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METHOD FOR CULTURE OF A BOVINE PULMONARY ENDOTHELIAL CELL STRAIN (CPAE) IN A SERUM-FREE MEDIUM AND ON MICROPOROUS MEMBRANES COATED WITH MATRIGEL™

by

SARAH BAATOUT (1), FABIENNE KINARD **, CLAUDE REMACLE **
and YVES-JACQUES SCHNEIDER *
* Laboratory of Cellular Biochemistry and ** Laboratory of Cell Biology,
University of Louvain, B-1348 Louvain-la-Neuve, Belgium

SUMMARY

In order to optimize culture conditions of bovine pulmonary endothelial cells (CPAE), we have compared different culture media, supports and extracellular matrices. Cell biomass was estimated by protein assay using Lowry's method. Different constituents including albumin, hydrocortisone, insulin, transferrin, triiodotyronine, Epidermal Growth Factor (EGF) and Fibroblast Growth Factor were separately added to Basal Defined Medium (BDM). Among them, BDM supplemented with EGF (1 ng/ml), hydrocortisone (100 nM), insulin (1 µg/ml), triiodotyronine (2 nM) and linoleic acid complexed to albumin (10 µg/ml) also named 'synthetic BDM' appeared to be the best serum-free nutritive medium and showed similar results as compared to DMEM supplemented with 10 % Fetal Bovine Serum (FBS).

Several supports have then been tested including tissue culture polystyrene (as a reference), teflon, polycarbonate or poly(ethylene terephthalate) track-etched membranes. Among them, cells cultivated on surface treated membranes in poly(ethylene terephthalate) exhibited the highest protein content with a significant increase in comparison to tissue culture polystyrene, probably because cells are fed on the two faces instead of one.

On treated poly(ethylene terephthalate) membranes, cells kept their endothelial morphology and ultrastructure.

Finally, cell biomass on several exogenous extracellular matrices was studied. Cells were cultivated in 'synthetic BDM' or DMEM supplemented with 10 % FBS and on poly(ethylene terephthalate) membranes. Among fibronectin, matrigel™ (solubilized tissue basement membrane), laminin, collagen (type I) and polylysine; matrigel™ appeared to be the optimal extracellular matrix.

(1) Correspondence to S. BAATOUT, Laboratory of Experimental Hematology and Oncology, Catholic University of Louvain, 1, rue G. Thérasse, B-5530 Mont-Godinne, Belgium.

In conclusion, we demonstrated herewith that bovine pulmonary endothelial cell cultures can be optimized in a serum-free medium and on microporous membranes with matrigel™ as an extracellular matrix without alterations of cell morphology.

Key words : CPAE cells, in vitro model, serum-free medium, microporous membranes, poly(ethylene terephthalate), extracellular matrix, matrigel.

INTRODUCTION

Alterations in the function of endothelial cells play an important role in the development of atherosclerosis (Ross *et al.*, 1974 ; Ross and Glomset, 1976). Cultivated endothelial cells provide a powerful approach with which to study the characteristics and function of the endothelium. However, the presence of serum in the nutritive medium hampers the precise determination of the effect of various components on their function. We have developed a new serum-free, chemically defined, Basal Defined Medium (BDM) which considerably increases the secretion of monoclonal antibody by hybridoma cells (Schneider, 1989 ; Schneider and Lavoix, 1990) ; with a low protein content it also allows the long-term (3 to 4 weeks) culture of rat hepatocytes (Jin *et al.*, 1989) or intestinal epithelial cells (Halleux and Schneider, 1991). Equally important is the nature of the support upon which cells rest. We have recently reported that the use of poly(ethylene terephthalate) microporous membranes improves the culture of various mammalian cells (Sergent-Engelen *et al.*, 1990). Moreover, the deposition of an extracellular matrix on the surface of the supports is necessary for the optimal growth of many adherent cells. Several kinds of coating have been proposed such as laminin, fibronectin, collagen (type I), polylysine or a solubilized tissue basement membrane gel matrigel™. These components have been shown to affect the ability of cells to proliferate (Timpl *et al.*, 1979 ; Jaffe, 1980 ; Kleinman *et al.*, 1981 ; Chazou *et al.*, 1981 ; Macarak and Howard, 1983).

Herewith, in order to optimize culture conditions of bovine pulmonary endothelial cells (CPAE), we present data about the effects of several components of the medium, different supports and several exogenous extracellular matrices on the biomass of bovine pulmonary artery endothelial (CPAE) cells. Based on these observations, we have developed a cell culture system using serum-free medium, poly(ethylene terephthalate) microporous membranes and matrigel™ as an exogenous extracellular matrix to grow and maintain endothelial cells under defined conditions.

Abbreviations used : BDM, Basal Defined Medium ; CPAE, pulmonary artery endothelial cells ; DMEM, Dulbecco's Modified Eagle's Medium ; EGF, Epidermal Growth Factor ; FBS, Fetal Bovine Serum ; FGF, Fibroblast Growth Factor ; Hydrocortis, Hydrocortisone ; Lin ac : alb, linoleic acid complexed to albumin ; mPET, membranes in Poly(ethylene terephthalate) ; nPC, native Polycarbonate ; nPET, native Poly(ethylene terephthalate) ; PET, Poly(ethylene terephthalate) ; sBDM, synthetic Basal Defined Medium ; T3, Triiodotyronine ; TCPS, Tissue Culture Polystyrene ; tmPET, treated membranes in Poly(ethylene terephthalate).

MATERIAL AND METHODS

Cell culture and subculture

The bovine pulmonary artery endothelial cell strain (CPAE) was obtained from the American Type Culture Collection (Rockville, Md). This cell strain has been characterized and shown to be endothelial in origin by angiotensin-converting enzyme activity, factor VIII related antigen and the presence of Weibel-Palade bodies (DEL VECCHIO and SMITH, 1981 ; DEL VECCHIO and LINCOLN, 1983). CPAE cells were maintained in 175-cm^2 flasks. For all experiments, cells were used between passages 11 and 20 and cultivated under water saturation and 5 % CO_2 (v/v) in air. CPAE cultures have been passed at a split ratio of 1:3 or 1:2 onto tissue culture polystyrene (Nunc, Intermed, Denmark). Daily phase contrast observations of morphology were used for assessing the culture state. Cell viability was assessed using trypan blue exclusion.

Culture medium

Basal Defined Medium (BDM ; SCHNEIDER, 1989) consists of a 5:5:1 (v/v/v) mixture of Iscove's Dulbecco's modified Eagle's, Ham's F12 and NTCT 135 media. To this, 25 mM glucose, 6 mM glutamine, 0.01 % (w/v) Pluronic F68, 50 µM ethanolamine, 25 mM HEPES, 3 g/l $NaHCO_3$ and 30 µg/ml penicillin and 70 µg/ml streptomycin were added. Routine culture was carried out in BDM supplemented with 1 % Fetal Bovine Serum (Gibco).

For some experiments, BDM was further supplemented with different substances : linoleic acid complexed to albumin I at two different concentrations (10 and 100 µg/ml, Gibco), insulin (1 µg/ml, Gibco), EGF (10 ng/ml, Gibco), FGF (50 ng/ml, Gibco), transferrin (10 µg/ml, Gibco), triiodotyronine or T3 (2 nM, Gibco), hydrocortisone (100 nM, Gibco), trace elements (Mn, Mo, Ni, Si, Sn and V) at the concentrations described by MCKEEHAN *et al.*, (1976). 'Synthetic BDM' (sBDM) corresponds to BDM supplemented with insulin, EGF, trace elements, ascorbic acid, alpha tocopherol, glucagon, prolactin, somatotropin, dexamethasone and linoleic acid complexed to albumin as previously used for rat hepatocytes (JIN *et al.*, 1989). Dulbecco's Modified Essential Medium (DMEM) supplemented with 10 % FBS was used as reference.

Biomass study by protein assay

Cells were harvested with trypsin (2.5 g/l) and EDTA (1.1 g/l) and seeded in each culture condition at an initial concentration of 50,000 living cells/cm^2. After different incubation periods at 37° C, cells were washed 3 times with PBS and dissolved twice in 1 ml of Na deoxycholate 1 % (w/v) previously adjusted to pH 11.3 with NaOH. Cell protein content was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard. Results are expressed in micrograms per cm^2 of growth area.

Preparation and coating of flasks

Cell cultures were carried out on different supports. For tissue culture polystyrene (TCPS), 6-well tissue culture plates (Nunc, Intermed, Denmark; growth area of 9.9 cm^2) were used. Polycarbonate and poly(ethylene terephthalate) exist as films (native polycarbonate nPC and native poly(ethylene terephthalate) nPET), as microporous (poly(ethylene terephthalate) membranes mPET or as treated poly(ethylene terephthalate) membranes tmPET. m and tmPET membranes were kindly provided by Whatman s.a. (Louvain-la-Neuve, Belgium). Inserts were prepared as previously described (HALLEUX and SCHNEIDER, 1991). Teflon flasks (Petriperm) were provided by Heraeus (Danau, FRG).

To assess the effect of exogenous extracellular matrices, culture substrata were precoated for 2 h at 37°C with laminin (10 µg/ml, Sigma), fibronectin (10 µg/ml, Gibco), polylysine (10 µg/ml, Sigma), type I collagen (30 µg/ml, Sigma) or matrigel™ (5 µl diluted 10 times in BDM, Flow Lab., Brussels, Belgium) and rinsed with PBS just before use.

Scanning electron microscopy

For ultrastructural studies, monolayers growing on microporous membranes were washed twice in PBS, fixed *in situ* with 2.5 % (w/v) glutaraldehyde in PBS for 1 h and rinsed twice with PBS, postfixed with osmium tetroxide (2 % w/v in PBS) for 1 h and rinsed 3 times with PBS. Samples were dehydrated with acetone in a critical point dryer (Balzers Union, Lichtenstein) and sprayed with gold (Balzers SCD040) before examination with a Hitachi S570 scanning electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

ANOVA 2 analysis and Newman-Keuls test were used. Results are expressed as the mean of 6 experiments ± SEM.

RESULTS

Effect of different medium components on CPAE biomass

The effect of different components classically added to nutritive medium on CPAE biomass was assessed by determining the protein content and compared to BDM, on one hand, and to DMEM supplemented with 10 % FBS, on the other hand (Fig. 1). When cells were cultivated in BDM supplemented with linoleic acid complexed to albumin, biomass was in the same order of magnitude as cells grown in the presence of BDM alone. Insulin and transferrin were found to be slightly mitogenic since biomass was significantly increased in their presence ($p < 0.05$). The addition of EGF or FGF to BDM augmented significantly CPAE growth in comparison to cells cultivated in the presence of BDM supplemented with trans-

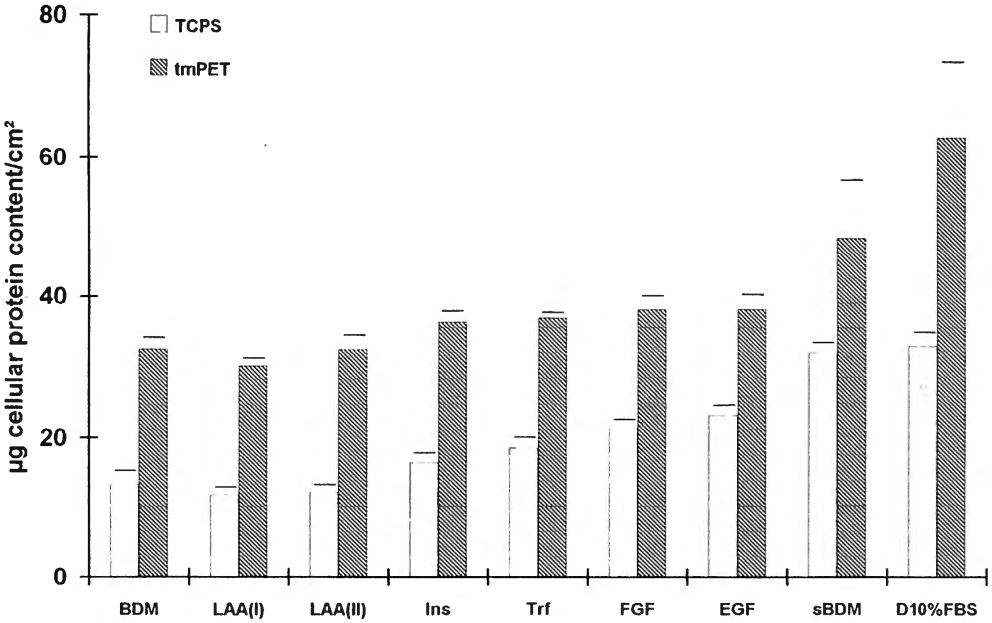

Fig. 1. — Influence of different components of the nutritive medium on the biomass of CPAE cells cultivated either on tissue culture TCPS or on tmPET. The final concentrations were linoleic acid complexed to albumin (10 µg/ml : LAAI; 100 µg/ml : LAAII), insulin (Ins : 1 µg/ml), transferrin (Trf : 10 µg/ml), FGF (50 ng/ml), EGF (10 ng/ml). Protein content (µg/cm^2) was measured after 36 h of culture. Synthetic BDM and DMEM 10 % FBS are abbreviated as sBDM and D10 % FBS, respectively.

ferrin or insulin ($p < 0.05$). The highest protein contents were obtained when the medium was either sBDM or DMEM supplemented with 10 % FBS ($p < 0.05$). DMEM was used as a control because this medium is known to be adequate for CPAE culture (GARCIA *et al.*, 1986).

In a second set of experiments, CPAE cells were cultivated in BDM but for each individual test, different components including some of the sBDM medium were added and a comparison was made with DMEM supplemented with 10 % FBS (Fig. 2). Results indicated that there was a significant difference between BDM alone and BDM supplemented with either triiodotyronine, trace elements, hydrocortisone or linoleic acid complexed to albumin ($p < 0.05$). EGF and insulin gave quantitatively similar results and were shown to be more effective than BDM supplemented with either triiodotyronine, trace elements, hydrocortisone or linoleic acid complexed to albumin ($p < 0.05$). Although there was a more important difference than in Fig. 1, the difference between sBDM and DMEM 10 % FBS was not significant. This difference can be explained by the fact that in Fig. 1, cells were at passage 12 and in Fig. 2, they were at passage 15.

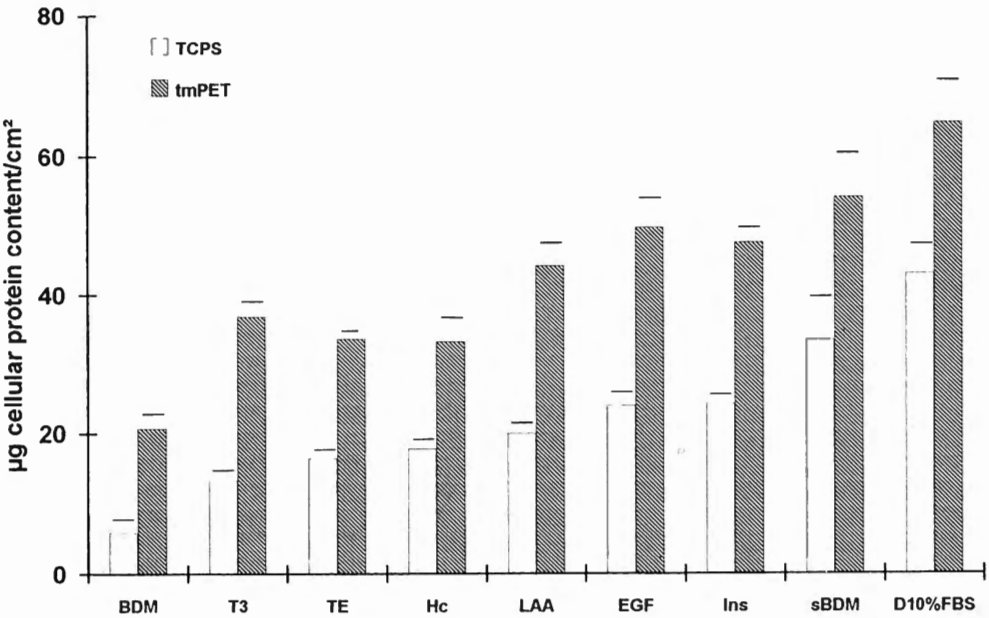

Fig. 2. — Influence of some components including sBDM constituents on the biomass of CPAE cells cultivated on TCPS or on tmPET. The final concentrations were hydrocortisone (Hc : 100 nM), linoleic acid complexed to albumin (LAA : 10 µg/ml), triiodotyronine or T3 (2 nM), insulin (Ins : 1 µg/ml), EGF (1 ng/ml). Protein content (µg/cm^2) was measured after 36 h of culture. Synthetic BDM and DMEM 10 % FBS are abreviated as sBDM and D10 %FBS, respectively.

Effect of different supports on CPAE growth

Figs 1 and 2 show that in every situation the cell yield was higher on microporous PET membranes than on TCPS dishes. Selective advantages of other substrata were investigated (Fig. 3) on CPAE cells at passage 17. Tissue culture polystyrene (TCPS) was used as reference. Teflon, polycarbonate (PC) or poly(ethylene terephthalate) (PET) in the form of film or microporous membranes were tested. Teflon gave the lowest protein content. No significant difference was detected when cells were cultivated either on TCPS, PC films or PET membranes. However, the PET films gave a significant higher protein content ($p < 0.05$). TmPET produced the highest CPAE biomass ($p < 0.05$). These results indicate that the use of tmPET considerably increased the protein content. Comparable results were observed for sBDM and DMEM supplemented with 10 % FBS.

Effect of different extracellular matrices on CPAE growth

To characterize the effect of different extracellular matrix proteins on the biomass of CPAE cells, protein assay measurements were performed on cells cultivated on fibronectin, matrigel™, laminin, type I collagen or polylysine. These

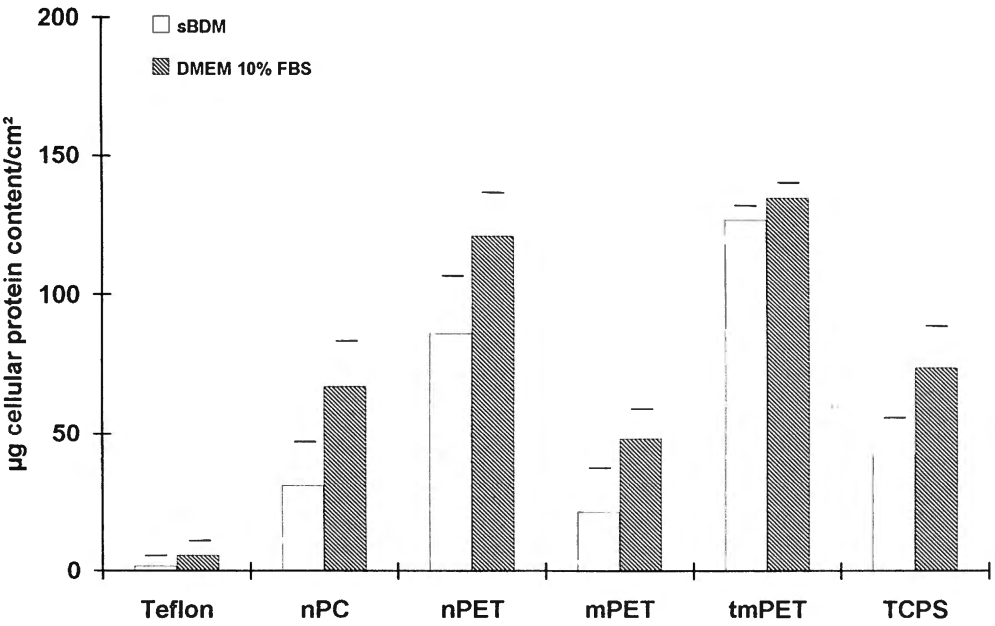

Fig. 3. — Comparison of CPAE cells biomass after 4 days of culture on different supports and either in sBDM or in DMEM supplemented with 10 % FBS. The supports were teflon membranes, polycarbonate (nPC) or PET (nPET) films, treated and nontreated PET membranes (tmPET or mPET, respectively) and tissue culture polystyrene (TCPS).

experiments were performed on TCPS (Fig. 4) and on tmPET (Fig. 5) and involved both sBDM and DMEM containing 10 % FBS. Extracellular matrices without cells were shown to exhibit a protein content which never exceeded 5 % of the total protein content.

When cells were cultivated on TCPS (Fig. 4) coated with laminin or matrigel™, no significant increase in protein content was detected in comparison to TCPS without precoating. A significantly enhanced protein content ($p < 0.05$) was detected with polylysine, fibronectin and collagen as compared to TCPS without precoating.

In comparison with noncoated tmPET, no significant increase was observed with fibronectin or laminin. In contrast, after precoating of tmPET with collagen or polylysine, a significant increase in protein content was observed as compared to noncoated tmPET ($p < 0.05$). The highest yield was recorded with matrigel™ as an exogenous extracellular matrix ($p < 0.05$).

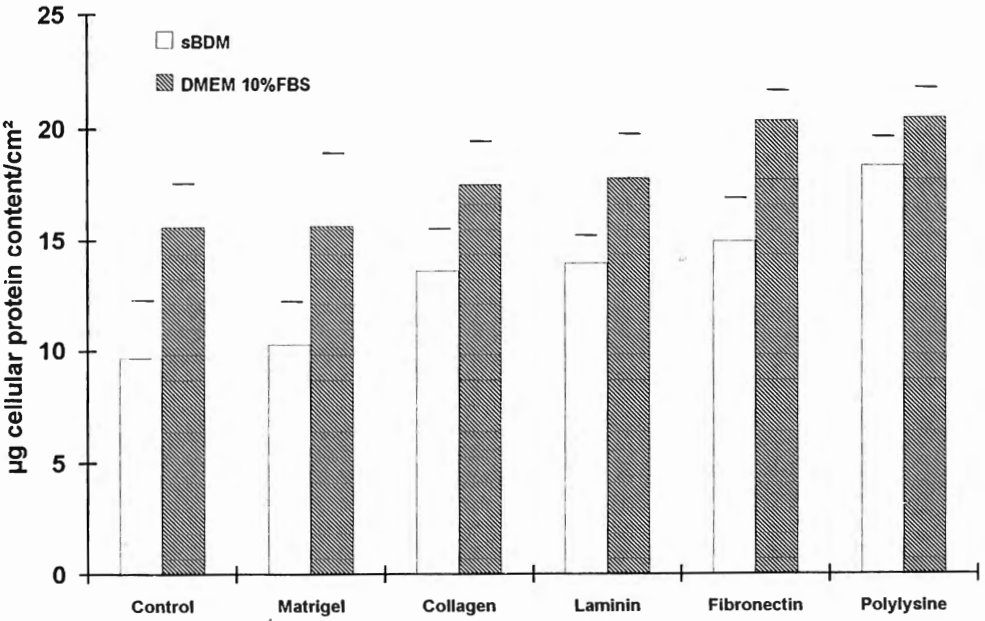

Fig. 4. — Comparison of CPAE cells biomass after a 36 h culture on TCPS precoated with different extracellular matrices and either in sBDM or DMEM supplemented with 10 % FBS. The used extracellular matrices were fibronectin (10 µg/ml), laminin (10 µg/ml), type I collagen (30 µg/ml), polylysine (10 µg/ml) and matrigel™ (dilution : 10 ×).

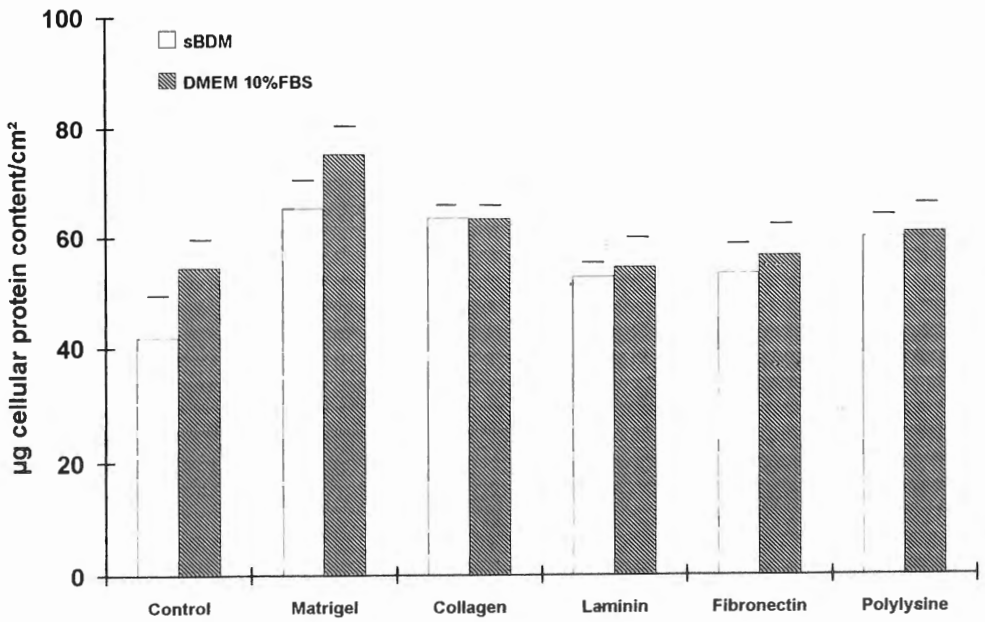

Fig. 5. — Comparison of CPAE cells biomass after a 36 h culture on tmPET precoated with different extracellular matrices, in either sBDM or DMEM supplemented with 10 % FBS. The used extracellular matrices were fibronectin (10 µg/ml), laminin (10 µg/ml), type I collagen (30 µg/ml), polylysine (10 µg/ml) and matrigel™ (dilution : 10 ×).

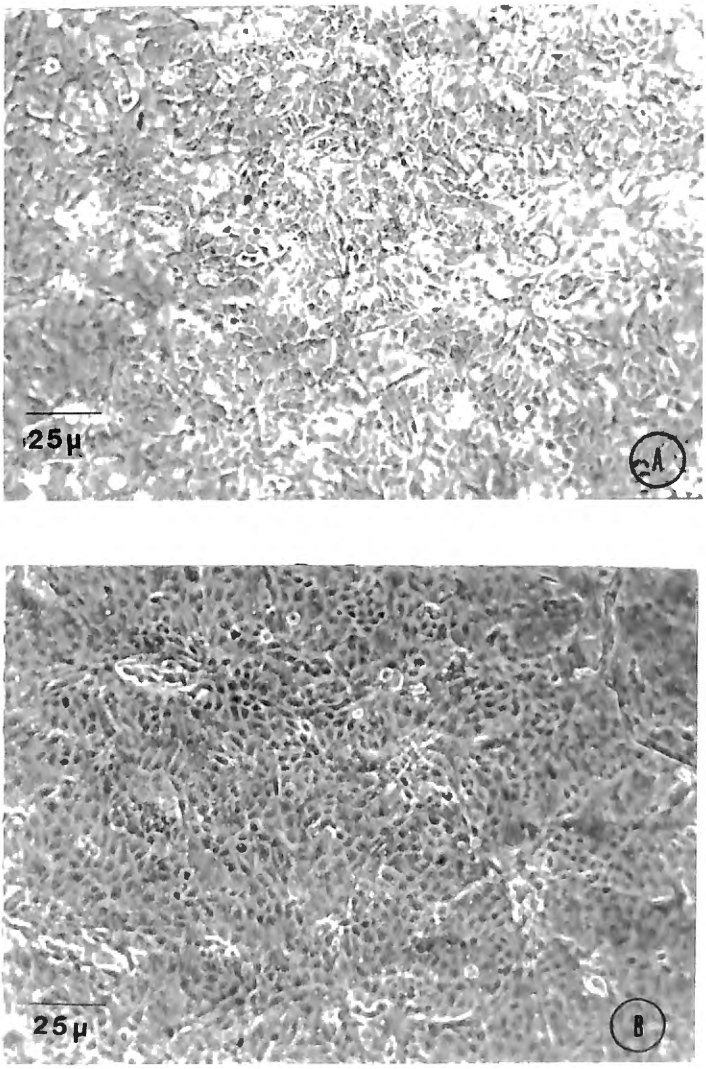

Fig. 6. — Phase contrast micrographs of CPAE cells cultivated for 5 days in BDM supplemented with 1 % FBS on TCPS (A) or tmPET (B).

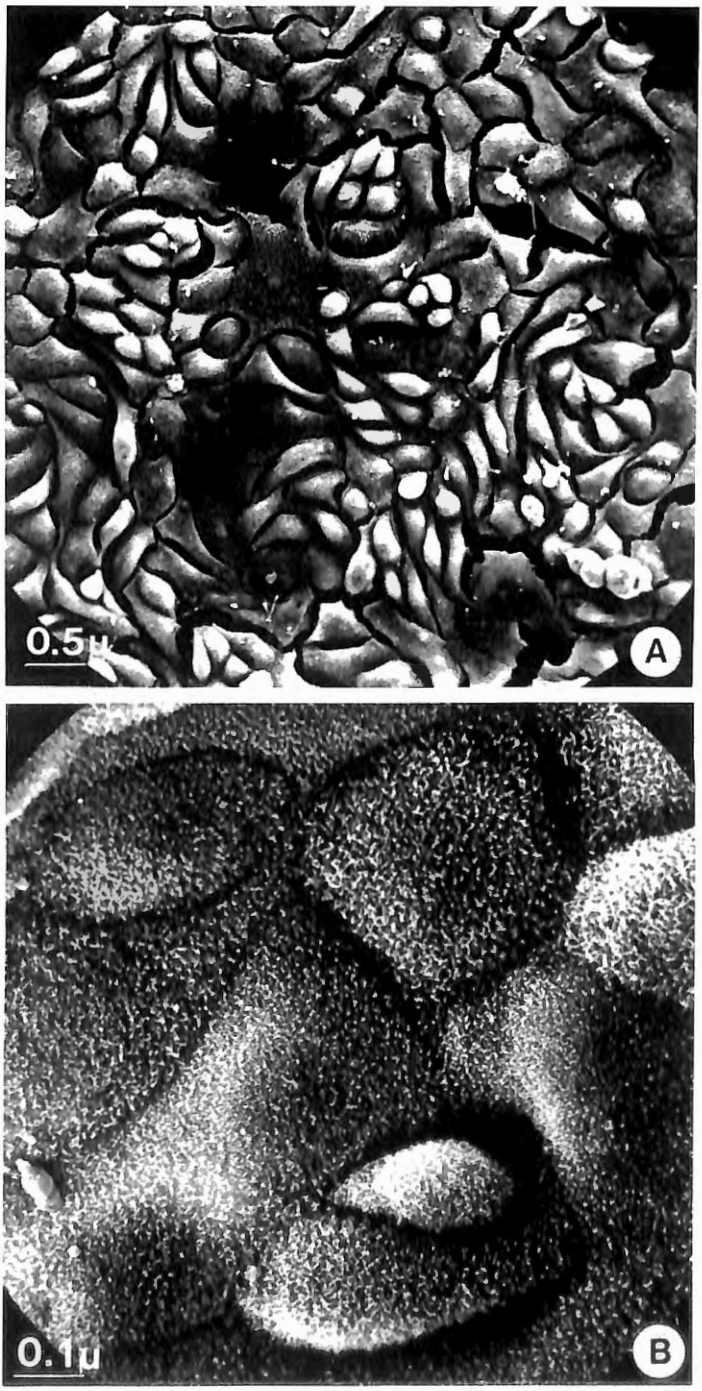

Fig. 7. — Scanning electron micrographs of CPAE cells cultivated for 5 days in BDM supplemented with 1 % FBS on tmPET.

Morphological and ultrastructural characterizations of CPAE cells on TCPS and on treated membranes of poly(ethylene terephthalate)

Fig. 6 gives representative pictures of CPAE cells after five days of culture in BDM containing 1 % FBS on TCPS (Fig. 6a) or tmPET (Fig. 6b). CPAE cells covered each substratum showing a confluent layer of cells with a characteristic cuboidal configuration.

Scanning electron microscopy of the surface of a complete CPAE cell monolayer grown on tmPET revealed an intimate association of the cells with the typical cobblestone appearance after five days of culture (Figs 7a and b), despite some diversity in the cell configuration.

DISCUSSION

The use of a cell culture system as an *in vitro* model of the vascular endothelium has a number of advantages, such as offering direct access to luminal and abluminal fluid for analysis, being highly simplified and limited to a single cell type and having an experimental medium that can be defined in terms of the chemical and biological composition. However, the factors that simplify the system also raise questions about its relevance to the real *in vivo* situation.

We varied several aspects of the culture conditions in an attempt to optimize CPAE culture. This cell strain is a well-characterized strain of bovine pulmonary endothelial cells. The effects of different conditions on the biomass of CPAE cells were measured by protein quantification (LOWRY *et al.*, 1951).

In order to optimize culture conditions of bovine pulmonary endothelial cells (CPAE), as a first step, the effect of different components of the nutritive medium on the biomass of CPAE was studied. Among all the media tested, albumin had only a minor effect, which agrees with the observations of DE GROOT *et al.* (1983). Albumin acts as a binding protein for fatty acid and also as a detoxifying agent in a manner similar to that postulated for transferrin (GUILBERT and ISCOVE, 1976). Hydrocortisone and triodotyronine displayed a slight effect on CPAE cell biomass. Hydrocortisone is known to improve the morphology of RF1 cells (a line of rat ovarian follicular cells) but does not affect their growth rate while it stimulates the epithelial cell line TM4 (HAYASHI *et al.*, 1987). The concentration of insulin required for stimulation of CPAE growth is high to reach an IGF-1 effect (HAYASHI *et al.*, 1987). Most of the stimulatory activity of transferrin is presumably related to its iron binding property. Insulin and transferrin are known to stimulate several cell types (BARNES and SATO, 1980). EGF and FGF have been widely used and are reported to stimulate growth of a variety of cell lines including endothelial cells, HeLa and BALB 3T3 cells.

CPAE cells grow well in a serum-free synthetic medium. sBDM medium is a synthetic nutritive medium originally developed for optimal growth of hybridoma cells and monoclonal antibody secretion (SCHNEIDER, 1989 ; SCHNEIDER and LAVOIX, 1990) ; it contains mineral cations, vitamins, coenzymes, nucleotide precursors, high

concentrations of glucose, amino acids, and antioxidant substances. After addition of appropriate hormones and growth factors, it also sustains growth and differentiation of anchorage dependent, polarized mammalian cells such as hepatocytes, MDCK, Hep G2 or CHO cells (JIN *et al.*, 1989).

The use of surface treated microporous membranes in PET as support significantly improves the biomass of CPAE cells. Culture on these membranes gives better biomass than on tissue culture polystyrene or polycarbonate (SERGENT-ENGELEN *et al.*, 1990). These results suggest that the improvement is due, on the one hand, to the physicochemical nature of the substratum surface since cell adhesion and biomass are enhanced on surface-treated PET as compared to the native polymer and, on the other hand, to the microporosity since for a same surface treatment, culture is improved on a microporous membrane as compared to the nonporous substratum. The PET membranes are also chemically inert and do not adsorb lipophilic solutes. In these culture conditions, CPAE cells expose their apical pole to the upper compartment of the insert, whereas their basal pole adheres to the PET membrane and is, therefore, in contact with the lower compartment. Such a model could therefore be appropriate for permeability studies of substances through the endothelial layer.

The substrate upon which cells rest when maintained in tissue culture is important for their biomass. The extracellular matrix provides a natural substrate upon which cells can undergo their characteristic changes in morphology occuring at mitosis as well as their differentiation. The endothelial cell is normally adherent to a complex basement membrane extracellular matrix consisting of type IV collagen, laminin, fibronectin, vitronectin, heparan sulfate proteoglycan, entactin, thrombospondin and von Willebrand factor (KRAMER *et al.*, 1987). We found that CPAE were able to proliferate on diverse extracellular matrices. Fibronectin is abundant in both vascular basement membrane and the surrounding interstitium and is a major extracellular connective tissue component. The protein content of CPAE cells was enhanced when culture occurs on TCPS precoated with fibronectin (10 μg/ml) and suggests that fibronectin is an important cell spreading factor. This substrate requirement of CPAE has also been found by others (GOLD and PEARLSTEIN, 1980). Either on surface treated PET membranes or on TCPS, we found an advantage in using collagen. Among the various components of the extracellular matrix, collagen is known to play an important role in promoting cell attachment, cell migration and cell biomass (GOSPODAROWICZ and LUI, 1981). Binding proteins for type I collagen and laminin have been observed in a bovine aortic endothelial cell-derived line. A laminin precoating either on TCPS or tmPET did not improve CPAE cells biomass. Polylysine has been found to improve attachment and clonal growth of human and chicken fibroblasts (MCKEEHAN *et al.*, 1976). In our experiments, polylysine gave a significant increase in the total protein content of CPAE cells. Matrigel™ is a solubilized basement membrane gel extracted from the mouse Engelbreth-Holm-Swarm sarcoma. Cultures of human endothelial cells can be induced to undergo extremely rapid morphological differentiation into capillary-like structures by matrigel™. When cells were cultivated on treated PET membranes, matrigel™

gave the highest protein content whereas precoating of TCPS did not affect CPAE cell biomass.

In light microscopy, the cells formed a continuous monolayer on TCPS and on surface treated PET membranes. The ultrastucture was similar on TCPS and on treated PET membranes.

In conclusion, in a synthetic hormono-defined nutritive medium, on treated PET membranes precoated with matrigel™, culture of CPAE cells gave excellent results. Moreover, under these conditions, cells keep their endothelial morphology and ultrastructure.

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REFERENCES

BARNES D. and G. SATO (1980) — Methods for growth of cultivated cells in serum-free medium. *Anal. Bioch.*, **102** : 255-270.

CHAZOU E.I., A.V. ALEXEEV, A.S. ANTONOV, V.E. KOTELIANSKY, V.L. LEYTIN, E.V. LYUBIMOVA, V.S. REPIN, D.D. SVIRIDOV, V.P. SVIRIDOV, V.P. TORCHILIN and V.N. SMIRNOV (1981) — Endothelial cell culture on fibrillar collagen : model to study platelet adhesion and liposome targeting to intracellular collagen matrix. *Proc. Natl. Acad. Sci. USA*, **78** : 5603-5607.

DE GROOT P.G, C. WILLEMS, M.D. GONSALVES., W.G. VAN AKEN and J.A. VAN MOURIK (1983) — The proliferation of human umbilical vein endothelial cells in serum-free medium. *Thromb. Res.*, **31** : 623-634.

DEL VECCHIO P.J. and J.R. SMITH (1981) — Expression of angiotensin converting enzyme in cultivated pulmonary artery endothelial cells. *J. Cell Physiol.*, **108** : 337-345.

DEL VECCHIO P.J. and D.W. LINCOLN (1983) — Phenotypes of cultivated endothelium expression of angiotensin-converting enzyme activity. In : *The endothelial cell — A pluripotent control cell of the vessel wall. D.G.S. Thilo-Korner and R.I. Freshney, eds. S. Karger Publishers, Basel* : 67-83.

GARCIA J.G.N., A. SIFLINGER-BIRNBOIM, R. BIZIOS, P.J. DEL VECCHIO, J.W. FENTON and A.B. MALIK (1986) — Thrombin-induced increase in albumin permeability across the endothelium. *J. Cell Physiol.*, **128** : 96-104.

GOLD L.I. and E. PEARLSTEIN (1980) — Fibronectin-collagen binding and requirement during cellular adhesion. *Biochem. J.*, **186** : 551-559.

GOSPODORAWICZ D. and G.M. LUI (1981) — Effect of substrata and fibroblast growth factor on the proliferation in vitro of bovine aortic endothelial cells. *J. Cell. Physiol.*, **109** : 69-81.

GUILBERT L.J. and N.N. ISCOVE (1976) — Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell culture. *Nature.*, **263** : 594-595.

HALLEUX C. and Y.J. SCHNEIDER (1991) — Iron absorption by intestinal epithelial cells : 1. $CaCo_2$ cells cultivated in serum-free medium, on poly(ethylene terephthalate) microporous membranes, as an in vitro model. *In vitro Cell. Dev. Biol.*, **27a(4)** : 293-302.

HAYASHI I., J. LARNER and G. SATO (1987) — Method for growth of TM4 cells in a serum-free medium. *In vitro*, **34** : 23-30.

JAFFE E.A. (1980) — Culture of human endothelial cells. *Transplant. Proc.*, **88** : 473-485.

JIN Y., A. BACQUET, A. FLORENCE and Y.J. SCHNEIDER (1989) — Desferrithiocin and desferrioxamine B : cellular pharmacology and storage iron immobilisation. *Biochem. Pharmacol.*, **38** : 3233-3240.

KLEINMAN H.K., R.J. KLEBE and G.R. MARTIN (1981) — Role of collagenous matrices in the adhesion and growth of cells. *J. Cell. Biol.*, **88** : 473-485.

KRAMER R.H., K.G. BENSCH and M.A. KARASEK (1987) — Isolation and characterization of human vascular endothelial cells with application to studies of the subendothelial matrix. In : *Cell separation : Methods and selected applications*, vol. 5. T.G. Pretlow II and T.P. Pretlow, eds Academic Press, Orlando, FL : 1-27.

LOWRY O.H., N.J. ROSEBROUGH and A.L. FARR (1951) — Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193** : 265-275.

MACARAK E.J. and P.S. HOWARD (1983) — Adhesion of endothelial cells to extracellular matrix proteins. *J. Cell. Physiol.*, **116** : 76-86.

MCKEEHAN W.L., W.G. HAMILTON and R.G. HAM (1976) — Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **73** : 2023-2026.

ROSS R., J.A. GLOMSET, B. KARIYA and L. HARKER (1974) — A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc. Natl. Acad. Sci. USA*, **71** : 1207-1210.

ROSS R. and J.A. GLOMSET (1976) — The pathogenesis of atherosclerosis (part one and part two). *N. Engl. J.* Med. **295,** 369-377 and 420-427.

SCHNEIDER Y.-J. (1989) — Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium. *J. Immunol. Methods*, **116 :** 65-77.

SCHNEIDER Y.-J. and A. LAVOIX (1990) — Monoclonal antibody production in semi-continuous serum- and protein-free culture : effect of glutamine concentration and culture conditions on cell growth and antibody secretion. *J. Immunol.*, **129** : 251-268.

SERGENT-ENGELEN T., C. HALLEUX, E. FERRAIN, *et al.* (1990) — Improved cultivation of polarized animal cells on culture inserts with new transparent polyethylene terephthalate or polycarbonate microporous membranes. *Biotechnol. Techniques*, **4** : 89-94.

TIMPL R., H. ROHDE, P. GEHRAN-ROBEY, S.I. RENNARD, J.M. FOIDARD and G.R. MARTIN (1979) — Laminin a glycoprotein from basement membrane. *J. Biol. Chem.*, **254 :** 9333-9937.