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Isolation of type II epithelial cells from rabbit fetal lungs by adherence on an IgG-coated surface

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Abstract. The lung is comprised of about 40 different cell types, of which only 15% are type II cells. These are the major, if not the sole, source of synthesis and secretion of lung surfactant. To date a large number of methods have been described for the isolation of pure populations of type II cells using a wide variety of techniques, but most of these have employed differential centrifugation methods and have used adult rodents. The present study reports the isolation of type II cells from fetal rabbit lungs by the immunoglobin G plating method. Pure populations of fetal type II cells in high yield and with good viability were obtained by the procedure for the first time from rabbit fetal tissue.
Key words. Pulmonary surfactant; lung type II epithelial cell isolation; adherence on IgG plates.

The mammalian lung is lined with a lipid-rich substance (mostly dipalmitoyl phosphatidylcholine) called pulmonary surfactant (SF), which has the capacity to reduce the surface tension during end expiration and thus prevent alveolar collapse at low transpulmonary pressures[1]. Insufficient amounts of surfactant, owing either to premature birth or to a maternal disorder, could be the immediate cause of the respiratory distress syndrome in newborns[2]. It is now well established that surfactant is a lipoprotein complex which is synthesized in the alveolar epithelial type II cell of the lung. Type II cells comprise 15% of all lung cells, but cover <15% of the alveolar surface[3,4].

In the past, methods have been described for the isolation of type II cells from a variety of different species[5]. Most of these methods have used a density gradient centrifugation step that separates cells on the basis of size and density. Since type II cells overlap in size with other lung cells[6], and since their size also varies both with species and with the development of the lung, methods of separation based on differential and density-gradient sedimentation could prove difficult, especially with regard to fetal rabbit type II cells. Dobbs et al.[7] have reported a novel approach using immunoglobin G (IgG) plating for type II pneumocyte isolation in a rat model.

In the present paper, we have isolated type II alveolar epithelial cells from preterm fetal rabbits by the IgG plating methods of Dobbs et al., with certain modifications, but without compromising the yield and purity of the cell populations.

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Materials and methods

Preparation of cell suspension from lung tissue. Rabbit fetuses at 27 days of gestation were aseptically removed by caesarean section from timed pregnant New Zealand white females under sodium pentobarbital anaesthesia (25 mg/kg b.w., Abbott Laboratories, India). The pregnant female rabbits from an inbred strain were obtained from the Central Animal House attached to this Institute. The rabbit fetuses thus obtained were also anaesthetized intraperitoneally with ketamine hydrochloride (10 mg/kg b.w., Parke-Davis, India) and heparin sodium (400 IU/kg b.w., Biological E., India). The inferior vena cava of the rabbit was cut, the trachea was cannulated and the lungs were perfused at 20 cm H_2O pressure with solution II (140 mM NaCl, 5 mM KCl, 2.5 mM sodium phosphate buffer, 10 mM HEPES (*N*-2-hydroxyethyl-piperazine) *N'*-2-ethanesulphonic acid, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$, pH 7.4, at 22 °C) via the pulmonary artery and then excised. About 10 to 15 ml of perfusion fluid was used to perfuse individual lungs. The lungs were lavaged to total lung capacity eight times with solution I (140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mm glucose, 0.2 mM EGTA [ethylene glycol-bis-(β-aminoethyl ether), *N,N'*-tetra-acetic acid], pH 7.4, at 22 °C) to remove the macrophages and were subsequently lavaged twice with solution II. For proteolytic digestion of the fetal lung, the filled lung method as described by Dobbs et al.[7] could not be used because of the difficulty of handling the tissue owing to its fragility and small size. Hence the lungs were minced and digested by incubating with

trypsin (Sigma) solution (0.1% in solution II) containing DNase I (Sigma) (a pinch/20 ml) for 20 min in a 37 °C water bath. A total of 20 ml of trypsin solution was used for each lung. The lungs were tested to small pieces with fine forceps and needle, and then 5 ml of 10% fetal calf serum (Gibco, Scotland) was added to stop the proteolytic digestion. The resulting lung minces and cell suspensions were filtered sequentially through two and then four layers of cotton gauge, followed by filtration through nylon mesh filters of 160 µm, 37 µm and 15 µm pore size. Then cells were kept on ice until all the cells had been filtered. No positive or negative pressure was applied during the filtration steps. Cells were centrifuged at 200 × g for 10 min and the supernatant, which contained the debris, was discarded. To remove any red blood cell (RBC) contamination the cells were subjected to hypotonic lysis by 0.83% NH_4Cl. The cells at this stage were referred to as 'before adherence'. The cell pellet was then resuspended gently in 5 ml of Minimum Essential Medium (MEM) (Sigma) without any calf serum. The cells were aspirated up and down with the help of a pasteur pipette (about 15 times) until they were in uniform suspension.

Adherence. Rabbit IgG was obtained from rabbit serum by affinity chromatography with protein A-sepharose CL-4B. Protein A-sepharose CL-4B was prepared by CNBr activation of sepharose CL-4B (Pharmacia) and subsequent binding of the protein A (Sigma) to the activated sepharose CL-4B[8]. Activated protein A-sepharose CL-4B was then packed in a small chromatography column and diluted rabbit serum [1:1 with 0.15 M phosphate buffered saline (PBS), pH 7.2] was passed through this. After washing the unbound protein with PBS, the bound IgG is eluted with glycine-HCl buffer, pH 2.8. IgG solution was made neutral with NaOH/Tris and extensively dialyzed against PBS[9].

For preparation of IgG-coated plates, IgG was taken in 50 mM Tris-base [TRIS (hydroxymethyl) aminomethane] at pH 9.5, at a concentration of 3 mg/5 ml of Tris/plate. The IgG was allowed to adhere for 3 h at room temperature. The solution was then removed from the plates and the plates were washed twice with PBS and once with MEM. The cells in MEM were added at a density of 20–30 × 10^6 cells/10 ml MEM/plate. Plates were incubated for 1 h in a 5% CO_2 atmosphere in an incubator at 37 °C. Cells were then panned to remove non-adherent type II cells, and these were centrifuged at 200 × g for 15 min.

Cell purity and viability. Purity of the cells was assessed by the modified Papanicolaou stain[10]. Cells were spun in a cytocentrifuge and the slides were dried for 15 h. Then these were sequentially stained with hematoxylin solution followed by lithium carbonate solution. After dehydration in graded ethanol, cells were mounted with distyrene plasticizer xylene (DPX). Viability was measured by the exclusion of trypan blue stain[11].

Table 1. Type II cell parameters in prematurely born rabbit pups (gestational age 27 days).

Parameter	
Fetal body weight (g)	28.01 ± 2.21
Fetal lung weight (g)	1.04 ± 0.11
Crude cell suspension	
Cell count (× 10^6/pup)	7.28 ± 2.69
Percentage type II cells	56.05 ± 2.50
Purified type II cell suspension	
Cell count (× 10^6/pup)	2.71 ± 1.43
Cell count (× 10^6/g lung tissue)	2.34 ± 1.50
Percentage viability	91.12 ± 2.95

Values are mean ± SD of 8 to 10 individual observations for each individual litter.

Electron microscopy. Cells in suspension were fixed for 2 h at room temperature in a solution of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. The cells were then pelleted at 10,000 × g for 5 min. The pellet was postfixed in 1% osmium tetraoxide in the same buffer at 4 °C for 1 h, followed by several changes of 100% alcohol at room temperature. The pellet was dehydrated in acetone and subsequently embedded in epon. Ultrathin sections were cut in an LKB (Sweden) Nova ultramicrotome, stained sequentially with aqueous uranyl acetate and alkaline lead citrate, and finally examined and photographed in a Jeol 1200 XE transmission electron microscope[12].

Results

Cell yield, purity and viability. As shown in table 1, cell populations isolated from fetal rabbits in the present study consisted of ~2.71 × 10^6 cells/pup or ~2.34 × 10^6 cells/g lung tissue, where the average lung weight was 1.04 ± 0.11 g. The cell yields obtained in the present study are comparable with or even better than an earlier study at Scott and co-workers[13] on fetal rabbits, although such studies on fetal rabbit type II cells are comparatively few. The cell populations obtained exhibited ~91% viability. The isolation procedures necessitate the exposure of the cells to proteolytic digestion and initial sedimentation steps which could be responsible for reduction in cell yield and viability of pure type II cells.

Light microscopy. A light microscopic photograph of the fetal type II pneumocytes as stained with a modified Papanicolaou stain is shown in figure 1. This figure clearly shows the presence of dark blue inclusion bodies in the cytoplasm of type II cells, which is a characteristic feature of these cells.

Electron microscopy. An electron micrograph of the cells after adherence is shown in figure 2. The cells are cuboidal in shape with microvilli mostly on the apical surfaces. A number of mitochondria, lamellar bodies and the nuclear membrane are clearly visible. However,

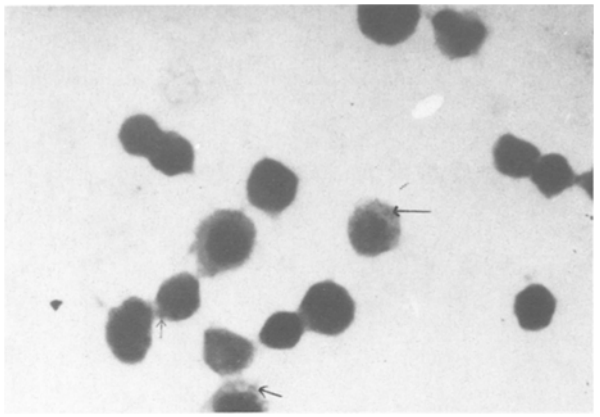
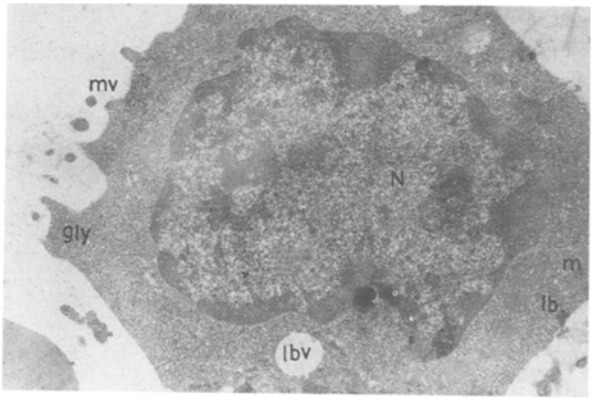


Figure 1. Type II epithelial cells of the rabbit fetal lung as seen under light microscope (× 1000).



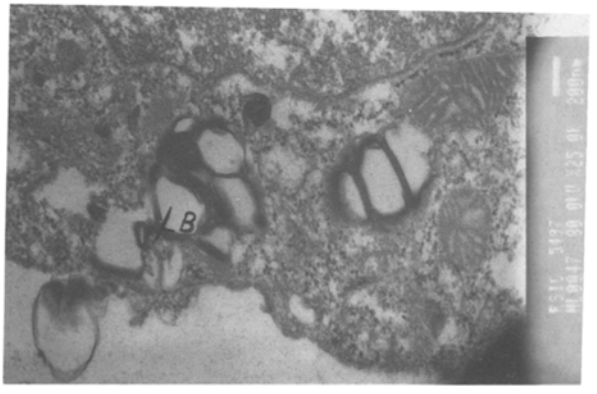


Figure 2. (*a*) Electron microscopic picture of the cell (× 22,000). gly: glycogen content, lb: lamellar bodies, lbv: lamellar body vesicles, n: nucleus, m: mitochondrion, mv: microvilli. (*b*) Higher magnification electron micrograph showing the lamellar structure (× 25,000).

lipids in some lamellar bodies were extracted during tissue processing, leaving behind empty vesicles. With it is shown a higher magnification picture of the dense lamellar bodies observed in the cells. The lamellar nature of this structure is apparent at higher magnification of the dense lamellar inclusion bodies.

Discussion

In order to attribute specific functions to a particular cell type, it is imperative to have a comparatively homogeneous population of the cells. Since lung tissue comprises more than 40 different cell types, and type II cells constitute only 15% of the total cell population in the normal lung, the task of isolating a homogeneous type II cell population becomes all the more difficult. Most previous studies isolated relatively pure populations of type II cells by culturing them for 24 to 48 h, but type II cells are thought to undergo both morphological and biochemical changes with time in culture[14,15].

Differential sedimentation using a wide range of gradients like ficoll, percoll and metrizamide[10] has been widely employed for the isolation of type II cells from adult animals. But because of the variations in cells with age and species[16], it is rather difficult to isolate alveolar type II cells from prematurely born fetal rabbits (gestational age 27 days) by directly applying the isolation procedures as described for the adult animals. It has been reported that fetal type II cells are difficult to recognize early in gestation because they contain few lamellar bodies and abundant glycogen, and probably have different sedimentation characteristics from the adult type II cells. There are comparatively few methods available of isolating fetal rabbit type II cells without prolonged tissue culture. Further, the time involved in cell sorting makes flow cytometry a less attractive technique[10].

For functional studies with a freshly isolated population of type II cells, we need an isolation procedure which gives a good cell yield and purity with minimum cell loss. IgG plating is one such method which can be used for isolating type II cells from fetal rabbits with high yield and purity. Most of the non-type II cells (macrophages, polymorphonuclear leukocytes and lymphocytes) have Fc receptors[17] and therefore bind to the IgG-coated plates. Type II cells lack the Fc receptor and hence remain non-adherent. Approximately 85–90% of these cells have been found to exclude vital trypan blue stain.

Development of such methods is likely to play a critical role in studies relating to the biochemistry, molecular biology and therapeutic applications of human pulmonary surfactant.

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