In Vitro Cell. Dev. Biol. 27A:843–852, November 1991

0883-8364/91 $01.50+0.00

CULTURE OF FETAL ALVEOLAR EPITHELIAL TYPE II CELLS IN SERUM-FREE MEDIUM

CAROLINE FRASLON, GAËLLE ROLLAND, JACQUES R. BOURBON[1], MICHEL RIEUTORT, AND CÉCILE VALENZA

Centre de Biologie Cellulaire, C.N.R.S. UPR3101, 67 rue Maurice Günsbourg, F-94205 Ivry sur Seine cedex, France

(Received 26 July 1990; accepted 17 May 1991)

SUMMARY

A serum-free culture medium (defined medium = DM) was elaborated by adding to Eagle's minimum essential medium (MEM), non-essential amino acids, transferrin, putrescine, tripeptide glycyl-histidyl-lysine, somatostatin, sodium selenite, ethanolamine, phosphoethanolamine, sodium pyruvate, and metal trace elements. This medium was tested for its ability to support sustained surfactant biosynthesis in fetal alveolar epithelial type II cells. For up to 8 days, ultrastructure was maintained with persistance of lamellar inclusion bodies. Thymidine incorporation into DNA was enhanced about 50% in DM as compared with MEM, whereas it was enhanced 300% in 10% fetal bovine serum. With DM, the incorporation of tritiated choline into phosphatidylcholine (PC) of isolated surfactant material was about twice that with MEM. Deletion experiments evidenced the prominent role of pyruvate, transferrin, and selenium in the stimulation of surfactant PC biosynthesis. The addition of biotin to DM enhanced surfactant PC biosynthesis slightly and nonsurfactant PC biosynthesis markedly. The presence of nucleosides seemed unfavorable to the synthesis of surfactant PC. Type II cells responded to the addition of epidermal growth factor and insulinlike growth factor-I both by increased thymidine incorporation into DNA and choline incorporation into PC. It is concluded that DM represents a useful tool for cultivating type II cells without loss of their specialized properties and for studying the regulation of cell proliferation and surfactant biosynthesis in a controlled environment.

Key words: lung; surfactant; pneumocyte type II; cell culture; defined medium; rat.

INTRODUCTION

Alveolar type II epithelial cell or type II pneumocyte is the source of pulmonary surfactant, the phospholipid-rich alveolar lining material. Surfactant plays a crucial role in alveolar dynamics. Maturation of type II cells before birth is of primary importance for the efficiency of respiratory function at birth (31).

Cultures of isolated type II cells represent an approach of choice for studying regulatory mechanisms of surfactant biosynthesis and lung maturational processes. To date, most studies have been conducted with cells maintained in serum-supplemented medium. A number of mediators present in serum including hormones and growth factors are involved, however, in the control of surfactant biosynthesis and in its onset during development, and are likely to have complex interactions with one another (2,31). Together with the variability of serum composition, this renders more difficult the study of the specific role of one particular factor. Moreover, serum enhances DNA synthesis and cell proliferation at the expense of surfactant biosynthesis and seems to participate in the loss of differentiated state observed in cell cultures (7,28). A serum-free, hormonally defined medium has recently been proposed for improved maintenance of adult rat alveolar type II cell differentiated state in vitro (18). Optimal surfactant biosynthesis was obtained in a medium supplemented with insulin, dibutyryl cyclic AMP, hydrocortisone, epidermal growth factor, and other additives. These various additives were undoubtedly beneficial for the biosynthesis of surfactant components, but again, the presence of several hormonal factors renders difficult the use of such a medium precisely for studying the mechanism of action of hormones, especially as far as their role in the ontogenesis of surfactant during fetal life is concerned.

The aim of the present study was to search for serum-free and hormone-free culture conditions adequate for limited cell growth but sustained surfactant biosynthesis in isolated fetal type II pneumocytes. In a previous study (9), Eagle's minimum essential medium (MEM) was the simplest medium among those that were demonstrated to support surfactant accumulation in fetal lung explants similar to that occurring in vivo during the same developmental period. Thus it was elicited as the basal medium in the present study. It was enriched with various additives previously shown to support survival and viability of various cell types including alveolar type II cells (Table 1). Serum-enriched MEM was used for providing a comparison basis for modifications induced by the additives. The criteria used for comparing cell behavior in the different media were cellular ultrastructure, the incorporation of thymidine into DNA, total protein biosynthesis, and the incorporation of labeled choline into phosphatidylcholine of surfactant and nonsurfactant material. Surfactant isolated by the method (27) used in the present work has been thoroughly characterized for its morphologic, biochemical, and functional features (12). The incorporation of a precursor into the major component of this material was therefore con-

[1] To whom correspondence should be addressed.

TABLE 1

COMPOSITION OF THE EXPERIMENTAL MEDIA AND ABBREVIATIONS USED IN THE TEXT[a]

Designation of Media	Composition	Concentration	References
Basal medium	Eagle's MEM		(9)
	Penicillin	100 IU/ml	
	Streptomycin	10 μg/ml	
DM	MEM containing Eagle's nonessential aminoacids		(35)
	transferrin	5 mg/liter	(35)
	putrescine	20 mg/liter	(35)
	GHL	10 μg/liter	(15)
	somatostatin	10 μg/liter	(15)
	sodium selenite	10 μg/liter	(15)
	ethanolamine	30 μg/liter	(34)
	phosphoethanolamine	30 μg/liter	(34)
	sodium pyruvate	110 mg/liter	(17)
	$ZnCl_2$	0.03 mg/liter	
	$MnCl_2$	0.01 mg/liter	
	$FeCl_3$	1 mg/liter	
	$CuSO_4$	0.025 mg/liter	
DM-GF	DM + EGF	100 μg/liter	
	+ somatomedine C (IGF-I)	50 μg/liter	
DM-L	DM + ascorbic acid	10 mg/liter	
	biotin	100 μg/liter	
	choline chloride	50 mg/liter	
	hypoxanthine	10 mg/liter	
	thymidine	10 mg/liter	

[a] Additives for DM were purchased from Sigma-France (L'Isle D'Abeau, France). EGF was from Serva (Heidelberg, Germany), and IGF-I was from Bachem (Bubendorf, Switzerland).

sidered as a specific criterion reflecting the maintenance of surfactant phospholipid biosynthesis in type II pneumocytes in vitro.

Materials and Methods

Animals. Virgin Wistar female rats (R. Janvier, St-Berthevin, France) were mated overnight in the lab, pregnancy being checked by palpation 14 days later. Fetuses were collected on gestational stage 19.5 days for type II cell isolation.

Cell cultures. Pregnant rats were killed by cervical dislocation. The fetuses were removed, decapitated, and the lungs were dissected out aseptically. Tissues were minced using a McIllwain tissue chopper (0.7 mm), and cells were dissociated with an enzymic solution of trypsin (Sigma, St. Louis, MO, 1 mg/ml), collagenase (Sigma, 1 mg/ml), and deoxyribonuclease (Sigma, 20 μg/ml) in Eagle's MEM. Complete dissociation was achieved by four successive 20-min periods of enzymic incubation at 37° C. After dissociation, two different methods usual for type II cell preparation were utilized.

The first method was as described by Post et al. (25) using a previous organotypic culture on Gelfoam pads (Upjohn Co., Kalamazoo, MI) according to Douglas and Teel (10) for enriching the preparation with type II cells. In brief, the cell suspension obtained after enzymic dissociation was filtered on nylon gauze (30 μm), then centrifuged (140 ×*g* for 10 min), supernatant was eliminated and cell pellet was resuspended in 10% fetal bovine serum (FBS)-supplemented MEM. Differential adhesion of fibroblasts was allowed for 1 h in culture flasks in the incubator. After harvesting of the nonadherent cells by a second centrifugation and resuspension in a small volume of medium, the cells were seeded onto 0.5-cm^2 Gelfoam pads placed in 3.5-cm culture dishes at a density of about 1.2×10^6 cells per pad. The pads were transferred to new dishes and medium was replaced the next day. The cells were cultivated on Gelfoam for 4 days in 2% FBS-supplemented MEM at 37° C in air 95%:CO_2 5%. The pads were then dissociated by collagenase (1 mg/ml MEM), cells were collected by centrifugation, and counted. They were then plated in 24-well culture plates (diameter 1.5 cm, Corning, NY) at a density of 3 to 4×10^5 cells per well, under a volume of 20 to 40 μl MEM, and the different media under study were added. This method is thereafter designated the Gelfoam technique.

The second method was as described by Batenburg et al. (4) except for the use of MEM instead of RPMI 1640 and the use of FBS 5% instead of FBS 10% for the adhesion steps. It was verified that this reduction did not affect significantly the yield of fibroblast adhesion. After enzymic dissociation as described above, cells were filtered sequentially through 42, 25, and 15-μm nylon gauze filters, then counted and brought to a concentration of

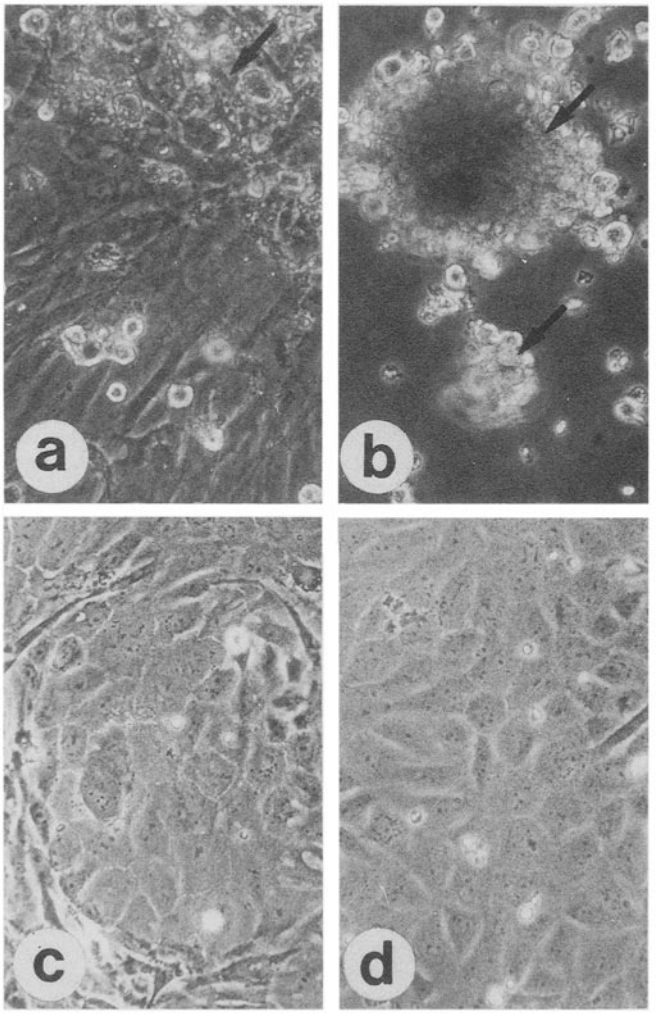

FIG. 1. Phase contrast microscopy of living cells. ×200. *a,b*, Cells obtained through the Gelfoam technique, cultivated 4 days as alveolarlike structures on Gelfoam, collected after Gelfoam enzymic solubilization, plated on multiwell plates and maintained 3 days in *a*, MEM + 10% FBS; *b*, defined medium + EGF + IGF-I (DM-GF). *Note* the proliferation of fibroblasts in presence of serum and their absence in DM-GF. Epithelial cells did not adhere nor spread on the bottom but remained as clusters (*arrows*) either floating or loosely attached to the bottom or to the underlying fibroblast layer. *c,d*, Cells obtained through the differential adhesion technique forming monolayers observed Day 4 after plating and grown for 3 days in *c*, MEM + 10% FBS; *d*, DM-GF. Fibroblasts were virtually absent in DM-GF; they formed bundles surrounding epithelial cells in MEM + FBS. Epithelial cells display the typical aspect described previously (3).

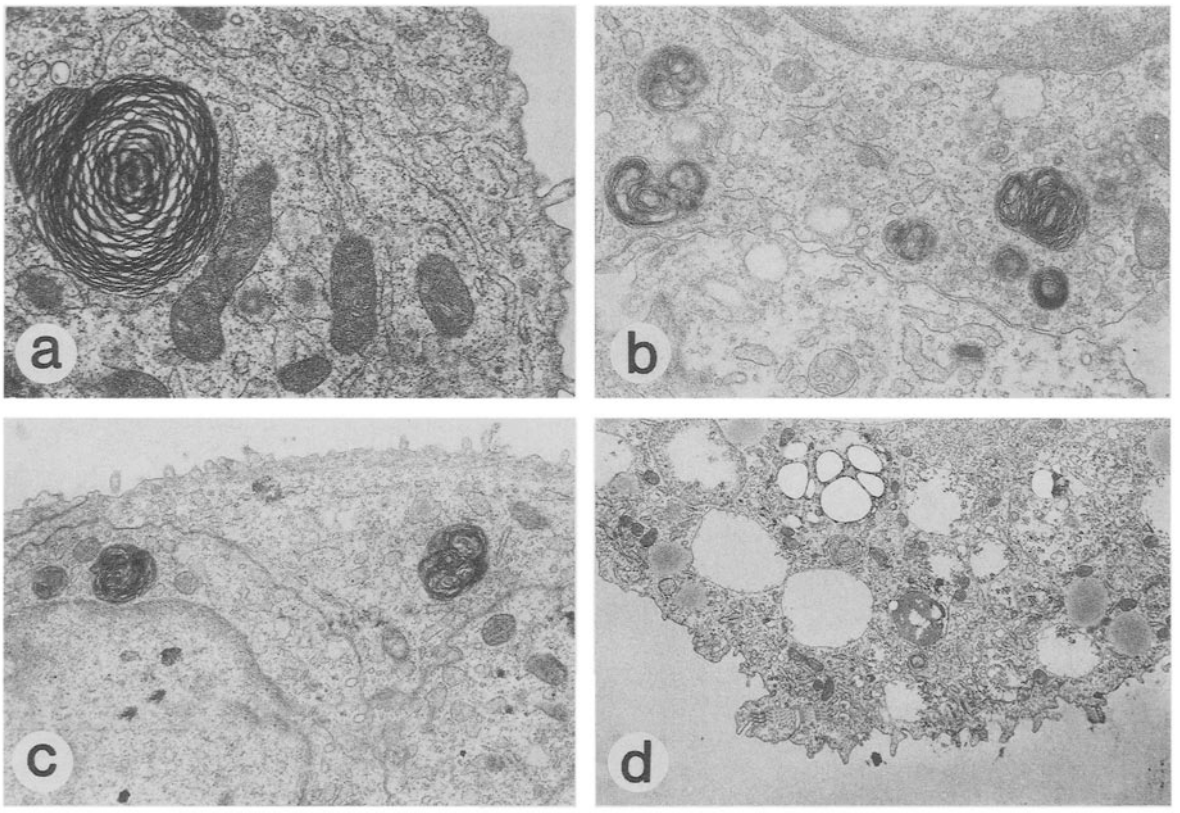

FIG. 2. Electron microscopic examination of cultivated cells prepared through the Gelfoam technique. Cells were cultivated 4 days as alveolarlike structures on Gelfoam (MEM + 2% FBS), collected by enzymic solubilization of Gelfoam, plated in the various media, and fixed after 3 more days. *a*, MEM without additive. Large monocentric lamellar body present in an epithelial cell. ×16 800. *b*, MEM + 2% FBS. Multicentric lamellar bodies in cells displaying the features of alveolar type II cells. ×16 800. *c,d*, Defined medium with growth factors. *c*, Type II cell with similar features as those in *b*. × 11 500. *d*, Epithelial cell with type I-like appearance. ×4400.

1.7×10^6 cells/ml. Two differential adhesion periods of 1 h in 10-cm cell culture dishes (20-ml suspension per dish) allowed removal of fibroblasts. The suspension of nonadherent cells was recovered and the cells seeded at a density of 4×10^5 cells per well of 24-well plates. The suspension was incubated overnight to allow epithelial cells to adhere. The following day, the medium and nonadherent cells were removed. The cells were rinsed with MEM and the various studied media were added. This method is thereafter designated the differential adhesion technique.

In both techniques, isolated pneumocytes were cultivated at 37° C under air 95%:CO_2 5%.

Culture media. Eagle's MEM with Earle's salts and α-MEM were purchased from GIBCO-BRL (Cergy-Pontoise, France). Fetal bovine serum was purchased from Eurobio (Paris, France). Fetal rat serum (FRS) was aseptically prepared in the laboratory from 21-day-old rat fetuses. Stripping of FBS was obtained by addition of 1 mg dextran (Sigma) and 10 mg of active charcoal (Sigma) per ml of FBS. The mixture was stirred 30 min at 55° C and filtered successively on Millipore filters 0.45 and 0.2 μm.

Defined medium (DM) and its variant DM-L were designated using a synthetic approach on the basis of various experimental data listed in Table 1. Additives purchased from Sigma were cell-culture tested. In addition, DM containing epidermal growth factor (EGF) and insulinlike growth factor-I (IGF-I) was tested (DM-GF). EGF (Serva, Heidelberg, Germany) was used because of its previously reported enhancing activity on surfactant biosynthesis in vitro (13); IGF-I (Bachem, Bubendorf, Switzerland) was used instead of insulin often present in defined medium formulas (3) because a) its presence has been evidenced in developing lung (30); and (b) insulin exerts a retarding action on lung maturation in vitro (29). Composition of DM, DM-L, and DM-GF is detailed in Table 1.

Morphologic studies. The aspect of epithelial cells was observed on living cells during and at the end of cultures. Binding of *Maclura pomifera* lectin (Sigma) was used as a criterion for identifying type II cells (8).

Monolayers or cell pellets obtained by centrifugation of cells scraped from their plastic support were fixed for electron microscopy examination. The samples were fixed in 2 vol of ice-chilled OsO_4 (1% in 0.1 *M* cacodylate buffer, pH 7.4), plus 1 vol of glutaraldehyde (2% in the same buffer) according to Hirsch and Fedorko (16). After postfixation in uranyl acetate, the samples were dehydrated in graded alcohol, infiltrated, and embedded in Epon. Ultrathin sections were prepared using a LKB ultramicrotome, then contrasted with uranyl-acetate and lead citrate, and examined with a Philips EM 300 electron microscope.

Incorporation of [^{14}C]thymidine. Cell DNA synthesis activity was estimated by measuring incorporation of 0.2 μCi/ml medium of [2-^{14}C]thymidine (50 mCi/mmol, C.E.A., Gif-sur-Yvette, France). The cells were incubated with the precursor in 24-h interval pulses starting at 0, 24, or 48 h of culture. The cells were subsequently treated as described by Rannels, et al. (26) for DNA extraction and determination of radioactivity. Briefly, after several washings with isotonic phosphate buffer, then 10 min fixation and rinsing with ice-chilled 5% trichloroacetic acid (TCA), each well received 200 μl NaOH (1 *N*) and, 5 min later, 200 μl acetic acid (1 *N*). The wells were then scraped with a rubber policeman, 200 μl of the mixture was recovered for determination of radioactivity in 10 ml of Dynagel scintillation cocktail (Baker, Deventer, The Netherlands), and 100 μl was used for protein determination (*see* below, biochemical determinations).

Biochemical studies. The amount of proteins was determined on cells used for thymidine incorporation with BioRad protein assay kit (BioRad Laboratories GmbH, Münich, Germany).

To determine the phospholipid composition of the cultivated cells, lipids were extracted by chloroform-methanol from nonradioactive samples (cul-

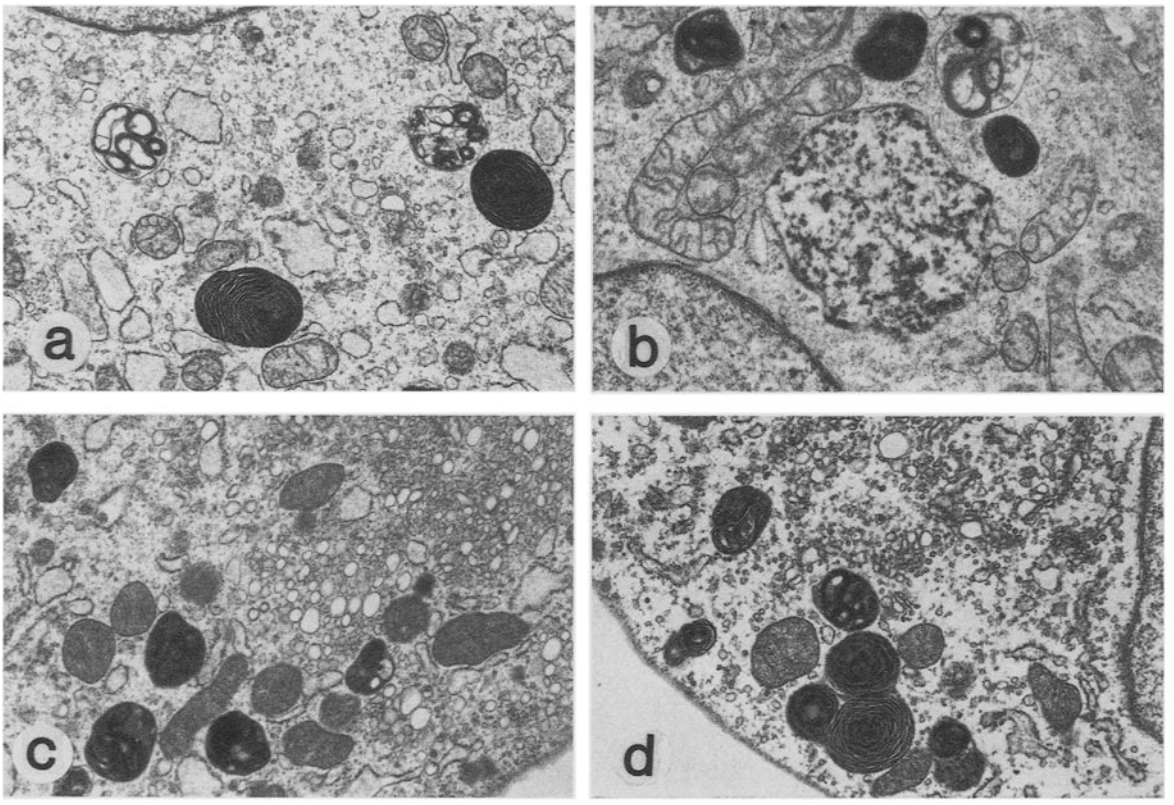

FIG. 3. Electron microscopic examination of cultivated cells prepared through the differential adhesion technique. After adhesion overnight in 5% FBS-containing MEM the cells were cultivated for 72 h in the various media before fixation. *a*, MEM without additives. Both monocentric lamellar bodies with normal aspect and multicentric figures of decaying appearance were observed. ×15 400. *b*, MEM + 2% FBS. Multicentric lamellar bodies in an epithelial cell. Lamellar bodies seemed generally less numerous than in MEM or DM. *Note* the abundance of well-developed mitochondria. ×15 400. *c*, Defined medium. Presence of numerous multicentric lamellar bodies (monocentric lamellar bodies were also observed on occasion). Vesicular structures presumably related to endoplasmic reticulum or Golgi apparatus were especially developed in the vicinity of lamellar bodies. ×19 400. *d*, Defined medium + growth factors. Simultaneous presence of mono- and multicentric lamellar bodies. Both rough endoplasmic reticulum and numerous vesicles are visible close to lamellar bodies. ×18 100.

ture media devoid of radioactive precursors). Phospholipids were separated by two-dimensional thin-layer chromatography (6). A trace amount of [^{14}C]dipalmitoyl phosphatidylcholine (CPC) (60 mCi/mmol, C.E.A., Gif-sur-Yvette, France) was added to each sample for determination of recovery during processing and analysis. The different phospholipids, visualized by iodine and identified with standards, were eluted from the gel. Material from PC spot was divided into three aliquot fractions used, respectively, to determine the extraction yield, the total PC content and disaturated phosphatidylcholine (DSPC) content after treatment with osmium tetroxyde (20). The different phospholipids were mineralized, and inorganic phosphorus was determined according to Ames and Dubin (1).

[Methyl ^{3}H]choline chloride incorporation and isolation of surfactant and residual fractions. As a quantitative index of surfactant biosynthesis in culture, the incorporation of 0.1 μCi/ml medium of [^{3}H]choline (80 μCi/mmol, C.E.A) into surfactant PC was determined after 72 h of contact with the precursor.

The surfactant (S) and residual (R) fractions were isolated by sucrose density gradient centrifugation according to Rieutort et al. (27). For each sample, cells from two or three culture wells were collected by scraping, and pooled in ice-cold TEN buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, 154 m*M* NaCl, pH 7.4), then homogenized in presence of nonradioactive carrier lung tissue (lungs from rat fetuses at term). The homogenates were laid on top of a solution of 0.75 *M* sucrose-TEN buffer. After ultracentrifugation at 48 000 ×*g*, 60 min, the material migrating at the interface was collected, diluted in buffer, and laid onto a discontinuous sucrose gradient (0.68 and 0.25 *M* in the same buffer). After a second ultracentrifugation (64 000 ×*g*, 60 min) the S fraction was recovered at the interface between 0.25 and 0.68 *M* sucrose layers, and stored at −25° C until further analysis. All the remaining material, including pellets and fluid from the two steps, was gathered as the R fraction (nuclei, microsomes, mitochondria, and cytosols). Evaluation of the recovery at the end of the procedure was performed by the addition at the time of homogenization, of a tracer-dose of previously prepared [^{14}C]-choline labeled surfactant fraction obtained from term rat fetuses whose mother had received 1 μCi [^{14}C]choline i.v. 24 h before.

Phospholipids were extracted from S and R fractions by a mixture of chloroform:methanol, 2:1 (vol/vol) and chromatographed in one dimension on silica gel 60 plates (Merck) by chloroform:methanol:water (65:45:5, vol/vol). The various phospholipids were visualized by iodine, and the PC spot was scraped and eluted from the gel according to Bligh and Dyer (5). PC was redissolved in methanol, and its radioactivity was determined in 10 ml Optiscint scintillation cocktail (L.K.B., St-Quentin-en Yvelines, France) with aid of an LKB β-radioactivity counter, using a double-channel desintegrations per minute (dpm) program.

Statistical analysis. Mean values of the experimental data are accompanied by SEM throughout the paper. Significance of differences between mean values was determined by analysis of variance and one-tailed *t* test.

RESULTS

Morphologic aspect of cultured cells. The morphologic features of cultured cells were analyzed by phase contrast light microscopy

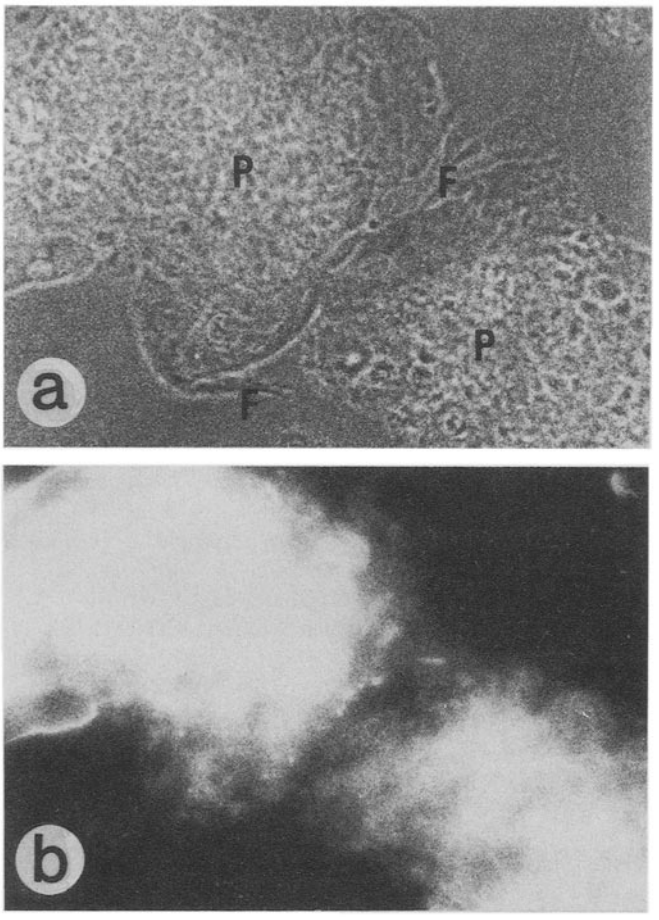

FIG. 4. Phase contrast (*a*) and fluorescence microscopy (*b*) of pulmonary cells isolated by the differential adhesion technique and grown for 72 h in DM, after binding of *Maclura pomifera* lectin coupled with rhodamin. *Note* intense fluorescence on pneumocyte islets (*P*) and lack of fluorescence on fibroblasts (*F*). ×480.

(Fig. 1) and transmission electron microscopy (Figs. 2 and 3). Identification of epithelial cells was confirmed by their ability to bind *Maclura pomifera* lectin (Fig. 4).

Phase contrast microscopy revealed that epithelial cells obtained through the Gelfoam technique rarely spread onto the bottom of plastic culture wells but generally remained as clusters of various sizes (Fig. 1 *a,b*), presumably representing organoid pseudoalveolar structures. Clusters either stayed in suspension in medium or loosely attached to the bottom. Consequently, for biochemical analyses, cells were recovered by centrifugation in their culture medium. A striking difference was seen between serum-supplemented media and defined media: in the former, remnant fibroblasts rapidly proliferated to form in 3 days confluent monolayers on top of which attached the clusters of epithelial cells (Fig. 1 *a*) whereas in the latter, fibroblasts were rare, with an optically empty plastic bottom between clusters (Fig. 1 *b*).

Epithelial cell populations obtained through the differential adhesion technique seemed very homogenous when grown in DM, with scarce remnant fibroblasts (Figs. 1 *d* and 4). Although being less numerous than with the Gelfoam technique, fibroblasts were observed in the presence of serum forming bundles which surrounded round-shaped epithelial-like sheets (Fig. 1 *c*).

Transmission electron microscopic examination evidenced the presence of lamellar bodies (LB) in epithelial cells obtained with both isolation techniques in all media (Figs. 2 and 3). Cells therefore retained a type II cell phenotype all over the culture period (4 days with differential adhesion technique and 8 days with Gelfoam technique). In all the defined media including minimum essential medium (MEM) without additives, the structure of LB was generally normal, whereas LB were most often multicentric in fetal bovine serum (FBS)-containing medium. LB and small vesicles seemed more abundant in DM and DM-GF. Mitochondria seemed more developed in the presence of serum.

Frequently with the Gelfoam technique and on occasion with the differential adhesion technique, cells were observed that were apparently epithelial in nature but were devoid of lamellar inclusions and presented membrane convolutions and vacuolization (Fig. 2 *d*). These features looked somewhat similar to those reported for alveolar type I cells (33), but may also translate cell dedifferentiation.

Thymidine incorporation into cell DNA. Results gained from cells prepared by the two isolation techniques were similar, although the incorporation was slightly higher on the average with the Gelfoam technique (Table 2).

As compared with MEM, the incorporation was strongly enhanced by sera and this effect of sera increased with time. The stimulating effect was stronger when serum concentration was ele-

TABLE 2

[2-^{14}C] THYMIDINE INCORPORATION INTO DNA OF TYPE II CELLS ON 24-h PERIODS STARTING AT 0, 24, OR 48 h OF THE CULTURE IN THE VARIOUS EXPERIMENTAL MEDIA (DPM/10^5 CELLS)[a]

	0–24 h		24–48 h		48–72 h	
Media	Gelfoam	Monolayer	Gelfoam	Monolayer	Gelfoam	Monolayer
MEM	643 ± 70	610 ± 10	850 ± 99	800 ± 104	693 ± 28	598 ± 71
2% FBS	961 ± 94	889 ± 98	1636 ± 141	1172 ± 100	1915 ± 72	963 ± 61
10% FBS	1565 ± 225	2134 ± 127	1914 ± 123	1821 ± 112	3105 ± 69	1548 ± 50
2% FRS	—	1082 ± 37	—	1748 ± 139	—	1927 ± 52
10% FRS	1427 ± 270	1494 ± 63	3407 ± 306	2051 ± 133	4292 ± 266	2980 ± 58
DM	912 ± 167	1367 ± 88	893 ± 55	1152 ± 58	852 ± 23	749 ± 16
DM-GF	1944 ± 471	927 ± 81	1526 ± 79	1179 ± 31	1175 ± 118	1066 ± 71

[a] Mean ± SEM on nine determinations.

TABLE 3

PROTEIN ACCUMULATION (μg PER CULTURE WELL) ON 72 h IN TYPE II CELLS OBTAINED BY THE MONOLAYER TECHNIQUE AND CULTIVATED IN THE VARIOUS EXPERIMENTAL MEDIA[a]

Media	24 h	48 h	72 h
MEM	9.9 ± 0.8	14.9 ± 1.0	16.7 ± 1.8
2% FBS	11.0 ± 0.4	17.3 ± 1.3	20.9 ± 1.5
10% FBS	12.3 ± 0.5	24.2 ± 3.5	27.8 ± 1.0
2% FRS	11.4 ± 0.7	13.6 ± 1.3	28.0 ± 0.8
10% FRS	12.5 ± 3.3	31.3 ± 4.0	35.2 ± 0.9
DM	11.5 ± 1.2	16.4 ± 1.8	21.9 ± 0.6
DM-GF	16.6 ± 1.4	34.9 ± 0.8	27.9 ± 1.3

[a] Mean ± SEM on six determinations (six culture wells all seeded with the same number of cells).

vated from 2 to 10%, and FRS seemed more stimulatory than FBS for a same concentration. The stimulation was as much as 5 to 6 times the incorporation in MEM.

Defined medium stimulated thymidine incorporation at the most to the same extent as 2% FBS (about 50 to 100%) but this effect tended to diminish with time. The addition of EGF and IGF-I to DM slightly enhanced thymidine incorporation.

Total protein synthesis in vitro. These determinations were possible only with cells obtained through the differential adhesion technique because the presence of remnant collagen after Gelfoam pad digestion interfered with cellular protein measurement. Proteins accumulated in cells all over the cultivation period in all media (Table 3). Both sera enhanced protein synthesis. In the latter instances, part of this increase was probably accounted for by fibroblast proliferation. FRS at a concentration of 10%, doubled protein concentration at 48 and 72 h as compared with MEM. With DM, protein accumulation was similar or slightly higher to that in basal MEM. The addition of growth factors (DM-GF) stimulated protein synthesis to an extent similar to that observed with 10% FBS.

Phospholipid composition of cultivated cells. Phospholipid composition determined on cells isolated through the Gelfoam technique maintained in three media, i.e. FBS 2 and 10%, and DM-GF is presented in Table 4. With all three media tested, the proportion of PC was similar although slightly lower as that reported for freshly isolated adult rat type II cells (21). The most noticeable difference between media was the lower proportion of DSPC in medium + 10% FBS (41% of total PC) as compared with other media (60% of total PC), which suggests a possibly better preservation of the high rate of DSPC production characteristic of type II cells in DM and 2% FBS than in 10% FBS, but may also result from proliferation of fibroblasts in the latter instance.

Tritiated choline incorporation into PC of surfactant and residual fractions. a) *Comparison between MEM and supplemented media.* Similar results were obtained with both cell-isolation procedures. As compared with MEM without additives, the addition of 2% FBS increased choline incorporation in S fraction about 50 to 100% depending on the culture experiment. The increase was lower for R fraction (about 25%) and significant only with cells from Gelfoam technique. Increasing FBS concentration from 2 to 10% led to a reduction of incorporation of labeled choline in both fractions, the apparent decrease being especially marked for cells prepared by the differential adhesion technique. FRS reduced labeled choline incorporation in both fractions as compared with MEM at concentrations 2 and 10% (Table 5). Stripping FBS with charcoal strongly reduced choline incorporation as compared with whole serum (Fig. 5). Again, the incorporation was lower when serum concentration was increased from 2 to 10%.

In DM, labeled choline incorporation into surfactant PC was increased about 60 to 80% as compared with DM. There was no significant change of incorporation into the residual fraction with the Gelfoam technique but a 45% increase with the differential adhesion technique. The addition of growth factors to DM slightly enhanced average choline incorporation in surfactant fraction but the differences were not statistically significant; a significant increase of incorporation into the residual fraction was observed in this instance with cells from the Gelfoam technique only (Table 5).

b) *Effects of deletions and additions to DM.* To further investigate the putative role of individual additives of DM in the stimulation of PC biosynthesis, media devoid of one or some additives were compared both to MEM and to complete DM. This study was conducted with cells prepared by the differential adhesion technique.

Only the deletion of pyruvate significantly reduced tritiated choline incorporation into PC of surfactant fraction without bringing it back to the level achieved in MEM, however. There was a trend toward a reduction of incorporation in absence of ethanolamine-phosphoethanolamine or of metal trace elements (8 determinations out of 12 below the mean level in DM). Deletions of transferrin, selenium, putrescine, somatostatin, or glycyl-histidyl-lysine (GHL) left incorporation unchanged as compared with complete DM (Table 6).

For residual fraction PC, none of the deletions significantly diminished the incorporation of the precursor as compared with DM, although a trend toward a decrease was at the limit of significance for pyruvate, somatostatin, GHL, and metal trace elements (Table 6).

In parallel, incorporation of tritiated choline was compared in cells maintained either in MEM, MEM plus transferrin and sodium selenite, α-MEM, and DM (Table 7). α-MEM contains pyruvate at the same concentration as DM but not the other additives of DM; in return, it contains biotin, ascorbic acid, and nucleosides not present in DM. The simultaneous addition of transferrin and selenium to MEM significantly enhanced the incorporation of the precursor into

TABLE 4

PHOSPHOLIPID PROFILE (PERCENTAGE OF TOTAL PHOSPHOLIPIDS) OF CELLS ISOLATED BY THE GELFOAM TECHNIQUE AND CULTIVATED FOR 72 h IN THREE CULTURE MEDIA[a]

	2% FBS	10% FBS	DM-GF
Sphingomyeline	10 ± 2.5	13 ± 4	15.5 ± 4
Phosphatidylcholine			
Total	47 ± 4.5	51 ± 2	46.5 ± 2
Disaturated	29 ± 4.5	21 ± 2.5	28 ± 3.5
Phosphatidylserine	6 ± 1.5	9 ± 4	8.5 ± 2
Phosphatidylinositol	7.5 ± 1.5	9 ± 4	8 ± 2
Phosphatidylethanolamine	23 ± 3	14 ± 2.5	18 ± 5
Phosphatidylglycerol	7.5 ± 2	4.5 ± 1.5	3.5 ± 1.5

[a] Mean ± SEM on five determinations.

TABLE 5

INCORPORATION OF [^{3}H]CHOLINE INTO PC OF S AND R FRACTIONS OF TYPE II CELLS CULTIVATED FOR 72 h IN THE PRESENCE OF THE PRECURSOR (dpm/10^5 CELLS/72 h)[a]

Media	MEM	2% FBS	10% FBS	2% FRS	10% FRS	DM	DM-GF
Gelfoam technique							
n	25	18	13	21	19	21	27
S fraction	245 ± 13	506 ± 45[d]	333 ± 41[c]	211 ± 15[b]	150 ± 12[d]	438 ± 42[d]	458 ± 18[d]
R fraction	2756 ± 152	3454 ± 448[b]	2999 ± 606	1965 ± 166[b]	1067 ± 238[d]	2647 ± 241	3660 ± 239[b]
Differential adhesion technique							
n	22	20	20	20	24	20	16
S fraction	185 ± 18	281 ± 44[b]	126 ± 10[c]	245 ± 25[b]	127 ± 11[c]	308 ± 25[c]	369 ± 39[d]
R fraction	1906 ± 198	2586 ± 527	636 ± 82[d]	2598 ± 630	1053 ± 76[d]	2785 ± 289[b]	2541 ± 282

[a] Mean ± SEM of the number of determinations indicated, gained from 4 different culture experiments. Significant differences with control medium for [b]$P < 0.05$; [c]$P < 0.005$; [d]$P < 0.001$.

surfactant PC, although in the previous experiment the individual deletion of each additive from DM had no effect. In α-MEM, the increase as compared with MEM was not significant for S fraction but was significant for R fraction.

Since MEM does not contain biotin, DM enriched with biotin (100 μg/liter) was tested in another experiment. Parallelly, a more complex medium containing ascorbic acid, hypoxanthine, and thymidine in addition to biotin (DM-L without elevated choline) was also essayed. Added biotin tended to increase choline incorporation into surfactant PC (127.9 ± 16.9% vs. 100.0 ± 8.8% in DM, $n = 8$) and significantly increased it into residual PC (141.5 ± 9.6% vs. 100.0 ± 8.1% in DM, $n = 8$, $P < 0.05$). Further addition of ascorbic acid, hypoxanthine, and thymidine tended by contrast to reduce surfactant PC biosynthesis (78.2 ± 5.2% of DM, $n = 8$) although it let incorporation into residual PC increase (136.3 ± 10.6% of DM, $n = 8$).

c) *Longer term experiments.* The experiments reported above indicate that DM ameliorates surfactant PC biosynthesis in vitro as compared to the reference MEM on short culture periods. Although the addition of biotin, ascorbic acid, hypoxanthine, and thymidine did not improve it further for freshly isolated cells, one can assume that their absence may become critical if culture was prolonged. Similarly, the low level of choline in MEM could represent a limiting factor. To test these hypotheses, the more complex formula designated DM-L was experimented. Cells isolated by the differential adhesion technique were maintained as monolayers for 1 wk (with one medium replacement) either in DM, DM-L, α-MEM, or MEM + 10% FBS. Then, nonradioactive media were replaced by media containing [^{3}H]choline and incorporation was allowed to occur for 72 h. Incorporation was conducted in DM for cells maintained in "cold" DM or 10% FBS, in DM or DM-L without additional choline chloride for cells maintained in "cold" DM-L and in α-MEM for cells maintained in "cold" α-MEM (Table 8). In cells maintained in DM for the whole culture duration, the incorporation of the precursor was enhanced as compared with cells used immediately after isolation, reaching 527 ± 41 and 3563 ± 331 dpm/10^5 cells in S and R fractions, respectively ($n = 12$). Culture in DM-L tended to reduce the incorporation as compared to DM (difference at the limit of statistical significance). The incorporation was considerably lower for both fractions in cells cultivated in α-MEM but clearly enhanced in cells maintained for 1 wk in presence of 10% FBS. In the latter instance, the similar increase in both fractions suggests that it may result from an enlargement of cell population.

Discussion

The investigations reported herein were undertaken in the goal to design a defined medium avoiding the disadvantages of using

TABLE 6

INCORPORATION OF [^{3}H]CHOLINE INTO PC OF S AND R FRACTIONS OF TYPE II CELLS (DIFFERENTIAL ADHESION TECHNIQUE) CULTIVATED FOR 72 h IN THE PRESENCE OF THE PRECURSOR EITHER IN MEM, DM, OR DM DELETED WITH ONE OR SOME ADDITIVES[a]

Medium	DM	MEM	Tr	Se	Pu	Som	GHL	EP	Pyr	MTE
S fraction	100.0 ± 8.4	50.2 ± 7.9[b]	99.3 ± 6.2	89.1 ± 6.4	103.2 ± 91	86.9 ± 8.4	89.0 ± 10.4	81.0 ± 8.1	67.7 ± 5.9[b]	85.0 ± 8.1
R fraction	100.0 ± 8.4	68.2 ± 6.6[b]	84.3 ± 6.7	80.9 ± 7.2	89.8 ± 9.3	77.9 ± 7.3	75.0 ± 8.5	81.5 ± 4.9	74.9 ± 4.5	78.1 ± 5.3

[a] Results are expressed as a percentage of the average incorporation in complete DM. Mean ± SEM on 12 determinations. [b] Significant difference with DM for $P < 0.05$. Key: DM without Tr: transferrin, Se: sodium selenite, Pu: putrescine, Som: somatostatin, GHL: glycyl-histidyl-lysine, E-P: ethanolamine and phosphoethanolamine, Pyr: sodium pyruvate, MTE: metal trace elements.

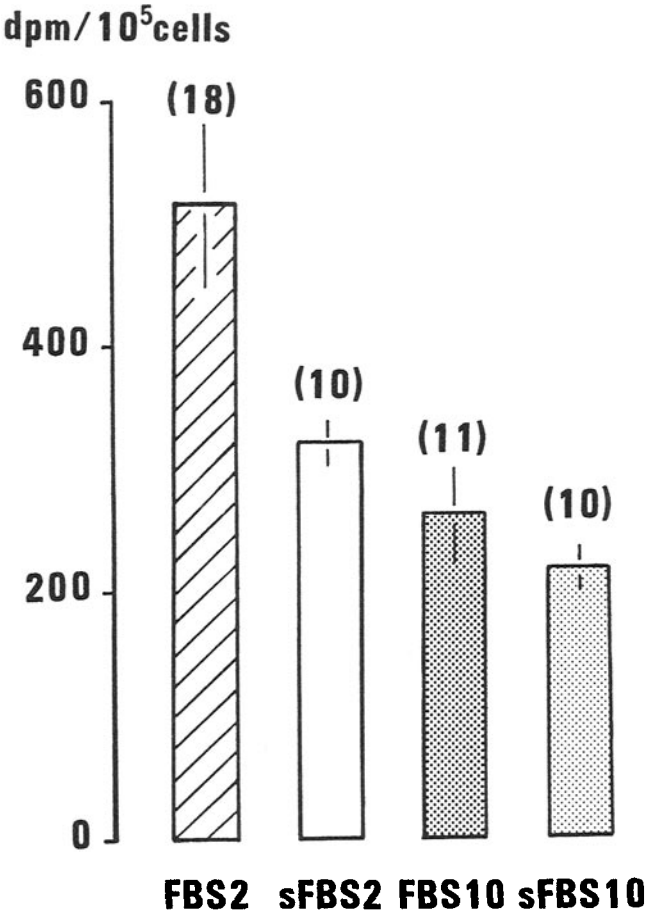

FIG. 5. Comparison of [^{3}H]choline incorporation in S-fraction PC of cells isolated by the Gelfoam technique and cultivated 3 days in the presence of whole or charcoal-stripped FBS in MEM. Mean ± SEM on the number of determinations *indicated in parentheses*. Significant difference ($P < 0.05$) between 2% FBS and 2% sFBS.

serum-supplemented medium and permitting sustained surfactant biosynthesis in isolated alveolar type II cells. The application to cultivated cells of the surfactant isolation procedure (27) in the presence of carrier tissue and labeled fraction for recovery estimate allowed us to achieve a specific evaluation of surfactant biosynthesis.

The ultrastructural examination of the cells indicated that at least a part of them retained a type II cell phenotype over the entire culture period because intracellular lamellar bodies were found in all media including MEM. A progressive trend toward the loss of lamellar bodies was noticed, however, after 1 wk in vitro, a tendency also illustrated by the relatively low level of DSPC in cultivated type II cells as compared with freshly isolated cells.

In the developing lung there seems to be a balance between type II cell multiplication and surfactant accumulation (23). Therefore, one of the objectives of the present study was to limit proliferation of type II cells without loss of their specialized properties. Consistent with previous findings (7,28), serum increased thymidine incorporation into cell DNA proportionally to serum concentration. As compared with MEM, 10% FBS increased incorporation about 3 times and 10% FRS up to 6 times. In comparison, the twofold increase and the 25 to 40% increase observed with DM after 24 and 72 h, respectively, seem rather modest. Moreover, the stimulation increased with time in serum but decreased in DM. The introduction of hypoxanthine and thymidine in DM or the use of α-MEM, which contains nucleosides and deoxy-nucleosides, did not enhance tritiated choline incorporation into surfactant PC whereas nonsurfactant PC biosynthesis was stimulated. This suggests that the absence in DM of components favorable to DNA synthesis is indeed advantageous for the expression of surfactant-producing activity. Another advantage of DM in this order was the limitation of growth of remnant fibroblasts in epithelial cell cultures.

Elevating serum concentration from 2 to 10% decreased tritiated choline incorporation into surfactant PC. This reduction is likely to be explained, at least in part, by isotopic dilution of choline present in serum at higher concentration than in MEM. This renders the comparison between DM and serum-supplemented MEM very difficult in terms of choline incorporation. It should be pointed out, however, that DM, which does not contain hormones, stimulated choline incorporation to the same extent as 2% whole FBS, which brought hormones likely to enhance surfactant biosynthesis, and more than 2% hormone-deprived charcoal-stripped FBS.

On the other hand, one could consider that the relatively low level of choline in DM represents at term a rate-limiting factor. In fact, the elevation of choline concentration in DM-L did not lead to increased labeled-choline incorporation after 1 wk of cultivation, whereas maintenance for the same duration in 10% FBS enhanced it. Consistently, in our previous study using lung explant culture, there was no correlation between the level of choline and the accumulation of surfactant material in various media (9). The low level of choline in DM therefore does not seem as unfavorable on the culture durations that were used but could probably be increased without inconvenience.

An important issue of the present study was to make evident the

TABLE 7

INCORPORATION OF [^{3}H]CHOLINE INTO PC OF S AND R FRACTIONS OF TYPE II CELLS (DIFFERENTIAL ADHESION TECHNIQUE) CULTIVATED FOR 72 h IN THE PRESENCE OF THE RADIOACTIVE PRECURSOR EITHER IN MEM, MEM PLUS TRANSFERRIN AND SODIUM SELENITE (SAME CONCENTRATION AS IN DM), α-MEM, OR DM[a]

Medium	MEM	MEM + Tr + SE	α-MEM	DM
S fraction	100.0 ± 10.0	148.0 ± 13.1[b]	139.0 ± 11.0	167.1 ± 18.7[b]
R fraction	100.0 ± 12.0	156.2 ± 22.8	187.9 ± 24.2[b]	208.1 ± 31.5[b]

[a] Results are expressed as a percentage of the average incorporation in MEM. Mean ± SEM on 12 determinations. [b] Significant difference with MEM for $P < 0.05$.

TABLE 8

INCORPORATION OF [^{3}H]CHOLINE INTO PC OF S AND R FRACTIONS ISOLATED FROM TYPE II CELLS (DIFFERENTIAL ADHESION TECHNIQUE) MAINTAINED FOR 7 DAYS IN NONRADIOACTIVE MEDIUM[a] THEN FOR 72 h IN PRESENCE OF THE RADIOACTIVE PRECURSOR[b,c]

Media	DM/[^{3}H]DM	DM-L/[^{3}H]DM	DM-L/[^{3}H]DM-L	αMEM/[^{3}H]αMEM	10%FBS/[^{3}H]DM
S fraction	100.0 ± 7.8	71.6 ± 8.4	69.2 ± 8.1	45.6 ± 6.6[e]	176.9 ± 12.4[f]
R fraction	100.0 ± 9.5	69.7 ± 6.8	74.2 ± 7.2	47.9 ± 5.7[e]	163.6 ± 14.1[d]

[a] DM, DM-L, α-MEM or MEM with 10% FBS; [b] in DM, DM-L without additional choline or α-MEM; [c] Results are expressed as a percentage of the average incorporation in the combination DM/[^{3}H]DM. Mean ± SEM on 12 determinations. Significant difference with reference condition (DM/[^{3}H]DM) for [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$.

stimulatory effect of pyruvate on PC biosynthesis, especially surfactant PC. This finding is consistent with the report that lactate is a much more efficient precursor than glucose for fatty acid and phospholipid biosynthesis in type II cells (11); providing the substrate which immediately follows lactate in the pathway had similar metabolic effects. Furthermore, pyruvate has been reported to stimulate growth and differentiation of other epithelial cells of the respiratory tract, namely, tracheal cells (32). Pyruvate alone, however, does not account for the whole stimulation observed in DM as compared with MEM because on the one hand, α-MEM, which contains pyruvate at the same concentration as in DM, failed to increase significantly choline incorporation into surfactant PC and on the other hand, the association of transferrin and sodium selenite in MEM was also stimulatory. Therefore, pyruvate may become rate-limiting for surfactant PC biosynthesis in the presence of the other components of DM, but these had together a supporting effect even if their individual deletion did not lead to significant loss of choline incorporation.

The complex interaction between additives is still illustrated by the individual deletions of sodium selenite and transferrin from DM which had no effect whereas their simultaneous addition to MEM enhanced choline incorporation. The trend toward a reduction of surfactant and nonsurfactant PC biosynthesis when metal trace elements and ethanolamine-phosphoethanolamine were deleted from DM probably illustrates their role in maintenance of cell viability (3). A similar trend was seen for nonsurfactant PC after deletion of GHL or somatostatin. The latter have been shown to be necessary for cell survival in fetal mouse primordium in vitro (15). Their mechanism of action is only conjectural: GHL could act to mediate transport of copper and other transition metals (24); somatostatin has poorly defined cytoprotective effects and may represent a differentiation-promoting agent through its inhibiting effect on cell proliferation (22). The absence of a marked effect of somatostatin removal from DM on surfactant PC biosynthesis would suggest deleting it from DM if a totally hormone-free environment is wanted.

Although the addition of biotin to DM seemed to enhance more nonsurfactant than surfactant PC biosynthesis, this vitamin should be added to the formula of DM, the most for prolonged culture experiments, the possible exhaustion of biotin stores then becoming a limiting factor for fatty acid biosynthesis by cultivated cells. By contrast, short- and longer-term experiments with DM-L indicated that ascorbic acid adjunction probably is not useful.

Type II cells maintained in DM responded to the addition of EGF and IGF-I, both by increased DNA and PC biosynthesis. These results are consistent with previous findings obtained for EGF either with lung explants or with isolated type II cells (13,14,19). Further analysis of the effects of each factor would be necessary. Nevertheless, these preliminary observations indicate that DM is adequate for testing putative stimuli for type II cell proliferation or surfactant biosynthesis and investigating their mechanism of action on isolated cells.

Finally, the defined medium elaborated in the present study is a useful tool for studying alveolar type II cell maturation and the control of surfactant biosynthesis, allowing one to overcome some of the disadvantages presented by the use of serum.

ACKNOWLEDGEMENTS

The authors thank Dr. Léa Marin for her invaluable advice and Mrs. Marie-Elizabeth Dufour for helpful technical assistance in electron microscopy. A preliminary step of this work was presented at the symposium Basic Research on Lung Surfactant held in Marburg, Germany, September 12–14, 1988.

REFERENCES

1. Ames, B. N.; Dubin, D. T. The roles of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235:769–775; 1960.
2. Ballard, P. L. Hormonal regulation of pulmonary surfactant. Endocrinol. Rev. 10:165–181; 1989.
3. Barnes, D. Serum-free animal cell culture. Biotechniques 5:534–542; 1987.
4. Batenburg, J. J.; Otto-Verberne, C. J. M.; Ten Have-Opbroek, A. A. W., et al. Isolation of alveolar type II cells from fetal rat lung by differential adherence in monolayer culture. Biochim. Biophys. Acta 960:441–453; 1988.
5. Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917; 1959.
6. Bourbon, J. R.; Rieutort, M.; Engle, M. J., et al. Utilization of glycogen for phospholipid synthesis in fetal rat lung. Biochim. Biophys. Acta 712:382–389; 1982.
7. Cott, G. R.; Walker, S. R.; Mason, R. J. The effect of substratum and serum on the lipid synthesis and morphology of alveolar type II cells *in vitro*. Exp. Lung Res. 13:427–447; 1987.
8. Dobbs, L. G.; Williams, M. C.; Brandt, A. E. Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. Biochim. Biophys. Acta 846:155–166; 1985.
9. Doucet, E.; Bourbon, J. R.; Rieutort, M., et al. Optimization of fetal lung organ culture for surfactant biosynthesis. In Vitro Cell. Dev. Biol. 23:189–198; 1987.
10. Douglas, W. H. T.; Teel, R. W. An organotypic *in vitro* model system for studying pulmonary surfactant production by type II alveolar pneumocytes. Am. Rev. Respir. Dis. 113:17–23; 1976.
11. Engle, M. J.; Brown, D. J.; Dehring, A. F. Lactate metabolism in fetal type II pneumocytes. Lung 164:155–164; 1986.
12. Farrell, P. M.; Bourbon, J. R.; Notter, R. H., et al. Relationships among surfactant fraction lipids, proteins and biophysical properties

in the developing rat lung. Biochim. Biophys. Acta. 1044:84–90; 1990.
13. Gross, I.; Dynia, D. W.; Rooney, S. A., et al. Influence of epidermal growth factor on fetal rat lung development *in vitro*. Pediatr. Res. 20:473–477; 1986.
14. Haigh, R. M.; Hollingsworth, M.; Micklewright, L. A., et al. The effect of human urogastrone on lung phospholipids in fetal rabbits. J. Dev. Physiol. 10:433–443; 1988.
15. Hilfer, S. R.; Schneck, S. L.; Brown, J. W. The effect of culture conditions on cytodifferentiation of fetal mouse lung respiratory passageways. Exp. Lung Res. 10:115–136; 1986.
16. Hirsch, J. G.; Fedorko, M. E. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and post-fixation in uranyl acetate. J. Cell Biol. 38:615–627; 1968.
17. Jozwiak, Z.; Snyder, C. E.; Murty, V. L. M., et al. Lipid composition of the secretion from human bronchial explant culture. Biochim. Biophys. Acta 802:282–286; 1984.
18. Kawada, H.; Shannon, J. M.; Mason, R. J. Improved maintenance of adult rat alveolar type II cell differentiation *in vitro*: effect of serum-free hormonally defined medium and a reconstituted basement membrane. Am. J. Respir. Cell Mol. Biol. 3:33–43; 1990.
19. Leslie, C. C.; McCormick-Shannon, K.; Robinson, P. C., et al. Stimulation of DNA synthesis in cultured rat alveolar type II cells. Exp. Lung Res. 8:53–66; 1985.
20. Mason, R. J.; Nellenbogen, J.; Clements, J. A. Isolation of disaturated phosphatidylcholine with osmium tetroxide. J. Lipid Res. 17:281–284; 1976.
21. Mason, R. J.; Williams, M. C.; Greenleaf, R. D., et al. Isolation and properties of type II alveolar cells from rat lung. Am. Rev. Respir. Dis. 115:1015–1026; 1977.
22. Moreau, J. P.; De Feudis, F. V. Pharmacological studies of somatostatin and somatostatin-analogues: therapeutic advances and perspectives. Life Sci. 40:419–437; 1987.
23. Nielsen, H. C.; Kirk, W. O.; Sweezey, N., et al. Coordination of growth and differentiation in the fetal lung. Exp. Cell Res. 188:89–96; 1990.
24. Pickart, L. The use of glycyl-histidyl-lysine in culture systems. In Vitro 17:459–466; 1981.
25. Post, M.; Torday, J. S.; Smith, B. T. Alveolar type II cells isolated from fetal lung organotypic cultures synthesize and secrete surfactant-associated phospholipids and respond to fibroblast-pneumonocyte factor. Exp. Lung. Res. 7:53–65; 1984.
26. Rannels, S. R.; Yarnell, J. A.; Fisher, C. S., et al. Role of laminin in maintenance of type II pneumocyte morphology and function. Am. J. Physiol. 253:C835–C845; 1987.
27. Rieutort, M.; Farrell, P. M.; Engle, M. J., et al. Changes in surfactant phospholipids in fetal rat lungs from normal and diabetic pregnancies. Pediatr. Res. 20:650–654; 1986.
28. Scott, J. E. The role of sera, growth factors, and hormones in the *in vitro* production of disaturated phosphatidylcholine and propagation of undifferentiated type II alveolar cells from fetal rabbit lung. Exp. Lung Res. 12:181–194; 1987.
29. Smith, B. T.; Giroud, C. J. P.; Robert, M., et al. Insulin antagonism of cortisol action on lecithin synthesis by cultured fetal lung cells. J. Pediatr. 87:953–955; 1975.
30. Snyder, J.; D'ercole, A. J. Somatomedin C/insulin-like growth factor I production by human fetal lung tissue maintained in vitro. Exp. Lung Res. 13:449–458; 1987.
31. Van Golde, L. M. G.; Robertson, B.; Batenburg, J. J. The pulmonary surfactant system: biochemical aspects and functional significance. Physiol. Rev. 68:374–455; 1988.
32. Wasilenko, W. J.; Marchok, A. C. Pyruvate regulation of growth and differentiation in primary cultures of rat tracheal epithelial cells. Exp. Cell Res. 155:507–517; 1984.
33. Weller, N. K.; Karnowsky, M. J. Isolation of pulmonary alveolar type I cells from adult rats. Am. J. Pathol. 124:448–456; 1986.
34. Willey, J. C.; Lechner, J. F.; Harris, C. C. Bombesin and the C-terminal tetradecapeptide of gastrin-releasing peptide are growth factors for normal human bronchial epithelial cells. Exp. Cell Res. 153:245–247; 1984.
35. Yamane, I.; Kan, M.; Hoshi, H., et al. Primary culture of human diploid cells and its long-term transfer in a serum-free medium. Exp. Cell Res. 134:470–474; 1981.