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Induction of Hypoxia in Glass versus Permanox Petri Dishes

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The survival of Chinese hamster ovary cells in culture following graded doses of X rays delivered under aerobic and hypoxic conditions, or treatment with the bioreductive drug SR 4233 under hypoxic conditions, was evaluated as a function of whether cells were plated onto glass or Permanox plastic petri dishes. In the case of treatment with SR 4233, the influence of varying the total volume of medium in the dishes was also studied. While the Permanox petri dishes were sufficient to yield "radiobiological" hypoxia, that is, oxygen enhancement ratios of approximately 3.0 were obtained for X irradiation, they were inferior to glass petri dishes with respect to the hypoxia-selective cytotoxicity of SR 4233. For a 90-min hypoxic exposure to 40 μM SR 4233, the surviving fraction of cells plated on plastic dishes averaged about 50-fold higher than that of cells plated on glass dishes. Although varying the total medium volume did affect the extent of SR 4233-induced cytotoxicity for glass dishes—drug toxicity decreased slightly with increasing medium volume—this was not the case for the plastic dishes, in which the cell survival following a fixed SR 4233 exposure was essentially constant as a function of medium volume. These results suggest, at least for SR 4233, and under these experimental conditions, that Permanox petri dishes are not satisfactory for such studies.

INTRODUCTION

Tumor control by radiotherapy, and in some cases chemotherapy, may be limited by the presence of clonogenic, hypoxic cells. The development of chemical sensitizing agents has been one of several approaches to attempt to cope with the problem presented by such resistant cells. During the 1960s and 1970s, hypoxic cell sensitizers were developed that, although less efficient than oxygen as radiosensitizers, were more effective at penetrating into hypoxic regions of tumors due to reduced rates of cellular metabolism (*1*). A more recent approach has been to exploit tumor hypoxia, rather than attempt to circumvent it, through the use of bioreductive drugs that exhibit selective toxicity to hypoxic cells (*2–4*).

The identification and characterization of such hypoxia-specific agents involve, among many other factors, extensive *in vitro* screening and a reliable means of rendering the chosen test system hypoxic. The experimental apparatus used to achieve low, reproducible, and verifiable oxygen levels is often awkward to work with, and the direct monitoring of oxygen concentration at a point of interest can present complex problems of physics (*5–9*); neither of these characteristics is particularly conducive to drug screening assays designed to be simple and rapid. One innovation has been the introduction of Permanox disposable tissue culture petri dishes, which are designed to facilitate the rapid removal of oxygen from cultures. These have been used by several investigators, including ourselves, for hypoxia studies (*10–15*). The availability of such disposable dishes with reduced oxygen permeability serves to simplify at least one aspect of such experiments, the inconvenience of having to treat with drug and/or irradiate hypoxic cells in a bulky suspension culture apparatus, or to trypsinize them from glass vessels, prior to replating for a survival assay or other analysis.

In the present study, we have compared the responses of hypoxic Chinese hamster ovary cells to X irradiation and exposure to the hypoxia-specific cytotoxic agent SR 4233 (*4, 16–19*) as a function of whether cells were grown on glass or Permanox petri dishes. In the case of treatment with SR 4233, the influence of varying the total volume of medium in the dishes was also evaluated. We report here that the Permanox petri dishes were sufficient to yield "radiobiological" hypoxia, that is, that oxygen enhancement ratios (OERs) of approximately 3.0 were obtained for X irradiation, suggesting hypoxia as it relates to radiological response. However, the Permanox dishes were inferior to glass petri dishes with respect to the hypoxia-selective cytotoxicity of SR 4233. This suggests, at least in the case of SR 4233, and under these experimental conditions, that Permanox petri dishes are not satisfactory for such studies.

MATERIALS AND METHODS

Cells

Chinese hamster ovary (CHO) cells, subline HA-1, were used for these studies. Cells were maintained in exponential growth by serial passage in αMEM supplemented with 10% fetal bovine serum (Hyclone, Sterile Systems, Logan, UT). For irradiation or drug treatments, cells were trypsin-

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ized from plastic T-flasks (Corning Glass Works, Palo Alto, CA) and diluted, and approximately 5×10^4 to 10^5 cells were plated into each 60-mm glass (Kimax, Kimble Products, Toledo, OH) or Permanox plastic (Lux Inc., Naperville, IL) petri dish. The experiment was then conducted 2–3 days later when the total number of cells per dish was between 1 and 2×10^6.

Irradiation and SR 4233 Treatment

For each radiation dose, one glass and one Permanox dish were selected, their dish lids were removed, the overlying medium was removed and replaced with 2 ml of prewarmed serum-free αMEM, and the dishes were sealed inside of a nylon gassing vessel (*10*). Each vessel was then gassed for 90 min at room temperature with either humidified, ultrapure N_2 (containing less than 10 ppm O_2) plus 5% CO_2 or air plus 5% CO_2. After the gassing period, vessels were sealed and irradiated using a 100-kVp Philips MG100 X-ray unit operating at 85 kV, 9.6 mA, at a dose rate of 0.8 Gy/min to the appropriate total dose. (More details of the dosimetry are given below.) The vessels were then opened, the dishes trypsinized independently, and the cells counted, diluted, and plated in appropriate numbers into Nunc polystyrene 60-mm petri dishes (Nunc, Inc., Naperville, IL) to assay for colony formation 10 days later.

For the SR 4233 treatments, glass and Permanox dishes were prepared as follows: one glass and one Permanox dish contained 1.0, 2.0, 3.0, 4.0, or 5 ml of serum-free medium and an equivalent set of dishes contained 40 μM SR 4233. After the original medium was removed and replaced with the desired volume of prewarmed drug-containing or control medium, six dishes with their lids removed were loaded into prewarmed nylon jigs and gassed with humidified, ultrapure N_2 containing 5% CO_2 for a total of 90 min while the vessels were submerged in a 37°C water bath. After the 90-min exposure time, vessels were removed and opened, the dishes were trypsinized independently, and the cells were counted, diluted, and plated into Nunc petri dishes to assay for colony formation 10 days later.

Each X-ray and SR 4233 experiment was repeated three separate times.

X-Ray Dosimetry and Hypoxia Induction in Nylon Vessels

The X-ray dose delivered to HA-1 cells plated onto the Permanox and glass petri dishes contained in the same nylon jig was expected to be different because of the different attenuation and backscatter characteristics of glass and plastic. Therefore, it was necessary to correct the dose delivered to the glass dishes relative to the known dosimetry for the Permanox dishes. The dose rate to plastic dishes had previously been determined to be 0.8 Gy/min, based on both ionization chamber and thermoluminescent dosimeter measurements. Since the purpose of these experiments was to determine whether it was necessary to change our existing irradiation protocol which used Permanox dishes, we did not initially perform dosimetric measurements for the glass dishes pending the outcome of these experiments. Instead, an approximate dose correction factor for the glass dishes was determined by direct comparison of the aerobic survival curves obtained for the glass and plastic dishes. A correction factor was applied to the dose axis of a plot of surviving fraction versus X-ray dose such that the resulting survival curve for cells grown on glass was superimposable on the curve for cells grown on plastic. Using this "biological dosimetry" approach, the glass-to-plastic dose correction factor was determined to be 1.75; that is, the dose to a glass petri dish within a particular nylon vessel was approximately 75% greater than that to a corresponding plastic dish. This value was quite reproducible in three separate irradiation experiments. This dose correction factor was applied to both the aerobic and hypoxic X-ray survival curves obtained for cells irradiated on glass dishes.

A different "dosimetric" problem was evident for the cells treated with SR 4233, insofar as it was uncertain at what point during the 90-min gassing time the drug would become activated, i.e., the oxygen tension in the liquid phase became sufficiently low to permit drug reduction to its toxic intermediate to proceed (*16, 18*). Furthermore, this time would be expected to vary depending on the volume of overlying medium, the amount of oxygen dissolved in the glass or plastic dishes themselves, the rate of cellular oxygen metabolism (which, in turn, would vary with temperature), and any possible effects of SR 4233 on cellular respiration (*5, 6, 8*). While estimating the influence of these parameters on the toxicity of SR 4233 was the main purpose of this study, it was nevertheless necessary to have some standard for comparative purposes. We chose to use a surviving fraction versus drug exposure time curve for hypoxic HA-1 cells treated with 40 μM SR 4233 as this standard. This cytotoxicity curve was generated using a different type of apparatus (*16–19*) in which cells in suspension were continuously stirred in glass vessels and gassed with nitrogen for an hour before the drug was added, thus allowing the surviving fraction of cells to be measured after a 90-min exposure to drug with "full" hypoxia throughout. The surviving fraction of HA-1 cells obtained under these circumstances, approximately 0.0016 (SEM 0.0010–0.0025), was used as our standard to estimate qualitatively the level of hypoxia achieved in the glass or Permanox petri dishes gassed in the nylon jigs; measured surviving fractions in the petri dish system that were higher than this value suggested that less stringent levels of hypoxia were obtained.

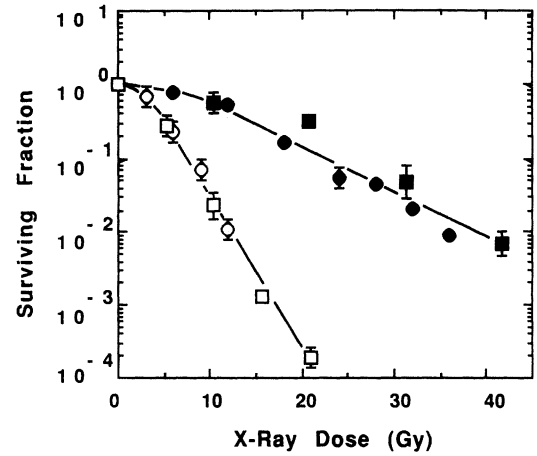

FIG. 1. Acute dose X-ray survival curves for hypoxic (solid symbols) and aerobic (open symbols) CHO cells irradiated as monolayers on glass (squares) or Permanox (circles) petri dishes. Each plotted data point is the geometric mean (±SEM) of three independent survival estimates. A common D_0 of approximately 2.2 Gy was calculated for the aerobic curves, and of 6.7 Gy for the hypoxic curves.

RESULTS

Irradiation Experiments

Figure 1 shows acute dose X-ray survival curves for hypoxic (solid symbols) and aerobic (open symbols) CHO cells irradiated as monolayers on glass (squares) or plastic (circles) petri dishes. The dose correction factor of 1.75 was applied to the survival curve data for glass dishes (see Materials and Methods). A common D_0 of 2.2 Gy (95% confidence limits: 1.6–3.4 Gy) was calculated for the aerobic survival curves, on the basis of a least-squares regression of pooled data for the exponential portions of the curves for glass and plastic dishes. The hypoxic survival curve for Permanox dishes had a D_0 of 6.2 Gy (95% confidence limits: 5.4–7.4 Gy), and that for glass dishes was 7.0 Gy (95% confidence

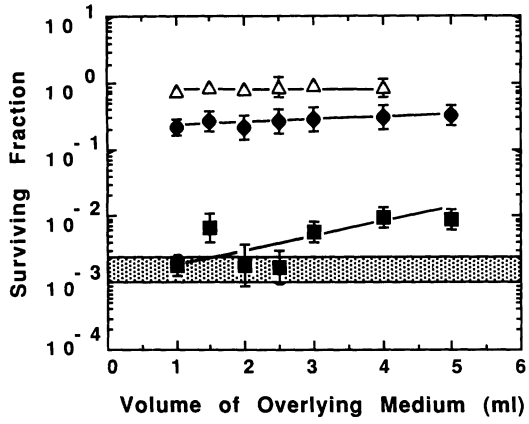

FIG. 2. The surviving fraction of CHO cells treated in Permanox (circles) or glass (squares) petri dishes following a 90-min exposure to 40 μM SR 4233 under hypoxic conditions, as a function of total medium volume per dish. Each plotted data point is the geometric mean (±SEM) of three independent survival estimates. The open triangles show pooled survival data for cells grown on glass and plastic that were treated in an identical manner, but with drug-free medium. The shaded region corresponds to the range of surviving fractions obtained (geometric mean ± SEM) for cells treated with SR 4233 in suspension culture, and rendered "fully" hypoxic before drug was added (see text for details).

limits: 4.2–10.6 Gy). There was no statistically significant difference between these D_0 values for glass and plastic dishes, on the basis of an analysis of variance between the pooled survival data for both types of dishes, and that for the two survival curves considered separately (although it should be noted, despite a lack of statistical significance, that in the case of the hypoxic survival curves, the data points for the glass dishes consistently fell above those for plastic). Thus the common D_0 for the pooled hypoxic survival curves was 6.7 Gy (95% confidence limits: 6.1–7.4 Gy), and the OER, expressed as the ratio of D_0's for the hypoxic and aerobic survival curves, was calculated to be 3.1 (2.7–3.5, SEM).

Hypoxic Toxicity of SR 4233

Figure 2 shows the surviving fraction of CHO cells treated as monolayers in Permanox (circles) or glass (squares) dishes following a 90-min exposure to 40 μM SR 4233 under hypoxic conditions, as a function of volume of drug-containing medium per dish. The open triangles show the survival of cells treated in an identical manner, except with different volumes of drug-free medium. Survival data for glass and plastic dishes were pooled in this case, and indicate that there was little or no toxicity associated with the general procedure of gassing and submersion in a water bath. Further, there was no systematic difference between the plating efficiencies, growth rates, and morphological appearance of HA-1 cells grown on glass or plastic dishes, suggesting that the type of dish *per se* did not influence the experimental outcome (data not shown). Also indicated with the shaded region is the "expected" survival range (geometric mean and one standard error) for cells treated with SR 4233 in suspension culture and rendered hypoxic before drug was added. For cells plated on plastic dishes, the surviving fraction after SR 4233 treatment averaged about 0.27 and did not vary with the volume of overlying medium. For cells plated on glass dishes, the surviving fraction was much lower, averaging about 0.005 for total medium volumes over the 1- to 5-ml range. At the smaller medium volumes of about 2.5 ml or less, the surviving fractions were nearly identical to those obtained for cells treated in suspension culture. Lines were fitted to these sets of data (control, Permanox and glass) using a least-squares regression of the individual survival values for three replicate experiments. Only in the case of the data set for glass dishes was the slope of the resulting curve significantly different from zero (level of significance: $P = 0.028$; correlation coefficient of regression line = 0.67).

DISCUSSION

In the present study, we have compared and contrasted the influence of two physical factors (glass versus plastic dishes and medium volume) on the survival of CHO cells to two toxic species: radiation, for which hypoxic cells should be resistant and aerobic cells sensitive; and SR 4233, a bioreductive drug for which hypoxic cells should be sensitive and aerobic cells resistant. For the radiation experiments, we found that there was no significant difference between the Permanox and glass petri dishes with respect to the OERs obtained for X rays (Fig. 1), suggesting that under both culture conditions, cells were sufficiently hypoxic for full radioresistance. This was not the case for SR 4233 cytotoxicity, however. Although the volume of overlying medium did exert a small influence on the hypoxic toxicity of the drug for cells plated on glass dishes (see below), the surviving fraction of cells on these dishes was nevertheless consistently lower than that on plastic dishes for a given medium volume (Fig. 2). This suggests a greater effectiveness of SR 4233 in the glass dishes, and, by inference, since the degree of drug activation is thought to be related to oxygen level (*17, 18*), that more stringent conditions of hypoxia were achieved.

As previously mentioned, there was a consistent and sizable difference in the average surviving fractions of cells that received identical SR 4233 exposures but were plated on different types of petri dishes. The approximate surviving fraction for cells grown on plastic was 0.27, compared to 0.005 for cells grown on glass, corresponding to a differential toxicity factor of 54. The survival of cells in the plastic dishes was essentially constant as a function of medium volume, whereas for cells in the glass dishes, there was reduced toxicity for increasing medium volumes. A possible explanation for the latter finding is that the presence of greater

depths of medium above the cell layer interfered with the equilibration process between the gas and liquid phases. As has been suggested by Boag (*5*), and more recently by Koch (*8*), a larger depth of medium over the cell layer would result in a longer effective time constant before gas–liquid equilibrium would be reached. This would be especially likely in the absence of constant mixing of the medium in the dishes, as was the case in the present study. The net effect of such a difference in equilibration rates for different medium volumes would be that the dissolved oxygen content following a fixed duration N_2 "degassing period" would be higher with larger total medium volume. As a consequence, the amount of SR 4233 reductive activation (*18*) would be decreased, and drug toxicity would be reduced.

In the case of the Permanox dishes, an essentially constant drug dose response as a function of medium volume was observed. Assuming that some oxygen was dissolved in the Permanox plastic, which would presumably not have been the case for glass, its gradual release (directly adjacent to the cell layer) during the 90-min degassing interval would have the same net effect as increased medium volumes added to the glass dishes, that is, reduced SR 4233 cytotoxicity. If this was occurring even for the smallest medium volume (1.0 ml), the addition of even more medium would not be expected to increase cell survival further, and a constant dose response would result. It has been suggested that a unique structural feature of the Permanox petri dishes may be responsible for the source of the oxygen contamination (C. J. Koch, personal communication). A small "lip" along the base of the dish may trap air underneath, thereby facilitating oxygen diffusion into the Permanox plastic and ultimately to the cell layer. The "continuous flow" gassing technique used in these experiments may not be particularly efficient at flushing out this air pocket, especially for short gassing times, and in the absence of movement of the dishes. In addition, the possible release of dissolved oxygen from the nylon gassing chamber into this air space may further exacerbate the situation.

Although direct measurements of oxygen concentration were not made in the present study, and it is therefore difficult to make inferences regarding mechanisms, two possible interpretations of these experimental findings come to mind. First, the oxygen dependence for the toxicity of SR 4233 may be more stringent than that for radioresistance. There is some precedent for differences in the oxygen tension dependence for radioresistance when compared to other end points such as cytotoxicity and chemosensitization by misonidazole (*15, 21*) or reductive activation of mitomycin C and analogs (*20, 22, 23*); these phenomena exhibit more stringent oxygen requirements than radioresistance. One report in the literature would support this possibility for SR 4233 (*20*), and other studies are in progress to address this issue further (C. J. Koch, personal communication), including one from our own laboratory (J. M. Brown, unpublished results). Indirect evidence from the present set of experiments would also support this possible interpretation. The cellular respiration rate was mentioned previously as one factor contributing to oxygen removal during the 90-min degassing period, and this process is itself temperature dependent. In the radiation experiments, the nitrogen gassing occurred at room temperature, whereas for the drug treatments, gassing took place at 37°C, suggesting that, all other factors such as cell density, medium volume, and dish type being equal, the cellular metabolic rate would be higher at the higher temperature, and oxygen tensions after 90 min in the irradiated dishes would be expected to be higher than those in the SR 4233-treated dishes. That "radiobiological hypoxia" was still achieved under these circumstances suggests that its oxygen dependence may well be less stringent than that for "SR 4233 hypoxia."

A second explanation for these results does not necessarily require that the oxygen dependence for bioreductive toxicity of SR 4233 be more stringent than that for radioresistance. Instead, the findings may be explained by timing differences in the N_2 gassing protocols for the drug versus radiation treatment groups. While the total gassing time was 90 min for both the SR 4233 and the X-ray groups, it should be noted that the irradiated cultures were not treated until the full 90 min of gassing was complete, whereas for the drug treatments, SR 4233 was added at the *start* of gassing, and toxicity then commenced at some (variable) point during the 90-min period. For the glass dishes, the elapsed time before drug activation occurred was presumably quite short (no more than a few minutes), especially for the smallest volumes of overlying medium, since the survival of cells at the end of total drug exposure time was nearly identical to that of the "reference" cells that were rendered fully hypoxic before SR 4233 was added (Fig. 2). For the Permanox dishes, the approximately 50-fold higher surviving fraction obtained following SR 4233 treatment was more consistent with a 30-min hypoxic exposure of the reference cells rather than a 90-min one (data not shown), suggesting that an hour of the total drug treatment time may have been "ineffective." Thus, had it been possible to add the SR 4233 after the 90 min of gassing rather than at the start of it, the toxicity to cells grown on the Permanox dishes may well have been greater, and with even longer gassing times, may have approached that of the reference cells. This interpretation of the experimental findings would also be consistent with the previously discussed possibility that an "oxygen trap" is formed under, or that oxygen diffuses from within, the Permanox dishes. Although such a trap would be present for dishes destined for irradiation as well as for drug treatment, the longer "effective" gassing time for the irradiated samples, coupled with the need to transport the gassing vessels to the irradiator (and thereby moving the dishes), may have served to dissipate the trapped oxygen.

In summary, while we have demonstrated a large difference in the hypoxic cytotoxicity of a bioreductive drug as a function of the type of petri dish used, glass or Permanox plastic, this result was not predictable on the basis of similar experiments assessing hypoxic radioresistance. While other manipulations of this same system could improve the gas exchange for the Permanox dishes, such as longer gassing times, use of a "vacuum manifold" rather than a "continuous flow" gassing system (*8*), agitation of the medium during gassing (*7, 9*), alteration of the "geometry" of the cell monolayer (*8*), or use of aluminum rather than nylon gassing chambers (*8*), these would be successful only insofar as the particular mechanism of action for the drug under study (be it bioreductive cytotoxicity, chemosensitization, binding to cellular macromolecules etc.) and its oxygen dependence are already known. Since this is obviously *not* the case during the initial screening of novel compounds, we would recommend that Permanox petri dishes not be used under these circumstances.

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