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CULTURE OF RABBIT PULMONARY CLARA CELLS

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Abstract: Rabbit pulmonary Clara cells isolated by centrifugal elutriation have been cultured for several weeks. Clara cells generally adhered poorly to plastic but the cells did attach to coated substrates. A selected medium supported serial subculture of Clara cells for 4-5 passages (1:2 split). The medium consisted of a basal nutrient medium, alpha MEM, supplemented with insulin, transferrin, epidermal growth factor, D-glucose, biotin, α-tocopherol, pituitary extract, trace elements and 2% Sephadex G-10-filtered FBS. Freshly prepared Clara cells showed high capacity to activate 2-aminofluorene (AF) to mutagenic products. However, after 6 weeks of culture the mutagenic activation of AF was reduced by 92,5% indicating loss of cytochrome P-450. © 1986 Society for Experimental Biology and Medicine

Introduction: The lung contains widely divergent cell types. In the epithelial lining of pulmonary airways a variety of cell types have been identified (1). Among them are the mucous-secreting cells, ciliated cells, Clara cells, basal cells, brush cells and neuroendocrine cells. The nonciliated epithelial (Clara) cells are thought to contribute to the secretory lining material of the pulmonary airways and they appear to be a major site of xenobiotic-metabolizing activity in the lung (2,3). Furthermore, Clara cells have a low capacity for DNA repair (4). The extreme cellular heterogeneity of the lung makes assignment of specific functions to individual cell types very difficult. Recently, techniques have been described for isolating Clara cells (3,5). However, methods for investigation of Clara cells *in vitro* are needed in order to define the nature of the Clara cells. We have developed a growth medium which supports the long-term serial cultivation of these cells. Herein we describe a growth medium which is effective in maintaining growth of Clara cells in culture.

Freshly isolated cells usually catalyze the various phase I and phase II reactions involved in drug biotransformation at rates closely resembling those observed *in vivo* (6). The major group of enzymes involved in phase I

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reactions (metabolic transformations which might lead to either activation to reactive toxic intermediates or to detoxification via phase II conjugation reactions) is the cytochrome P-450. The concentration of cytochrome P-450 usually declines upon cultivation of cells in vitro (7). Studies with hepatocytes have shown that there is a marked heterogenicity in the rates at which individual cytochrome P-450 forms are lost in culture, and that the cell culture conditions exert dramatic effects on this enzyme complex (8).

In the rabbit pulmonary Clara cells, two major forms of cytochrome P-450 are found, form 2 and form 5, in about equal amounts (9, 10). Only form 5 is active in transformation of the carcinogenic arylamine 2-aminofluorene (AF) to a mutagen (11). Consequently, AF is an excellent substrate for testing the maintenance of this major cytochrome P-450 during cultivation of Clara cells.

Materials and Methods: Clara cells (85% purity, 90% viability) were isolated from lungs of male New Zealand white rabbits with protease and centrifugal elutriation as described previously (3). Eluted cell fractions were concentrated by centrifugation at 4^{0}C and resuspended in HEPES buffered saline (HBS) before culture. Clara cells were identified by nitroblue tetrazolium staining (5).

Various basal growth media and supplements were tried. The following media were used: Medium 199 with Hank's salts (SIFF Media Unit, Oslo, Norway), Dulbecco's modified Eagle's medium (SIFF), RPMI 1640 (SIFF), F-12 (SIFF), MEM alpha medium (GIBCO Grand Island, N.Y.) and LHC-medium (12). Furthermore, various supplements were tested. Culture dish surfaces were coated with collagen, fibronectin or a mixture of fibronectin, collagen and crystallized bovine serum albumin (FVB) (12). Primary cultures were dissociated into single cells using a trypsin solution (1% polyvinylpyrrolidine (PVP), 0.02% ethylene glycol bis (β-aminoethyl ether) N,N,N^{1},N^{1}, tetraacetic acid (EGTA) and 0.02% trypsin prepared in HBS) (12). Bovine pituitary extract was prepared by homogenizing freshly collected bovine pituitaries as previously described (13).

Maintenance of the important enzyme complex cytochrome P-450 was tested by studying changes in the Clara cells ability to activate AF to mutagens.

Freshly isolated Clara cells or Clara cells cultured for six weeks were incubated in a special chamber, separated from the tester strain Salmonella typhimurium TA 98 by a Nucleopore membrane (pore size 0,20 µm) as previously described (3). Briefly, the chambers containing the Clara cells were placed in sterile 20 ml vials where HEPES buffer (pH 7.4) with 1% bovine albumin, bacteria and AF were added. After incubation for 2 hrs the bacteria were washed in sterile buffer and plated on Vogel-Bonner minimal plates for scoring of induced revertants.

Results:
Growth of Clara cells in medium 199 was poor, as it was in most of the other media tested. Attachment of cells in medium 199 was 1 % or less. However, we found that a modified alpha-MEM medium supported growth of Clara cells and enhanced the attachment efficiency by nearly 20%. The alpha-MEM medium was supplemented with $NaHCO_3$ (0.5 g/liter), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

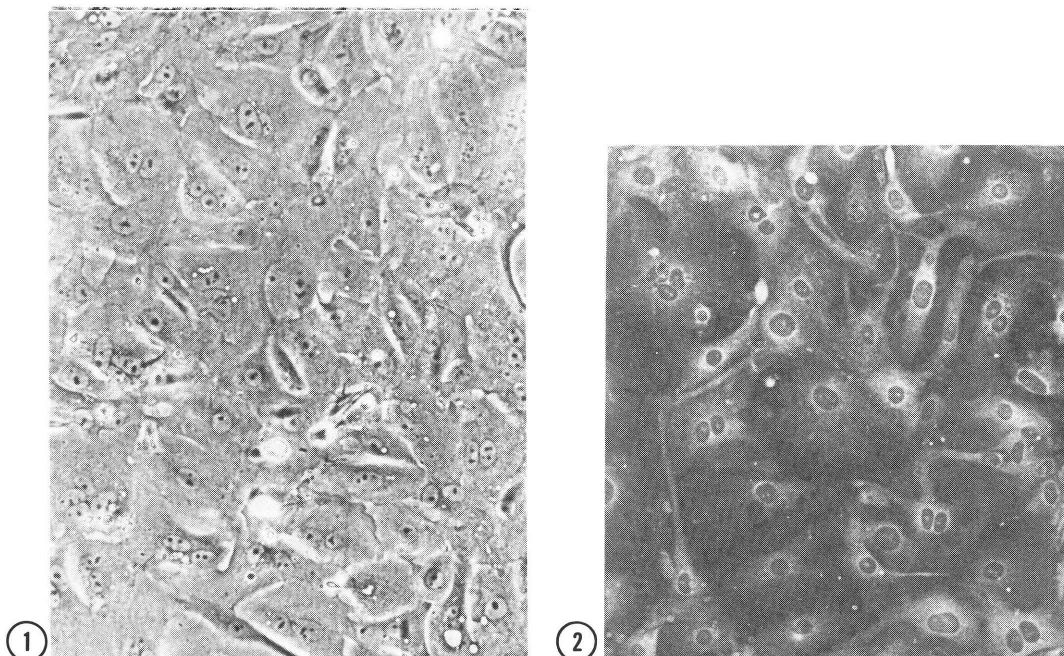

Figure 1. Phase contrast micrograph of confluent culture of Clara cells, x 200.

Figure 2. Clara cells stained by the nitroblue tetrazolium technique, x 200.

(3.6g/liter), insulin (5 mg/liter), transferrin (5 mg/liter), epidermal growth factor (EGF) (10 µg/liter), D-glucose (2 g/liter, biotin (0.4 mg/liter), α-tocopherol (0.01 mg/liter), pituitary extract (35 µg protein/ml), trace elements (12) and 2% Sephadex G-10-filtered FBS. Furthermore, the presence of antioxidants such as ascorbic acid (5 µg/ml) and glutathione (5 µg/ml) during the elutriation procedure, enhanced attachment efficiency of Clara cells 2 fold.

Active growth of Clara cells with a typical epithelial appearance began about 2-3 days after seeding (Fig. 1). Continuous proliferation was maintained for 4-5 passages with subculture every 7-14 days. FVB provided a good substrate for attachment. Split ratios greater than 1:3 were not successful. Population doubling times ranged from 72 to 96 hrs or longer. The Clara cell specific nitroblue tetrazolium assay was positive (Fig. 2) after six weeks of culture. In the chamber mutagenicity assay Clara cells showed a marked ability to activate AF to mutagenic products (Table I), even though they had lost most of their activation capacity.

Discussion: **Mammalian alveolar type II lung cells have been grown successfully *in vitro* (14). This study shows that Clara cells isolated from rabbit lung also can be maintained in pure culture.**

However, cultivation of Clara cells required the use of various hormones and growth factors. The cells formed an epithelial monolayer after 7-14 days in culture and we were able to subculture and maintain these

Table 1

Mutagenic Activation of AF by Freshly Isolated and Cultured Clara Cells*

Cells	Culture time	Substrate	Revertants/plate	Per cent 0
Clara	6 weeks	AF (20 μg/ml)	233 ± 72	8.5
Clara	0 "	AF (20 μg/ml)	2,754 ± 48	100

*Incubations in triplicate

cells through 4-5 passages. The isolation and culture of a pure population of Clara cells permits the in vitro assessment of factors which might modulate Clara cell function and will be useful for studies of Clara cell physiology, growth, differentiation and pathobiology. However, the data suggest a significant loss of cytochrome P-450 content during cultivation since after 6 weeks of culture there was a reduced ability to activate AF to mutagenic products. Consequently, an important task will be further modification of the culture medium in order to develop a medium that will better maintain the cells' metabolic functions.

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