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Differential protective effects of varying degrees of hypoxia on the cytotoxicities of etoposide and bleomycin*

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Summary. Oxygen is thought to be involved both directly and indirectly in the mechanisms of action of several anticancer agents. We studied the effects of various oxygen concentrations on the cytotoxicities of the following drugs: bleomycin (BLM), etoposide (VP-16), doxorubicin (DOX), and mitomycin C (MMC). Human sarcoma cells, MES-SA, were exposed to drug for 1 h at one of several oxygen concentrations: less than 1%, 2.5%, 5%, 21%, and 95%. Cytotoxicity was assessed by cellular incorporation of ^{3}H-thymidine into DNA 5 days after drug exposure. Control experiments varying oxygen concentration without drugs demonstrated toxicity only at the highest concentration (95%). Three different responses of drug sensitivity to varying oxygen tensions were observed. BLM, which has been shown to utilize oxygen as a substrate in generating free radicals and producing DNA scission, demonstrated a progressive increase in cytotoxicity over the entire range of increasing oxygen concentrations. This is consistent with the model of a BLM-cation-oxygen complex and catalytic reduction of oxygen. VP-16, which also produces DNA strand breakage but by interaction with topoisomerase II, exhibited a threshold response. VP-16 toxicity was ameliorated by anoxic conditions (less than 1% O_2), but not by oxygen concentrations of 2.5%–95%. The reason for this protective effect of anoxia with VP-16 is not clear. In contrast, acute anoxia had no effect on the cytotoxicities of DOX and MMC. We conclude that acute hypoxia protects cells from both BLM and VP-16 but that the nature of that protection is different. VP-16 toxicitiy is blunted only by severe anoxia, whereas BLM exhibits a dose response effect over the entire range of oxygen concentrations.

Introduction

The oxygenation state of cells has been shown to be a major determinant of the cytotoxic efficacy of several anticancer drugs [14, 18, 27, 28, 30, 31, 37, 39, 40]. The role of molecular oxygen may be particularly important for bleomycin (BLM), a mixture of glycopeptides important in the therapy of lymphomas, germ cell cancers of the testis and ovary, and squamous carcinomas [36]. BLM is known to intercalate partially into DNA, and to form a complex with reduced cations (primarily ferrous ions) and oxygen [1, 6, 10, 20, 33]. This BLM-Fe^{2+}-0_2 complex is thought to act as a minienzyme, and has been shown in vitro to catalytically reduce oxygen and generate superoxide and hydroxyl free radicals [6–8, 16, 23, 26]. The BLM complex cleaves deoxyribose at the C_{3-4} position, resulting in liberation of base propenals [12, 16, 23, 34] and single- and double-strand breaks in DNA [11–12, 26, 38].

It has been shown that cells are protected from BLM toxicity by acute hypoxia [30–31, 40]. However, the relationship of this protection to various levels of hypoxia is not known. In this paper, we examine the effects of graded concentrations of oxygen, ranging from 0% to 95%, on the cytotoxicity of BLM. The epipodophyllotoxin drug etoposide (VP-16), which also produces DNA strand breakage but by a mechanism not thought to involve molecular oxygen, was also studied in parallel experiments [17, 21, 22, 24, 42]. The experiments were designed to evaluate the effects on drug action of short-term (4-h) acute exposure to various oxygen concentrations, in order to minimize the effects of hypoxia on cell cycle traverse [3–4, 19, 35, 40]. Two other drugs whose actions are also reported to be different in hypoxic cells also served as controls: mitomycin C (MMC), which is activated by preincubation under anaerobic conditions [18, 28], and doxorubicin (DOX), which is reportedly less active in hypoxic cells [14, 27, 39].

Materials and methods

Drugs and chemicals. BLM was a gift from Dr. William Bradner of Bristol Laboratories Syracuse, NY. The other drugs used, VP-16, DOX, and MMC, were provided by the Pharmaceutical Resources Branch, Developmental Therapeutics Program, National Cancer Institute Bethesda, Md (courtesy of Ruth Davis, Toxicology Branch, and Dr. John Douros). Fresh solutions of these drugs were prepared for each experiment. BLM, DOX, and MMC were dissolved in Hanks' balanced salt solution (HBSS). VP-16 was dissolved at 0.1 mg/ml in ethanol and then further diluted in sterile 0.9% NaCl. ^{3}H-Thymidine (methyl-^{3}H, 77 Ci-mmol) was purchased from the New England Nuclear Corp., Boston, Mass.

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Cell culture. These experiments were performed with the human sarcoma cell line MES-SA, which has recently been developed in our laboratory [13]. Monolayer cultures of MES-SA cells were grown in tissue culture flasks (Corning Glass Works, Corning, NY) containing a 1 : 1 mixture of Waymouth's medium and McCoy's 5A medium designated W/M medium (Grand Island Biological Co., Grand Island, NY) supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), insulin (5 μg/ml), and 15% newborn calf serum (NBCS). Cells were grown at 37 °C in humidified room air containing 5% CO_2 and subculted every 5–7 days. Cells were harvested by brief (3–5 min) exposure to 0.06 *M* EDTA, washed with HBSS plus 10% NBCS, and counted by hemocytometer and trypan blue dye exclusion. The cells were then washed and resuspended to a concentration of 1.0×10^4 cells/ml in W/M medium and 15% NBCS.

Controlled atmosphere conditions. A sealed plexiglass chamber, 60 × 100 × 50 cm (Germ Free Laboratories, Inc., Miami, Fla), was used for exposure of tumor cells to various atmospheric conditions. The chamber was supplied with gas inlet and outlet ports, two pairs of glove ports with heavy rubber gloves to allow manipulation of cells under controlled atmospheric conditions, and pans of sterile distilled water for humidification. Tanks of premixed gases all contained 5% CO_2, with the O_2 content varying as 0, 2.5%, 5%, or 95%, and the remainder being N_2 (Liquid Carbonics Corp., Chicago, Ill). The chamber was placed in a warm room (37 °C) during the experiments. A period of 2 h was allowed for temperature and atmospheric equilibration before the experiments were begun. Oxygen tension in the chamber was measured every hour during the course of an experiment by sampling with a glass syringe from the gas outlet port and measurement in a Corning 178 oxygen analyzer (Corning Medical and Scientific Corp., Corning Glass Works, Medfield, Mass).

Cells were maintained in the environmental chamber for 4 h. The first 2 h were used to obtain an equilibrium of the atmosphere and media with the desired oxygen concentration. Drug exposure occurred during the 3rd h. After exposure to drugs for 1 h, the cells were washed 3 times with 0.6 ml W/M medium plus 15% NBCS, by pipetting through rubber gloves within the chamber without altering the atmospheric environment. The 4th h in drug-free medium was included to allow for some drug efflux from cells. The cells were washed again prior to transfer to the 95% air, 5% CO_2 incubator for 4 days of growth prior to the thymidine incorporation assay.

Thymidine incorporation cytotoxicity assay. Prior to each experiment, tumor cell suspensions were adjusted to a concentration of 1.0×10^4 cells/ml in W/M medium plus 15% NBCS, and then 2×10^3 cells (0.2 ml) were seeded into each well of a 96-well microtiter tissue culture plate (flat-bottom wells with lids; Falcon Plastics, division of Becton Dickinson, Inc., Oxnard, Calif). Cells were seeded 24 h before being placed into the environmental chamber to allow for attachment and initiation of proliferation after exposure to the various oxygen tensions described in the previous section. The plates were incubated for 4 days at 37 °C in a humidified incubator containing 95% air and 5% CO_2. Approximately 96 h after drug exposure, the cells were labeled for 24 h with ^{3}H-thymidine at a final thymidine concentration of 10^{-4} *M* in the medium containing 0.5 μCi per well. All experiments were done in six wells for each drug concentration. In addition, six separate experiments were performed and pooled for each atmospheric condition. After labeling, the cells were harvested with 0.05% Pronase (Sigma Chemical Co., St. Louis, Mo), and then adsorbed to a glass filter using a cell harvester (PHD Cell Harvester, Cambridge Technology Inc., Cambridge, Mass). The cells from each well were placed into scintillation vials and treated with 0.2 ml Protosol tissue solubilizer (New England Nuclear Corp., Boston, Mass) for 30 min at 50°. Scintillation fluor (Econofluor, New England Nuclear Corp., Boston, Mass) was added, and radioactive was counted by a scintillation counter (Beckman LS-9000), and expressed as disintegrations per minute.

We have previously shown that this type of thymidine incorporation assay, performed several days after drug exposure as a measure of surviving cell number, correlates very well with cytotoxicity as determined by clonogenic assay for B16 melanoma cells [43] and for the MES-SA cell line (B. I. Sikic, unpublished results).

Statistical analysis. The data from six separate experiments were pooled for each experimental point in Figs. 1–4. and Table 1. All comparisons between the groups were made using the two-tailed Student's *t*-test. In addition, the significance of oxygen concentration as a factor affecting cytotoxicities at various drug concentrations was asessed by one-way analysis of variance.

Results

Ambient partial pressures of oxygen were monitored hourly while the cells were in the environment chamber for 4 h. Preliminary gassing of the chamber was undertaken to establish the desired atmosphere. For the experiments with anoxia, the chamber was gassed with 95% N_2 : 5% CO_2 and the oxygen concentration was maintained as close to zero as possible. Actual measurements of oxygen under these conditions were 5–7 torr (less than 1% O_2). For the other gas mixtures, the desired partial pressure of oxygen was achieved by the preliminary gassing and maintained at 20 torr (2.5% O_2), 40 torr (5% O_2), or 720 torr (95% O_2).

Preliminary experiments established that control cells maintained an exponential rate of growth throughout the 4 days after initial seeding into microtiter plates and exposure to the various oxygen concentrations (data not shown). The doubling time of the cells in these preliminary experiments was approximately 22 h, consistent with the initial characterization of the MES-SA cell line [13].

Extreme hypoxia for 4 h had no effect on cell viability and proliferative capacity in the controls without drug treatment, as illustrated in Fig. 1. In contrast, the hyperoxic controls exposed to 95% O_2 : 5% CO_2 demonstrated a 90% inhibition of thymidine incorporation assessed 96 h after hyperoxic exposure, compared to the four groups of controls exposed to lower oxygen concentrations (Fig. 1). The effects of the drugs at 95% O_2 are normalized to this baseline 90% inhibition of control thymidine incorporation in Figs. 2–4.

The effects of varying concentrations of ambient oxygen on the cytotoxicity of BLM are illustrated in Fig. 2. Increasing oxygen concentrations from 0% to 95% resulted in increasing cytotoxicity in the range of BLM concentra-

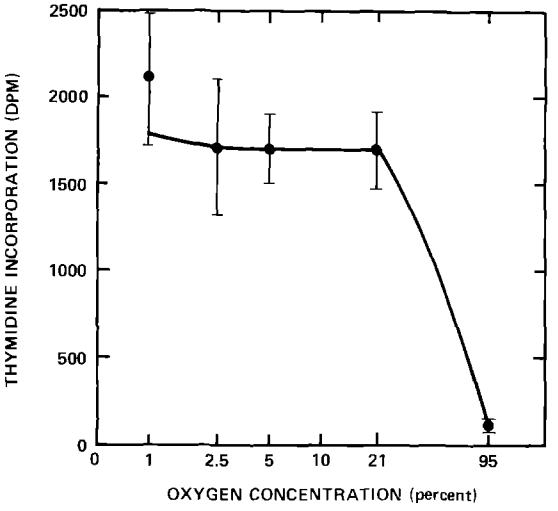

Fig. 1. The effect of exposure to various concentrations of oxygen on cell proliferation, assessed by ^{3}H-thymidine incorporation in MES-SA cells. Cells were exposed to oxygen concentrations for 4 h and cellular proliferation was assessed 4 days later. Each point represents the mean ±SD of six different wells, with 2×10^3 cells seeded initially per well

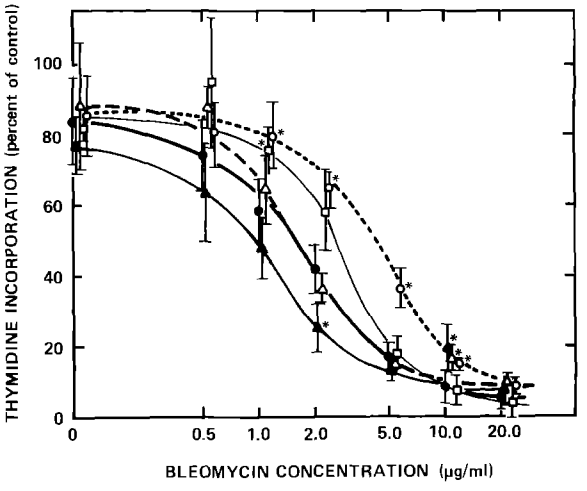

Fig. 2. The effects of various concentrations of ambient oxygen on the cytotoxic dose-response curves of BLM in MES-SA cells. Cytotoxicity is expressed as the percent of control thymidine incorporation, with the controls being cells not treated with BLM. Concentrations of BLM range from 0.1 to 20 µg/ml. The concentrations of oxygen used are as follows: 0% (○); 2.5% (□); 5% (△); 21% (●); 95% (▲). Each point represents the mean ±SD of six wells. Significant differences (according to Student's *t*-test) from cells exposed to 21% O_2 are indicated by an *asterisk. The effect of % O_2* on the toxicities of 0.5, 1.0, and 2.0 µg/ml BLM was also significant ($P < 0.001$) by one-way analysis of variance

tions from 0.1 to 20 µg/ml. The control level of thymidine incorporation used for each curve was similar, except for the 95% O_2 groups, which exhibited a baseline 90% inhibition superimposed on the drug effect (see Fig. 1). Significant differences in BLM cytotoxicity compared to air were found in all the atmospheric groups, although not at every drug dose (Fig. 2).

Figure 3 demonstrates the protective effect of acute anoxia on the cytotoxicity of VP-16. Significant differences from the air (21% O_2) control were found with the

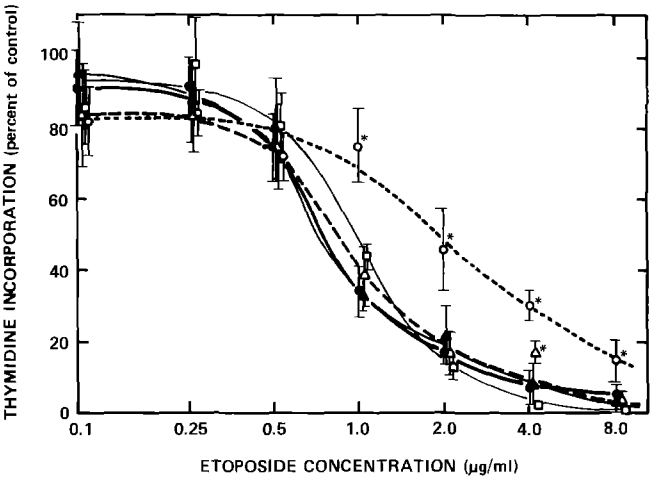

Fig. 3. The effects of various concentrations of ambient oxygen on the cytotoxic dose-response curves of VP-16 in MES-SA cells. Cytotoxicity is expressed as the percent of control thymidine incorporation, with the controls being cells not treated with VP-16. Concentrations of VP-16 range from 0.1 to 20 µg/ml. The concentrations of oxygen used are as follows: 0% (○); 2.5% (□); 5% (△); 21% (●); 95% (▲). Each point represents the mean ±SD of six wells. Significant differences (according to Student's *t*-test) from cells exppsed to 21% O_2 are indicated by an asterisk. The effect of % O_2 on the toxicities of 1.0 and 2.0 µg/ml VP-16 was also significant ($p < 0.001$) by one-way analysis of variance

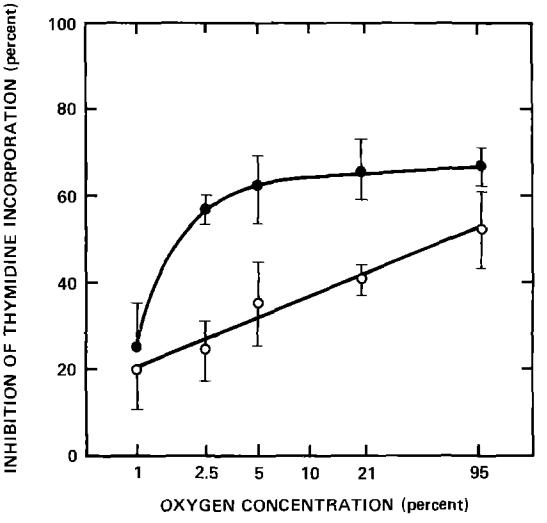

Fig. 4. The effect of increasing concentrations of oxygen on the cytotoxicity of BLM and VP-16, as assessed by the degree of inhibition of thymidine incorporation, when the drug concentration is held constant. The drug concentrations used for both BLM (○); and VP-16 (●) are 1.0 µg/ml. Each point represents the mean ±SD of six wells. The effects of % O_2 on the cytotoxicities of both agents are highly significant by one-way analysis of variance ($p < 0.001$ for BLM regardless of which oxygen concentrations were included in the analysis, $p < 0.001$ for VP-16 only when the 1% O_2 data was included

anoxic group at all VP-16 concentrations from 1.0 to 8.0 µg/ml. The drug dose-response curves at 2.5%, 5%, 21%, and 95% O_2 concentrations were virtually superimposable.

The contrast between the effects of varying oxygen concentrations on the cytotoxicity of BLM vs VP-16 is shown in Fig. 4. The data from the 1.0 µg/ml drug concen-

Table 1. Effects of hypoxia and anoxia on cytotoxicities of bleomycin, etoposide, mitomycin C, and doxorubicin on MES-SA cells

Drug	Percent ambient oxygen		
	21% (160 mmHg)	5% (40 mmHg)	1% (5 mmHg)
Bleomycin (1.0 μg/ml)	41 ± 3	35 ± 9	20 ± 10*
Etoposide (1.0 μg/ml)	66 ± 7	62 ± 8	25 ± 11*
Mitomycin C (1.0 μg/ml)	77 ± 4	–	77 ± 5
Doxorubicin (0.1 μg/ml)	61 ± 7	–	64 ± 2

Results expressed as percent inhibition of thymidine incorporation compared to untreated control MES-SA cells
* $p < 0.01$, anoxic vs normoxic conditions (21% O_2)

trations in Fig. 2 and 3 was re-expressed as the percent inhibition of thymidine incorporation in order to discern the relationship between ambient oxygen and drug cytotoxicity. Two different patterns are evident: There is a progressive increase in BLM cytotoxicity across the entire range of oxygen concentrations from 0% to 95%. In contrast, VP-16 exhibits a threshold phenomenon in relation to oxygen. The cytotoxicity of VP-16 exhibits a threshold phenomenon in relation to oxygen. The cytotoxicity of VP-16 was similar at all oxygen concentrations from 2.5% to 95%, with decreased toxicity evident only in the anoxic group.

The cytotoxic effects of DOX and MMC were also examined under anoxic conditions. In contrast to the results with BLM and VP-16, there was no discernible protection against or augmentation of DOX and MMC during the short-term drug exposure and anoxia in our experiments (Table 1). Since the results with extreme hypoxia did not differ from air controls, intermediate degrees of hypoxia were not tested.

Discussion

These results provide further evidence for the central role of molecular oxygen in the action of BLM, and reveal a protective effect of acute anoxia against the cytotoxicity of VP-16. Such transient severe hypoxia is known to be common in human solid tumors [5, 15, 40, 41], and could serve as an important mechanism of resistance to these drugs.

These experiments were designed to minimize the antiproliferative or cytostatic effects of longer-term exposure to hypoxia in the experimental tumor cell population. Four hours of anoxia has previously been shown not to inhibit the traverse of cells through the proliferative cell cycle [40]. Thus, the protective effects of short-term anoxia against the actions of BLM and VP-16 are probably not mediated by delays in cell cycle progression or changes in cell cycle distribution in the tumor cell population.

Our results demonstrate that BLM cytotoxicity is related to oxygen concentration over the entire range of 0%–95% O_2 atmosphere under which the cells were exposed to the drug (Fig. 2). This is consistent with the hypothesis of BLM as a mini-enzyme, chelating ferrous ions and binding to DNA, with molecular oxygen serving as a substrate in the generation of DNA strand breakage [1, 6–8, 10–12, 16, 20, 23, 26, 33, 34, 38]. The existence of a BLM-$Fe^{2+}O_2$ complex has been inferred from circular dichroism and absorption spectra and from studies of the role of oxygen in DNA degradation [1, 6–8, 10, 20, 25, 33, 34]. It is likely that the mechanism of the DNA scission involves direct abstraction of a hydrogen atom from the C_{3-4} bond of deoxyribose in DNA, rather than production of superoxide and hydroxyl free radical species as previously proposed [12, 16, 23, 26, 29]. The liberation of base propenals from DNA by this BLM complex has recently been proposed as an additional toxic mechanism [12].

Although the concentration dependence of oxygen in BLM cytotoxicity suggests a direct involvement by molecular oxygen, possible indirect effects cannot be ruled out. Two such possibilities would be progressive decreases in drug inactivation or in repair of drug effects with increasing oxygen concentrations, both of which we consider to be unlikely.

The protective effect of anoxia in VP-16 cytotoxicity was an unexpected finding Figs. 3, 4). Oxygen has not been implicated directly in the mechanism of DNA scission by VP-16, which appears to be mediated by an interaction of the drug with the breakage-reunion reaction of the enzyme topoisomerase II [9, 17, 21–22, 24, 32, 42]. However, topoisomerase II is known to be highly ATP-dependent, and it is possible that 4 h of acute anoxia may have depleted ATP levels in our cells sufficiently to inhibit the interaction of VP-16 with the enzyme.

We were unable to demonstrate an effect of acute anoxia on the cytotoxicities of MMC or DOX (Table 1), in contrast to other reports [14, 18, 27–28, 39]. In the case of MMC, a period of preincubation under anaerobic conditions appears to enhance metabolic activation and toxicity [18, 28], and we minimized the period of hypoxia to examine possible direct interactions of drug with oxygen. In the case of DOX, the lack of protection by anoxia may again reflect our acute rather than chronic hypoxic conditions, or perhaps unknown metabolic differences between our cells and those previously reported [14, 27, 39]. Several mechanisms of action have been proposed for anthracyclines, including DNA intercalation and strand breakage, free radical generation, and surface membrane effects. Our data would suggest that DOX is acting via a non-oxygen-dependent process in these cells, rather than by microsomal activation and generation of free radicals from oxygen, which may occur in other cells or tissues [2].

In summary, acute hypoxia protected our cells from the toxicities of both BLM and VP-16 although the nature of that protective effect appears to be different for the two drugs. For BLM, there was increasing toxicity with increasing oxygen concentration, consistent with the concept of a BLM-O_2 complex mediating cytotoxicity. The presence of substantial numbers of hypoxic cells in human tumors may represent an important source of resistance to these drugs.

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