A Flow-Through Tissue Culture System with Fast Dynamic Response

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A flow-through system in which monolayer cells, growing on a 50-cm² glass surface, are in contact with a film of medium with a thickness of 0.14 mm, is described. For murine B16 melanoma cells, the loading capacity is $8 \cdot 10^6$ cells. The flow-through principle permits frequent off-line or on-line detection of medium constituents for a period on the order of days. The system has a fast dynamic response. With off-line radiochemical detection, the system was applied to the uptake of uridine and excretion of uracil over a period of 45 h. With on-line fluorescence detection, the interaction between the cells and two anthracycline analogs was monitored. The cells can be easily observed with a light microscope.

Information on the course of uptake or release of compounds by cells can be obtained by repeated sampling of culture medium in contact with the cells. Continuous refreshing of the medium reduces the danger of accumulation of products or depletion of nutrients. When culture flasks are used, frequent replacing of medium is time consuming, can lead to errors due to cell handling, and may result in detachment of monolayer cells. Moreover, the latter method requires a relatively large volume of medium, which increases the detection limit for changes in the medium. To allow rapid monitoring, the volume of the medium in contact with the cells must be small relative to the total volume of all cells. When immobilized cells were used, monitoring of changes in medium composition was reported for periods on the order of hours (1,2). If conditions permitting cell growth are used, the duration of measurement can be increased. McGee *et al.* (3) described a system in which cells were grown in monolayer in round 200- μ l glass capillary tubes. Compared with the latter method, the system described below has the following advantages: (i) due to the lower maximum diffusion distance, the response of the system is faster; (ii) for the same number of cells, the amount of medium in contact with cells is

smaller, which means that changes in medium concentration are greater; (iii) for the same linear velocity of the medium the amount of medium containing the compound under study is greater; (iv) the flat surface gives better conditions for attachment of new cells; and (v) the cells can easily be visualized with a light microscope.

MATERIALS AND METHODS

Cells. A murine B16 melanoma cell line was used to test the system. The cells were grown in Dulbecco's minimum essential medium containing 15% dialyzed fetal calf serum and antibiotics (100 U/ml penicillium and 100 μ g/ml streptomycin). The atmosphere contained 5% carbon dioxide. For plating of cells within the system, the cells were suspended in trypsin (1 mg/ml) plus 5 mM EDTA in Hanks' buffered salt solution, free of Ca²⁺ and Mg²⁺. After resuspension in culture medium, 10^6 cells were allowed to attach to the glass surface for 2 h before medium was pumped through the system. To avoid wastage of medium before the experiment was started, the medium was pumped from and returned to a container. Cells could be removed from the system by using the suspension conditions mentioned above.

FIG. 1. Construction of the flow-through system. The inlet and outlet tubes are cemented into the upper glass plate, and the strip of silicone glue forms a cavity when the two glass plates are pressed together. The dots represent the cellular layer. For low flow rate conditions, 120 holes with a diameter of 4 mm were made in the lower plate (not shown) and covered with polypropylene. The upper plate was then turned over and the cells were plated on it.

Apparatus. The system, as shown in Fig. 1, consists of two plates of borosilicate glass measuring $260 \times 50 \times 4$ mm. One of these plates holds the inlet and outlet tubes (3 cm long, made of stainless steel, internal diameter 0.5 mm), cemented in place with epoxy resin (Araldite AV 9, obtained from Ciba-Geigy) under heating (180°C for 40 min). The distance between the holes is 22 cm.

The chamber for the cells (2.5 cm wide and 17 cm long) was formed by applying a 3-mm-wide strip of silicone glue (Perfecta Chemistry, Goes, The Netherlands) to the lower plate, the thickness of the glue determining the thickness of the film of medium in the chamber. For all measurements mentioned here, the thickness was 0.14 mm. The required thickness was obtained by the use of a sheet of adhesive Teflon of the desired thickness, from which a space representing the walls of the chamber was cut out. The glue was applied to this space and smoothed to the level of the Teflon film by drawing the edge of a cover glass for microscopy over the glue. After that, the glass plates were pressed together. To avoid shortage of oxygen at low flow rates (less than 0.2 ml/min), 120 holes with a diameter of 4 mm were made in the

lower plate (not shown), to which a sheet of polypropylene (30 μm thick) was adhered with silicone glue, to allow oxygen transfer to the cells in the system. Under these conditions, the cells were plated on the upper plate (Fig. 1).

Connections were made with silicone-rubber tubing (inner diameter 0.5 mm), and a peristaltic pump (Gilson, type minipuls 2) was used. Before the system was put into operation, the glass surface was thoroughly cleaned with laboratory soap (Liquinox, obtained from Alconox, New York), rinsed with diluted hydrochloric acid (2%), and sterilized in an autoclave for 20 min at 120°C . For experiments with cells the complete system was placed into an incubator at 37°C with an atmosphere containing 5% carbon dioxide.

Chemicals. [^{14}C]Uridine (Urd)¹ (specific activity 52.4 Ci/mol) was obtained from the Radiochemical Centre, Amersham, United Kingdom.

[^{14}C]Uridine and [^{14}C]uracil were separated by paper electrophoresis, using chro-

¹ Abbreviations used: Urd, uridine; Ura, uracil; DX, doxorubicin; CMM, carminomycin; PALA, *N*-(phosphonacetyl)-*L*-aspartic acid.

matography paper 3 (Whatman, Maidstone, Kent, United Kingdom), at 3000 V for 30 min. The paper was wetted with a solution containing 25 mM disodium tetraborate and 1 mM EDTA (pH 9). As doxorubicin source, the clinical preparation produced by Farmitalia (Milan, Italy) was used. Carminomycin was kindly provided by Bristol Laboratories (Syracuse, N.Y.). Cell-culture medium and fetal calf serum were obtained from Grand Island Biological Company, Grand Island, New York. The fetal calf serum was dialyzed before use.

For all experiments use was made of purified water prepared with a Milli-Q water purification system (Millipore, Bedford, Mass.). All reagents were of analytical grade.

RESULTS AND DISCUSSION

When the medium flows through the system, biochemically active compounds interact with the cells. When the uptake is rapid, the concentration of these compounds decreases rapidly as a function of the distance from the inlet point. For the limiting case of infinitely rapid uptake for a surface covered with cells, the prediction of the uptake as a function of liquid film thickness, flow rate, distance from the inlet point, and diffusion coefficient has already been described for this geometry (4). When the uptake by the cells in the system is slow, the concentration will decrease less rapidly and in the limiting case will not change at all. The change in concentration can then be increased by decreasing the flow rate. To avoid shortage of oxygen at low flow rates, holes were made in one of the two glass plates and covered with polypropylene sheet. The lower limit of the flow rate is determined by inadequacy of the supply of nutrients to the cells or an injurious increase in the concentration of catabolytes.

Dynamic Response of the System

For the assessment of changes in medium composition attributable to viable cells, the dynamic response of the system must have a certain rapidity. On the basis of the average

cell diameter (about 10 μm) and the flatness of the glass surface, the liquid film could be made thinner than 0.14 mm to obtain a more rapid dynamic response. However, the amount by which the thickness of the film of medium can be reduced is limited by the following factors: (i) the increased risk of total nutrient depletion or an injurious increase of the increased concentration of poisonous catabolytes in the medium after the requisite stopping of the flow for 2 h to permit settling of new cells; (ii) the increased danger of entrapped gas bubbles which tend to spread out and occupy too much space; and (iii) for the same linear velocity, greater shear forces (resulting from a steeper velocity gradient, perpendicular to the surface of the plates) can be expected to increase the risk of detachment of cells.

An increase of the film thickness results in poorer dynamic behavior. Analogous to the measurement of the contribution to peak width by detectors for HPLC, the response to injection of a small volume of fluid was measured with a manual loop injector. For this purpose, 20 μl of a uridine solution (10^{-4} M) was injected, and Hanks' buffered salt solution was used as the eluant. The volume of the chamber containing the cells was 0.7 ml. The response was measured with a uv monitor (Perkin-Elmer, type LC75) at a wavelength of 254 nm. Peak broadening, shown in Table 1, was determined by measuring half the width at six-tenths of the maximum peak height. Values were corrected for peak broadening attributable to the detector (contribution 11–23%). The theoretical plate number (N) was calculated according to the formula $N = (t_d/\sigma_t)^2$ in which t is the time for passage through the system and σ_t is half the peak width at six-tenths of the maximum height.

The asymmetry ratio of the peaks, measured at one-tenth of the maximum height, was 1.4.

Uptake of Uridine

[^{14}C]Uridine (medium concentration 10^{-6} M) was used to measure the radioactivity in

TABLE 1
DYNAMIC RESPONSE OF THE SYSTEM
TO A PULSE SIGNAL^a

Flow rate (ml/min)	Peak broadening ^b		Residence time (min)	Number of theoretical plates ^c
	(s)	(μ l)		
0.07	42	49	13.2	355
0.09	34	51	10.3	330
0.19	17	54	4.5	252
0.28	14	65	3.1	176
0.40	11	74	2.2	144

^a Conditions: the contribution to peak broadening was measured at six-tenths of the maximum peak height. Injection: 20 μ l of uridine (10^{-4} M). The peak was obtained by monitoring the uv absorbance at the outlet of the system at a wavelength of 254 nm by the use of a HPLC detector (Perkin-Elmer, type LC 15) and is represented by

in which w is the peak width and $h_{\max}$ is the maximum peak height. All values were corrected for contribution of the detection system. The volume of the chamber containing the cells was 0.7 ml.

^b Multiplying the peak broadening, obtained in seconds, by the flow rate resulted in peak broadening, expressed in microliters.

^c The number of theoretical plates was calculated according to chromatographic theory.

the effluent as a function of flow rate (Fig. 2). The measurements were performed over a 90-min interval. Electrophoretic analysis of the effluent showed that during this period radioactivity representing Ura was negligible (for a longer period, see below). Under the assumption of infinitely rapid uptake, the fraction of Urd taken up by the cells attached at sites within a distance of 5 cm from the inlet point was calculated, with a formula described elsewhere (4), to be greater than 0.5. Because the observed decrease is much

lower, it may be concluded that the rate of decrease of Urd is mainly determined by the cellular uptake rate. If the film thickness were increased, the contribution of uridine transport in the medium to the rate of decrease of Urd would become higher. Because culture-flask experiments showed that the uptake of Urd is proportional to the medium concentration (up to $2 \cdot 10^{-6}$ M), the uptake can be described either by

$$\frac{dQ}{dt} = -kpc$$

in which Q is the amount of Urd taken up per 10^6 cells, k is the first-order clearance constant (from culture-flask measurements $4 \cdot 10^{-4}$ liters/h, 10^6 cells), p is the number of cells, C the medium concentration, and t is time, or by

$$V \frac{dC}{dt} = -kpc$$

in which V is the volume of the chamber containing the cells. With the use of the right boundary conditions for the flow-through system, this equation develops to

$$\ln \frac{C_e}{C_0} = -\frac{kpt_d}{V}$$

in which t_d is the residence time in the system and C_e and C_0 are concentrations at the end and the beginning of the system, respectively. Because $t_d = V/W$, in which W is the flow rate, we have

$$\ln \frac{C_e}{C_0} = -\frac{kp}{W} \quad [1]$$

Calculated values, obtained with Eq. [1], and measured values for several values of W are shown in Fig. 2. It is evident that the difference between the two sets of data is less than 10%. The results indicate a rate constant similar to that found in the culture-flask experiments. To study the long-term uptake of Urd, radioactivity of the perfusate was measured over a 45-h interval during which Urd was constantly present in the medium at an initial concentration of 10^{-6} M. The amounts of Urd and Ura in the perfusate during this

period are shown in Fig. 3. The clearance constant, calculated with Eq. [1], was $3.4\text{--}3.7 \cdot 10^{-4}$ liter/h, 10^6 cells, which is in fairly good agreement with the value found for the short-term uptake and in the culture-flask studies (see above). The production of Ura by these cell increased gradually up to a plateau of $0.45 \cdot 10^{-10}$ mol/ 10^6 cells h^{-1} , which was reached after 20 h and persisted for the rest of the 45-h period.

Uptake of Anthracyclines

Doxorubicin (DX), a drug belonging to the group of anthracyclines, is used in the treatment of various types of tumor. To investigate the antitumor properties and systemic toxicity of other anthracyclines, analogs such as carminomycin (CMM), which has lately undergone clinical trials, have been synthesized. To assess the interaction of such compounds with the cells in the system, a manual HPLC loop injector was placed just upstream from the inlet tube for the injection of 20 μl of various concentrations of DX or CMM. The outlet tube was connected to a fluorescence monitor which could detect the compound selectively. The peak heights, starting with the lowest amount of both compounds

FIG. 2. Uridine concentration at the outlet of the system (C_e) after exposure to B16 cells in relation to the initial concentration (C_0), according to calculated (---) and experimental (O) values. Conditions: uridine concentration in medium, 10^{-6} M; specific activity, 52.4 Ci/mol; number of cells, 5×10^6 .

FIG. 3. Time course of uridine (Urd) and uracil (Ura) concentrations at the outlet of the system. Conditions: radioactivity of uridine and uracil was measured in 2-ml fractions collected during a 45-h period after perfusion in the presence of B16 cells; the initial radioactivity of Urd in the medium is shown. Fractions were collected for 15 min at intervals of 1.25 h; Urd medium concentration, 10^{-6} M; specific activity 52.4 Ci/mol. The cell number was stationary during this period (1.5×10^6 cells) and the medium stock was kept at 0°C during the experiment.

in the sequence DX, CMM are shown in Table 2. It is evident that the height of the peak for DX is proportional to the injected amount, whereas for CMM the low signal indicates a relatively high uptake. With the amounts injected, however, saturation of the uptake occurred between 0.2 and 2 mg.

As an extra check, the signal of CMM was measured after removal of the cells from the system (see right-hand column).

In general, the intensity, the position, and the peak shape convey information about the interaction between a compound and cells. The intensity supplies information about the participation in cellular metabolism. The position gives information concerning the equilibrium or quasi-equilibrium parameters, such as the distribution of the injected

TABLE 2
MAXIMUM PEAK HEIGHT AFTER INJECTION
OF TWO ANTHRACYCLINES

Amount injected (mg)	Peak height ^a		CMM (without B16 cells)
	DX	CMM	
0.02	25	0	21
0.2	220	9	220
2.0	—	80	—

^a Conditions: the peak height was measured after injection of 20 μ l cell culture medium containing doxorubicin (DX) or carminomycin (CMM); flow rate: 0.1 ml/min; detection: fluorescence based on excitation/emission wavelengths of 474/604 nm (DX) and 490/550 nm (CMM), respectively, for maximum sensitivity. Height is expressed in arbitrary fluorescence units (sensitivity for DX and CMM was 210 and 195 units $\cdot$ l $\cdot$ g⁻¹, respectively). The peak height is $h_{\max}$, as represented in Table 1. A HPLC fluorescence monitor (Perkin-Elmer, type M3000) was used for the measurements.

compound over medium and cells. Peak broadening in excess of that predicted by simple chromatographic theory can be attributed to slow mass transfer within the cells or through the cell walls. Cases where slow metabolism occurs require ample discussion, which will be given in a subsequent paper.

CONCLUSIONS

The system described here can be used to simulate pharmacokinetic phenomena *in vitro*. Knowledge of the uptake of Urd and the amount converted to Ura in intact cells is useful for the study of rescue of drugs inhibiting *de novo* pyrimidine synthesis (PALA, pyrazofurin). A steady low concentration of Urd gives a better approximation of physiological conditions (2–4 μ m) than can be obtained with culture flasks.

The demonstrated high rate of CMM uptake suggests that a relatively low CMM concentration prevails in the center of the poorly vascularized B16 tumor *in vivo*. Studies on this phenomenon can be extended by measuring the outlet concentration as a function of volume flow rate, mass flow rate, or the

effect of sequential injections on the output signal. The release of drug after cell death can be studied as well. Use of the system to compare differences in the interaction between cells and various analogs can be helpful for the selection of drugs with respect to antitumor activity or toxicity.

Because in studies performed with the present system the same cells can be exposed successively to different compounds, the number of variables is lower than for studies done with different cells in different flasks. However, the sequence of the injections must be chosen such that the preceding injections have a minimal effect on cell physiology. This must be checked by observations made after repeated injections.

The number of theoretical plates (Table 1) also indicates the potential use as a "living column," which can be applied for separation on the basis of different interactions with the cells (e.g., stereoisomers).

The system can also be used to measure cell lysis in response to drugs over a period on the order of days (e.g., by assessing radioactivity after incorporation of radiolabeled thymidine or uridine).

To increase the capacity of the system, cells can be allowed to settle on the other plate as well.

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