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Gaseous Oxide Toxicity Evaluated with Cell Monolayers on Collagen-Coated, Gas-Permeable Teflon Membranes

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A system was developed to evaluate the cytotoxic potential of gaseous oxides *in vitro*. Target cells were MRC-5 human lung fibroblasts cultivated as monolayers on gas-permeable, FEP-Teflon membranes. Membranes were secured in Chamber/Dishes with a 25 mm diameter well. To promote attachment of fibroblasts to the membranes, the latter were incubated in collagen (Vitrogen) solutions for 10 min prior to plating the cells. The collagen pretreatment was significantly more effective than poly-L-lysine, fetal calf serum, polybrene and bovine serum albumin. Several types (mouse and calf) of acid-soluble and alcohol-soluble collagen fractions were evaluated, and all of them promoted cell attachment with equivalent efficiency. Cells on membranes were exposed to gases in a Plexiglass chamber with a gas flow of 2 L/min. Sulfur dioxide caused a marked loss in cell viability (as indicated by ATP content of the monolayer) after 30 min exposure to 0.01% and 0.005%. A level of 0.001% did not affect viability, and none of the levels tested caused a sloughing of the monolayer after 90 min. Nitrogen dioxide induced a more modest drop in cell viability after 30 min exposure to 0.1%, while 0.005% and 0.05% were nontoxic. No cell sloughing occurred with NO_2 exposures, and exposures to CO_2 at levels of 20% for 90 min were nontoxic. This system, with cell culture monolayers on gas-permeable Teflon membranes, is simple and convenient. As such, it has potential application to cytotoxicity evaluations with numerous gases.

Introduction

Gaseous pollutants present a significant potential health hazard because of constant exposures and the difficulties encountered in containing, removing, or neutralizing the agents. In addition, the respiratory tract has an exceptionally large surface area with sensitive cells, the loss of which can lead to secondary effects such as infection. Gaseous oxides (CO_2, NO_2, and SO_2) and ozone (O_3) are common in the atmosphere of industrialized areas, and each has cytotoxic activity. Data from human and animal inhalation studies indicate that in high concentrations they can retard mucociliary clearance (*1*), increase airway resistance (*2*), cause lipid peroxidation (*30*, and induce overt cytonecrosis (*4,5*).

In vitro cell culture models (*6–8*) often have been used to gain some insight into the mechanisms which may help explain respiratory toxicity. These model systems provide improved control over environmental variables and greater precision in exposures. Unfortunately, the amount of gaseous agent within an exposure chamber is not necessarily equivalent to the dose experienced by the target cells. This is because the depth and composition of the fluid medium can affect the gas. Only the dissolved gas which is unbound and not neutralized can reach the cell monolayer. This technical problem can be circumvented by cultivating the cells on porous mem-

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brane filters (*9*) or by rotating standard culture vessels to directly expose the cells to the gaseous phase (*8*).

As an alternative solution to the exposure problem, we have developed a system in which cell monolayers are established on etched FEP–Teflon membranes. These are optically clear and gas-permeable (*10*). Gaseous agents can reach cells directly, without dilution or inactivation by the culture media. Here we describe the mechanics and operation of the system, and present data illustrating the toxicity of sulfur dioxide and nitrogen dioxide for lung fibroblasts.

Methods

The MRC-5 strain of human lung fibroblasts were originally obtained from the American type Culture Collection (#CCL 171). They were maintained in alpha MEM medium containing newborn calf serum (10%). They were routinely seeded at a density of 1×10^5 cells/mL, and were incubated at 37°C in 5% CO_2 in air (98% humidity). Stocks of cells were propagated in 25 cm^2 or 75 cm^2 tissue culture flasks. For the exposure studies, the fibroblasts were trypsinized, washed, and 1.5 mL was added to Bionique Chamber/Dishes (*11*) with FEP–Teflon membranes (both currently available from Corning Glass Works, Corning, NY). To promote attachment of the fibroblasts, the membranes were pretreated with collagen (Vitrogen, Flow Laboratories, McLean, VA) for 10 min at room temperature, followed by three rinses with sterile distilled water.

In addition to Vitrogen, the commercially available collagen, several fresh preparations of collagen were prepared. Calf skin collagen (type III, Sigma, St. Louis, MO) was soaked in 70% ethanol (100 mg/20 mL) for 24 hr at room temperature. After centrifugation to pellet out the undissolved material, the supernatant solution was removed and saved for use (calf skin collagen-alcohol soluble). The pelleted collagen was next mixed with 20 mL 0.1% sterile acetic acid and incubated at 4°C for 48 hr. After centrifugation at 29,000*g* for 1 hr, the supernatant solution was removed aseptically and stored for use (calf skin collagen-acid soluble). Mouse tendon collagen (acid-soluble) prepared with a similar procedure (*12*) was generously provided by D. Byrne and R. Church of this institution.

Monolayers were incubated for 48 hr prior to use (i.e., at the point when the monolayer was approximately 80% complete to confluent. For gaseous treatments, three Chamber/Dishes were placed directly over 1-in. diameter holes in a

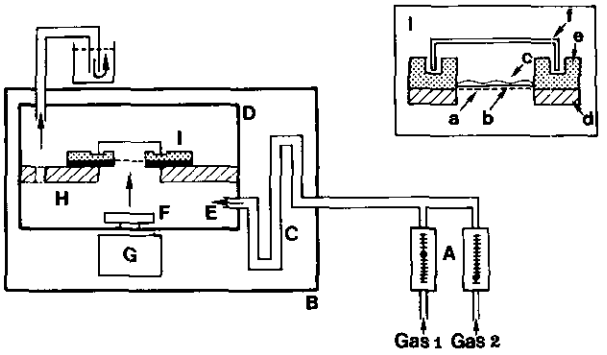

FIGURE 1. Diagram of the system used to expose monolayers to toxic gases: (A) flow meters; (B) incubator; (C) warming coil for incoming gas mixture; (D) Plexiglass chamber; (E) gas inlet; (F) mixing propeller; (G) magnetic stirrer; (H) shelf; (I) Chamber/Dish with Teflon membrane cell substrate. Inset reveals details of Chamber/Dish: (a) gas–permeable Teflon membrane; (b) collagen coating on membrane; (c) cell monolayer; (d) metal base plate; (e) Chamber/Dish core unit of sold Teflon; (f) plastic Petri dish (35 mm) cover.

Plexiglass shelf. The shelf was inserted into a Plexiglass chamber (Bellco Glass, Vineland, NJ), approximately 1 ft^3 in volume (Fig. 1). Immediately below the shelf was a propeller-type gas mixer driven by a magnetic stirrer outside of the chamber. The entire unit was placed inside a 37°C incubator. Gases were proportioned from tanks outside of the incubator, passed through a warming coil, and entered the Plexiglass chamber near the mixer. Gases passed under the Chamber/Dishes with monolayers on the membranes, and left through a liquid trap above the incubator. The gas flow rate was standardized at 2 L/min. Due to the relatively short exposure periods and the fact that the gases never saturated the media, the pH of the cultures did not fall below 7.0 during exposures. Hence, pH effects were apparently negligible in their contribution to toxicity.

Viability assays were based on ATP content, as determined with a luciferin-luciferase assay (*13*). Monolayers were washed three times in PBS prior to extracting the ATP with 0.5 mL of 90% MDSO. The chamber next was rinsed with 0.5 mL Tris-EDTA buffer which was pooled with the MDSO, diluted, and assayed photometrically with commercial reagents (LKB Wallac, Paramus, NJ). Total protein was determined with the BioRad (Richmond, CA) modification of the Lowry assay using a lysozyme standard.

Results

Initial studies were devoted to developing a protocol for the preparation of the target cells.

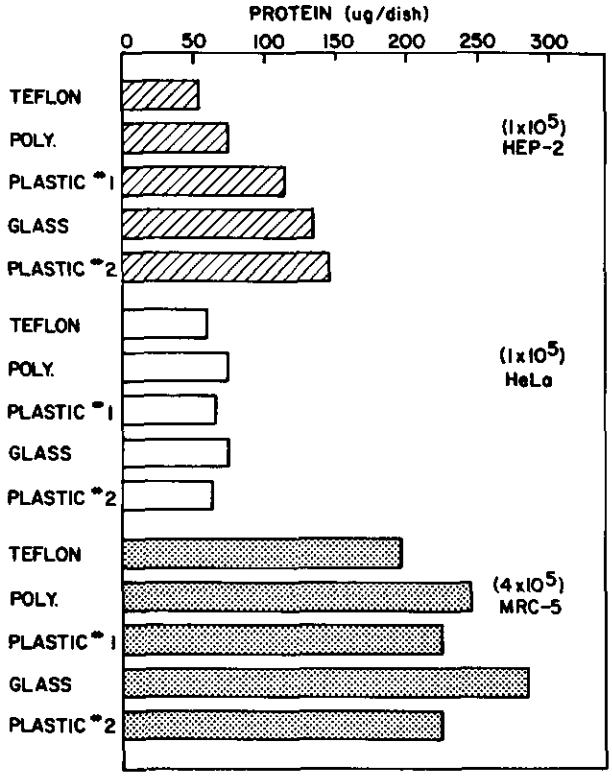

FIGURE 2. Relative attachment of three cell types (MRC–5 lung fibroblasts, HeLa and HEP–2) to various substrates (Teflon membranes; polystyrene; glass coverslips) and two types of commercially available 35 mm tissue culture Petri dishes. Each substrate was placed in a Chamber/Dish with a 25 mm diameter well. Mean data from five replicates.

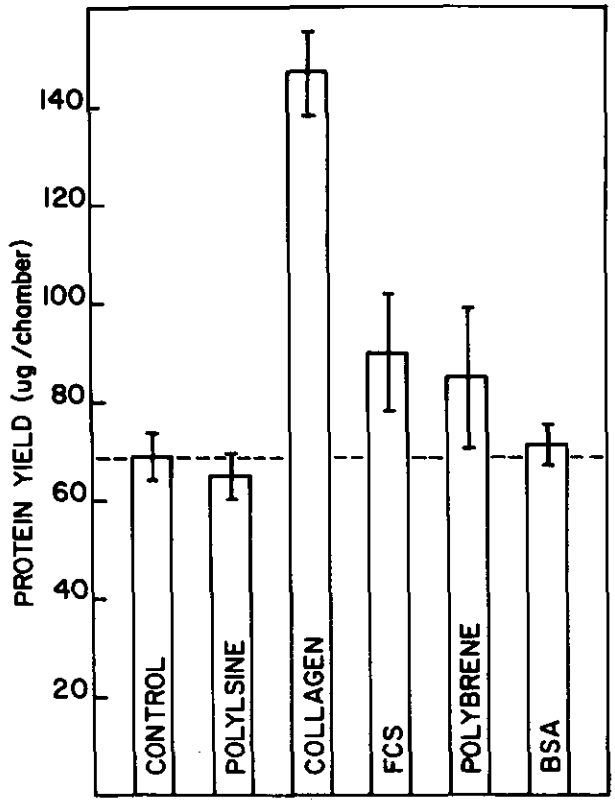

FIGURE 3. Effect of various pretreatments of Teflon membranes on cell yields. Mean data from four replicate, ± standard deviation. Treatments included 10 min exposure to PBS (control), 0.1% polylysine, collagen (Vitrogen), FCS (fetal calf serum), 0.1% polybrene, and bovine serum albumin (BSA, 100 mg/mL).

Fibroblasts of respiratory tract origin (MRC-5 cells, originally derived from human fetal lung tissue) are well characterized and are known to be sensitive to respiratory pathogens (*14*). However, their attachment rate to most substrates is quite low (Fig. 2). Compared to other cells such as HeLa or HEP-2, relative yields of protein were approximately one-half when evaluated on a per cell basis. Attachment of the lung fibroblasts to Teflon membranes was consistently lower than that noted with glass or plastic substrates. This same effect was also noted with HEP-2 cells, but it was not prominent with the HeLa cells (Fig. 2).

In an attempt to increase attachment to the Teflon membranes, several protein pre-treatments were evaluated. The data (Fig. 3) indicate that a brief (10 min) collagen rinse of the membrane was optimal. The collagen was significantly better than purified proteins, serum, or poly-L-lysine. Protein yield after 48 hr of incubation were approximately double those seen with the untreated control membranes or those rinsed with other proteins.

Table 1. Comparison of various collagen types used to promote attachment of MRC-5 lung fibroblasts to etched FEP-Teflon membranes in chamber/dishes.[a]

Collagen preparation	ATP content of monolayer, μg/dish
PBS control	1.48 ± 0.72
Vitrogen	7.05 ± 1.33
Calf skin (acid-soluble)	7.79 ± 0.82
Calf skin (ethanol-soluble)	6.72 ± 0.22
Mouse tendon	6.40 ± 1.87

[a]Mean data ± standard deviation from seven replicate dishes.

Because collagen was clearly superior to the other protein treatments, various collagens were evaluated to determine if any were especially effective. The results (Table 1) indicate that all four of the collagens were roughly equivalent in promoting attachment to Teflon membranes. Both ethanol-soluble and acid-soluble preparations were capable of promoting attachment so that ATP yields (i.e., viable cells) were severalfold

greater than those from control, untreated preparations. For convenience, the remainder of the experiments described here all used Vitrogen as the cell attachment promoter.

The apparatus constructed for the gassing of cell monolayers on Teflon membranes was shown to be effective when used with Chamber/Dishes containing FEP–Teflon membranes. To illustrate gas transfer through membranes, a series of dishes with normal medium were subjected to a 60-min flow of 10% CO_2 in air. Initial pH of the medium was 8.0. Chamber/Dishes with sealed lids and glass substrates did not change, while those with membranes had a mean pH of 7.8. After 120 min, the glass samples had a pH of 7.8, while that in the membrane dishes was 7.5. Samples were run in triplicate, and interdish variaiton typically was ≤ 0.01 pH unit. Clearly, the apparatus and protocol does allow for the passage of low molecular weight gaseous dioxides into the interior of the cell culture vessels.

When sulfur dioxide was evaluated with this lung cell culture system, it was cytotoxic at concentrations of 0.005% and higher (Fig. 4). Levels of 0.1%, 0.001% and 0.005% did not affect the cell mass attached to the membrane since protein contents were static (or even increasing slightly, as with the lowest concentration). Hence, there was no sloughing of the cell monolayer over the relatively short time period (2 hr) of exposure. Measurements of ATP provide a more accurate index of actual cell viability. These data show that 0.001% was without effect, but 0.005% and 0.01% both caused marked decreases after 60 min. By the end of the 120-min exposure period, both concentrations of SO_2 caused decreases in total ATP of at least 50%.

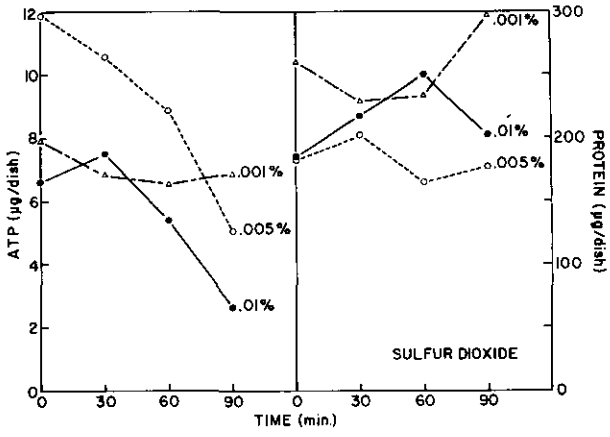

FIGURE 4. Effect of sulfur dioxide exposures on viability of lung fibroblast monolayers cultivated on collagen-coated Teflon membranes. Mean data from three replicates for each point.

Nitrogen dioxide, when tested in this *in vitro* device, was considerably less toxic than sulfur dioxide. Tenfold higher concentrations (NO_2, relative to SO_2) produced only marginal responses. Levels of 0.1%, 0.05% and 0.005% did not cause overt sloughing of the monolayer as indicated by assays of total protein (Fig. 5). Levels of ATP were nearly constant over the 120 min period with exposures to 0.05% and 0.005% NO_2. The highest level tested, 0.1% NO_2, did cause a decrease in ATP after 60 min. By 120 min at 0.1% NO_2, the ATP in the monolayer fell to approximately 2.3 µg, or a drop of in excess of 40% of the control levels.

Not all gaseous dioxides were toxic, however. The data obtained from carbon dioxide exposures (Fig. 6) revealed no sloughing of cells and no loss of viability. Levels of CO_2 as high as 20% did not

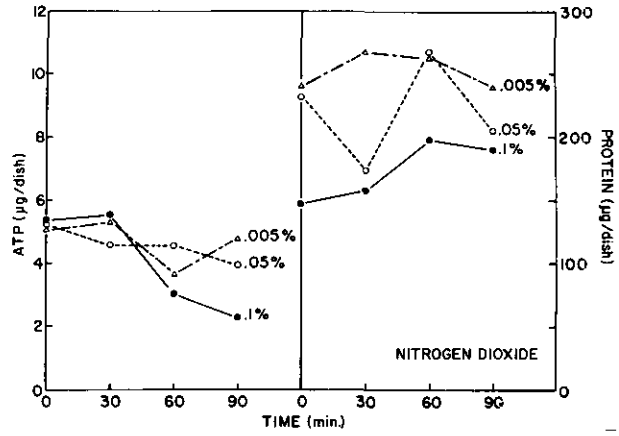

FIGURE 5. Effect of nitrogen dioxide exposures on viability of lung fibroblast monolayers cultivated on collagen-coated Teflon membranes. Mean data from three replicates for each point.

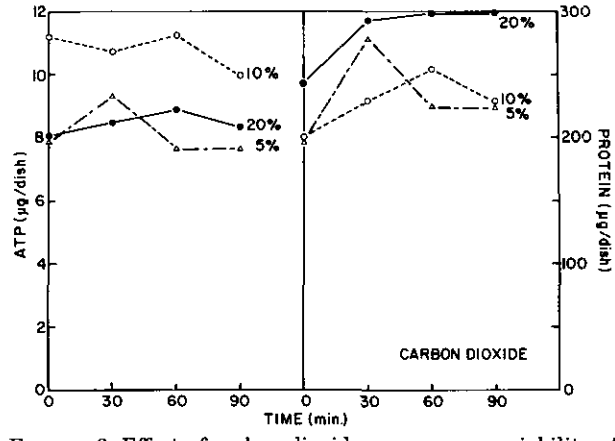

FIGURE 6. Effect of carbon dioxide exposures on viability of lung fibroblast monolayers cultivated on collagen-coated Teflon membranes. Mean data from three replicates for each point.

affect cells, whereas levels of 0.1% NO_2 and 0.01% SO_2 did cause significant decreases in ATP levels.

Discussion

FEP-Teflon (fluorinated ethylene propylene, made by DuPont, Wilmington, DE) is a high density, flexible material which is resistant to most laboratory reagents. Because of its high heat resistance, it can be autoclaved without inducing any change in structure or form. When prepared in thin films (e.g., 25 μm), the material is optically clear. When chemically etched, the surface becomes hydrophilic and can act as a substrate for cell attachment (*10,11*). The unique feature of this material is that it is permeable to most gases in the size range of carbon dioxide. Such membranes have been used with gas-sensing electrodes (*14*) and for delivering oxygen to cells in batch culture (*15*). Most recently they have been used to evaluate the *in vitro* cytotoxic potential of ozone, a common gaseous pollutant (*16,17*). Ozone was shown to decrease both viability and plating efficiency after a 2.5 hr exposure to 0.1 to 0.2 μg ozone/12 cm^2 of membrane covered with alveolar Type II cells.

Our data indicate that common gaseous dioxides represent a spectrum of cytotoxic potential. With human lung fibroblasts and exposure times of 30 to 120 min, SO_2 was highly toxic, NO_2 had minor toxicity, and CO_2 was nontoxic. Levels ranged from 0.001% to 20%. The system which we used was simple to construct and use, and several replicates could be run simultaneously. Toxicity was verified objectively, through the use of protein and ATP assays. The former was a convenient, quantitative index of cells attached to the substrate, while the latter indicated the ratio of viable to nonviable cells when compared with controls. For example, when the protein levels remain near constant and ATP levels fall relative to controls, this means a greater fraction of nonviable cells in the monolayer population. All three dioxides tested failed to alter the cell mass adherent to the membrane, though viability was affected to various degrees.

Since ATP and protein values measure two very different parameters (cell mass versus cell metabolism) direct comparisons between ATP and protein are not as informative as the trends in individual curves. For example, falling protein values indicate monolayer sloughing (none was observed here), while static values mean no detachment, and rising values mean active metabolism and generation of cell mass. Note that because the observation time (90 min) was much shorter than the generation time (17–24 hr under optimal conditions) one is not able to observe increases in ATP which come from cell multiplication. Cases of falling ATP values with static protein values (e.g., 0.005% SO_2) indicate loss of viability without cell detachment. Cases of static values or slight increases in ATP and/or protein mean no loss of viability and continued metabolism and synthesis of cell mass. The speed of the ATP loss is intriguing, but at this stage it would be premature to ascribe it to either cause or effect. Further studies on the kinetics of the ATP decrease will be necessary to determine whether it plays an active or a passive role in pathogenesis.

Our experimental design resembled that of Alink et al. (*17,18*), in that both used FEP-Teflon membranes in chambers situated in a flowing gas environment. Significant technical differences do exist, however. The membranes used here were chemically etched, and it was not necessary to physically abrade the surface. We also used a collagen treatment to promote cell attachment and to establish a monolayer with the fastidious lung fibroblasts. In our system, the mixer used to prevent gradient formation was magnetically driven by an electric motor outside of the chamber. This prevented the accumulation of ozone which would affect the cells in addition to the test gases. Both systems, however, serve well to evaluate gas toxicity *in vitro* since exposures are direct and gas is not diluted or absorbed by the liquid media.

Such *in vitro* experimental approaches represent the most direct route to defining the biochemical mechanisms by which gases affect cells. Early studies on general toxicity involved inhalation experiments on immobilized or anesthetized animals. That type of protocol serves to delineate the physiological response to agents with respiratory tract toxicity but does little to explain cellular pathology and the mechanisms responsible for it. *In vitro* systems allow one to use homogenous cell populations and to accurately control exposures and related environmental factors such as humidity and temperature.

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