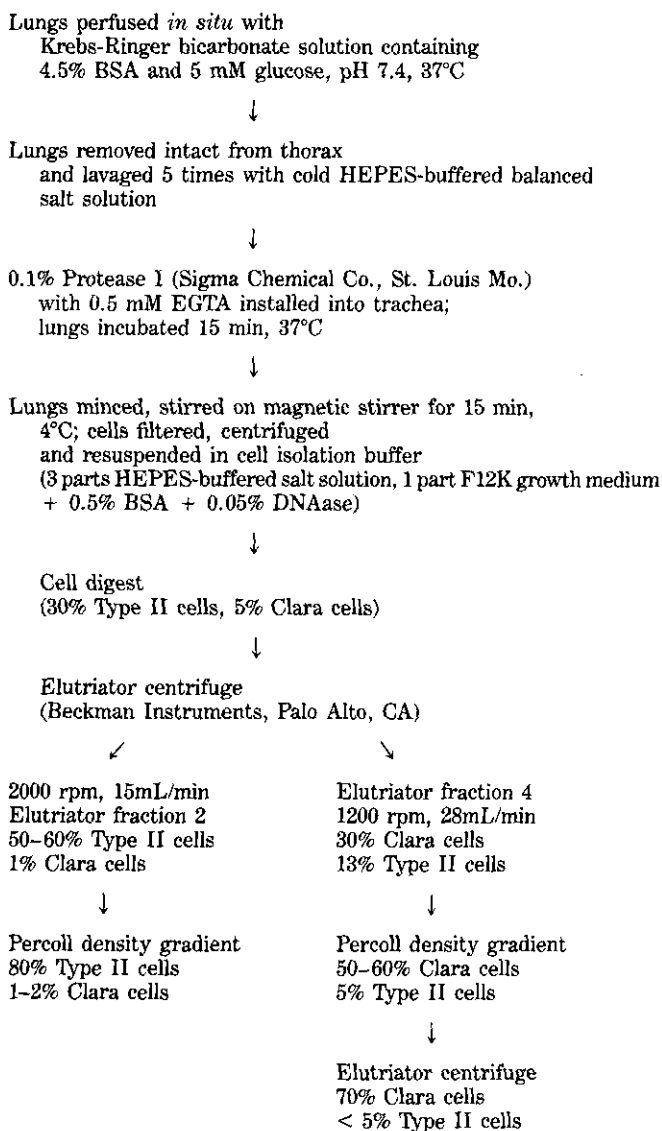


FIGURE 1. Preparation of alveolar Type II and Clara cells from rabbit lung. Detailed methods described elsewhere.

tor will separate up to 10^9 cells on the basis of size into two to ten discrete fractions in less than an hour. This method is especially applicable to Clara cell separation because of the large size of this cell type. However, certain precautions must be observed to reduce clumping; 0.5% bovine serum albumin and 0.05% deoxyribonuclease are added to the elutriator buffer to prevent clumps of smaller cells from eluting with the Clara cells. Density gradients are useful for Type II cell separation from other cells because of the uniform size and low density (due to high phospholipid content) of these cells compared to other cells. Alveolar Type II cells from rat and rabbit have been separated on albumin, Ficoll, Metrizamide and Percoll gradients in different laboratories (3-5,21,26). Differential plating/adherence rates of cells can also be used for Type II cell separation in combination with density gradients when these cells are placed in primary culture (5). Macrophages attach much more quickly (3-4 hr) than Type II cells (18-20 hr) when placed in culture.

Clara cells are more difficult to isolate because of their variable sizes and densities and small numbers per lung. Following the first elutriation, a two-polymer aqueous phase system has been used to separate Clara cells from other cells (26). Although fractions containing 70% Clara cells could be achieved, the total cell yield was quite low. The Percoll density gradient fractionation and second elutriation of Clara cells (illustrated in Fig. 1) results in two to four times the total cell yield obtained with the phase separation technique.

The freshly isolated cells maintained at 4°C will retain about 90% of their mixed-function oxidase activity for at least 18 hr. However, since most cultured cells quickly lose their *in vivo* levels of cytochrome P-450 and monooxygenase activities (27), studies of xenobiotic metabolism in lung at present are limited to the freshly isolated cells. The spontaneous loss of cytochrome P-450 in cultured hepatocytes can be prevented in part by the presence of nicotinamide (28), metyrapone (29) or other substances in the culture medium. These factors are now being investigated in pulmonary cell culture.

Cell Identification

Alveolar Type II cells isolated from rabbit lung appear to retain their characteristic ultrastructure (Fig. 2). The lamellar bodies, composed principally of phospholipid, are easily identified with the electron microscope (Fig. 2b) as well as with the light microscope using a modified Papanicolaou stain (Fig. 2a) (4). Phosphine 3R fluorescent dye also stains phospholipid and is used for type II cell identification (5).

The ultrastructural features of the isolated Clara cell appear similar to those observed *in situ* (Fig. 3) (15). However, in many isolated cells a rearrangement of the cellular ultrastructure occurs so that the orientation of most organelles is lost (26). Under the light microscope, rabbit Clara cells are identified by staining with nitroblue tetrazolium after a short treatment with 10% formalin (26). Figure 4 illustrates nitroblue tetrazolium

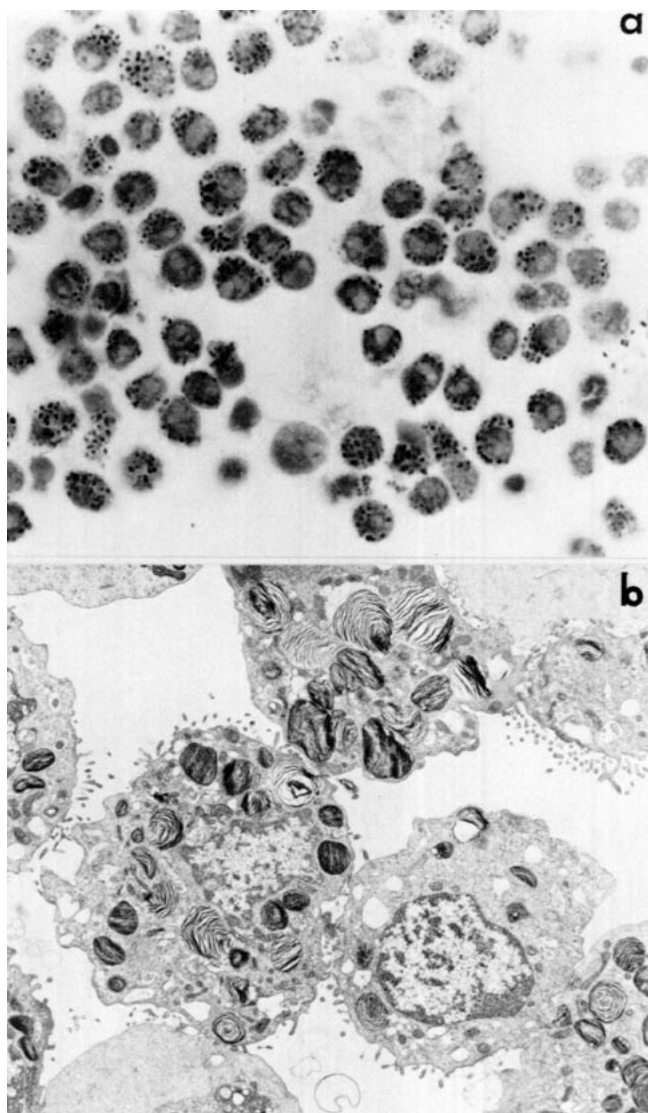


FIGURE 2. Alveolar Type II cells isolated from rabbit lung: (a) modified Papanicolaou stain, magnification 650 \times ; (b) electron micrograph, magnification 4500 \times .

staining of various fractions during the cell isolation procedure. Only 5% of the cell digest is composed of Clara cells (Fig. 4a). Elutriator fraction 4 contains about 30% Clara cells (Fig. 4e). Figure 4f shows the final fraction of the second elutriation which consists of 70% Clara cells.

Xenobiotic Metabolism in Isolated Pulmonary Cells

Alveolar macrophages, isolated from lung lavages, exhibit little, if any, foreign compound metabolism (30-32). However, considering the cellular heterogeneity of lung and the relatively high activity of some mixed-function oxidase enzymes in rabbit pulmonary microsomes (33), it seemed likely that certain cells

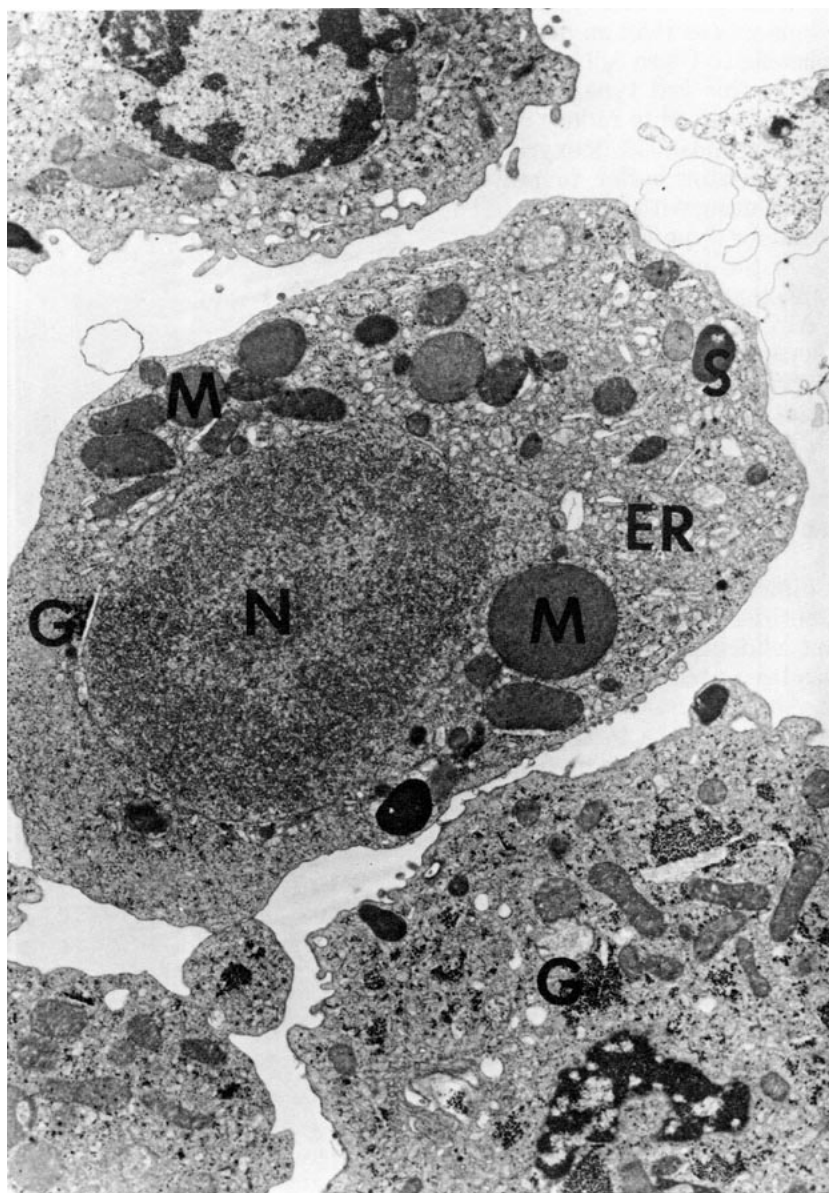


FIGURE 3. Electron micrograph of Clara cell isolated from rabbit lung: (N) nucleus; (M) mitochondrion; (G) glycogen granules; (S) secretory granule; (ER) endoplasmic reticulum. Magnification 7500 \times .

might be unusually active. In fact, the Clara cell of the bronchiolar epithelium was the first pulmonary cell shown to contain high concentrations of cytochrome P-450 monooxygenase activity (17). Table 1 illustrates some of the foreign compound metabolism activities which have been measured in Clara cells isolated from rabbits. The Clara cells have high metabolic activities for all these substrates. Alveolar Type II cells isolated from rabbit also metabolize most of these substrates (Table 1) (2). Freshly isolated Type II cells metabolize 7-ethoxycoumarin to umbelliferone at a rate of about 41 pmole/mg protein/min. This 7-ethoxycoumarin deethylase activity cannot be accounted for by the 1–2% contamination of these cells by Clara cells. However,

coumarin hydroxylase activity seems to be specifically enriched into the Clara cells. The trace amounts of this activity observed in the Type II cell fraction are probably due to Clara cell contamination. Although the Type II cells appear to have lower mixed-function oxidase specific activities than either the Clara cells or the cell digest, they could still account for a significant portion of the total xenobiotic biotransformation which occurs in lung.

The Type II and Clara cells isolated from rabbit have also been used to identify the presence of the rabbit pulmonary cytochrome P-450 isoymes, P-450_I and P-450_{II}, in these two cell types (22). Whereas immuno-histochemical analysis of tissue sections has localized

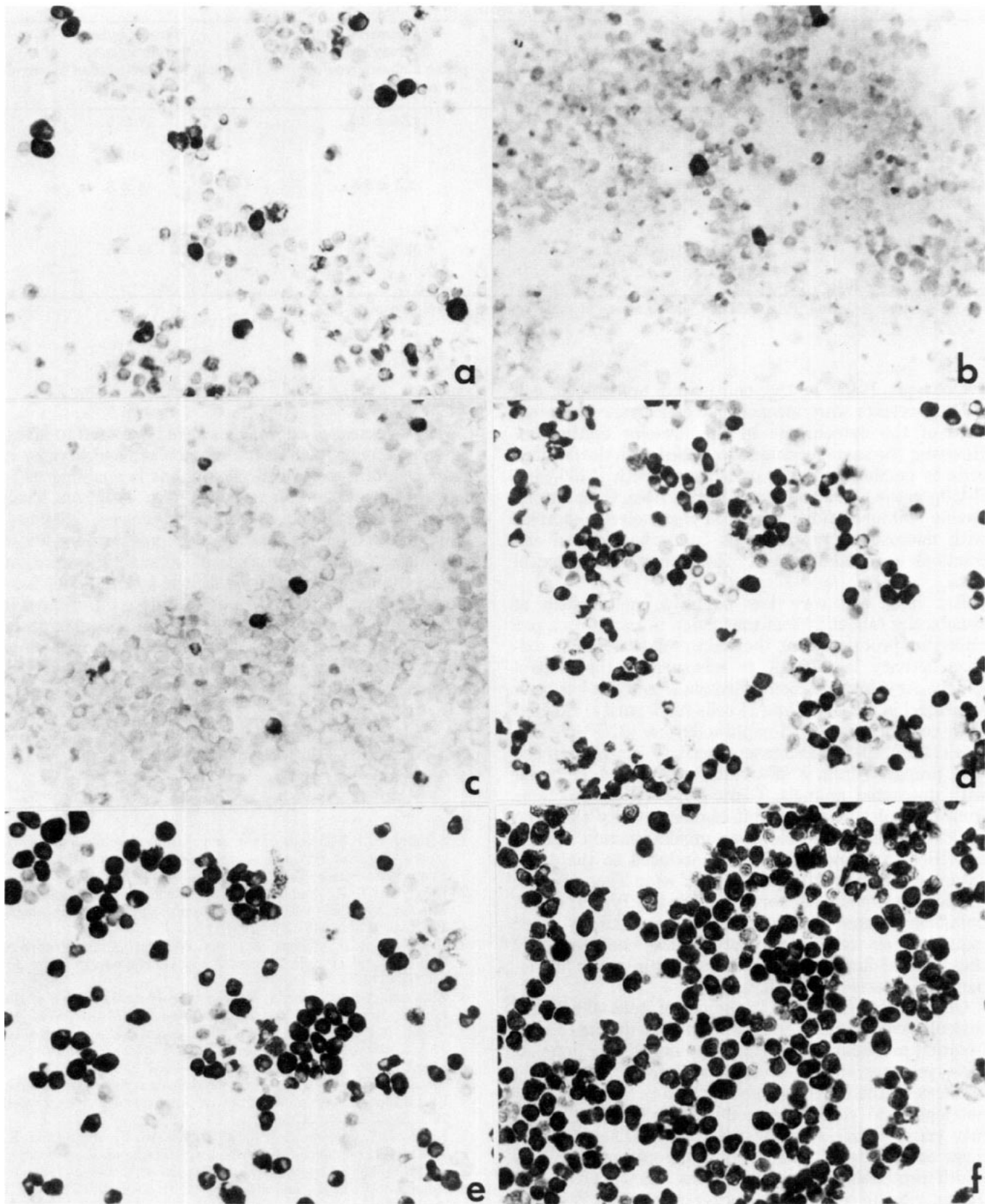


FIGURE 4. Nitroblue tetrazolium staining of cell digest, elutriator fractions and final Clara cell enriched fraction (70% purity): (a) cell digest; (b) elutriator fraction 1; (c) elutriator fraction 2; (d) elutriator fraction 3; (e) elutriator fraction 4; (f) final Clara cell fraction after second elutriation. Dark stained cells are Clara cells. Magnification 250 \times .

Table 1. Enzyme activities in cell fractions.

	7-Ethoxycoumarin deethylase, pmole umbelliferone/mg protein/min ^a	Coumarin hydroxylase, pmole umbelliferone/mg protein/min ^a	Benzo(a)pyrene hydroxylase, pmole benzo(a)pyrene-OH/mg protein/min ^a
Cell digest (30% Type II, 5% Clara cells)	109 ± 13	12.0 ± 2.5	17 ± 5
Alveolar Type II (80% Type II, 1-2% Clara cells)	41 ± 9	1.0 ± 0.5	18 ± 3
Clara cells (50-70% Clara, < 5% Type II cells)	141 ± 49	32.0 ± 5.8	43 ± 6

^a All samples sonicated; 1mM NADPH added; means of three or more determinations ± SE.

cytochrome P-450 in the nonciliated bronchiolar cell (19,22), results were inconclusive concerning the presence of the cytochrome in the alveolar epithelium. However, the same technique was used with the isolated cells to demonstrate the presence of both P-450_I and P-450_{II} in the Clara and Type II cells. Also, Ouchterlony double immunodiffusion and SDS-gel electrophoresis with microsomal preparations from the isolated cell fractions were used to verify the immunohistochemical data.

Rat lung has very low oxidative metabolism of xenobiotics (about 20% of that found in rabbit on a per milligram protein basis); therefore, mixed-function oxidase activity is difficult to measure in the control animal. Aryl hydrocarbon hydroxylase activity has been measured in alveolar Type II cells (90% purity) isolated from control rats and β -naphthoflavone (β NF)-treated rats (11). A 90-fold increase in activity is seen in these cells prepared from β NF-treated animals as compared with untreated animals. Clara cell-enriched fractions from control animals exhibit higher activity than the purified Type II cells. However, mixed-function oxidase activities in Clara cells are not induced to the same extent (only about 20-fold) by β NF as in Type II cells. These experiments support a role for the Type II cell in pulmonary xenobiotic metabolism, particularly after induction, as well as emphasizing the need to study these mixed-function oxidase systems in other pulmonary cells besides the Clara cell.

One possible limitation to the use of isolated cells for toxicological studies is enzyme alteration during the cell isolation procedures. Although the mixed-function oxidase activities of intact cells are unaltered by the presence of the Protease I, other factors such as loss of nutrients and cell-cell communication and regulation may cause changes in these activities. These factors must be considered when comparing enzyme activities in different isolated cell populations and when comparing activities in cells to those observed in other research models.

Comments

Various research approaches have been used to investigate pulmonary xenobiotic metabolism and toxicity in lung. Use of isolated cell populations is enabling us to identify those cells in lung which have significant levels of cytochrome P-450 and monooxygenase enzymes. Individual cell populations are also important models in the study of cellular coupling of xenobiotic activation/deactivation systems. Data with the isolated cells have shown qualitative as well as quantitative differences in the ways different pulmonary cells metabolize chemicals. Studies of these differences may help us discover which cells might be susceptible to the toxic effects of certain chemicals and understand why these toxicities occur.

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