System for Studying Uninfected and Virus-Infected Cell Cultures in Hyperbaric Chambers

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Equipment and techniques previously used to investigate the effect of hyperbaric gases on bacteria were modified to permit comparable investigations with uninfected and virus-infected tissue cell cultures. This report describes the modified equipment and related methodology. Use of the system is illustrated with findings on the effect of oxygen-helium mixtures at 68 atm on cell physiology and virus growth in two cell types. Our results suggested that, under those experimental conditions, several synthetic processes in chick fibroblast monolayers are inhibited but that Sindbis virus growth in the cells is increased. Growth of Japanese encephalitis virus in porcine kidney cells was found to be unaffected by oxygen-helium gas at partial pressures of oxygen between 0 and 700 mm Hg, but morphological alterations in the cells occurred at low and high pO₂ levels.

Specialized pressure equipment and methodology have recently been developed in this laboratory for the study of microorganisms under conditions resembling those encountered by personnel in experimental deep sea habitats. As shown by several earlier reports, the system proved to be suitable for determining the effects of hyperbaric helium or oxygen-helium gas mixtures on bacteria. It was found, for example, that exposure of Staphylococcus aureus to such environments resulted in reduced susceptibility to several antibiotics (3, 4) and in decreased activity of a cell wall synthesizing enzyme, murein transpeptidase (6). Similar conditions were also observed to increase the uptake and rate of transport of β-galactosides in Escherichia coli (1, 5). Because these results suggested that the environmental conditions under study caused alterations in the cellular physiology of bacteria, it became of interest to conduct similar experiments with uninfected and virus-infected cell cultures. For these experiments, modifications in the pressure equipment and tissue culture methodology had to be made. With such modifications, the system is now considered to have some unique features and a brief report seems warranted. This paper presents a detailed description of the pressure equipment and techniques used for cell cultures under these conditions and describes some experiments with two ribonucleic acid viruses having different growth rates.

MATERIALS AND METHODS

Pressure equipment. The pressure equipment was designed at this laboratory and manufactured by American Instruments Co., Silver Spring, Md. Fig. 1 is a schematic illustration of the apparatus. The water bath has a capacity of 190 liters and is equipped for water circulation and temperature control over a range of −10 C to +60 C. The bath contains sheet metal racks that hold four rows of six stainless steel pressure cylinders with outside measurements of 30 cm in height and 6 cm in diameter. The pressure cylinders are equipped with removable stainless steel inner sleeves which can be loaded with as many as 18 plastic tissue culture plates, 10 by 35 mm in size. For pressurization, the cylinders are sealed with threaded stainless steel caps fitted with neoprene O-rings and then attached to a manifold by quick-disconnect devices. The manifold-cylinder system is pressurized by compressed gas from supply tanks to pressures up to 68 atm (1000 lb/in²), which is equivalent to the pressure of seawater at a depth of 670 m. The cylinders can be pressurized and depressurized singly or simultaneously in any numbers. Depressurization is accomplished by release of gas at a metered flow rate over a 30-min period. The released gas from cylinders containing infected cultures is channeled through glass-wool filters and into a 290 C furnace. In addition to the pressure cylinders described above, a

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Fig. 1. Schematic drawing of an apparatus for studying the effect of hyperbaric gases on normal and infected cell cultures.

set of larger cylinders with outside measurements of 32 cm in height and 13 cm in diameter, was used. These accommodate 60-mm, rather than 35-mm, tissue culture plates and also have ports through which a vacuum may be drawn for purging the gas phase with experimental gases before pressurizing. Plastic instead of metal sleeves are used to avoid excessive weight.

Cell cultures. Chicken embryo cells (CEC) derived from 10-day embryonated eggs were suspended at a concentration of about $5 \times 10^5$/ml in Hanks lactalbumin hydrolysate medium with 10% heat-inactivated (56 C for 30 min) calf serum and dispensed in 2-ml samples into plastic petri plates (10 by 35 mm). After overnight incubation at 37 C in a 5% CO$_2$-air incubator, the confluent monolayers were washed twice with Hanks balanced salt solution and covered with 1.5 ml of L-15 (Leibovitz) medium with 1% serum either immediately or after virus adsorption.

Porcine kidney cells, designated as PS(Y-15) by Inoue and Yamada (2), were grown as described by Zebovitz et al. (9) in plastic petri plates (10 by 60 mm). These cells, too, were washed and overlaid with Leibovitz medium (5 ml) containing 1% serum. All tissue culture media were purchased from Grand Island Biological Co., Grand Island, N.Y. Media were routinely supplemented with penicillin (100 U/ml) and streptomycin (100 gg/ml) before use.

At the completion of the experiments, the cells were suspended by standard trypsinization procedures. Numbers of viable cells were determined by the trypsin blue exclusion staining technique.

Viruses. The CEC monolayers were inoculated with Sindbis virus (SV) strain AR339 by overlaying the cells with a 0.2-ml suspension containing $2 \times 10^8$ plaque-forming units (PFU). After a 30-min adsorption period, the monolayers were washed twice and covered with Leibovitz medium, as described above. The PS(Y-15) monolayers were inoculated with 0.1 ml (2.0 $\times 10^8$ PFU) of the Nakayama strain of Japanese encephalitis virus (JEV). The adsorption period was 2 h. Virus assays were done by standard plaqueing techniques, by using CEC for SV and PS(Y-15) cells for JEV.

Pressurization of cell cultures. Just before pressurization, plates containing CEC and the small cylinders were placed separately into a large plastic box equipped with glove ports and inlet-outlet ports for gases from supply tanks. After purging the box with a gas mixture of helium and oxygen at a pO$_2$ of 150 mm of Hg (normal O$_2$/He) and flushing for 20 min to displace room air, the culture plates were placed in their cylinders and sealed. The cylinders were next attached to manifolds in the pressure apparatus and pressurized to 68 atm with pure helium. At that pressure, the partial pressure of helium was about 51,500 mm of Hg, and that of oxygen was at the approximately normal level of 150 mm of Hg. Nonpressurized (1 atm or 14.7 lb/in.²) control cultures were held in cylinders containing either normal O$_2$/He or room air. All cultures were incubated at 37 C.

The cylinders which accommodate 60-mm plates, by contrast, were loaded, sealed, and attached to manifolds before the purging procedure. A vacuum of about 260 mm of Hg was drawn and each cylinder was purged for 30 min with normal O$_2$/He. The cylinders were then either pressurized to 68 atm with pure helium or held at 1 atm as controls. For certain experiments, the large cylinders were purged with helium containing various concentrations of oxygen ranging from 0 to 90% in intervals of 10%, or with pure oxygen. In the cylinders that were pressurized to 68 atm with helium, the corresponding final pO$_2$'s ranged from 0 to 700 mm of Hg in intervals of 70 mm of Hg. Several plates containing distilled water were usually introduced in each cylinder to maintain high humidity. All PS(Y-15) cultures were incubated at 35 C. In most experiments, replicate cultures were held in a standard air incubator as additional controls.

Procedures with radioactive compounds. The radioactive compounds were obtained from New England Nuclear Corp., Boston, Mass. The compounds were added to the media at the beginning of each experiment as shown in a footnote to Table 1. Incorporation into cells was determined by removing the medium and covering the monolayers with cold 10% trichloroacetic acid. After 20 min, the trichloroacetic acid precipitable material from each plate or a pool of two or more plates was collected on membrane filters with a pore size of 3.0 gm and washed with 10% trichloroacetic acid. Radioactivity remaining on the filters was determined in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Incorporation of substrate was expressed as counts per minute per 10$^4$ CEC.

RESULTS

While conducting some preparative experiments, we found that growth media containing bicarbonate buffers were not able to maintain proper pH for tissue cells in a closed or pressurized system. Leibovitz medium, however, which is buffered with phosphate, provided satisfactory control of pH for both uninfected and infected CEC or PS(Y-15) cells during the incubation period in pressure cylinders. It was also shown that CEC were able to develop confluent monolayers from suspension on plates in pressure cylinders with normoxic O$_2$/He at 1 or 68 atm as well as in a standard CO$_2$-air...
incubator. The monolayers withstood rapid decompression (10 min) from 68 atm to 1 atm without apparent adverse effects.

**Substrate incorporation by CEC.** A comparison was made on substrate incorporation among cultures exposed to normal O₂/He or to room air. All cultures were maintained at 1 atm for 24 h and included uninfected or SV-infected cultures, and infected cultures to which actinomycin D had been added. The data resulting from these experiments are not presented. We observed that incorporation of alanine, glycine, thymidine, and uridine was higher in the O₂/He-exposed cells than in the air-exposed cells, but the differences were small and of doubtful significance.

Table 1 illustrates the difference in substrate incorporation between cultures maintained in normal O₂/He at 1 and 68 atm. In these experiments the range of incorporation was broad, but in almost every case there was a reduction in incorporation in the pressurized cultures. The weight of the evidence therefore suggests that this overall difference is significant.

**Virus growth in CEC.** SV titers were measured 24 h after infection and exposure to normal O₂/He and 1 and 68 atm (Table 2). Although differences in this case also were small, consistently higher titers were obtained in the pressurized cultures both in the absence and presence of actinomycin D. Since the rate of growth of the virus under the conditions of these experiments has not been determined, we cannot compare the results with those shown in Table 1 in which there appears to be a slight reduction in actinomycin D-resistant uridine incorporation in the pressurized infected cultures.

**Virus growth in PS(Y-15) cells.** In contrast to growth of SV in chick cells, the replication of JEV in PS(Y-15) cells in normal O₂/He was unaffected by pressurization. Figure 2, as representative of typical results, shows that JEV growth, when measured once daily for 3 days in normal O₂/He, was essentially the same at 68 atm as at 1 atm.

JEV growth was also studied in cells after a 48-h exposure to gases other than those with normal pO₂. Figure 3 presents data on virus growth at 1 and 68 atm in gases having 10 different concentrations of oxygen and in comparison with data from cells in normal O₂/He. As can be seen, virus growth was retarded in the absence of oxygen to the same extent at 1 and 68 atm. Intermediate oxygen concentrations or increased helium pressure had negligible effects on JEV replication.

**Cell morphology.** Microscope examination showed that exposure to normal O₂/He at 1 or 68 atm had no effect on numbers, viability, or morphology of normal or infected CEC and PS(Y-15) cells. However, morphological alterations were noted to occur in PS(Y-15) cells subjected to gases with a very low (0 mm of Hg) or very high (630 and 700 mm of Hg) partial

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**Table 1.** Incorporation of amino acids and nucleotides by chicken embryo cells after a 24-h exposure to 68 atm of oxygen-helium gas as compared with 1 atm of gas mixture

| Labeled compound | Infection | Expt showing decrease/total expt | Decrease (%) | Range | Sample mean |
|------------------|-----------|---------------------------------|--------------|-------|-------------|
| Alanine          | –         | 4/4                             | 10–18        | 13    |
| Glycine          | –         | 4/4                             | 10–19        | 14    |
| Thymidine        | –         | 2/2                             | 10–21        | 16    |
| Uridine          | –         | 7/7                             | 4–13         | 9     |
| Uridine (+ac-    | –         | 5/5                             | 5–33         | 19    |
| thinitomycin D)  |           |                                 |              |       |
| Alanine          | +         | 3/3                             | 6–13         | 9     |
| Glycine          | +         | 3/3                             | 2–12         | 8     |
| Thymidine        | +         | 2/2                             | 2–11         | 7     |
| Uridine          | +         | 6/7/c                           | (3–21)       | 9     |
| Uridine (+ac-    | +         | 7/7                             | 6–20         | 13    |
| thinitomycin D)  |           |                                 |              |       |

*Radioactivity per milliliter of medium was: alanine, 0.1 μCi; glycine, 0.25 μCi; thymidine and uridine, 0.05 μCi. Specific activities, respectively, were 137, 101, 56, and 53 mCi/mmol. The approximate ranges of incorporation in counts per minute of these compounds by uninfected and infected cells respectively were: alanine, 15,000–30,000, 3,000–8,000; glycine, 15,000–30,000, 6,000–15,000; thymidine, 30,000–45,000, 12,000–21,000; uridine, 30,000–45,000, 18,000–30,000; uridine and actinomycin D, 800–1,000, 7,000–19,000.

*Five replicate cultures were used in each experiment for each compound.

*In one experiment there was a 3% increase in uridine incorporation.

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**Table 2.** Sindbis virus production in chicken embryo cells after a 24-h exposure to 68 atm of helium-oxygen gas as compared with 1 atm of gas mixture

| Actinomycin D | Expt showing increase/total expt titered | Increase (%) | Range | Sample mean |
|---------------|------------------------------------------|--------------|-------|-------------|
| –             | 6/6                                      | 5–50         | 18    |
| +             | 4/4                                      | 16–56*       | 32    |

*In one experiment there was a 2.3-fold increase (not included in average) in titer, and in all but one experiment virus titers were higher in actinomycin D-treated than in nontreated cultures.
pressure of oxygen. Under such conditions, most cells appeared to be somewhat smaller than control cells with sharply defined nuclei and inconspicuous cell outlines (Fig. 4). Alterations in the morphology of cells were also observed with $O_2/He$ at $pO_2$'s between 350 and 560 mm of Hg, but a smaller proportion of the cells was affected. Altered cell morphology was observed both in uninfected and infected cultures and at either 1 or 68 atm.

**DISCUSSION**

The results obtained in these investigations clearly indicate that our methods are adequate for the detection of relatively small changes in the physiology of uninfected and virus-infected animal cells exposed to gases of various composition and pressure. Previous work using the same apparatus has yielded considerable information on the physiology of several bacterial species (1, 3–6). Most of the above experiments have been of a short-term nature (1–3 days), but the cylinders equipped for continuous gas flow can be used for experiments extending over a period of a week, or until the culture medium needs to be changed. Modifications could be introduced, however, to replace the culture medium, a step which would further increase the time limit of experiments.

The results obtained, portrayed in part in Table 1, suggest that helium is not toxic for cells at 1 atm but may become so at higher pressures. The procedures can be used to test the effect of other gases and of other pressure levels. Conversely, the observations shown in Table 2 and Fig. 2 and 3, that helium has small or negligible effects on virus growth, indicate that these studies could now be extended to a series of other experimental conditions.

Although our experiments were not designed to provide definitive information, it is appropriate to cite work of others who showed that various gases and altered hyperbaric pressure can have significant effects on uninfected and infected tissue cells. Taylor et al. (8), for example, reported that L-cell growth was optimal at a $pO_2$ of 68 mm of Hg but was reduced by almost 20% at 38 or 150 mm of Hg. Segre (7) found that exposure to high $pO_2$ did not change uninfected porcine kidney cells, but that the usually noncytopathogenic hog cholera virus produced cytopathic effects in cells at high $pO_2$. In studies with gases of the helium-xenon series, Schreiner (Fed. Proc. 27:872–878) also showed that exposure to helium caused increased growth in uninfected HeLa cells.

Because of the numerous variables involved, the response of aquanauts to the various stresses introduced by their environments, including response to infectious agents, cannot be accurately gauged. The methods here described offer a simple approach to a study of the individual
FIG. 4. Effect of a high oxygen tension on the morphology of PS(Y-15) cells. Uninfected cells were grown at 1 atm either in helium with a $pO_2$ of 150 mm of Hg (A) or in pure oxygen, $pO_2$ of 760 mm of Hg (B). Magnification: 120×.
USE OF HYPERBARIC CHAMBERS

variables at the cellular level and may lead to the rapid detection of conditions which have a high potential for being detrimental.

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