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OPTIMIZATION OF FETAL LUNG ORGAN CULTURE FOR SURFACTANT BIOSYNTHESIS

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SUMMARY

Lung organ culture has been a widely used system for studying differentiation and maturation of alveolar epithelium through various culture conditions. The purpose of this work was to carefully characterize in vitro lung biochemical differentiation through isolation of surfactant fraction from tissue and to search for optimal culture conditions. Fetal rat lung was explanted on the 18th gestational day for studying glycogen storage, and on the 20th gestational day for studying surfactant accretion, and cultivated for 48 h. Morphologic differentiation was studied by electron microscopy on tissue explanted on the 17th or 18th gestational days and cultivated for various times. Glycogen storage was greater on fluid medium, although less than occurring in vivo. Cellular integrity and surfactant accumulation were maximal on a semisolid medium containing 0.5% agar. Use of O_2-CO_2 instead of air-CO_2 for gassing the explants slightly decreased phospholipid accumulation. Among media used in previous lung culture studies, Waymouth MB 752/1 was the only one to allow net glycogen accumulation in vitro. The most favorable media for surfactant phospholipid accretion were Waymouth MB 752/1, Eagle's minimum essential and its Dulbecco's modification, CMRL 1066, and NCTC 109. They allowed a 12- to 14-fold increase of surfactant fraction phospholipids in vitro, which is similar to the increase occurring in vivo during the same period. Ham's F10 and F12 media allowed a six fold increase. RPMI 1640 and medium 199 (M199) allowed only a three fold increase. Phospholipid concentration in nonsurfactant fraction only doubled during culture, and differences between various media were much less marked. DNA concentration changed little during culture. Morphologic differentiation of epithelial cells was advanced as compared with in vivo timing in a medium allowing maximal surfactant accretion (Waymouth MB 752/1) but not in a medium allowing low surfactant increase (RPMI 1640). The possible role of compositional differences between media is discussed.

Key words: lung; fetus; maturation; surfactant; glycogen; culture media.

INTRODUCTION

Organ culture has been a widely used approach for studying lung metabolic functions and biosynthetic activities in vitro, especially those aspects related to maturation of type II pneumocytes, the cells responsible for surfactant production. A number of data dealing with morphologic (17,33,37,45,47) and biochemical (8,16, 28-30,47) features of maturational processes and with their regulatory (2,13,18,20-22,27,33) or pathologic (8, 20) aspects have been gained through this technique.

A variety of different conditions, especially different culture media, have been used in these experiments. This causes great difficulty in comparing the various published results, especially from a quantitative point of view. Indeed, a controversy has been raised about the possibility of reproducing authentic lung biochemical maturation in vitro including production of surfactant with normal phospholipid composition (3,29).

The purpose of the present work was: (a) to characterize more carefully lung biochemical maturation in vitro through isolation of surfactant from cultivated tissue and (b) to search for optimal conditions for type II pneumocyte maturation in lung explants cultivated on chemically defined media. Three criteria were considered: glycogen storage during the phase preceding type II cell differentiation, accumulation of surfactant phospholipids in fetal lung tissue after type II cells have started to differentiate, and morphologic type II cell differentiation in vitro.

Three aspects of culture conditions were successively considered. First, we examined the consequences of using different substrata by comparing explants resting on a cellulose-ester filter and a grid, with explants lying on a semisolid medium containing agar gel. Second, nine commercially available media previously used for lung cultures were compared. Finally, atmospheric conditions

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were assessed by comparing cultures grown in 95% oxygen:5% carbon dioxide vs. 76% nitrogen:19% oxygen:5% carbon dioxide.

MATERIALS AND METHODS

Explant culture preparation

Wistar female rats (Centre d'Elevage R. Janvier, St. Berthevin, France) were used. Animals were mated overnight in the lab, and the next morning was considered the first gestational day. Depending on the type of experiment, fetal lungs were collected either at 16.5, 17.5, or 19.5 d of gestation, i.e. at 10:00 a.m. on the 17th, 18th, or 20th gestational day.

Pregnant rats were killed by a blow to the head and their fetuses aseptically removed. All tissue handling and culture initiation was done under aseptic conditions. Fetal lungs were rapidly removed, pooled in ice-chilled Waymouth MB 752/1 medium, and then cut into 0.7 mm pieces with a McIllwain tissue-chopper (The Mickle Laboratory Engineering Co., Gomshall, Surrey, UK). Tissues were kept in ice-chilled Waymouth medium until all cultures could be initiated.

Lungs were explanted on Day 17.5 for glycogen storage studies, and cultivated for 48 h to reach a stage equivalent to 19.5 d of gestation, the stage when glycogen concentration is maximum in the course of normal development (7, 31).

For phospholipid determinations, fetal lungs were explanted on Day 19.5 when the first differentiated type II cells can be observed (48), and also cultivated for 48 h to reach a stage equivalent to term of gestation.

For electron microscopy (EM) analysis of in vitro cell differentiation, explantation of tissue was performed at 16.5 or 17.5 d and explants maintained in culture for different durations.

Eight different culture media previously used in recent studies on lung organ or organotypic cultures were used: Eagle's minimum essential medium (MEM) (35,38), Dulbecco's modification of MEM (26), Medium 199 (13), Ham's F10 (46) and F12K (12,14,19), Waymouth's MB 752/1 (16,18,20,28,45), RPMI 1640 (8,42,43), and CMRL 1066 (47). A ninth medium, NCTC 109, was added to the list because of the large number of constituents. All media were supplied by Eurobio, Paris, France, along with comprehensive information about the precise composition of each medium.

For glycogen studies, the glucose concentration in all media was adjusted to 5 mg/ml (28 mmol/ml), the glucose concentration in Waymouth's medium, to avoid differences due to this substrate whose stimulating action on glycogen biosynthesis (24) is well established. The method for lung explant culture on cellulose-ester filters (Millipore-France, Velizy, France) was as described by Gross et al. (19). Each filter bearing about 20 lung explants was placed on a stainless steel grid at gas-medium interface in a single-well plastic culture dish (Falcon Plastics, Los Angeles, CA), containing 1 ml of fluid medium.

Semisolid media containing 0.5% agar (49) were prepared by mixing 3 vol of commercial fluid medium with 1 vol of heated agar solution (2% agar powder, purchased from Sigma, St. Louis, MO, dissolved in Krebs-Ringer bicarbonate solution containing 1 mg/ml glucose). While still warm, the mixtures were dispensed into Lindbro (Flow Laboratories Inc., Hamden, CT) multiwell plates. Each well received 1.5 ml of semisolid medium. At 37° C, the mixtures formed a soft gel and the explants were laid on top.

Because the duration of culture was short, and based on preliminary experiments dealing with morphologic and biochemical maturation, replacement of the media was unnecessary. Except for those experiments in which the atmosphere composition was especially studied, the explants were maintained in a disposable mixture of 76% nitrogen:19% oxygen:5% carbon dioxide (supplied by Air Liquide Co., Vitry, France) at a temperature of 37° C. Cultures were terminated by pooling the explants and freezing them in liquid nitrogen. Tissues were stored at −25° C until further utilization.

Subcellular fractionation technique

Surfactant (S) and nonsurfactant (R = residual) fractions were isolated by sucrose density gradient ultracentrifugation according to the method first described by Frosolono et al. (15), revised by Sanders and

TABLE 1

CHANGES IN GLYCOGEN CONCENTRATION IN LUNG EXPLANTS FROM 17.5 D-OLD FETUSES CULTIVATED FOR 48 H EITHER ON FLUID OR SOLID MEDIUM[a]

	Initial Content	Waymouth		CMRL 1066		RPMI 1640	
		Fluid	Solid	Fluid	Solid	Fluid	Solid
Microgram of glycogen per milligram tissue	9.6 ± 0.5	13.0 ± 0.4[b,c]	9.1 ± 0.2[c]	9.4 ± 0.3	9.5 ± 0.5	4.7 ± 0.3[b,c]	2.8 ± 0.3[b,c]
Microgram of glycogen per milligram protein	139 ± 8	153 ± 4[b,c]	123 ± 6	126 ± 4	138 ± 5	74 ± 4[b,c]	28 ± 2[b,c]

[a]Mean ± SEM on 10 culture experiments.
[b]Significant difference as compared with initial content for $P < 0.001$.
[c]Significant difference between fluid and solid media for $P < 0.001$.

Longmore (40), and adapted to small-size samples by Rieutort et al. (36). Tris-hydroxymethyl aminomethan, EDTA, and sucrose necessary for preparing isoosmotic buffers, pH 7.4, were purchased from Sigma. Hydrochloric acid and sodium chloride were purchased from Prolabo (Paris, France).

Biochemical analyses

Glycogen assay. The method of Chan and Exton (10) was used. Tissues were homogenized in distilled water; homogenates were dropped onto pieces (4 × 1.5 cm) of Whatman chromatography paper 3MM (Whatman Ltd, Maidstone, England). Glycogen was precipitated and washed three times in 66% ethanol. After rinsing with acetone, papers were dried and placed in tubes containing α1.4–1.6 amyloglucosidase (Boehringer, Mannheim, FRG) in acetate buffer (pH 4.5) for glycogen hydrolysis. Glucose was then determined by the glucose oxidase method (GOD-Perid,Boehringer). Oyster-glycogen (Sigma) treated the same way was used as a standard.

Protein and DNA determination. Proteins were determined on an aliquot fraction of lung-water homogenates by the method of Schacterle and Pollack (41) using bovine serum albumin (BSA) (fraction V, Sigma) as a reference. DNA was precipitated by trichloracetic acid (Prolabo, Paris, France) from an aliquot part of residual fraction, and measured by the diphenylamine method of Burton (9) using calf-thymus DNA (Sigma) as a reference. Other chemical reagents were provided by Prolabo.

Phospholipid analysis. Lipids were extracted overnight from whole lung tissue or from S and R fractions by chloroform:methanol (2:1 vol/vol). A trace amount of [^{14}C]dipalmitoyl phosphatidylcholine (about 10 000 dpm, 60 mCi/mmol, Amersham International PIC, Amersham, UK) was added to each sample before extraction for determination of recovery after processing. An aliquot fraction was saved for total phospholipid content determination. The rest was chromatographed by two-dimensional, thin-layer chromatography as previously described (8). Individual phospholipids were eluted from silical gel by the Bligh and Dyer method (5). Phosphatidylcholine (PC) was divided into three aliquots: one for disaturated phosphatidylcholine (DSPC) determination by the osmium tetroxide method of Mason et al. (32), another for phosphorus assay, and the third for determination of recovery. Recovery was calculated from radioactivity of aliquot fractions of total phospholipids, PC and DSPC, determined by scintillation counting (Dynagel, Baker Chem., Deventer, Holland). It was assumed that recovery rates of other individual phospholipids were the same as that of total PC. All solvents of analytical grade were provided by Prolabo.

Morphological studies

Lung explants taken at random at the start and at different times (*see* Results) of the culture period were fixed according to Hirsch and Fedorko (25) in a mixture of 2 vol of osmium tetroxide (1% in cacodylate buffer, 0.1 *N*, pH 7.4) and 1 vol of glutaraldehyde (2% in the same

TABLE 2

CHANGES IN PHOSPHOLIPID CONCENTRATION (nmolPi/mg Tissue, whole tissue analysis) IN LUNG EXPLANTS FROM 19.5 D-OLD FETUSES CULTIVATED FOR 48 H EITHER ON FLUID OR SEMISOLID MEDIUM[a]

	Initial Content	Fluid Medium	Solid Medium
Total phospholipids			
Waymouth		12.2 ± 1.9(5)	23.6 ± 2.0(3)(5)
CMRL 1066	10.6 ± 0.8	14.9 ± 0.6(2)	19.9 ± 2.3(3)
RPMI 1640		12.9 ± 1.3	14.6 ± 0.8(2)
Total PC			
Waymouth		6.8 ± 0.2(2)(5)	9.7 ± 0.9(3)(5)
CMRL 1066	4.7 ± 0.5	6.8 ± 0.5(2)(5)	9.3 ± 0.6(3)(5)
RPMI 1640		6.1 ± 0.5(1)[b]	6.1 ± 0.4(1)
DSPC			
Waymouth		3.0 ± 0.2(6)	6.4 ± 1.0(3)(6)
CMRL 1066	2.6 ± 0.4	4.2 ± 0.1(2)(4)[c]	6.0 ± 1.0(3)(4)
RPMI 1640		2.3 ± 0.3	2.8 ± 0.5
PG			
Waymouth		0.12 ± 0.02(1)(6)	0.67 ± 0.14(3)(6)
CMRL 1066	0.06 ± 0.02	0.13 ± 0.02(1)(5)	0.59 ± 0.11(3)(5)
RPMI 1640		0.14 ± 0.03(1)(5)	0.40 ± 0.08(3)(5)

[a]Mean ± SEM on 10 culture experiments.
[b]Significant difference as compared with initial content for: (1) $P<0.05$; (2) $P<0.01$; (3) $P<0.001$.
[c]Significant difference between fluid and solid media for: (4) $P<0.05$; (5) $P<0.01$; (6) $P<0.001$.

buffer). Tissues were then dehydrated in graded acetone and embedded in Epon (Serva, Heidelberg, FRG). Thin sections were prepared with an LKB ultramicrotome, stained with uranyl-acetate and lead citrate, and examined under a Hitashi H S9 electron microscope. Chemicals were provided by Merck, Darmstadt, FRG.

Statistical methods

Means of the different experimental series were compared by Student's t test for unrelated values. A threshold of $P < 0.05$ was considered for statistical significance.

RESULTS

In the first step, nature of substratum was investigated with only three different media, namely Waymouth MB752/1, CMRL 1066, and RPMI 1640. Glycogen storage, phospholipid accumulation in whole tissue, and morphologic appearance of cells were successively examined.

Table 1 summarizes the results for glycogen storage. With Waymouth's medium, glycogen concentration increased in explants placed on filters in fluid medium, but not on semisolid medium. With CMRL 1066, no change was observed, either with fluid or semisolid medium. With RPMI 1640, glycogenolysis occurred in both conditions but was more especially marked on solid medium. As a whole, fluid medium seemed to be favorable for glycogen accumulation in fetal lung in vitro during the period when glycogen storage is occurring in vivo. Fluid medium was therefore used for further screening analysis of media concerning their ability to support glycogen storage.

Concentration of total phospholipids, total PC, DSPC, and phosphatidylglycerol (PG), the most representative phospholipids of surfactant (39), was measured in whole lung explants. They all seemed to increase strongly on semisolid substrata with all three media (Table 2), except for DSPC in RPMI 1640. By contrast, changes with fluid media were either less marked or absent. Semisolid medium containing agar was therefore used for further screening analysis of media concerning their ability to support surfactant accretion.

Morphologic study in electron microscopy (Fig. 1 and 2) was limited to Waymouth's medium, the one most often used in previous studies dealing with in vitro lung differentiation. Cultures starting from Day 17.5 were maintained for 72 h. In explants cultured on filters in liquid medium, necrosis of mesenchymal cells occurred to a variable extent, whereas epithelium appeared healthy (Fig. 1 *a*). Ultrastructure of epithelial cells was normal (Fig. 2 *c*) except for the structure of lamellar bodies, which appeared loose, i.e. devoid of usual tight laminar substructure as shown in Fig. 2 *c'*. In explants maintained on semisolid medium, mesenchymal cells seemed healthy (Fig. 1 *b*) and type II pneumocytes seemed very similar to those in vivo at the corresponding developmental stage (gestational Day 20.5); numerous lamellar bodies of normal aspect were seen (Fig. 2 *d*).

Comparison of the nine selected media was successively analyzed for glycogen storage and phospholipid accumulation in tissue. For glycogen storage (data summarized in Fig. 3), the only medium allowing some glycogen accumulation was Waymouth MB752/1. However, the 30% increase achieved in vitro was far from reproducing the 300% increase which occurs during the same time in vivo (7). No change occurred, i.e. the initial concentration was maintained, in explants cultivated in MEM, CMRL 1066, NCTC 109, and Ham's F12. Glycogenolysis occurred to variable extent in other media, the largest being observed in RPMI 1640. It should be mentioned that in parallel experiments designed for other purposes, when

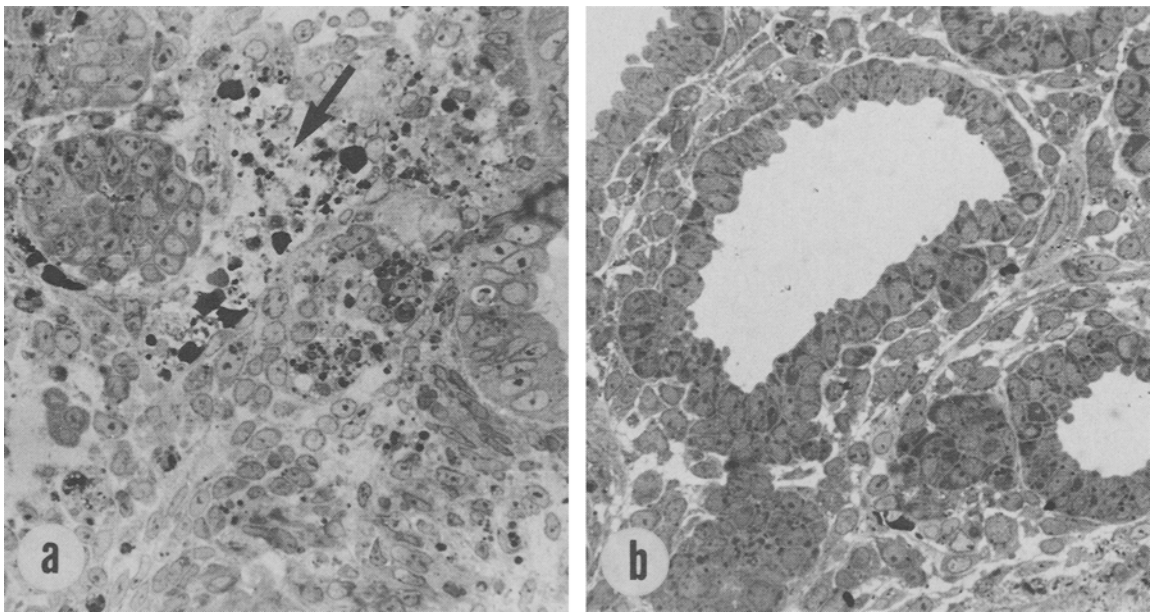

FIG. 1. Morphologic aspect of fetal rat lung explants cultivated on *a*, filter and fluid Waymouth MB 7752/1 or *b*, semisolid (agar gel) Waymouth MB 752/1. Semithin sections observed in light microscopy, final magnification ×1500. Note necrosis of mesenchyme (*arrow*) with fluid medium and its healthy appearance with gel medium.

tissue was explanted at 19.5 d of gestation, i.e. the stage when glycogen breakdown is starting in vivo, glycogenolysis occurred in all media, including Waymouth's, with a slightly faster rate than that in vivo (data not shown).

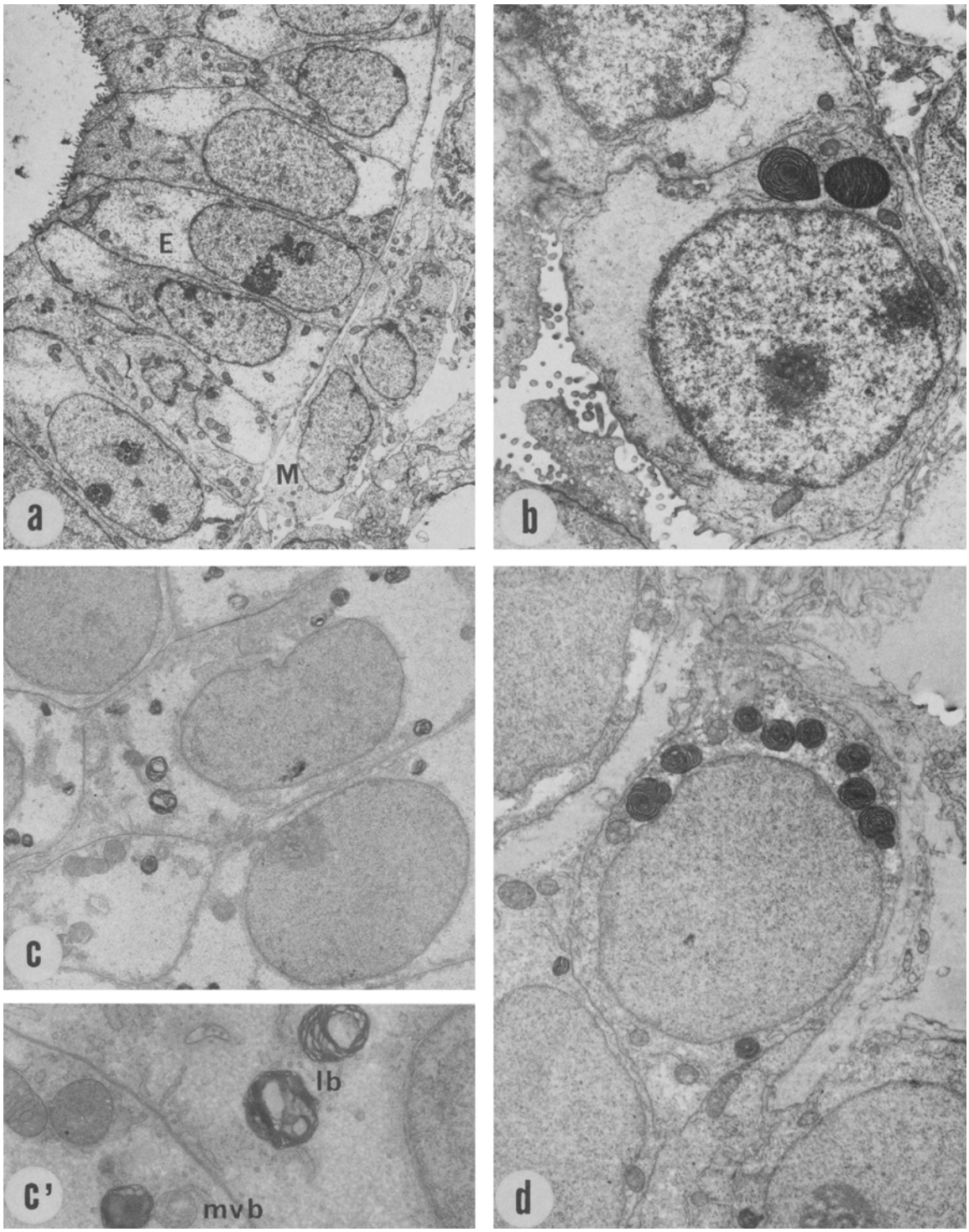

FIG. 2. Morphologic aspect of fetal rat lung explants cultivated on filter and fluid Waymouth or on semisolid agar-gel Waymouth for 72 h from 17.5 d of gestation and comparison with in vivo corresponding stages. Observation in electron microscopy. *a*, Initial state (17.5 d-old rat fetus). *E* = undifferentiated epithelium; *M* = mesenchymal cell. ×2381. *b*, Differentiated type II cell in the lung of 20.5 d-old rat fetus, in vivo stage corresponding to cultivated tissue. ×7143. *c*, Culture on filter with fluid medium. The general aspect of epithelium is normal except for the altered structure of lamellar bodies. Note abundance of glycogen. ×4762. *c'* = higher magnification ×14 286. *lb* = lamellar bodies; mvb = multivesicular body. *d*, Culture on semisolid medium. Note the similarity with in vivo aspect (*b*) of this differentiated type II pneumocyte. ×7143.

Phospholipids of S and R fractions were referred to tissue wet weight (because subfractionation is performed on wet tissue) and DNA. Changes in DNA content during culture were not statistically significant, except in RPMI 1640 which induced an increase (Table 3). S fraction (Table 3 and Fig. 4) was very low at the time of culture initiation. It increased markedly in all media. However, three groups of media could be distinguished:

Group 1 (MEM, Dulbecco MEM, Waymouth MB752/1, CMRL 1066, and NCTC 109). In these media, all phospholipids of S fraction reached the same concentrations in vitro as those previously observed on corresponding gestational age in vivo, i.e. 21.5 d (6). The increase was about 12- to 14-fold as compared with initial content for entire S fraction as well as for different individual phospholipids. Phosphatidylglycerol, however, increased about 30-fold.

Group 2 (Ham's F10 and F12). The increase was about half that in media of group 1, i.e. about a six fold increase.

Group 3 (RPMI 1640 and M199). The increase was only about half that in group 2 and one fourth that in group 1, i.e. a threefold increase.

R fraction changes (Table 3) were less striking in culture than those in S fraction. Total phospholipids and total PC only were determined. Media of group 3, which allowed the lowest accretion of S fraction, were also those that gave the smallest increase of R fraction phospholipids. However, the differences with other media were less marked than for S fraction. Total phospholipids of R fraction increased about 1.8 times in media RPMI 1640, M199, and NCTC 109, and 2.2 to 2.5 times in other media.

The study of in vitro morphologic differentiation of lung cells was limited to two media, one from group 1 (Waymouth MB 752/1) and one from group 3 (RPMI 1640). Fetal lung was explanted on Day 16.5 and observed after different culture durations. In Waymouth's medium, differentiation of type II pneumocytes occurred earlier than expected from normal in vivo timing; lamellar bodies were already observable in epithelial cells after 24 h in vitro, i.e. 48 h in advance as compared to normal stage of differentiation in vivo (Fig. 5 *a*). This was never seen in medium RPMI 1640 in which lamellar bodies were observed only after 96 h (equivalent to 20.5 d) and exhibited an abnormal structure often similar to lysosomal structures (data not shown). Furthermore, cells in mesenchyme differentiated to lipofibroblasts with characteristic lipid droplet inclusions after 72 h in Waymouth MB752/1 (Fig. 5 *b*) but not in RPMI 1640.

For testing the role of atmospheric composition, explants were cultivated on semisolid Waymouth MB752/1 and CMRL 1066 media in presence of 95% oxygen:5% carbon dioxide. As compared with results obtained with the ternary gas mix, total phospholipids, but not PC DSPC, and PG, were slightly lower in S fraction, and total phospholipds, but not PC, were decreased in R fraction in both media (Table 4); DNA content was not modified (Table 4). As a whole, presence of high oxygen concentration seemed less favorable than air for phospholipid biosynthesis, but the difference was small.

Discussion

The results reported here characterize fetal lung maturation in vitro at a specific and sensitive biochemical level, namely, surfactant fraction isolation and analysis. The surfactant fraction analysis provides advantage in allowing greater ability to detect changes in phospholipids associated with lung maturation than does whole tissue analysis (36). Furthermore, the surge in culture of a large surfactant fraction with normal phospholipid composition when compared with in vivo development (6) demonstrates that an authentic maturation of type II pneumocytes can occur in vitro, contrary to previous assumption (3). Disaturated PC content of S fraction after cultivation was similar, even a little higher, than that determined at a corresponding stage in vivo (6). By contrast, in a recent study (29), DSPC was not increased in lamellar bodies extracted from cultivated fetal rabbit lung explants. It is difficult, however, to compare both studies, because of the species difference and that 7 d of culture were used for fetal rabbit experiments.

In the present study, glycogen storage in vitro was investigated because of the close temporal relationship between utilization of fetal lung glycogen stores and production of surfactant phospholipids (7,8,31). More specifically, glycogen content increases until Day 20 of gestation, then decreases precipitously as the rate of phospholipid biosynthesis is enhanced (7,31). A

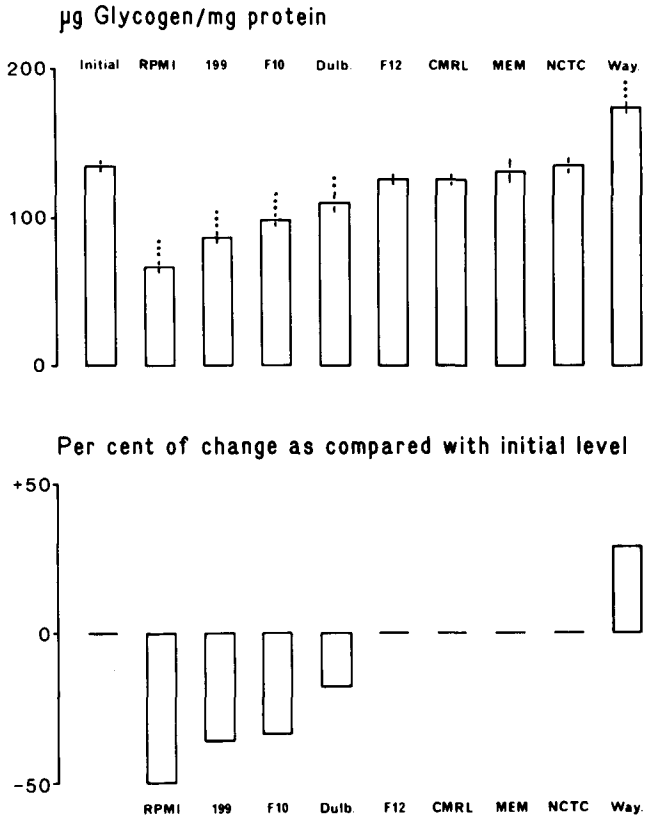

FIG. 3. Changes in glycogen concentration in fetal lung explanted at 17.5 d of gestation and cultivated for 48 h (fluid medium) on nine selected culture media. Increased glycogen content was seen only with Waymouth's medium.

TABLE 3

DNA CONTENT AND CONTRAST BETWEEN CHANGES IN PHOSPHOLIPID CONCENTRATIONS IN THE SURFACTANT FRACTION AS COMPARED TO THE RESIDUAL FRACTION EXTRACTED FROM FETAL LUNG EXPLANTS FROM 19.5 D-OLD FETUSES CULTIVATED FOR 48 H ON NINE DIFFERENT MEDIA (SEMISOLID)[a]

	Initial Content	MEM	Dulbecco	NCTC	Waymouth	CMRL	F10	F12	M199	RPMI
DNA (μg per mg tissue)	6.3 ± 0.3	7.1 ± 0.3	7.5 ± 0.7	7.7 ± 0.6	7.7 ± 0.6	7.3 ± 0.7	6.7 ± 0.8	6.8 ± 0.4	7.1 ± 0.6	9.7 ± 0.7
S fraction TPL	0.51 ± 0.01	7.06 ± 1.21	6.90 ± 1.10	7.00 ± 1.20	6.22 ± 0.59	5.60 ± 0.57	3.06 ± 0.66	3.41 ± 0.32	1.92 ± 0.22	1.76 ± 0.36
S fraction PC (nmol Pi/mg tissue)	0.33 ± 0.04	4.60 ± 0.90	4.20 ± 0.50	4.28 ± 0.66	3.75 ± 0.31	3.24 ± 0.49	1.94 ± 0.37	2.34 ± 0.33	1.16 ± 0.17	0.96 ± 0.16
R fraction TPL	8.0 ± 0.4	17.3 ± 1.8	18.4 ± 2.1	14.7 ± 1.1	20.2 ± 2.3	18.1 ± 2.8	16.0 ± 1.5	17.6 ± 2.6	14.4 ± 1.5	14.0 ± 1.1
R fraction PC (nmol Pi/mg tissue)	3.1 ± 0.5	8.0 ± 1.5	7.8 ± 1.1	8.3 ± 0.7	6.4 ± 1.0	6.5 ± 1.0	6.0 ± 0.7	8.1 ± 1.6	5.6 ± 1.0	5.4 ± 0.6

[a]Mean ± SEM on six culture experiments.

precursor-product relationship between fetal lung glycogen and lipids has been demonstrated previously in vitro (8). Culture on a filter with liquid medium seemed a better condition than culture on semisolid medium for glycogen synthesis (or at least for maintaining initial glycogen content). This might result from a better uptake of glucose by the explants in liquid medium. However, it seems that even with the most favorable medium, i.e. Waymouth MB 752/1, in vivo glycogen accumulation in developing fetal lung was not reproducible in vitro. Hormones are possibly needed for maintaining active glycogen accumulation. The ability of fetal lung to store glycogen in vitro seems age-dependent because it is glycogen breakdown that was observed when 19.5 d-old tissue was explanted on Waymouth's medium in parallel sets of experiments.

By contrast with glycogen-storage capacities, lung tissue seemed morphologically healthier and phospholipid biosynthesis was higher on semisolid medium than with fluid medium. This is possibly due to better oxygenation of cells or to better biophysical properties of substratum. Therefore, except if glycogen accretion is required, lung explants should be cultivated on a semisolid medium.

Comparison of ability of previously used media to support fetal lung maturation in vitro was a major goal of this study. Medium Trowell T8 which had been used in previous studies (19,27) was excluded because it contains insulin, a hormone that has been demonstrated to delay lung maturation in vitro (20,44). The reasons for behavorial differences of lung explants depending on the medium are not clear when composition of media are compared. Waymouth MB 752/1 was the only medium to allow some glycogen accretion in vitro. This cannot be accounted for by the major compositional difference of this medium, i.e. its higher glucose concentration, because for this study glucose concentration was adjusted to the same level in all nine media. The higher content in some amino acids or in several cofactors like choline chloride or thiamine in Waymouth's medium may be involved.

Regarding the ability to support surfactant biosynthesis, media could be divided into 3 groups, but comparison of compositions within each group or between groups does not bring clear insights. Despite the evident precursor role of choline chloride for PC biosynthesis, choline chloride concentration does not seem to be a limiting factor, inasmuch as CMRL 1066, which has the lowest concentration, and Waymouth MB 752/1, which has the highest (500 times more), gave similar results for PC and DSPC accretion in S fraction as well as in R fraction.

The capacity of a given medium to support surfactant biosynthesis does not seem to correlate with its complexity, because MEM, the simplest medium, belongs to group 1. Presence of nucleotides and their precursors (CMRL 1066 and NCTC 109) seems to have no influence on phospholipids nor on DNA. In some instances, complexity could even be a negative factor: the presence of metallic ions (Cu, Fe, Zn) in Ham's media only was

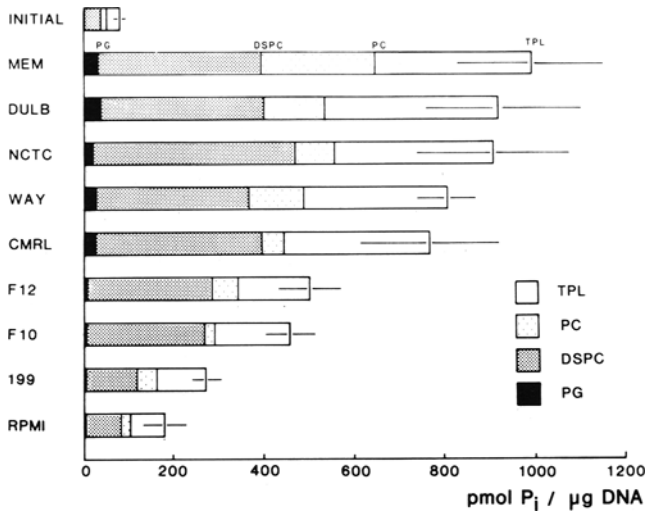

FIG. 4. Changes in phospholipid concentrations (pmol Pi/µg DNA) in surfactant fraction extracted from fetal lung explants from 19.5 d-old fetuses cultivated for 48 h on nine selected semisolid media. Mean ± SEM on six culture experiments. Abbreviations: TPL = total phospholipids, PC = total phosphatidylcholine, DSPC = disaturated phosphatidylcholine, PG = phosphatidylglycerol. For clarity of the figure, SEM is given only for TPL. All changes occurring in culture were significant as compared with initial contents.

possibly related to the comparatively lower storage of surfactant by tissue in these media than in those of group 1.

Concerning group 3, M199 is rather deficient in vitamins and cofactors, but this is not true for RPMI 1640 which gave similar results. It should be emphasized that MEM and RPMI 1640, which gave different results, have somewhat similar compositions. The major differences are a lower concentration of tryptophan, presence of *p*-amino benzoic acid and much higher concentration of inositol in RPMI 1640. Inositol concentration in this latter is the highest among the nine media. Taking into account the role of inositol in acidic-phospholipid biosynthesis (23) and the role of inositol and phosphatidyl-inositol in membrane signal transduction functions (4,34), a toxic or at least a negative effect of excessive inositol cannot be ruled out.

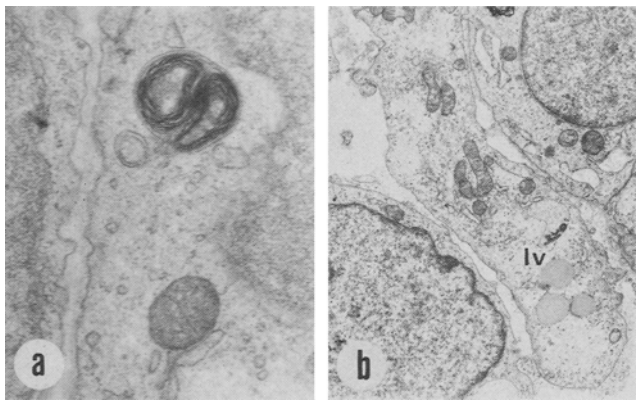

FIG. 5. In vitro differentiation of fetal lung cells. (EM study). *a*, 16.5 d-old fetal lung cultivated for 24 h on Waymouth's medium. Lamellar bodies have appeared in epithelial cells 48 h before the stage when they normally do so in vivo. ×21 429. *b*, 16.5 d-old fetal lung cultivated for 72 h on Waymouth's medium. Some mesenchymal cells have differentiated into lipofibroblasts with characteristic lipid-droplet inclusions (*lv* = lipid vacuoles). ×5357.

TABLE 4

DNA CONCENTRATION (µg/mg tissue) AND PHOSPHOLIPID CONCENTRATIONS (nmol Pi/mg tissue) IN SURFACTANT AND RESIDUAL FRACTIONS EXTRACTED FROM FETAL LUNG EXPLANTS FROM 19.5 D-OLD FETUSES CULTIVATED FOR 48 H ON SEMI-SOLID WAYMOUTH OR CMRL 1066 MEDIA UNDER AN ATMOSPHERE OF 0_2 95%:CO_2 5%[a]

	Waymouth MB752/1	CMRL 1066
DNA	7.3 ± 0.5	5.8 ± 0.4
S Fraction		
Total phospholipids	4.64 ± 0.29[b]	3.80 ± 0.48[b]
Total PC	3.04 ± 0.17	2.57 ± 0.36
DSPC	2.51 ± 0.13	2.15 ± 0.27
Lyso PC	0.03 ± 0.01	0.04 ± 0.01
PG	0.10 ± 0.02	0.09 ± 0.02
PI	0.20 ± 0.02	0.20 ± 0.03
R Fraction		
Total phospholipids	13.0 ± 0.5[b]	11.0 ± 0.1[b]
Total PC	7.0 ± 0.5	5.3 ± 0.2

[a]Mean ± SEM on five culture experiments.
[b]Significant difference with explants cultivated under N_2 76%:O_2 19%:CO_2 5% for $P<0.05$.

To summarize, the ability to support surfactant biosynthesis in vitro seems to be the consequence of a complex equilibrium between medium components rather than the presence or absence of a definite constituent.

Electron microscopic analysis of tissue explanted on Day 16.5 of gestation permitted observation of accelerated type II cell differentiation in vitro. This observation in the rat agrees with previous experiments performed with human fetal lung obtained after abortion (33,45). However, the advantage of the present study is that unstressed fetuses were rapidly removed and their lungs processed for culture immediately. It is of particular interest that precocious development occurred without exposure to hormones in vitro. This raises the issue about factors controlling the timing of differentiation in vivo. It seems that total information for differentiation was present early in the organ itself but could not be expressed before a definite stage. The present study does not answer this specific problem, but lung organ culture seems a suitable model to study this question. It should be pointed out that this precocious morphologic differentiation only occurred in a medium supporting a large surfactant accretion in vitro. Therefore a good correlation exists between morphologic maturation and surfactant content of the tissue. The healthy appearance

of connective tissue in Waymouth MB752/1 and its decay in RPMI 1640 is consistent with the putative crucial role of epithelial-mesenchymal interrelationships for pneumocyte differentiation (27). Although the present study does not provide quantitative morphologic data for supporting this hypothesis, observations reported here suggest that Waymouth MB 752/1 allowed improved surfactant biosynthesis at least partly because it maintained the mesenchymal cell population.

Composition of atmosphere above the explants does not seem to be a crucial factor for surfactant biosynthesis, although the slightly lower accretion of total phospholipids was probably accounted for by some toxicity of oxygen in the O_2-CO_2 mix. This is consistent with previous observation (18). On the other hand, the present results disprove the previous assumption (11) that in vitro maturation of lung explants could be an effect of oxygen toxicity.

The present work points out the importance of choosing medium for studying type II pneumocyte maturation in vitro, an aspect that was largely neglected in previous studies. The results also permit the recommendation of optimal conditions for surfactant biosynthesis in lung organ culture: semisolid medium, use of a medium of group 1, and an atmosphere composed of carbon dioxide-enriched air.

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