CELL PROPAGATION ON FILMS OF POLYMERIC FLUOROCARBON AS A MEANS TO REGULATE PERICELLULAR pH AND pO_2 IN CULTURED MONOLAYERS

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1. Introduction

In the intact animal, the pH, pCO_2 and pO_2 of the cell environment is under exquisite biochemical and physiological control. This state is not approximated by hitherto accessible techniques of monolayer cell culture; while bulk pH of the medium might be measured and even regulated electronically, this is rarely practised, and no means have been available to sense and control the pH, pCO_2 and pO_2 in the *cell layer*. We are concerned that inadequate regulation of these parameters might introduce toxic effects and unwanted selective pressures into in vitro cell propagation, as well as clouding the biological significance of culture-dependent phenomena such as contact inhibition of movement, growth and biosynthesis [1–3]. The cellular effects of pCO_2 (e.g. via formation of protein–carbamates) remain to be specified, but the need for precise regulation of pericellular pH is obvious. Moreover, it is established that oxygen tensions greater than that of air (hyperbaric) are acutely toxic to whole animal tissues [4], as well as lymphocyte cultures [5] and can increase tumor incidence [6–11]. The toxic effects are attributed to oxidation of protein-SH and polyenoic fatty acids in membrane phospholipids [12]. Only in pulmonary alveoli does the physiological oxygen level approach the pO_2 of air, which most tissue culture systems attempt to approximate, while cells typically reside at much lower pO_2s, depending upon their vicinity to the proximal ends of systemic arterioles [13]. In most cells oxidative phosphorylation functions maximally already at pO_2s not greater than 4 mm Hg [14]. The pO_2 of air is thus potentially 'hyperbaric' to many cultured cells, and these exist empirically somewhere between toxic and inadequate oxygen levels, depending upon variables such as the depth of the fluid layer, medium viscosity and interfacial mixing [15]. We wish to avoid these hazards, and at the same time propagate diverse cells (other than tumors growing in suspension culture) in quantities and under conditions appropriate for biochemical and biophysical studies, including membrane characterization. Accordingly we have developed a new approach to monolayer cell culture, suitable for small and large scale operation, and allowing regulation of pH, pCO_2 and pO_2 in the cell layer. We here present the essential features of the method and will subsequently re-

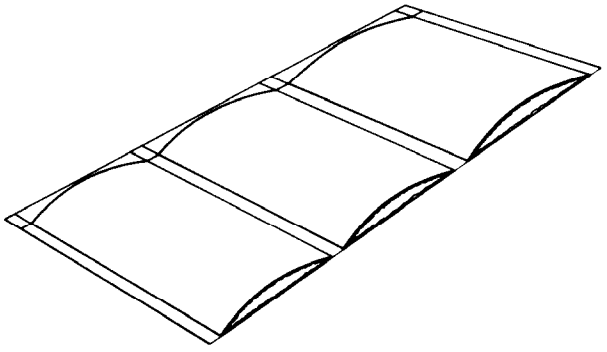
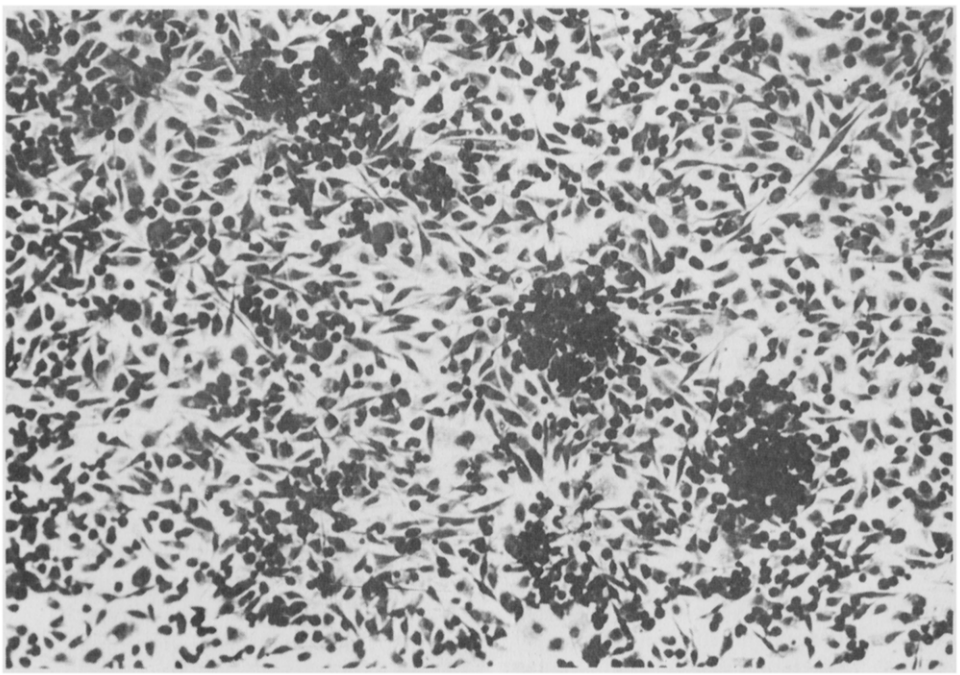


Fig. 1. A typical cell culture container.

(A)



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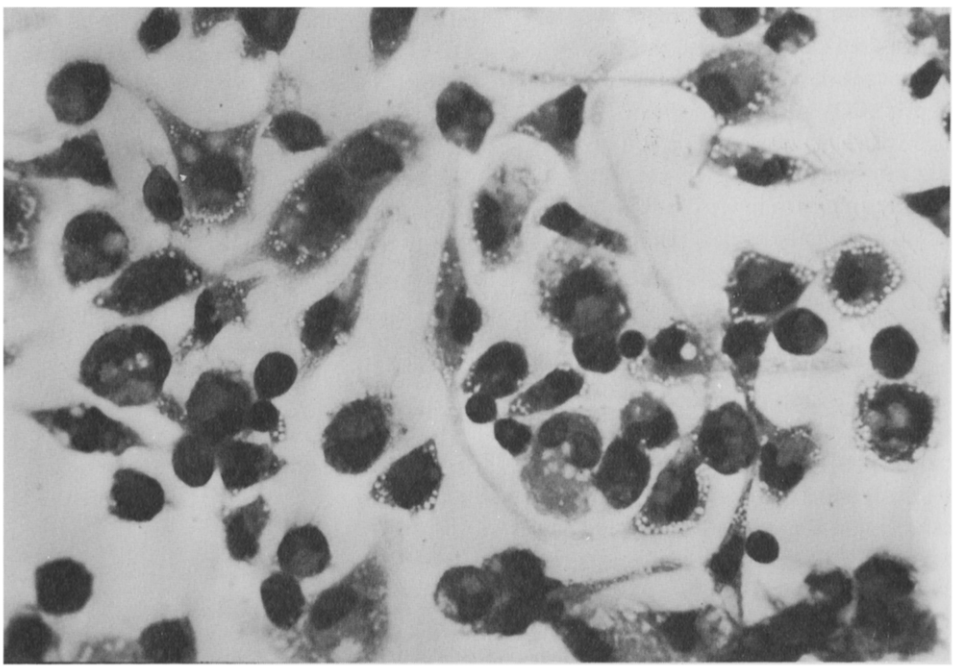
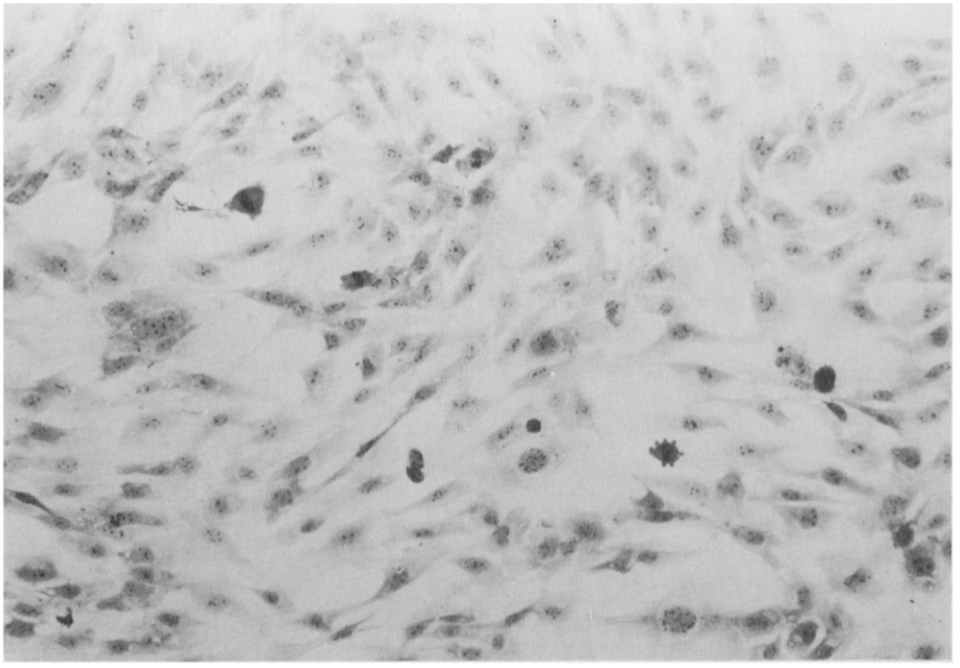


Fig. 2. Microscopic appearance of cells grown on FEP films. (A) Mouse (NMRI/Han) peritoneal cells after 12 days in Eagle's medium + 20% fetal calf serum, pH 7.1. This is a macrophage monolayer with 3 clusters of proliferating lymphocytes; Wright-Giemsa stain; 40 ×. (B) Mouse peritoneal cells after 3 days in Eagle's medium + 20% inactivated O Rh + human serum, absorbed with sheep erythrocytes; pH 7.1. Some sheep erythrocytes were included in the inoculum to stimulate the phagocytic activity Evident in the photograph. Wright-Giemsa stain; 160 ×.

(C)



(D)

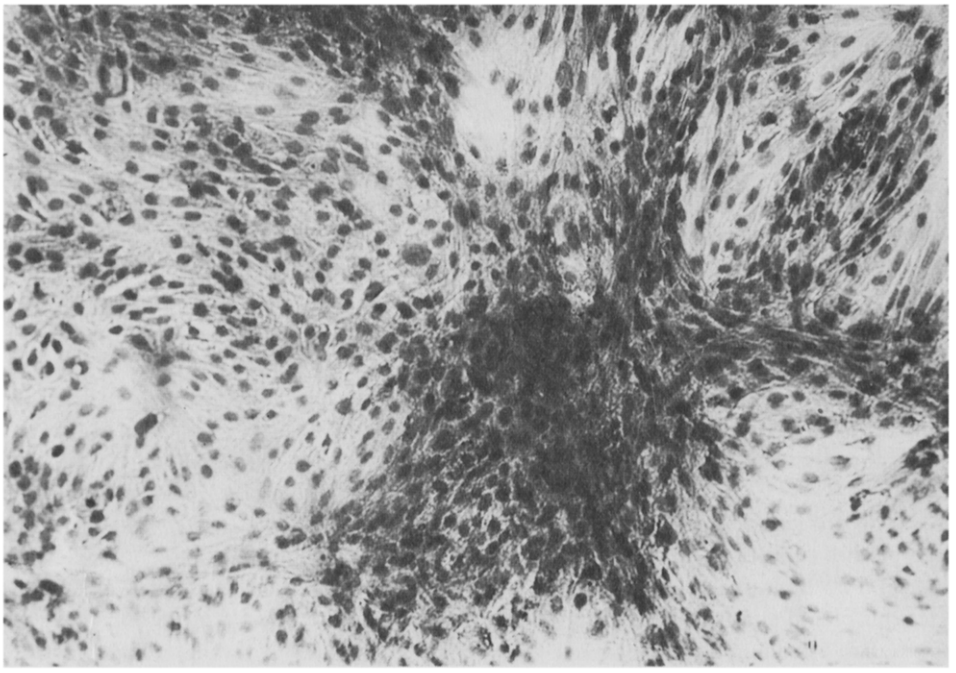


Fig. 2 (continued). (C) Monolayer of primary rat fibroblasts (Lewis), 3 days after inoculation. Eagle's medium + 20% inactivated calf serum; pH 7.3; Wright-Giemsa stain; 40 × . (D) Bovine mammary tissue. The cells were dispersed with collagenase prior to inoculation. The photograph shows development of epithelial nests after 8 days of culture in Eagle's medium + 20% fetal calf serum; pH 7.2. Fixed in methanol and stained with hematoxylin-eosin; 40 ×.

port on the effects of these parameters on various cultured cells.

2. Experimental

We replace conventional cell supports by biologically-inert, transparent, gas-permeable membranes of thermoplastic fluoro-ethylene-propylene copolymer (FEP-Teflon, DuPont), 25 μm thick. The films, chemically etched to become sufficiently hydrophilic for cell attachment, are so supplied as 30 cm wide rolls by Carl Huth and Söhne (712 Bietigheim/Württ., Germany). Before use the membrane is washed overnight with laboratory detergent (7X, Serva, 69 Heidelberg, Germany), rinsed three times in deionized water, twice in double-distilled water and dried. The film is then diathermically sealed into bags or tubes of desired shapes and dimensions (fig. 1). Growth areas and area/volume relationships are accurately reproduced. We commonly use rectangular bags, 5–30 cm in width and length, containing fluid layers 2–10 cm thick.

The films lack absorption bands in the visible and UV (Cary spectrophotometer, Model 15). Light scattering is also slight, contributing an absorbance of 0.1 at 235 nm and less at higher wavelengths. The containers are thus conveniently sterilized by germicid UV light (Philips lamp TLADK 30 W/05 at 30 cm, for 15 min). Their optical clarity makes the films suitable for microcinematographic, UV spectrophotometric and spectrofluorometric measurements on the attached cells. Media, cells, etc. are currently injected into a corner of a container via a 0.7 mm (o.d.) needle. The elasticity of FEP prevents leakage, but to assure sterility, we reseal diathermically. The cell/surface and surface/volume relationships are altered slightly by this step, but remain identical for each bag. Medium volume depends upon the planned duration of uninterrupted culture.

For growth on one surface, we let the cells attach for 60 min under culture conditions, but without agitation. For growth on both internal surfaces, the bags are inverted after this period and the process repeated. The containers are then placed on a gauze-covered screen, stacked in a 37° incubator. These are rotated back and forth by an external, eccentrically placed motor at 20–120 cycles per min, with 1 min resting intervals following 5–10 sec rotation in a given direction. Removal of cells can be by trypsinization, EDTA or mechanically; gentle folding and stretching of the pliable membranes readily detaches confluent cells as extended sheets. The films can be reutilized after washing and very large numbers of cells can be grown in little space. Thus, a typical incubator of dimensions 60 cm × 80 cm × 120 cm with screens spaced 5 cm apart, has a growth surface exceeding that of the largest conventional 'roller machines', when cells are grown on both membrane surfaces.

3. Results and discussion

The diffusion characteristics of the membrane for O_2, CO_2 and H_2O for typical operating conditions are given in table 1.

Table 1

Gas	Pressure gradient (mm Hg)	Diffusion rate* (μmoles/hr/cm^2)
O_2	137	6.3
CO_2	38	2.2
H_2O	47	4.7

* Values are for 37°

The diffusion rates per atmosphere difference of partial pressure were 35, 44 and 76 μmoles/hr/cm^2 of O_2, CO_2 and H_2O respectively. The values for O_2 were obtained polarographically, those for CO_2 by the weight gain of bags of known surface area, containing a CO_2 absorbant (Soda Asbestos, Merck) and those for H_2O) from the weight loss of water-filled bags of known surface area located in dry air. These diffusivities fall into perspective through the extreme example of alveolar macrophages, which respire rapidly, utilizing 2.5 μmoles O_2/hr/10^7 cells. At confluence there are 1.2–1.7 × 10^6 cells/cm^2; substituting their oxygen demand of about 0.4 μmole into Fick's 'first law' [16], using the diffusivities given above and assuming all oxygen to be used in the cell layer, the pO_2 across the film will be ~ 9 mm Hg; lower respiration and cell density bring the pO_2 of the cell layer close to that of the gas phase. Assuming a RQ of 1.0 and CO_2 generation solely in the cell layer gives a pCO_2 across the FEP film ~7 mm Hg; lower cell densities,

respiration and RQ will diminish this diffusion gradient.

Because many cells lower pH by lactic acid production, we maintain pH through feedback control of pCO_2, using the Henderson-Hasselbach relation [17]:

$$pH_{37^\circ} = 6.06 + \log \frac{[HCO_3^-]}{0.024 pCO_2}$$

$[HCO_3^-]$ is typically 0.024 M and for our macrophage example we set pH = 7.1 ± 0.05 for optimal phagocytosis, yielding an initial pCO_2 near 100 mm Hg. To effect pH regulation, one container is used both to grow cells and to maintain pH. For the latter, the sterile tip of a micro glass electrode (Ingold no. 225-92-M5) is sealed in by the aid of a sleeve and the circuit to a remote Ag/AgCl reference electrode completed by a salt bridge, isotonic with the culture medium. The pH electrode controls a titrator (Radiometer no. TTT 1b), whose output operates a gasflow valve (Herion no. 2009) regulating the input of CO_2. When cells produce no 'strong' metabolic acids, the pCO_2 remains constant. However, with phagocytosing macrophages pH maintenance requires a drop in gas phase pCO_2 from 100 to 33 mm Hg during the 72 hr following inoculation of 8×10^6 cells/cm^3. Our method of pH control is unique in maintaining the ionic strength of the medium and insuring sterility. Substitution of buffers other than bicarbonate does not constitute pH regulation, since acid production still changes pH. Also such systems lack the 'nutritive' function of CO_2 and HCO_3^-.

In the reported studies we have monitored the gas phase pO_2 with a Clark-type oxygen electrode, but have not regulated it. In the cited macrophage example the pO_2 (at H_2O saturation and 37°) rises from 128 to 142 mm Hg during the 72 hr after inoculation; the increment is due to the drop in pCO_2. There are transient fluctuations in pO_2 (~ 4 mm Hg) during the operation of the CO_2 valve.

Fig. 2 shows representative photomicrographs of several cell types cultured at constant pH and pO_2 125–145 mm Hg. Under the conditions employed, embryonic fibroblasts and peritoneal macrophages behave similarly on FEP teflon as on conventional supports, but the lymphocytes and mammary epithelial cells fare much better. Thus, the number of lymphocytes in a 4-day mixed-macrophage–lymphocyte culture is typically *twice* that of the inocolumn, whereas published experiments report a 55–65% lymphocyte *loss* in an equivalent period [6]. Also plaque forming cells and antibody production is regularly found even after 10 days. Finally, the epithelial nests which organize during cultivation of dispersed bovine mammary tissue could not be obtained under conventional culture conditions.

In our present work on the effects of pO_2 on cultured cells, the pO_2 electrode regulates pO_2 by driving reciprocally-linked N_2 and O_2 flow valves. To maintain pCO_2 also, an in-line pH electrode is used to operate the CO_2 valve, and an in-line pCO_2 electrode switches medium, when the pCO_2 drops below desired limits.

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