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Novel Culture and Exposure System for Measurement of Effects of Airborne Pollutants on Mammalian Cells

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■ A novel method for exposing mammalian cells in vitro to air pollutants under conditions resembling those in vivo is described. Cells grown on membrane filters are nourished and protected from drying during exposure by perfusion of nutrient medium from the opposite surface of the filter. The cells are covered by a thin layer of fluid comparable in thickness to the mucus layer overlying the respiratory epithelium. Exposure to NO_2 at levels commonly measured in the ambient air of urban settings (0.1–0.2 ppm) produces a pronounced cytotoxic effect after less than 4 h of pollutant encounter. It is proposed that this cell exposure system will have utility in the study of effects of airborne pollutants on mammalian cells.

The first biologically significant contact of airborne pollutants with sensitive animals or humans usually involves the epithelial cells that line the respiratory tract. Cells of the respiratory epithelium are directly exposed to pollutant species under conditions that resemble those of the ambient air, but which differ in at least two important aspects. The inhaled air is immediately warmed and humidified to near saturation; secondly, any pollutant species must penetrate a thin layer of mucus before encountering the cells.

The methods available to assess the nature of events that occur at the cellular level include histology, studies of enzyme activity, and functional analysis at the cellular and organ level. Long-term effects such as tumor development (*1*) and chronic lung disease (*2*) may be shown in animal models. However, these studies do not allow conclusions regarding the initial biochemical events that occur at the first contact of cells and pollutants, and in addition, are complex and expensive to carry out. Short-term effects such as reduction in lung clearance and enzyme induction in lung or other body tissues show that airborne pollutants can produce prompt effects on cellular organelles and metabolism (*3*). These short-term effects can be specified as to target cells in intact lung by histochemical or autoradiographic methods. However, the analysis of biochemical events in the various classes of cells is not possible because of difficulties presented in their separation from each other.

Programs for the isolation and in vitro maintenance of lung cells by class have been aimed at overcoming the deficiencies experienced in the analysis of short-term effects. However, the in vitro studies have been conducted with cell cultures maintained beneath a layer of serum-containing medium several millimeters deep. This physical conformation is unnatural because it requires the pollutant to be absorbed by the medium before contacting the cells. Rather than experiencing a relatively direct exposure to the pollutant, the cells are exposed to products resulting from the interaction of the pollutants with medium components. These may be as simple as soluble ions (NH_4^+, SO_3^{2-}) or may be addition products with serum components of unknown structure and reactivity. Interpretation of observed effects on cellular activity then becomes difficult.

The problem is well illustrated by studies of the effect of NO_2 on conventionally grown cell cultures (*4*). When covered by a few millimeters of serum-containing medium, the cells were resistant to killing by NO_2 in concentrations up to 4100 ppm. When the cell culture flasks were inverted so that the NO_2 containing atmosphere could contact the cells more directly, the cells were rapidly killed at concentrations less than 100 ppm. By comparison, in vivo effect-producing concentrations are on the order of 1 ppm. In animals, histological changes in the lungs were seen after a few hours exposure to about 2 ppm (*5*), and loss of bactericidal capability by pulmonary macrophages occurred after a 17-h exposure to 0.7 ppm (*6*).

The results noted above suggest that in experiments conducted in vitro, the thickness of the media overlying the cells must be strictly limited to simulate effectively the concentrations and effects of airborne pollutants that occur in vivo. The present paper reports on the development and testing of a cell culture and exposure system for studying monolayers of mammalian cells in vitro to air pollutants under conditions that closely resemble those in vivo.

Materials and Methods

Growth Medium and Cell Cultures. In all studies reported below, the cell growth medium was Eagle's minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL Fungizone). All cell culture media and reagents were obtained from Grand Island Biological Co., Grand Island, N.Y.

Cell cultures of mixed morphology were established from near-term golden hamster embryos (*Mesocricetus auratus*) and from adult hamster tracheas using conventional techniques (*7*). In brief, the tissues were minced and incubated at 37 °C with 0.25% trypsin. The freed cells were harvested by centrifugation and seeded into plastic culture flasks (Falcon Plastics, Oxnard, Calif.) with growth medium. Cell line V-79 (Chinese hamster lung) was obtained from E.H.Y. Chu (University of Michigan, Ann Arbor) and line CHO (Chinese hamster ovary) from J. E. Cleaver (University of California, San Francisco).

Cell Culture Method. Various methods were explored in the present study to expose cell cultures in vitro under conditions similar to those in vivo. Included were roller bottle cultures in which the bottles were continuously flushed with the test atmosphere, and devices that periodically immersed cell-bearing glass slides in medium (including simple tilting of the culture flasks) between exposures to the test atmosphere. Loss of cell viability due to drying was difficult to control in these systems, especially for extended exposure of many hours. In addition, movement of the medium across the cell layer tended to dislodge the cells and make quantification of the effects of the pollutant difficult. (For example, dislodgment of cells precluded clonal experiments in which the survival and retention of single cells or their progeny could be used to measure toxicity in terms of the subsequent formation of macroscopic colonies.) Also, the periodic immersion in a moving layer of growth medium likely washed away or

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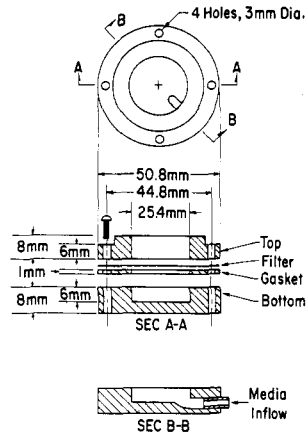

Figure 1. Cell culture assembly

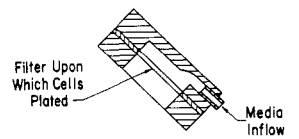

Figure 2. Orientation of cell culture assembly during exposure

otherwise disrupted the complex, mucopolysaccharide-lipid-protein interface layer between the atmosphere and the cell interior, and allowed accumulation in the medium of toxic products formed by pollutant absorption from the test atmosphere.

With guidance from these early experiments and strict adherence to simulating the in vivo conditions, a novel design for a cell culture method evolved that satisfies the following criteria:

- The cells be covered by a layer of fluid medium about as thick as the mucus layer over the respiratory epithelium
- The cells be nourished and protected from drying during the exposure
- The medium flow toward the cells from below at a very low rate to avoid dislodgment of cells or cellular surface exudate
- Exposures be carried out in a biologically sterile atmosphere to avoid contamination with microorganisms
- The cells be readily recoverable from the exposure system to accommodate tests for mutagenesis and malignant transformation, or biochemical analysis.

The novelty of the cell culture method stems from the design and use of the filter holder assembly shown in Figure 1. The principle of operation involves first planting cultures as a monolayer or as thinly dispersed cells on a cellulose membrane filter. Cultures are seeded by gentle suction onto 47-mm diameter Millipore filters (type HA) that have been treated to remove surfactants used in manufacture. The treatment involves heating to 80–90 °C in a large volume of Hanks' balanced salt solution (Grand Island Biological Co.) followed by a thorough rinsing with distilled water and autoclaving while immersed in distilled water. The cell-bearing filters are then transferred to the uniquely designed filter holder assembly (Figure 1).

Autoclavable plastic (Lexan, General Electric Co.) is used for construction of the assembly. To ensure against leakage, a thin coating of sterile silicone stopcock grease is applied to the silicone rubber gasket and around the nylon screws that secure the assembly. The assembly is fitted with a tubing connection to allow perfusion of the filter with nutrient medium from the side opposite the cells at a controlled rate of approximately 0.02 mL/cm^2/h using an infusion pump (Harvard Instruments, Inc., Model 930, Cambridge, Mass.).

For exposure to gaseous media, each assembly is positioned with the cell-bearing surface facing downward at a slight angle from horizontal (Figure 2) so that the growth medium runs off as waste and does not accumulate over the cells. The result is a continuously renewed, thin layer of medium overlaying the cells corresponding to the thin mucus layer in vivo.

Exposure System. The system designed to generate, control, and monitor pollutant atmospheres is shown in Figure 3. At the heart of the system are two chambers, an NO_2 exposure chamber and a control chamber. Each chamber has a volume of approximately 28.3 L (1 ft^3) and can accommodate up to four cell culture assemblies. An exposure temperature corresponding to that prevailing in the respiratory tract (37 °C) is maintained by enclosing the chambers in temperature-controlled incubators.

The inlet stream to the chambers is composed of air with 5% CO_2. (CO_2 is introduced to maintain the proper pH in the bicarbonate buffer containing cell culture media.) Air filtration, sterile airways, and sterile chamber walls are maintained to eliminate contamination by airborne microorganisms. All fittings are stainless steel. Tubing is either Teflon or stainless steel flame sterilized prior to installation.

Compressed house air and a cylinder of compressed CO_2 are used as the sources for the inlet stream mixture. The compressed house air is purified by a 5.0-μm filter and a 0.4-μm filter in series. The flows of air and CO_2 are metered by use of stainless steel rotameters. A flow rate of 4 L/min is maintained to each chamber although the flow rate may be varied to allow alternate residence times. With a flow rate of 4 L/min, approximately eight volume changes per hour occur.

Before entering the chambers, the air/CO_2 mixture is prewarmed to 37 °C by passage through a coil of stainless steel tubing within each incubator. The mixture may also be passed through two gas washing bottles in series, should elevated humidification be desired. Just prior to entering the exposure chamber, the appropriate amount of NO_2 is injected. The main components of the pollutant generation system include

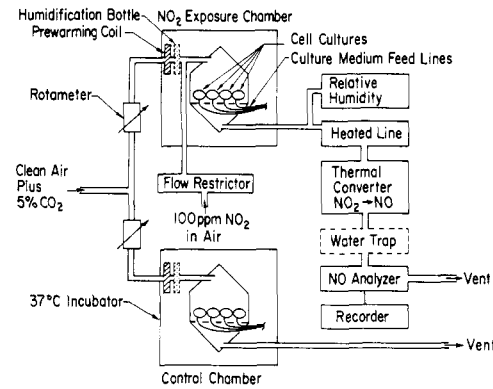

Figure 3. Exposure system

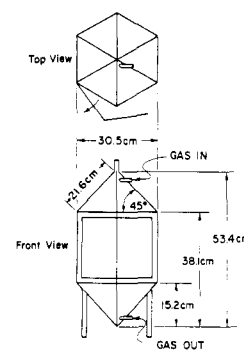

Figure 4. Chamber

a cylinder of compressed NO_2 in synthetic air (approximate concentration of 100 ppm NO_2) and a flow restrictor (porous metal structure) to maintain precise control of flow into the diluting mainstream. Changes in exposure concentrations may be accommodated with ease.

The design of the exposure and control chambers is patterned on the "Rochester chambers" used in the exposure of whole animals (*8*). The chambers are hexagonal with conical top and bottom sections as shown in Figure 4. The gas entry and exit ports are at the top and bottom, respectively. The gas enters in a tangential manner to promote uniform mixing into the central portion of the chambers where the cell cultures are positioned. The chamber bodies are constructed of autoclavable Lexan. The interior of the exposure chamber is coated with silicon grease to minimize NO_2 absorption by the Lexan.

Continuous monitoring of the chamber atmosphere is necessary to ensure that deviation from the set exposure conditions does not occur. The primary method for continuously monitoring NO–NO_x is a Beckman 952 chemiluminescent analyzer. The Saltzman method (*9*) is used as the backup and verification method. Relative humidity is monitored continuously using a Brady Array Model SC1021-M. Internal probing is conducted periodically to ensure that the NO_2 concentration is uniform throughout the chamber.

Monitoring oxides of nitrogen in an airstream that may experience elevated relative humidities requires precautions to prevent condensation of water and the subsequent absorption of NO_2. After passing through the exposure chamber, the gas stream flows through a heated vent line to an external NO_2 to NO converter. The temperature of the vent line is kept slightly higher than that of the incubators to prevent condensation and the associated loss of NO_2. After conversion of NO_2 to NO, which is relatively insoluble in water, the gas is passed through a water trap. The dry gas is then fed to the chemiluminescent analyzer and analyzed for the NO concentration. A recorder provides a permanent record of the NO_2 concentration (as NO) during the course of an experiment.

Experimental Results

Selection of Membrane Filters. Several filters of various pore sizes and manufacturers were evaluated for use as substrates for cell growth (Table I). Primary considerations were the ability to support an active increase in cell number and maintenance of morphology similar to that observed in cell cultures on the usual glass or plastic surface. Secondary considerations were strength and retention of flatness while subject to the pressure of the infusion pump.

Two hamster cell strains (embryo, tracheal) were planted at an initial density 1×10^5 cells per filter in 35-mm culture dishes, and samples were fixed, stained, and mounted on days 3 and 5 after planting.

Good morphology was judged as a flat, even appearing cytoplasm as opposed to a rounded or spindly appearance (3–6 cytoplasmic extensions from the main cell body extending at times into the pores of the filters). Cells on filters with pore sizes of 3.0 μm or greater developed a spindly appearance. By comparison, cell morphology on the Millipore 0.45-μm filters and the Sartorius 0.1-μm filters was acceptable. The 0.45-μm Millipore filter was selected for use in the subsequent experiments based upon the production of a cell distribution more uniform than that produced using the Sartorius filter.

Table II. Test for Effect of Perfusion on Cell Attachment

Cells ($\times 10^{-5}$ planted 491 mm^2	Cells ($\times 10^{-6}$) recovered from same area after perfusion		Cells ($\times 10^{-6}$) recovered from controls
	Counts	Means	
Hamster cell line V-79			
1	0.42	0.38	0.45
	0.34		
5	2.2	2.2	2.3
10	5.3	6.6	5.5
	7.8		
20	4.8	6.0	6.1
	7.2		
Hamster cell line CHO			
1	0.60	0.68	0.72
	0.75		
5	0.80	0.76	0.69
	0.71		
10	1.1	1.4	1.7
	1.7		
20	3.0	2.9	3.2
	2.8		

Effect of Perfusion on Cell Attachment. A series of tests was conducted to ensure that the cells remained attached to the filters during perfusion and could be quantitatively recovered from the filters following perfusion. Hamster cells lines V-79 and CHO were suction planted on Millipore filters at four densities and maintained in 60-mm dishes for approximately 6 h. The filters bearing cells were assembled into filter holders and allowed to remain under an overlying layer of medium comparable to a petri dish culture for an additional 18 h before perfusion was begun. The filter holder chambers were then inverted to a "cells down" position, and the cultures were perfused for 6 h. The filters were then removed from the holders and incubated with trypsin. The cells were collected by centrifugation, resuspended in medium, and counted in a hemocytometer. Control cultures were mounted in filter holder chambers but held in a "cells up" position and were not perfused. The results presented in Table II show that the cells remained attached to the filters during perfusion and were

Table I. Membrane Filters Evaluated as Substrates for Cell Growth

Brand	Type	Av pore size,[a] μm	Composition[a]	Av thickness,[a] μm
Sartorius	S113-09	0.1	Nitrocellulose	115
Sartorius	S113-02	3.0	Nitrocellulose	170
Sartorius	S113-01	8.0	Nitrocellulose	170
Millipore	HA	0.45	Mixed esters of cellulose	150 ± 10
Millipore	SS	3.0	Mixed esters of cellulose	150 ± 10
Millipore	SM	5.0	Mixed esters of cellulose	130 ± 10
Millipore	SC	8.0	Mixed esters of cellulose	130 ± 10

[a] Manufacturer's data.

Table III. Effect of Humidification of V-79 Cells Exposed as Microcolonies

Air/CO_2, not humidified	Air/CO_2, humidified	Incubator immersed
No. of colonies in exposed areas		
90 ± 14.4	73.5 ● 9.7	116.25 ± 12.6

recoverable from the filters to the same extent as from the nonperfused controls.

Effect of Humidity on Cell Survival. Loss of cell viability due to drying of the cell cultures exposed to ambient atmospheres is a potential handicap in studies of the kind proposed. The exposure chamber was developed with provisions for maintaining a humid atmosphere during the exposure to polluted air. To determine whether humidification is important to cell survival, two exposure chambers were set up with atmospheres at 5% CO_2/95% air. One chamber was maintained at about 90% relative humidity, while the other was operated at ambient humidity which ranged from 40 to 60%. Filters were planted with either 5×10^5 (mass level) or 200 (colony level) line V-79 cells. Filters planted with cells but not mounted in holders were placed in petri dishes covered with growth medium, and placed in a separate incubator to serve as controls. After a 6-h exposure of the cells, the filters supporting the colony level plantings were removed from the holders and transferred to petri dishes. In the case of the mass level plantings, the cells were removed from the filters with trypsin and replanted at colony level in petri dishes. After one week, the number of macroscopic colonies formed in each case was determined. The results of this test are presented in Table III and indicate that high humidity during exposure of microcolonies on the perfused filters of up to 6 h is not required for subsequent formation of macrocolonies. The values are the mean number of colonies ± SD and are based on four or five replicate plates. Table III suggests a slight loss of colony-forming cells during perfusion. However, this loss was small, and in subsequent experiments such losses did not confound toxicity studies since exposed and control cells were identically perfused. Table IV shows results demonstrating that perfusion of mass cultures at high or intermediate humidities did not alter the plating efficiency or colony-forming ability of the cells. These data also show that the colony-forming ability of the V-79 cells recovered from filters ranged from 50 to 70%. This value is lower than that observed under optimal culture conditions, where 100% may be approached (*10*). The reason for this lowered value is not known.

These findings were unexpected and suggest that this system has latitude for operation under conditions simulating lower humidity over the epithelium of the upper respiratory tract (for example, the upper trachea during mouth breathing) as well as the high humidity that exists over the epithelium in the lower respiratory tract.

Cytotoxic Effects of NO_2 Exposure. Concurrent with the development of the filter holder assembly, tests of the toxicity

Table IV. Effect of Humidification on V-79 Cells Exposed as Near-Confluent Monolayers

Air/CO_2, not humidified	Air/CO_2, humidified	Incubator immersed
No. of colonies per 200 cells plated		
106.2 ± 9.6	118.2 ± 10.7	116.2 ± 5.8
No. of colonies per 500 cells plated		
248.2 ± 14.2	286.6 ± 52.2	354.4 ± 18.9

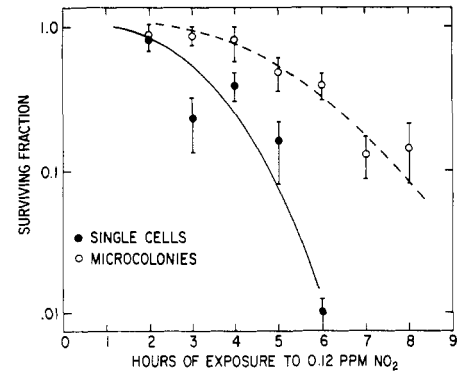

Figure 5. Survival of V-79 cells exposed to 0.12 ppm NO_2

of NO_2 on cultured cells were conducted in cell culture flasks. The flasks were flushed with the NO_2/air mixture under study, and then held for 30 min to 1 h so that the cells remained covered with the nutrient medium. The highest concentration of NO_2 tested in these experiments was 100 ppm. No effect was observed when the cells were covered by a 5-mm layer of medium. When the overlaying medium was removed, the cells were quickly killed. These findings confirmed observations reported by others (*4*).

Cell cultures were then exposed to NO_2 using the cell culture assemblies and the exposure system developed during the present study. It became immediately apparent that cells covered with a thin layer of overlying media are highly sensitive to killing by NO_2 at low concentrations. Exposure of cells to only 1 ppm of NO_2 for 6 h killed nearly all the cells as judged by their ability to form macroscopic colonies upon subsequent incubation. The concentration of NO_2 was therefore lowered to 0.12 ppm for subsequent experiments.

Figure 5 shows the cytotoxic effect of NO_2 on line V-79 cells that were either planted on the filters and immediately exposed to the pollutant or allowed to remain in submerged culture overnight before exposure. The surviving fraction is the number of cells surviving the NO_2 exposure divided by the number of cells surviving a similar exposure to clean air at the same time and for the same length of time. In clean air, only a slight loss of colony-forming cells (less than 10%) occurred during exposures up to 8 h. The difference in the two curves shown in Figure 5 is attributed to the fact that during the overnight incubation, the singly planted cells underwent one or two divisions and produced microcolonies of 2–4 cells. The curve obtained by the immediate exposure of cells following planting has a slight shoulder, which suggests some level of resistance or perhaps a repair process. Since the mechanism of killing in this system is completely unknown, any conclusion would be premature. A possible mechanism is peroxidation of unsaturated lipids in the cell membrane (*11*). A trivial explanation for the killing, such as lowering of the pH of the culture medium by absorption of NO_2, does not seem likely since the phenol red indicator in the cell culture medium did not indicate such a pH change. Attempts to lower the pH of the growth medium by bubbling a mixture of 100 ppm NO_2 in air through a small volume of medium gave no indication of change even after 30 min of vigorous bubbling.

Summary

The in vitro analysis of the events surrounding the interaction of airborne pollutants and cells in culture, including cultured cells from respiratory epithelium, has been obliged to deal with unrealistically thick overlays of medium. Data are of limited value because of the inability to maintain the cells in a state similar to that in vivo during the exposure to the pollutant.

A novel cell culture and exposure system has been developed which allows exposure to test atmospheres for prolonged periods without loss of significant numbers of cells by dislodgment or loss of cell viability due to drying. The system also permits recovery of the cells for further analysis. In addition, the cell culture and exposure system allows evaluation of the mode of interaction of gases with cells under conditions that are relevant to the in vivo experience.

The value of such a system has now been demonstrated as a result of observations made during the design, building, and testing of a prototype system. These observations show that NO_2, a toxic and possibly mutagenic agent for mammalian cells, prevents growth of clones from single cells at 0.12 ppm when the medium layer covering the cells is very thin but not when the medium is in a layer of conventional thickness (i.e., about 5 mm). The toxic effect is time dependent and occurs without an apparent change in the pH of the medium. It may represent a "hit" on cells that is the result of nearly direct interaction of NO_2 with cell surfaces. Not only does this appear to be relevant to the mode of injury that occurs in vivo, but this particular physical configuration of the in vitro system is necessary to produce data analogous to the in vivo response of respiratory epithelial cells.

Acknowledgment

This study was a joint activity of the Department of Community and Environmental Medicine and the School of Engineering at UCI. The authors acknowledge the valuable technical contributions of Marcia Witte and John Taylor in the design and development of the cell culture system and exposure system, respectively, and for the operation of the facility.

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Statistical Evaluation of Trends in Ambient Concentrations of Nitric Oxide in Los Angeles

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■ The aerometric data on nitric oxide at several air monitoring sites in Los Angeles County for the period of 1959–1974 are statistically analyzed to determine trends in the concentration levels. Time series and intervention techniques are used to filter out the effects of seasonality, serial correlations, and short-term trends in weather variables. The ambient impacts of the 1966 and 1971 automobile exhaust programs are evaluated in terms of the trend parameters.

The effectiveness of a pollution control measure may be a posteriori judged by the step changes and trends induced in the pollutant concentration. Such information is vital for future policy decisions. Graphical techniques may be used to obtain preliminary estimates of the control effects. Results of such analyses for various atmospheric contaminants in Los Angeles County have been reported (*1–4*). However, for precise estimation of these effects, one must duly account for the seasonal behavior and the random fluctuations in the pollutant concentration. The intervention methods previously described (*5–7*) provide a useful tool for estimation of these effects.

Nitric oxide (NO) is a primary input in the formation of photochemical smog in Los Angeles. Beginning with the 1966 automobile model year, California state air quality standards called for hydrocarbon emissions not to exceed 275 ppm and carbon monoxide emission not to exceed 1.5 vol % (*8*). American automobile manufacturers attempted to meet these standards with a series of engine modifications. It was suspected (*9*) that these modifications had the undesirable effect of increasing substantially the emissions of NO. Consequently, a new set of air quality standards was implemented with the introduction of the 1971 model vehicles calling for a maximum level of NO emissions of 4 g/mile. As the proportion of new cars in the car population meeting these standards increases over time, one would certainly expect a down trend in the ambient concentration level of this pollutant.

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