Response of Aerobic and Hypoxic Cells in a Solid Tumor to Adriamycin and Cyclophosphamide and Interaction of the Drugs with Radiation

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ABSTRACT

I have assessed the relative sensitivity of aerobic and hypoxic cells to Adriamycin (ADR) and cyclophosphamide (CY) in the drug-sensitive murine 16/C tumor. The end point used was tumor response to subsequent radiation given under aerobic or acutely hypoxic conditions.

Delay in tumor growth following 15 gray radiation alone to ~0.4 g tumors was about 3 days longer after aerobic than after hypoxic radiation. Prior treatment with CY had little effect on this difference, implying little selectivity of CY for killing aerobic or hypoxic cells, and the effects of CY and radiation were additive.

Treatment with ADR abolished the difference in response to aerobic and hypoxic radiation given from 0.5 to 2 hr after the drug, suggesting that most of the cells which survived treatment with ADR were hypoxic. Difference in response to aerobic and hypoxic radiation at 6 to 24 hr after ADR was equal to or greater than that in non-drug-treated mice, implying rapid reoxygenation after ADR. Experiments on small, nonpalpable tumors with a low proportion of hypoxic cells showed that ADR was slightly more effective than against larger tumors and that some aerobic cells were spared by the drug when the hypoxic fraction was small. Misonidazole is known to be selectively toxic for hypoxic cells, and a high dose of misonidazole gave a small increase in antitumor effects of ADR without increased toxicity.

My results suggest that ADR (but not CY) may spare hypoxic cells in a solid tumor and are consistent with limited diffusion of ADR from tumor blood vessels.

INTRODUCTION

Cells in solid tumors that are distant from functional blood vessels may be hypoxic and resistant to radiation therapy. Such cells might also be resistant to chemotherapy for the following reasons: (a) drug concentration in their vicinity may be low because of limited diffusion from blood vessels and drug uptake by intervening cells; (b) nutritionally deprived cells have been found to be slowly proliferating (12, 27, 28) and most anticancer drugs are more active against rapidly dividing cells (29); (c) nutritionally deprived cells may have different metabolism from their well-nourished neighbors, and factors such as hypoxia or decreased cellular energy production might influence the uptake or toxicity of drugs.

There have been few studies of drug distribution within tumors. Ozols et al. (17) found limited penetration of ADR2 into tumor nodules in the peritoneum of mice bearing an ovarian tumor. Poor penetration of ADR and other drugs into spheroids (spherical aggregates of tumor cells maintained in tissue culture) has been observed (5, 15, 16, 26, 36) and cells which are sensitive to ADR in suspension or monolayer may be resistant when grown as spheroids or solid tumors (14, 26).

These results suggest that distribution of ADR in solid tumors may be as important as intrinsic drug sensitivity in determining tumor response.

Assessment of the relative response to drugs of aerobic and hypoxic cells within a solid tumor may be attempted by using the methods of radiation biology to estimate the proportion of hypoxic cells in drug-treated and control tumors. This technique has suggested that hypoxic cells may be spared by the rapidly metabolized alkylating agents nitrogen mustard and 1,3-bis(2-chloroethyl)-1-nitrosourea (10, 11), while data for CY have implied selective toxicity for aerobic cells in a rat mammary tumor but no such selectivity in the murine B16 melanoma (6, 11). Data for ADR and other drugs are not available and are hindered by the lack of experimental solid tumors which respond to ADR. I now report studies of the response of aerobic and hypoxic cells to ADR and CY in the drug-sensitive 16/C mouse mammary carcinoma.

MATERIALS AND METHODS

Experimental Design. The design of experiments to assess the effect of anticancer drugs on aerobic and hypoxic cells in solid tumors is illustrated in Chart 1. C3H/He mice at least 10 weeks old were given implantations in the left hind leg of a suspension of 2 × 10^6 cells from the syngeneic 16/C mammary carcinoma (4, 30). Animals were coded with numbered ear tags and randomized groups received various treatments (Chart 1) usually given when mean tumor diameter was in the range of 8.5 to 9.5 mm (weight, 0.3 to 0.4 g).

Radiation was delivered to the tumor-bearing leg of awake, nonanesthetized animals (aerobic radiation) or to mice anesthetized with sodium pentobarbitol whose tumor blood supply was occluded by a clamp applied to the thigh for 5 min prior to and during irradiation (hypoxic radiation, 25). The radiation source was a double-headed 100-kVp X-ray unit which delivered a tumor dose at a dose rate of 11.4 gray/min (19).

Standard parenteral formulations of ADR (Farmitalia, Milan, Italy) or CY (Horner, Mount Royal, Quebec, Canada) were diluted in 0.9% NaCl solution injected i.p. into mice in volumes of 0.01 ml/g body weight either alone or at various times before or after tumor irradiation. MISO (Roche, Welwyn Garden City, England) was used in a few experiments, and because of limited solubility was injected in a larger volume of 0.05 ml/g body weight.

Response of tumors to treatment was assessed by regrowth delay. Tumor diameter was recorded to the nearest 0.5 mm by passing the leg through a series of graded holes drilled in Lucite, and tumor weight was estimated from this measurement by a calibration curve defined previously. Tumor diameter was recorded at intervals of 2 to 3 days by an observer who was unaware of the treatment history. The end point used to compare response of the tumor to various treatments was mean delay (compared to controls) in the time for tumors to grow to 1 g.

1 Supported by a Research Grant from the National Cancer Institute of Canada.
2 The abbreviations used are: ADR, Adriamycin; CY, cyclophosphamide; MISO, misonidazole; LD₅₀, dose lethal to 50% of animals.

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RESULTS

Radiation. As expected, radiation alone caused a greater delay in tumor growth when delivered under aerobic conditions, as compared to irradiation of acutely hypoxic tumors (Charts 2 to 4). Anesthesia alone had no effect on the aerobic radiation response of the 16/C tumor.

CY. The LD<sub>50</sub> following i.p. injection of single doses of CY into C3H mice was about 250 mg/kg body weight. In 2 experiments, groups of mice were given injections of CY alone (100 or 150 mg/kg), or received 15 gray aerobic or hypoxic radiation 0.5 hr after injection of CY using the experimental design shown in Chart 1. Growth curves obtained in one experiment are shown in Chart 2, and data from both experiments are summarized in Table 1. Effects of CY were at least additive to those of radiation, and the difference in growth delay caused by aerobic or hypoxic radiation was equal or greater after treatment with CY than in non-drug-treated animals. There is no suggestion that the drug has selective toxicity for aerobic cells of the 16/C tumor.

ADR. The LD<sub>50</sub> following i.p. injection of single doses of ADR into C3H mice was found to be about 16 mg/kg (Table 3). ADR caused dose-dependent growth delay of the 16/C tumor (Charts 3 and 4).

Using the experimental design of Chart 1, several experiments were performed in which various doses of ADR were followed 0.5 hr later by various doses of radiation delivered to the tumor under aerobic or acutely hypoxic (clamped) conditions. Anesthesia and application of the clamp without radiation had no effect on tumor response to ADR. Typical growth curves are shown in Chart 3, and the relationship between delay in tumor growth and dose of radiation given after a fixed dose of ADR (10 mg/kg) is shown in Chart 5. There was no difference in the response to aerobic or hypoxic radiation at 0.5 hr after ADR from 5 to 15 mg/kg, suggesting that most of the cells which survived drug treatment were hypoxic.

In further experiments, the interval between ADR and radiation was varied between −0.5 hr (i.e., radiation given first) and +24 hr (Chart 6). ADR given 0.5 hr after radiation was equal in effect to the drug given 0.5 or 2 hr before radiation, so there was no evidence that ADR sensitized the tumor cells to radiation; at these times, there was no significant difference in response to radiation given under aerobic and hypoxic (tumor clamped) conditions. However, at 4 to 24 hr after ADR, radiation was more effective under aerobic than under hypoxic conditions, and the difference in growth delay was equal to or greater than that in non-drug-treated mice (Chart 6). A probable explanation is that hypoxic cells are spared by ADR in normal air-breathing mice, but that such cells reoxygenate and become sensitive to radiation by 4 hr after drug treatment.

Tumor growth delay following combined treatment with ADR and radiation was often greater than the sum of growth delays for either treatment alone (Chart 3). To determine whether there was a superadditive interaction between these 2 treatments, I constructed isobolograms (i.e., envelopes of equal effect) from the dose response data for aerobic and hypoxic radiation alone and for ADR alone (Chart 4) as described by Steel and Peckham (24). These isobolograms, drawn for the end point of regrowth delay equal to 10 days, are shown on Chart 7. Experimental data, indicated by X on Chart 7 were obtained from the dose-response curve for combined treatment (Chart 5) and from other experiments. For a 0.5-hr interval between ADR and radiation, the data are consistent with an additive relationship for ADR and irradiation under aerobic conditions, but may indicate a superadditive relationship when low-dose ADR is followed by irradiation delivered to the clamped, hypoxic tumor. For a 6-hr interval between ADR and aerobic radiation, we found consistently a greater antitumor effect than in the sum of growth delays for either treatment alone.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Delay to grow to 1 g (days)</th>
</tr>
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<tbody>
<tr>
<td>CY alone</td>
<td>5.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aerobic radiation</td>
<td>7.5 ± 1.0</td>
</tr>
<tr>
<td>Hypoxic radiation</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>CY + aerobic radiation</td>
<td>12.6 ± 1.4</td>
</tr>
<tr>
<td>CY + hypoxic radiation</td>
<td>8.1 ± 1.1</td>
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</tbody>
</table>

<sup>a</sup> Mean ± S.E.
Hypoxia and Chemotherapy

Chart 3. Growth curves for the 16/C tumor treated with radiation (XRT) (15 gray) under aerobic or hypoxic conditions, ADR (Adria) (5-15 mg/kg), and ADR followed 0.5 hr later by radiation. Δ, □, hypoxic radiation. Points, means for groups of 5 to 8 mice; bars, S.E. Broken line at lower right is subject to uncertainty because of death of some of the mice.

Chart 4. Dose-response curves relating the delay in time for tumors to grow to 1 g following treatment with ADR (10 mg/kg) and 15 gray radiation (Radn.) given under aerobic (A) or hypoxic (○) conditions. Data for radiation alone are shown for comparison. Points, means for groups of 6 to 8 mice; bars, S.E. Gy, gray.

Chart 5. Dose-response curves relating the delay in time for tumors to grow to 1 g following treatment with ADR (10 mg/kg) followed 0.5 hr later by radiation under aerobic (O) or hypoxic (△) conditions. Lines drawn in Chart 4 for radiation alone are shown for comparison. Points, means for groups of 6 to 8 mice; bars, S.E. Gy, gray.

Experiments with Small, Nonpalpable Tumors. In 2 experiments, the mice were treated at 5 or 6 days after implantation when the tumors were not yet palpable, and results are compared with pooled data for larger tumors in Table 2. Aerobic radiation was much more effective against small tumors, and some tumors did not grow after irradiation. The difference in growth delay caused by aerobic as compared to hypoxic tumor irradiation was also greater for these microscopic tumors ±3.7 days (S.E.) than for larger tumors [3.4 ± 0.8 days]; this result is expected because small well-vascularized tumors usually contain a low proportion of hypoxic cells. ADR was slightly more effective against small tumors than against larger tumors. ADR followed 0.5 hr later by aerobic radiation cured most of these microscopic tumors and was more effective than ADR followed by hypoxic radiation (Table 2).

Adriamycin plus Misonidazole. I have reported previously that the simultaneous injection of high-dose MISO (1 mg/g) with ADR may lead to a small increase in activity against the 16/C tumor without a detectable increase in toxicity (29). Since my current results suggest sparing of hypoxic cells by ADR, while MISO has selective toxicity for hypoxic cells, I have studied further the interaction of ADR and MISO.

The LD_{50} following i.p. injection of single-dose MISO in C3H mice is about 1.4 mg/g body weight. Toxicity data for ADR...
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with and without MISO (1 mg/g) in mice bearing the 16/C tumor are shown in Table 3. MISO had no effect on the acute LD₅₀ of ADR. The effects of adding MISO to ADR for treatment

Chart 7. Isobolograms constructed from the data in Chart 4 by the method of Steel and Peckham (24). The shaded areas define envelopes of additivity for ADR combined with aerobic radiation (top) or hypoxic radiation (bottom). Experimental data have shown a mean growth delay of 10 days for ADR (10 mg/kg) followed by 5 gray radiation (from dose-response curves of Chart 5) and for ADR (5 mg/kg) followed by 15 gray radiation. These results were independent of whether radiation was given under aerobic or hypoxic conditions and are labeled X. The point labeled Y represents data for aerobic radiation (10 gray) given 6 hr after ADR (5 mg/kg) which led to tumor growth delay of 11.3 ± 0.8 days.

Table 2
Comparison of effects of ADR and/or radiation against microscopic tumors treated on Days 5 or 6 after transplantation and against larger (~0.4 g) tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of growth delay</th>
<th>Fraction of mice with local tumor control</th>
<th>Days of growth delay</th>
<th>Fraction of mice with local tumor control</th>
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<tbody>
<tr>
<td>ADR 7.5 mg/kg</td>
<td>6.8 ± 1.8</td>
<td>0/8</td>
<td>3.2 ± 1.4</td>
<td>0/9</td>
</tr>
<tr>
<td>10.0 mg/kg</td>
<td>9.3 ± 1.4</td>
<td>1/7</td>
<td>6.0 ± 0.6</td>
<td>0/34</td>
</tr>
<tr>
<td>Aerobic radiation (15 gray)</td>
<td>17.0 ± 3.1</td>
<td>6/18</td>
<td>7.0 ± 0.7</td>
<td>0/29</td>
</tr>
<tr>
<td>Hypoxic radiation (15 gray)</td>
<td>8.5 ± 2.1</td>
<td>3/18</td>
<td>3.6 ± 0.6</td>
<td>0/29</td>
</tr>
<tr>
<td>ADR (7.5 mg/kg) + MISO (1 mg/g)</td>
<td>9/9</td>
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DISCUSSION

My results imply that ADR is selectively toxic to aerobic cells of the drug-sensitive 16/C murine tumor, but that CY had no selective toxicity. The data also suggest that hypoxic cells which survive ADR may reoxygenate by 4 hr later, and that radiation is more effective if delayed until reoxygenation has occurred after treatment with ADR.

Data for CY are similar to those of Hill and Stanley (11), who found no selectivity of the drug for aerobic and hypoxic cells of B16 melanoma, but differ from those of Dixon et al. (6), who showed that CY spared hypoxic cells of a transplanted rat tumor. Most other investigators have reported an additive relationship of CY with radiation (35, 37). I have studied previously the in vitro toxicity of metabolites of CY for aerobic and hypoxic Chinese hamster ovary cells and found equal sensitivity (31).

The effects of ADR on the 16/C tumor are probably due to selective lethal effects on aerobic cells in vivo, although the end point of growth delay does not allow exclusion of selective nonlethal effects such as cell cycle progression delay. The
possibility that ADR interacts with radiation to give selective radiosensitization of acutely hypoxic cells (or selective radioprotection of aerobic cells) can be rejected because (a) a similar response to combined treatment is observed when radiation is given first, and (b) the half-life of ADR in tumors has been reported to be 2 to 3 days (20), so that loss of a selective interaction with radiation should not occur in 4 hr. Rather, the results of Chart 6 imply rapid reoxygenation of cells that survive ADR.

At least 3 factors might contribute to a differential effect of ADR on aerobic and hypoxic cells in vivo. Previous experience from this laboratory and others has shown that hypoxic cells may be more resistant to ADR than aerobic cells in vitro (14, 22, 31), although this result is not universal (8, 33). Hypoxic cells in vivo also tend to be slowly proliferating, and like most drugs ADR is more toxic to rapidly proliferating cells (1, 18). Third, there is evidence for limited diffusion of drugs into tumor tissue, as detected by fluorescence, from vascular surfaces in vivo (17), or from the surface of spheroids in drug-containing medium (5, 16, 26).

ADR was only slightly more effective against small nonpalpable tumors than against larger tumors (Table 2). In contrast, radiation given under air-breathing conditions was much more effective against small tumors, and the large difference in response of small tumors to aerobic as compared to hypoxic radiation is consistent with a much smaller proportion of hypoxic cells in early tumors. If the selective toxicity of ADR for aerobic cells in larger tumors were due to differences of intrinsic drug sensitivity under aerobic or hypoxic conditions, I would have expected differences in the response of small and large tumors to ADR to be similar to those for aerobic radiation. The data obtained fit better with a model of limited penetration of ADR from the tumor blood vessels. The model requires different diffusion characteristics for ADR and oxygen, but this is to be expected.

Other investigators who have studied the in vivo interaction of ADR and radiation used to treat experimental tumors have reported additive effects (7, 21, 34). However, their results may have limited relevance to humans because the tumors studied were all resistant to ADR, as are most solid tumors of mice. My studies with a tumor that is more sensitive to ADR also show additive effects of ADR with aerobic radiation when the interval between treatments is short, but this additive relationship may conceal 2 opposing effects.

The selectivity of ADR and radiation for the same subpopulation of aerobic or well-nourished cells would be expected to produce subadditive effects for treatment of tumors in air-breathing mice, but isobologram analysis of this interaction shows that it lies within the envelope of additivity (Chart 7). Thus, there may be an additional interaction between ADR and radiation that is of unknown cause, does not depend on the order of administration, and might lead to superadditive effects with radiation given under hypoxic conditions (Chart 7). This effect cannot be explained by the observation of some investigators that one modality may interfere with repair of sublethal damage caused by the other (2, 3, 9), since this interaction is included within the concept of additivity of isobologram analysis.

The results of this study have some implications for the development of effective combination chemotherapy with ADR. If ADR spares poorly nourished or hypoxic cells in human tumors (but not in better vascularized normal tissue), it should be possible to improve therapeutic index by combining the drug with agents that have selective activity for nutritionally deprived cells. The data of Tables 3 and 4 illustrate this possibility for MISO, but this drug gives hypoxic cell toxicity only at doses close to those which cause lethal toxicity. Other nitroimidazoles, bioreductive alkylating agents such as mitomycin C, glucose analogs such as 5-thio-D-glucose, or drugs that are activated in regions of low pH might give selective toxicity of nutritionally deprived cells (13, 23, 32), but none of the currently available agents have high activity at nontoxic doses. Unless such drugs also have toxicity for well-nourished tumor cells, they may fail currently used drug screens and will require testing in combination with radiation or ADR. Development of drugs designed to attack nutritionally deprived cells of solid tumors deserves careful study.

ACKNOWLEDGMENTS

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REFERENCES


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