Microrespirometer Chamber for Determinations of Viability in Cell and Organ Cultures

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The effects of chemical, physical, and infectious cytotoxic agents on primary and cultured cells were evaluated by measurements of oxygen uptake for various time periods. A newly developed respirometer used a Clark oxygen electrode in a 1.0-ml chamber, with provisions for constant mixing and for temperature control of both the sample and electrode chambers. The device was unique because the electrode and instrumentation were provided by a clinical blood-gas analyzer. Oxygen uptake by blank controls was negligible, whereas cells and tissue consumed oxygen at rates of approximately 1 to 5 $\mu\text{l/h}$ in a dose- and temperature-dependent fashion. Cyanide, heat, and freeze-thaw lysis reduced the oxygen uptake to less than 0.6 $\mu\text{l/mg}$ per h. Infection of trachea organ cultures with *Mycoplasma pneumoniae* significantly reduced relative ciliary activity, tetrazolium reduction capacity, and oxygen consumption in a coordinated fashion.

Cell and organ cultures are being used with increasing frequency for the isolation of microorganisms and for the study of host-parasite interactions. Such systems are relatively simple, but can provide the cellular environment necessary for the growth of fastidious organisms. However, difficulties encountered in the objective assessment of cell viability in cell and organ cultures tend to diminish their potential usefulness for the clinical laboratory.

One parameter which could circumvent this problem is kinetic data on oxygen consumption. Measurements of respiratory activity or oxygen uptake by microbes, cells, or tissues have served as a convenient index of metabolic activity (16). Such data have also served to help define the oxygen flux across a surface (12), measure the activity of enzyme systems (4), establish the oxygen requirements of cells (3), and determine the effects of pathogens on host tissue (6). Although values for gross utilization can be informative, an understanding of the rate of utilization, i.e., kinetics, would generally be more helpful in establishing the mechanism of alterations in respiratory activity. The study described here concerns the development of a new respiratory kinetic assay for cytotoxicity in cell and organ cultures.

Since the development of the Clark electrode (9), which gives an electrical signal directly proportional to oxygen activity (18), numerous respirometer chambers have been developed (10, 17, 19, 20). The apparatus presented here is unique in that: the sample chamber has a micro

(1 ml) working volume with provisions for constant stirring; the electrode and sample chamber are water jacketed; and the electrode and instrumentation are provided by a clinical blood-gas analyzer. As such, the respirometer acts as a conversion unit for a blood-gas analyzer. The data demonstrate its potential for application to a wide variety of cell and tissue systems and offer new information on the consequences of a host-parasite interaction.

MATERIALS AND METHODS

The chamber, fabricated from plexiglass, consisted of a water-jacketed electrode holder and sample chamber with provisions for a magnetic stir bar and an inlet and outlet for medium (Fig. 1). The complete assembly was 5.0 cm wide by 7.5 cm long. A scale, cross section drawing of the respirometer (Fig. 2) shows: (i) tube for the oxygen electrode from the blood-gas analyzer; (ii) outlet from the water jacket; (iii) gum rubber gasket between the upper, detachable electrode holder and the lower sample chamber; (iv) medium outlet; (v) a 1.5-cm-diameter stainless-steel screen on which the tissue sample was placed and which covers a well (1.25-cm diameter by 0.3 cm deep) for the miniature stir bar (0.16 by 0.79 cm); (vi) medium inlet; (vii) water jacket inlet; (viii) polyethylene film gasket; and (ix) the base plate. The three separate plexiglass sections separated by gaskets were secured with brass machine screws in tapped holes. Plexiglass bonds for the electrode chamber-water bath were made with chloroform, and the tubing was secured in the inlets and outlets with silicone sealer.

The base plate was fastened to the bottom of the sample chamber, and the stir bar was placed be-

FIG. 1. Photograph of (A) the respirometer chamber components and (B) the assembled chamber.

neath the removable screen. The tissue sample was placed on the latter, and the sample block was capped by a gasket and the electrode holder section. The Clark electrode was then inserted so that its measuring tip sealed the 0.4-cm opening in gasket "C." The inlet and outlet for the water jacket were then connected to the water pump of the blood-gas analyzer. Temperature equilibration was reached in approximately 5 min. Medium was injected into the sample chamber, all bubbles were carefully expelled, and the medium tubes were clamped off with hemostats. The chamber was then placed on a magnetic stirrer, and readings were noted on the analyzer and/or recorder. When cell suspensions were used, they were introduced directly into the assembled sample chamber through the medium inlet. A temperature setting of 39 C was used on the blood-gas analyzer water bath, since some heat loss occurred via the plastic tubing. Separate trials indicated that this would hold the respirometer steady at 37 C.

Tyrode balanced salt solution (8) was sparged with air and prewarmed to 37 C before use. Oxygen solubility was determined by the method of Dixon and Kleppe (4) with catalase (200 $\mu\text{g}/\text{ml}$) and hydrogen peroxide (0.03%) so that the readings could be converted from millimeters of pressure to microliters per milliliter.

Instrumentation consisted of a digital readout

FIG. 2. Cross-sectional, scale drawing of the assembled respirometer. (A) Electrode chamber; (B) water jacket outlet; (C) gasket; (D) medium outlet; (E) stainless-steel screen to support tissue; (F) medium inlet; (G) water jacket inlet; (H) gasket; (I) base cover plate. For reference, the width of the base is 5.0 cm.

blood-gas analyzer (model 213, Instrumentation Laboratory Inc., Lexington, Mass.) connected to a strip chart recorder (Omniscrite, Houston Instrument Div., Bausch & Lomb Inc., Bellaire, Tex.). The

Clark electrode was standardized daily with 0.00% and 12.00% oxygen as recommended by the manufacturer.

Tissue samples of trachea, liver, or kidney were from adult BALB mice or Golden hamsters. They were aseptically removed, rinsed in chilled phosphate-buffered saline, and cut into 1- to 2-mm slices. Samples, 5 to 10 mg each, were given a final rinse in phosphate-buffered saline and were used in the respirometer within 3 to 5 min after removal.

Primary cells (kidney, African green monkey) and an established cell line (HeLa, S3) were provided by the Grand Island Biological Co. (GIBCO, Grand Island, N.Y.). Cells were received as suspensions of 10^5 to 10^6 cells/ml in Eagle minimal essential medium. Cells were collected by centrifugation at $10,000 \times g$ for 10 min and were washed in phosphate-buffered saline prior to being resuspended in Tyrode solution at 5×10^6 cells/ml.

Organ cultures of hamster trachea were prepared as described previously (5). In addition to these trachea "ring" (i.e., slice) cultures, explants of quarter-sections of trachea were maintained using a new method. Tracheas were removed as in the ring procedure but were cut in half longitudinally and across, thereby forming four large, nearly flat or slightly curved pieces approximately 5 by 10 mm. Three such pieces were placed (epithelial side up) on a bed of agar (10 ml per 60- by 15-mm petri dish) and overlaid with 0.7 ml of medium. The agar base consisted of 2 g of Ionagar (Colab Laboratories, Glenwood, Ill.) and 1.2 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma Chemical Co., St. Louis, Mo.) in 160 ml of water. After being autoclaved, the agar was mixed with 20 ml of medium 199 (GIBCO), 20 ml of fetal calf serum, 1.5 ml of 0.1% phenol red, and penicillin G (final concentration, 500 units/ml). The liquid overlay was medium 199 with 10% fetal calf serum and 500 units of penicillin G per ml. The pH of both was previously adjusted to 7.6 with 7.5% sterile sodium bicarbonate. Cultures were incubated at 36°C in 5% CO_2 in air. Such cultures will be designated as "explants," whereas slices of trachea grown in liquid medium only will be designated as rings. Tetrazolium chloride reduction, a measure of cellular dehydrogenase activity, was measured as previously described (8, 13).

RESULTS AND DISCUSSION

Preliminary experiments with mouse liver slices indicated that solid tissue used detectable amounts of oxygen in a regular fashion. Data presented in Fig. 3 show that the rate was approximately $5 \mu\text{l/h}$ with 20.3 mg of slices (1- to 2-mm thick). After 12 min of incubation in the respirometer, the medium was expelled, and the 1.0-ml chamber was purged with 10 ml of fresh Tyrode solution. The tissue remained in situ, and the fluids were continually stirred. When the flow of medium was terminated, the electrode restabilized and oxygen uptake continued in a linear fashion at essentially the

same rate as that noted before the 2-min wash-out.

To determine the limits of oxygen usage by tissues, hamster trachea and mouse kidney tissues were compared with the respirometer (Fig. 4). Mouse kidney displayed a very rapid utilization of oxygen, even when tested at 5.5 mg. Larger amounts of tissue (e.g., 10 mg) rapidly used the available oxygen in a linear fashion for 10 min ($>6 \mu\text{l/h}$), but afterwards uptake became nonlinear. Hamster trachea tissue, at 5.3 mg, had a respiratory rate slightly in excess of the blank (zero tissue) control. At 10.0 mg, uptake was similar to that noted with approximately 5 mg of mouse liver (i.e., $2.4 \mu\text{l/h}$).

To ascertain effectiveness with cultured cells, monkey kidney and HeLa cell suspensions were used. Both cell types used more than $1.5 \mu\text{l/h}$ when concentrations of 2×10^6 to 5×10^6 cells/ml were tested, although monkey kidney cells

FIG. 3. Oxygen uptake by mouse liver slices in respirometer. After 10 min (arrow), the medium was replaced with Tyrode solution.

FIG. 4. Comparison of respiration rates by comparable amounts of tissue from mouse kidney and hamster trachea.

had a somewhat faster rate. The supposition that such oxygen uptake was related to cellular respiration was confirmed by blocking such activity with a respiratory-enzyme poison. Cultures of monkey kidney cells ($5 \times 10^6/\text{ml}$) were placed in the respirometer and were either left untreated or were exposed to cyanide (Fig. 5). When $5 \mu\text{l}$ of 2 M NaCN was injected into the 1-ml chamber, oxygen uptake ceased almost immediately. The control culture continued to use oxygen in a nearly linear fashion well after activity had stopped in the sample treated with the cyanide.

Oxygen uptake could be curtailed by physical inactivation as well as by chemical cytotoxicity. Explants of hamster trachea, maintained at an agar-liquid interface, were tested for oxygen utilization after exposure to heat or freeze-thaw inactivation (Table 1). Explants that were untreated maintained metabolic activity as shown by the tetrazolium reduction assay. Cellular

FIG. 5. Oxygen uptake by monkey kidney cells ($5 \times 10^6/\text{ml}$), either untreated (curve B) or exposed to $5 \mu\text{l}$ of 2 M NaCN injected into the 1.0-ml chamber at $t = 15 \text{ min}$ (curve A).

enzymes, especially dehydrogenases, can reduce the colorless tetrazolium chloride into a red, insoluble formazan. When extracted with acetone and measured spectrophotometrically, the value of the optical density at 490 nm per milligram of dry weight was greater than 0.4. Explants that were heat inactivated (56 C , 15 min) or freeze-thaw (three cycles of -20 to $+36 \text{ C}$) inactivated had optical density per milligram values of less than 10% of the control (0.024 and 0.31, respectively). This decrease in cellular enzymatic activity was similarly reflected in respiration rates. Control explants consumed $1.35 \mu\text{l}/\text{mg}$ per h, whereas the heated or frozen and thawed cultures used 0.30 or $0.58 \mu\text{l}/\text{mg}$ per h.

The effects on infectious cytotoxic agents were evaluated with mycoplasma-infected trachea ring cultures, which could be tested for ciliary activity, tetrazolium reduction, and oxygen utilization. Control (i.e., uninfected) cultures showed high ciliary activity, with the relative activity (percentage of the ring remaining intact times the vigor of beating; maximum value of 300) of more than 240 after 6 days in culture (Table 2). Infected trachea organ cultures showed a marked reduction in ciliary activity, with relative activity values of less than 20.

The deleterious effect of *Mycoplasma pneumoniae* on the ciliary motion of the epithelium was also observed in significantly decreased tetrazolium reduction capacity. The mean values (obtained with 36 rings in two experiments) of tetrazolium reduction, expressed as the optical density at 490 nm per milligram of dry weight, were 0.554 for control tissue and 0.113 for infected tissue, a drop of 80%. Respiration rates also showed the significant effect of mycoplasma infection on the metabolism of epithelium. Normal trachea rings consumed approximately $1.19 \mu\text{l}/\text{mg}$ per h when nine rings were evaluated in the 1-ml respirometer chamber. Mycoplasma-infected tissue had a mean consumption rate of $0.42 \mu\text{l}/\text{mg}$ per h, approximately a 65% decrease in respiration.

The results of this study indicate that the

TABLE 1. Effect of cell viability on tetrazolium reduction and oxygen consumption of trachea explant cultures^a

Treatment	Wet wt (mg)	Tetrazolium reduction		Respiration	
		OD ₄₉₀ ^b	OD ₄₉₀ /mg of dry wt	mm/h	$\mu\text{l}/\text{mg}$ per h
Control	3.24	1.370	0.423	157	1.35
Freeze-thaw (3 $\times$)	2.17	0.066	0.031	45	0.58
Heat (56 C, 15 min)	2.57	0.063	0.024	27	0.30

^a Mean data from three replicate experiments.

^b OD₄₉₀, Optical density at 490 nm.

TABLE 2. Effect of *Mycoplasma pneumoniae* infection on the ciliary activity, tetrazolium reduction capacity, and respiration of tracheal-ring organ cultures

Treatment	Expt ^a	Relative ciliary activity		Tetrazolium reduction (OD ₄₉₀ /mg of dry wt ^b)	Respiration (μ l/mg/h)
		Day 0	Day 6		
Control	1	275	242	0.460	1.07
	2	278	258	0.647	1.31
Infected	1	293	16	0.189	0.59
	2	297	0	0.037	0.25

^a Each experiment involved nine trachea rings per group.^b OD₄₉₀, Optical density at 490 nm.

kinetics of oxygen consumption by viable cells can be used to assess the degree of cytotoxicity induced by various agents. This potential is significant in light of the fact that cell and organ cultures are being used with increasing frequency (e.g., microbiology [1, 7]; toxicology [5, 15]; clinical diagnosis [2]). In addition, the effects of metals on the metabolism of cell (14) and organ cultures (D. Adalis, D. Gardner, F. Miller, and D. Coffin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, B49, p. 19) suggest that this method may also be of value for detecting environmental contamination by heavy metals. A major advantage of this respirometer is that it utilizes the electrode and instrumentation available in a clinical blood-gas analyzer. This increases the versatility of the instrument, avoids duplication of major components, and avoids the necessity of having to fabricate oxygen-sensitive electrodes (9) and the accompanying electronic apparatus. This configuration also allows the readout to consist of actual units (e.g., millimeters of Hg) instead of percentages or relative indices. The plexiglass components were relatively simple to construct, assemble, and operate, so the device should be useful in a variety of situations. It should be of special value when immediate changes are of interest, since infectious agents or products with cytotoxic activity can be introduced into the assay system while respiration measurements are being taken.

The greatest potential for the device may well be in pathogenesis studies, since differences in overall oxygen uptake have recently been noted between infected and normal trachea organ cultures (6). The data presented here verify that finding by showing that the rate of oxygen utilization is constant but decreased when trachea organ cultures are infected with an infectious agent such as *M. pneumoniae*. These results show that general metabolism in ciliated respiratory epithelium is altered in a measurable fashion by at least one pathogen. After 6 days in culture, the cili-

ary activity, tetrazolium reduction (dehydrogenase) activity, and rate of oxygen utilization were all coordinately depressed. Methods such as those presented here should provide a basis on which to develop objective, physiological data on cytotoxic responses of cells and tissues.

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