Environmental Health Perspectives
Vol. 16, pp. 83-88, 1976

Isolation of Cells That Retain Differentiated Functions *in Vitro*: Properties of Clonally Isolated Type II Alveolar Pneumonocytes

by William H. J. Douglas* and Philip M. Farrell†

We have isolated by clonal culture techniques a diploid cell strain (L-2) from adult rat lung. These cells appear to retain differentiated functions that are present in type II alveolar epithelial cells of intact lung. The L-2 cells are diploid, epithelial cells, they contain osmiophilic lamellar bodies in their cytoplasm and they synthesize lecithin by the same *de novo* pathway as whole lung.

Introduction

The terminal respiratory passages of mammalian lung consists of numerous cell types including: ciliated epithelial, goblet, Clara, argentaffin, mucous, endothelial, fibroblast, smooth muscle, macrophage, mast, type I and type II alveolar cells. Because of this cellular heterogeneity, it is impossible to study the response of individual cell types in the lung to toxicological insult. This cellular heterogeneity present in whole lung persists when pulmonary tissue is enzymatically dissociated into single cells (*1*). On the other hand, if suspensions of single, viable cells are cloned, each cell has the potential of generating a clonal cell population, thus assuring purity of cell type. This approach permits isolation of cells which retain differentiated functions, and insures that the cells will not be overgrown by fibroblasts. Culture conditions may then be developed which permit replication of these cells without loss of differentiated function and diploid karyotype.

* W. Alton Jones Cell Science Center, Lake Placid, New York 12946.

† Developmental Biology and Clinical Nutrition Section, Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development (NICHHD), Bethesda, Maryland 20014.

Cloning directly from primary cell suspensions has several unique advantages. Since colonies are derived from single cells, overgrowth of slow growing cell types by more rapidly proliferating ones is avoided. Various lines can be isolated from the same specimen, each with maximal epigenetic purity. Clonal growth of cells at low population densities avoids rapid depletion of essential components from the medium and thus allows closer control of cell nutrition (*2*).

Within the past decade, cell culture techniques have been developed that permit differentiated cells derived from normal tissues to retain specialized characteristics while undergoing a useful number of replicative cycles *in vitro*. This was first accomplished by the application of clonal culture techniques to enzymatically dispersed suspensions of cells derived from differentiated skeletal muscle of chick embryo (*3*) and subsequently extended to cartilage (*4*), pigmented retina (*5*), thyroid (*2*), liver (*6,7*), and pituitary (*8,9*). In all of these cases, cells were isolated that retained organ-specific functions *in vitro*. Cell strains like these are potentially extremely valuable for *in vitro* toxicity testing.

These successes prompted efforts in our laboratory to isolate a homogeneous population of type type II alveolar epithelial cells from the terminal

alveoli of rat lungs. Type II cells synthesize, store, and secrete a surface-active phospholipid (pulmonary surfactant) which coats the terminal respiratory passage ways and lowers surface tension at the air-alveolar interface and thus aids in stabilizing the air sacs (*10*).

We applied clonal culture techniques to primary cell suspensions of normal adult rat lung and isolated a cell strain (L-2) that synthesizes a highly saturated lecithin which is the major component of pulmonary surfactant. The pathway of lecithin synthesis utilized by L-2 cells *in vitro* (*11,12*) is the same pathway used by type II cells in whole lung (*13*), and this function (monitored by conversion of labeled precursors into lecithin) is easily quantitated in the cultured cells.

Thus the L-2 cell system provides a unique opportunity to investigate the effects of environmental pollutants on a vital lung function, i.e., production of pulmonary surfactant. Future consideration should be given to development of an *in vitro* toxicity testing system utilizing the L-2 cell to examine the influence of a variety of environmental pollutants on the production of pulmonary surfactant by the L-2 cell.

Materials and Methods

Dissociation Procedures

Pulmonary tissue, obtained from respiratory disease-free, adult, female, Lewis strain rats, was cut into 1-cm^3 pieces and washed in several changes of 1% chicken serum in Moscona's saline without calcium and magnesium (CSS). The lung was then minced into 1-mm^3 fragments and 2g of mince transferred to a 50-ml Erlenmeyer flask and washed several times in CSS. With each wash, the fragments of tissue were allowed to settle and the solution removed with a 14-gauge cannula affixed to a syringe.

The lung was then dissociated into single cells by treatment with a trypsin:collagenase mixture (CTC). This solution consisted of 0.1% trypsin (1:300, Nutritional Biochemicals, Inc.) and 0.1% collagenase (Worthington, CLS) dissolved in CSS.

Ten volumes of CTC were added, and the flask agitated gently in a reciprocal shaker bath for 20 min at 36.5°C. Agitation was at a rate sufficient to keep the fragments in gentle motion. After the fragments had settled briefly, the supernatant solution containing the suspended cells was removed and transferred to a chilled centrifuge tube. An equal volume of chilled nutrient medium was added and the tube maintained in an ice bath. This extraction was carried out three times. The cells were pooled, collected by centrifugation (400*g* for 6 min), and resuspended in fresh chilled medium.

Sometimes the viscosity of the solution impeded rapid settling of unsuspended tissue fragments and inhibited removal of the dissociated cell suspensions. This effect is due to release and uncoiling of deoxyribonucleic acid from ruptured cells and is readily mitigated by crystalline deoxyribonuclease (DNase). Addition of DNase (0.4 mg/ml) to the CTC and the brief incubation (1-2 min) at 36.5°C usually reduced the viscosity and permitted rapid settling of undigested tissue (*14*).

The nutrient medium was a modified Ham's F12, F12K (*6*), with optional antibiotics (penicillin G potassium, 100 units/ml; kanamycin, 100 μg/ml; amphotericin B, 6.25 μg/ml), supplemented with 10% fetal calf serum. The fetal calf serum was selected by including it in media for cloning a frozen stock of liver cells, at 5, 10, and 15% serum concentrations. The serum lot giving the highest plating efficiency and the healthiest appearing clones, was chosen and stored frozen at −70°C until used. With most lots, 10% fetal calf serum gave satisfactory results with adult rat lung cells, both in primary culture and subsequent passage. All lots of serum were filtered (0.22 μm, Millipore) and stored at −70°C. Grand Island Biological Company fetal calf serum, lot A0204H and A344420, were used for the isolation and for the experiments reported here.

Isolation and Passage of Clones

Petri dishes (100 mm, Falcon Plastics) containing 6 ml of nutrient medium were allowed to reach equilibrium in a humidified incubator at 36.5°C continually flushed with sufficient CO_2 to maintain the medium at pH 7.4. Viable cells, i.e., those excluding trypan blue, were counted in a hemocytometer, and each dish received 10^4 viable cells. After inoculation, the dishes were incubated undisturbed for 1 week. The medium was then replaced three times a week.

When the clones were between 1 and 2 mm in diameter, single clones were encircled with porcelain cylinders (Fisher Scientific) coated with silicone grease (Dow-Corning). The selected colony was washed with CSS and 0.1 ml of CTC was added. For this purpose, it was convenient to use a 1 ml plastic syringe with a 20-gauge needle. The plate was returned to the incubator. At intervals, the cells were examined at low power. When most of the cells were rounded up, usually within 15-30 min, the excess CTC was removed with the

syringe, taking care not to dislodge the loosely attached cells. A drop of nutrient medium (0.1 ml) was then added. The cells were gently drawn into the syringe and transferred to a 35-mm Petri dish containing 2 ml of nutrient medium. These dishes were incubated until they were nearly confluent. At this time the cells were subcultured to 60 mm dishes.

Freezing of Cell Lines

Healthy, semiconfluent cultures were selected for storage in liquid nitrogen. The cells were removed from the plates with CTC and suspended in medium at 5×10^6-1×10^7/ml. An equal volume of nutrient medium containing 20% dimethyl sulfoxide was added dropwise. Ampoules containing 1 ml of this suspension were frozen using the Linde controlled rate freezer. Initially the temperature was reduced 1°C/min down to −30°C. Then the cooling rate was increased to 5°C/min until a temperature of −100°C was reached. At this point, the ampoules were stored in liquid nitrogen.

Electron Microscopy

Lung cells growing in plastic culture vessels were fixed for 1 hr in 2.5% glutaraldehyde in 0.1*M* cacodylate buffer, pH 7.4. After a brief rinse in 0.2*M* buffer, the cells were postfixed for 30 min in 1% osmium tetroxide in 0.1*M* cacodylate buffer, pH 7.4. The cells were dehydrated in 90, 95, and 100% hydroxypropyl methacrylate and embedded in Epon-Araldite *(15)*. After an initial polymerization of the embedding resin at 60°C for 24 hr, the plastic Petri dish was separated from the embedded monolayer of cells *(15)*, and the clones were flat-embedded in fresh Epon-Araldite. This embedding technique permitted vertical sectioning through the tissue. After staining with uranyl and lead ions, sections were examined in a Phillips EM-300 electron microscope for the presence of osmiophilic lamellar bodies in the cytoplasm of the cultured cells. An alternative method of specimen preparation was to dissociate monolayers enzymatically into single cell suspensions and then prepare a cell pellet for electron microscope observation.

Isolation of Cell Fractions

Lamellar body fractions were prepared by differential centrifugation of clone L-2 cells utilizing the Page-Roberts technique *(16)*.

^{14}C-Choline Incorporation Studies

Cells were grown in 75-cm^{2}plastic tissue culture flasks (Corning) until they reached a subconfluent stage. On the day of the assay, after removal of the medium, 20 ml of fresh media was added to each flask. ^{14}C-Choline [1,2-^{14}C] at 100 μCi/ml was diluted 1:10, and 0.1 ml of this solution (1 μCi) added to each flask. Cells were incubated for 3 hr at 37°C, the medium removed, and cells rinsed with 10 ml of CSS. The cells were then harvested with CTC, washed in CSS and counted.

The lipid fraction of the cells was extracted for 20 min with approximately 20 volumes of a 2:1 chloroform-methanol solution (1.5 ml for 4-7 $\times 10^6$ cells). A 1-ml aliquot of the mixture was removed from each sample and exactly 0.2 volumes of water added. After vigorous shaking, separation of the two fractions was accomplished by spinning the mixture at 400*g* for 10 min. Then 0.1 ml of the upper aqueous phase was removed and placed in a scintillation counting vial. Duplicate samples were taken. The remainder of the aqueous phase was discarded. Similar samples were obtained from the lower chloroform: methanol phase. The samples were dried and 10 ml of Aquasol (New England Nuclear) added to each vial and they were counted in a liquid scintillation counter (Beckman LS 100).

A similar protocol for ^{14}C-methionine incorporation was used in studies designed to evaluate the different pathways of lecithin synthesis in L-2 cells.

Results

Application of clonal culture techniques to primary cell suspensions derived from normal, adult rat lungs resulted in the isolation of four rat lung cell strains (L-1, L-2, L-3, L-4), that appear to have originated from type II alveolar pneumonocytes. These four strains possess two ultrastructural markers that are present in type II cells of whole lung: osmiophilic lamellar bodies and peroxisomes. Osmiophilic lamellar bodies are the cellular organelles in which the pulmonary surfactant is stored prior to secretion onto the alveolar surface *(17-20)*. Peroxisomes are organelles that possess catalase activity and are also present in type II cells in whole lung *(21)*.

One of these clones, L-2, has been more thoroughly characterized than the other three *(11,12,22)*. L-2 cells grown in monolayer culture retain the normal, female, rat, diploid karyotype after 50 population doublings, and they continue

to exhibit an epithelial morphology *in vitro*. These cells also fail to form colonies in soft agar or agarose (*23,24*), suggesting that they are nontransformed or normal cells. Routine sterility tests on the L-2 cells were uniformly negative, and there was no evidence of mycoplasma contamination (*25,26*).

Osmiophilic lamellar bodies are present in the cytoplasm of the L-2 cells throughout 50 population doublings *in vitro* (Fig. 1). Ultrastructurally, these organelles resemble similar structures present in the type II cells of intact rat lung (*27*). Occasionally lamellar bodies are observed being secreted from the L-2 cells into the surrounding culture medium. This process resembles the secretion of lamellar bodies into the alveolar space by type II cells in whole lung.

To substantiate that the osmiophilic lamellar bodies in L-2 cells were indeed the surfactant containing lamellar bodies present in whole lung, subcellular fractions were prepared from L-2 cells by differential centrifugation utilizing Page-Roberts technique for whole lung (*16*). In a similar manner, subcellular fractions were prepared from intact rat lung and from cloned rat liver cells. Lamellar body fractions isolated from the L-2 cells and intact lung were virtually identical. No lamellar body fractions could be isolated from an equivalent number of rat liver cells grown under identical culture conditions.

O'Hare and co-workers (manuscript in preparation) have demonstrated a correlation between the presence of pulmonary surfactant and certain nonspecific esterases that are identifiable bio-

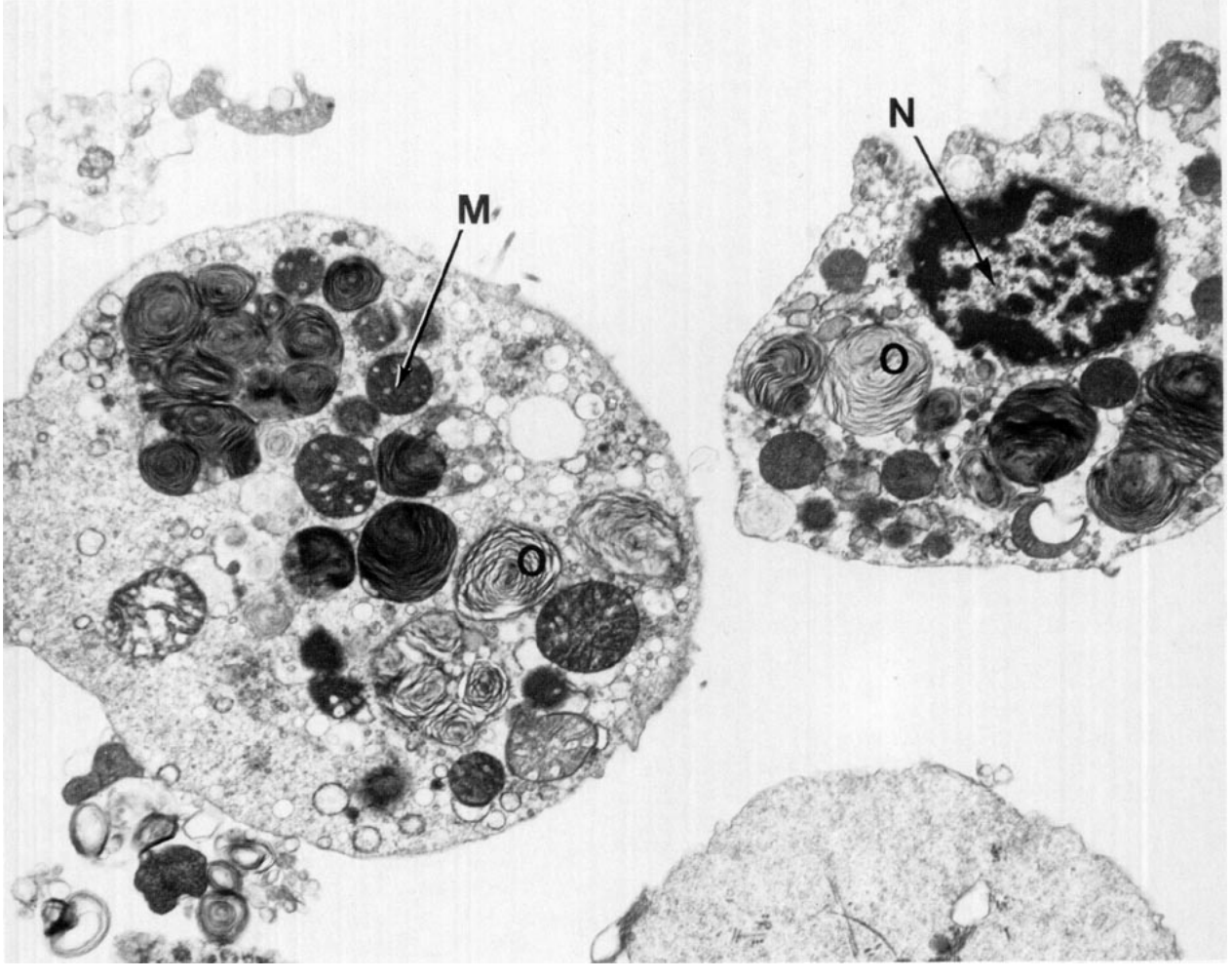

FIGURE 1. Electron micrograph of clone L-2 cells fixed after enzymatic dissociation of the monolayer into single cells and fixation in a pellet. Osmiophilic lamellar bodies (O) are present in cell cytoplasm. These organelles resemble lamellar bodies present in type II alveolar epithelial cells of intact lung. A nucleus (N) and mitochondria (M) are also present in the cytoplasm of the cultured cells. 20,860×.

chemically, electrophoretically, and cytochemically. These surfactant-associated esterases which are readily distinguishable from other pulmonary esterases are present in surface active preparations obtained from pulmonary lavage fluid. These surfactant-associated esterases are also present in L-2 cells (manuscript in preparation).

Monolayer cultures of L-2 cells produce lecithin *(11, 12)* via the same biosynthetic pathways utilized by type II cells in intact lung *(13)*. There are two *de novo* pathways for lecithin synthesis: pathway I, the choline incorporation route and pathway II, the phosphatidylethanolamine methylation route. Isotopic measurement of these two pathways in L-2 cells reveals predominance of pathway I, the choline route (Fig. 2). The conversion of ^{14}C-choline and ^{14}C-methionine to lecithin as a function of incubation time is shown in Figure 2. The linear generation of radioactive lecithin product can be maintained for at least 5 hr and the choline incorporation route was 200-400 times more active than the methionine incorporation pathway. Lecithin synthesis in whole lung also occurs via the choline incorporation route *(13)*.

In pathway I, the choline incorporation route, there are three enzymes that can be measured with radiosubstrates. Measurement of specific enzyme activity of choline kinase, cytidyl transferase and choline phosphotransferase (Fig. 3) clearly indicates that the L-2 cells are enriched in

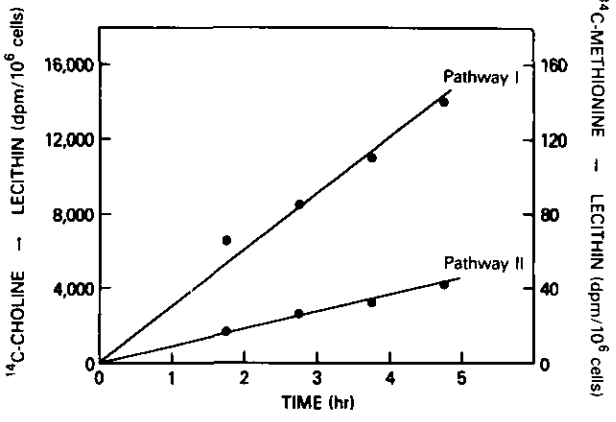

FIGURE 2. Graph comparing the rate of lecithin synthesis in L-2 cells by pathways I and II. The rate of pathway I is measured by the uptake of ^{14}C-choline, while the rate of pathway II is measured by the rate of ^{14}C-methionine uptake. Results indicate a 400-fold increase in the rate of production of lecithin by pathway I when compared to pathway II. This confirms that the L-2 cells in culture synthesize lecithin via the same biosynthetic pathway as type II cells in whole lung.

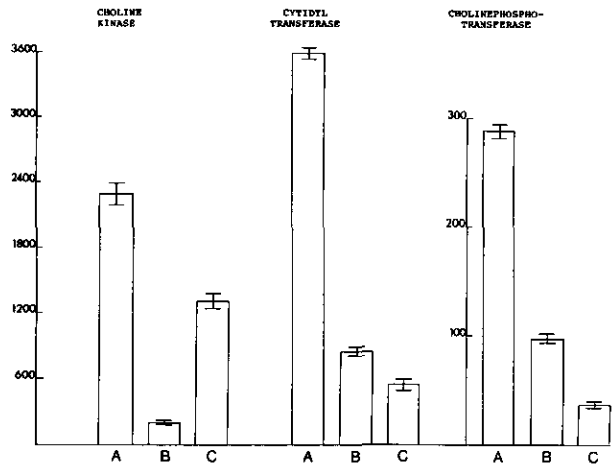

FIGURE 3. Enrichment of L-2 cells in choline pathway enzymes: (*A*) L-2 cells in culture; (*B*) whole lung; (*C*) WI-38 cells in culture. The specific enzymatic activities of choline kinase, cytidyl transferase, and cholinephosphotransferase were determined on homogenates of adult rat lung and cultured L-2 cells isolated from rat lung using radio-assay procedures described elsewhere *(13,28)*. Saturating concentrations of each substrate were utilized. Homogenates were prepared by grinding tissue or cells in a Potter-Elvehejm apparatus using 4 volumes of 50*mM* Tris-HCl, pH 8.0, containing 5*mM* EDTA and 10*mM* dithiothreitol. Activities are expressed as picomoles product produced per min per mg protein. Mean ± S.E. are shown. ($p<0.001$ as compared to either whole lung homogenates or WI-38 cells). WI-38 cells are human embryonic lung fibroblasts that do not retain lung-specific functions *in vitro*.

choline kinase and cytidyl transferase (10-fold) and choline phosphotransferase (5-fold). This magnitude of enrichment is interesting since it correlates with the observation that type II pneumonocytes comprise 10% of the cellular population in whole lung.

Preliminary studies suggest that L-2 cell extracts demonstrate the presence of surface-tension lowering material, i.e., surfactant, which is similar to that found in lavage fluids from whole lung. Extracts of WI-38 cells (human diploid lung fibroblasts that do not demonstrate any lung-specific functions *in vitro*) contained no detectable surface-active material.

Discussion

Relevant, rapid screening systems for *in vitro* toxicity testing are clearly needed. Mammalian cell cultures provide a convenient and relatively inexpensive method for predicting certain toxic parameters, but the specific cell cultures utilized for toxicity evaluation must be carefully selected. Cells that retain specialized, differentiated characteristics *in vitro* are logical candidates for use

in toxicity testing; they possess the advantage inherent in tissue culture systems, and also retain functions possessed by differentiated cells *in vivo*.

The biochemical, enzymatic, morphological, and cytochemical evidence accumulated to date on the L-2 cell (*11,12,22*) clearly supports the hypothesis that one can clone directly from adult rat lung. It has also been shown that some of the isolated clones retain differentiated properties of type II alveolar pneumonocytes *in vivo* (*22*). Since the L-2 cell strain retains many type II cell-specific functions it provides a unique opportunity to evaluate the influence of toxic compounds on a vital lung function, i.e. the production of pulmonary surfactant.

Future consideration should be given to development of an *in vitro* toxicity testing system utilizing the L-2 cell to examine the influence of a variety of toxic agents on the production of pulmonary surfactant by these cells.

This research was supported by funds from the American Lung Association, The National Heart and Lung Institute (Grant HL-17110-01) and The W. Alton Jones Cell Science Center.

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