The Response of Hypoxic B16 Melanoma Cells to \textit{in Vivo} Treatment with Chemotherapeutic Agents\textsuperscript{1}

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SUMMARY

Survival curves are presented for the treatment of B16 melamomas with a range of single doses of cyclophosphamide (CY), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), or 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea (MeCCNU). When these four drugs are assessed in terms of the tumor cell kill at the lethal dose to 10\% of the mice, MeCCNU is found to be much the most effective, followed by CCNU, and then CY and BCNU together. The superiority of MeCCNU is possibly related to the fact that it seems to be longer lived in the mice than are the other drugs. Combined drug and irradiation experiments have indicated that CY kills both oxygenated and hypoxic cells in the tumor, leaving proportions equal to those in the tumor prior to treatment, whereas BCNU preferentially spares the hypoxic cells. Since hypoxic cells constitute a population of cells that is at a distance from blood vessels, this result suggests that CY treatment of B16 melamomas is not limited by an inability of the drug to diffuse to cells away from blood vessels.

INTRODUCTION

Although recent studies (9, 10) have indicated that it is possible to cure some large solid tumors in animals with chemotherapy alone, most of such tumors are resistant to nontoxic doses of the drugs that are presently available. In contrast, present-day chemotherapy is an increasingly effective treatment for leukemias and lymphomas (3). One possible explanation for the resistance of solid tumors is that, because of inadequate vasculature, the drug may never become equally distributed throughout the tumor. Some tumor cells may then always survive by virtue of not being exposed to a large enough concentration of the drug before the level in the blood reaches the limit of toxicity to the host.

It has been found by irradiation studies that many cells in solid animal tumors are at very low oxygen levels, indicating the inadequacy of the blood supply in such tumors. Similarly, morphological studies have indicated that at least some tumors contain cords of apparently viable tumor cells close to the blood vessels, with necrotic regions beginning at a distance of 100 to 150 \( \mu \)m from the nearest blood vessel. This distance is approximately equal to the calculated diffusion distance of oxygen (11, 12) and is so small largely because of rapid metabolism. However, other factors such as degradation or tissue binding may well reduce the distance that a drug will diffuse away from a blood vessel.

The morphological studies suggest that hypoxic cells are located close to the boundary between the viable and necrotic regions. It seems likely therefore that the hypoxic cells are the clonogenic cells in the tumor that are farthest from any blood supply. Thus, if the effect of a chemotherapeutic agent on the tumor is limited by the distance it can diffuse from the blood vessel, it is the hypoxic cells that would be expected to have the best chance of survival. It should be noted, however, that they may also survive by virtue of being inherently more resistant to the drug. The experiments described in this paper were designed to study the response of B16 melanoma cells to single doses of CY,\textsuperscript{2} BCNU, CCNU, or MeCCNU and to investigate whether, for CY and BCNU treatment, the hypoxic cells survive preferentially. To this end, 2 types of experiment were performed. Firstly, s.c. growing tumors were treated with a single dose of 1 of the drugs and were then given a dose of irradiation, either under air-breathing or anoxic conditions, to determine whether or not the cells surviving the chemotherapy were predominantly hypoxic. Secondly, tumors were given a dose of irradiation, which would be expected to kill predominantly the well-oxygenated cells, and then were treated with 1 of the 2 drugs to determine the drug sensitivity of the hypoxic cells that had survived the irradiation treatment. The results of the various treatments were assayed by using the lung colony technique, which gives the surviving fraction of cells in the tumor.

MATERIALS AND METHODS

Tumor and Mice. The tumor used in these experiments was the B16 melanoma, a transplantable tumor that arose originally in a C57BL mouse. Tumors were transplanted by

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injecting $5 \times 10^5$ or $10^5$ tumor cells s.c. into both flanks of recipient mice. The tumors were allowed to grow for about 2 weeks before being used in the experiments, by which time they were between 100 and 300 cu mm. Female C57BL mice, 8 to 12 weeks of age and weighing 18 to 21 g, were used throughout the study. These mice were bred at the Institute of Cancer Research breeding center, and experiments have failed to demonstrate any antigenicity of the tumor in this subline. In such experiments the mice were pretreated with 2 i.p. injections of $2 \times 10^8$ HR cells 1 week apart. One week later the mice were given s.c. inoculations with small numbers of morphologically viable tumors mixed with $10^8$ HR cells. An inoculum of 10 viable cells gave 16 of 18 tumor takes in control mice and 17 to 20 takes in immunized mice, while an inoculum of 1 viable cell gave 4 of 20 takes and 3 of 20 takes in control and immunized mice, respectively (G. G. Steel, private communication).

**Preparation of Cell Suspensions and Assay System.** Cell suspensions for both i.v. and s.c. injection were prepared in the following way. The tumors were dissected out of the animals, freed from the fibrous capsule, and minced finely with scissors. The mince was then shaken with 25 ml PBSA and allowed to settle. The supernatant was discarded and the mince was incubated for 10 min at $37^\circ$ in 25 ml PBSA with 0.2% trypsin (Bacto-trypsin; Difco Laboratories, Inc., Detroit, Mich.) and DNase (0.1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) added. The tumor material was again allowed to settle and the supernatant was discarded. It was then resuspended in 25 ml PBSA and incubated with fresh enzymes as above for 45 min, after which the mixture was firmly shaken to free cells loosened by the digestion. A small volume (2 ml) of fetal calf serum was then added and, after the remaining lumps of tumor had settled, the supernatant was poured off and filtered through a 200 mesh stainless steel screen. It was then centrifuged twice and resuspended in Eagle's basal minimum essential medium with 10% fetal calf serum added. Following this, dilutions were prepared for counting in a hemocytometer.

This technique resulted in a suspension of single cells in which 90 to 95% excluded the dye erythrosin, with a yield of 3 to $4 \times 10^6$ cells/g of tumor. Cells were assayed for survival using a lung colony assay (6, 7). Suitable dilutions were made of the cell suspension, and then the cells were mixed with HR cells and 15 $\mu$m plastic microspheres (3M Co., St. Paul, Minn.). A small volume (0.2 ml) was then injected i.v. into each of 7 to 10 recipient mice, resulting in the required number of viable cells, $10^6$ HR cells, and about $7 \times 10^6$ microspheres being injected per animal. Approximately 3 weeks later the mice were sacrificed, the lungs were removed and fixed in Bouin's fluid, and the macroscopic tumor nodules that had formed in the lungs of recipient mice were counted. Initial studies indicated that this count gave a measure of the number of surviving cells in the original cell suspension (7). The surviving fraction of cells for treated tumors was then determined by dividing the number of tumor nodules obtained per cell injected (colony efficiency) for cells from treated tumors by the colony efficiency for cells from untreated control tumors.

**Irradiation.** For irradiation the mice were unanesthetized and confined to perforated Perspex containers. All the irradiations were given to the whole body using $^{60}$Co $\gamma$-rays. Half-way through each irradiation the mice were rotated through $180^\circ$ to achieve better dose uniformity in the tumor. For anoxic irradiations the animals were killed 5 min before the start of irradiation. Dosimetry was performed using a Baldwin-Farmer standard dosenmeter, and different doses were achieved by exposing the mice at different distances (25 to 40 cm) from the source. Dose rates in the range of 100 to 300 rads/min were used.

**Cytotoxic Drugs.** CY was obtained from Ward, Blenkinsop and Co., London, England (Endoxana), while the 3 nitrosoureas were obtained from the Cancer Chemotherapy National Service Center, NIH, Bethesda, Md. The CY was dissolved in PBSA, while BCNU was initially dissolved in 100% ethanol and then diluted with PBSA to give 5% ethanol in the final solution. Both CCNU and MeCCNU were injected as a finely divided suspension in PBSA with a few drops of Tween 80 added. The mixture was homogenized before being injected. The particles were fine enough to pass easily through a 26-gauge needle. All the drugs were injected i.p. in a volume of 0.5 ml within 15 min of preparation.

**RESULTS**

**Drug Treatment.** The initial experiments were designed to determine the in vivo response of the B16 melanoma cells to the 4 drugs and also to establish information on which to base the study of the specific response of the hypoxic cells in the tumor. Since the fraction of the hypoxic cells surviving was to be determined using irradiation treatment, it was desirable that the tumors be irradiated as soon as possible after the drug treatment so that a significant degree of reoxygenation (transfer of surviving hypoxic cells into an oxygenated environment) would not have occurred before irradiation. It was necessary, however, that drug action should be complete before the irradiation because, for anoxic irradiations, the animals had to be killed just before irradiation. Other studies (1, 8) have suggested that the antitumor half-lives of CY and BCNU are less than 1 hr, and our studies (unpublished observations) indicated only a small amount of reoxygenation in the B16 melanoma by 2 hr after a dose of 1000 rads. Consequently, to check that drug action was complete by 2 hr it was decided to investigate the response of the tumor to single doses of the 4 drugs, either at 2 hr or at 22 hr after drug injection, to see whether additional cell killing could be detected in the interval of 2 to 22 hr.

In all the experiments the mice were divided into groups of 3 or 4 (6 to 8 tumors) with tumors of approximately equal volume. The groups were then assigned to a treatment or kept as controls as required. In the initial series of experiments the response of B16 melanomas to single doses of a range of concentrations of each of the drugs was investigated. The results for the 4 drugs are shown in Chart 1. The closed circles and the open squares are for tumors assayed 2 or 22 hr after the drug injection, respectively. Linear regression lines have been fitted to the data points.
Response of Hypoxic Cells to Chemotherapeutic Agents

Surviving fraction of tumor cells in air-breathing animals at a given dose/surviving fraction of tumor cells in dead animals (anoxic) at the same dose

Assuming that the dead animals have 100% hypoxic tumor cells, this ratio from Chart 2 gives a value of approximately 12% for B16 melanomas used in this study.

Treatment with Drug followed by Irradiation. The purpose of the combined drug-and-radiation treatment was to determine whether the hypoxic cells in the tumor preferentially survive the drug treatment. Because of the reasons discussed at the beginning of “Results,” the time interval between drug and irradiation treatment was chosen to be 2 hr, and only the drugs CY and BCNU were tested.

Accordingly, groups of tumor-bearing mice were given 3 mg CY or 0.5 mg BCNU, which would be expected to reduce tumor cell survival to about 6%, followed 2 hr later by irradiation under either anoxic or air-breathing conditions. A large group of tumor-bearing mice were all treated with the drug and then some were kept as controls while the remainder were treated with various doses of irradiation. The tumor cells were then assayed for viability using the lung colony technique, and the results of these treatments are shown in Chart 3. In these figures the reduction in survival attributable only to the irradiation treatment is plotted. The killing due to the drug treatment was normalized to unity by using drug-treated controls for calculating the survival due to irradiation. The closed triangles in these charts are for tumors irradiated under anoxic conditions and the closed circles for tumors irradiated in air-breathing conditions.

and only for MeCCNU is there a significant difference in the results for these assay times, although the results for CCNU are rather scattered. This indicates that no additional cell killing is seen from 2 to 22 hr after single doses of CY and BCNU, and probably CCNU.

Irradiation Treatment. Before irradiation studies could be used to investigate the fraction of hypoxic cells surviving drug treatment, it was necessary to establish the response of B16 melanomas to single doses of irradiation. Accordingly, groups of tumor-bearing mice were irradiated with doses in the range of 500 to 3500 rads under either anoxic or air-breathing conditions. The results of these irradiations are shown in Chart 2. The closed triangles are for irradiation of tumors in dead animals (anoxic) and the open circles are for tumors irradiated in air-breathing mice. For doses above 1000 rads, linear regression lines were calculated for the 2 sets of data. These lines had slopes that were not significantly different (p > 0.5), indicating that, at these doses, the response of the tumor in air-breathing mice is governed by the hypoxic cells in the tumor. With a number of assumptions (2, 5), a measure of the fraction of hypoxic cells in the tumor can be obtained by taking the ratio where the 2 lines are parallel.

Chart 1. Surviving fraction of cells in B16 melanomas as assayed 2 or 22 hr after in vivo treatment with various doses of 1 of 4 cytotoxic drugs. a, CY; b, BCNU; c, CCNU; d, MeCCNU.

Chart 2. Surviving fraction of cells in B16 melanomas following γ-irradiation under either anoxic (tumor-bearing animals killed 5 min before irradiation) or air-breathing conditions.

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mice. These points are the means of the least 2 independent survival determinations. The lines on the diagrams are taken from Chart 2 for tumors irradiated without prior treatment.

To investigate whether reoxygenation in the 2 hr following the drug treatment was affecting the results, some groups of tumor-bearing mice were treated with drugs and then irradiated immediately under air-breathing conditions before any reoxygenation could occur. The tumors were assayed 2 hr later so that the total time available for drug action was the same as previously. The results of these irradiations are shown in Chart 3 as the open squares. For CY (Chart 3a) these survival values are not different from those observed when irradiation was given 2 hr after drug treatment, and thus it is unlikely that any significant reoxygenation occurred in the 2 hr between drug and irradiation treatment. For BCNU (Chart 3b) a small difference may be present, but the data are not good enough to be certain of this.

It is seen in Chart 3a that the data for irradiation following CY treatment are a very good fit to the survival curves for irradiation alone, and an analysis of covariance has confirmed that there are no significant differences. The similarity of the anoxic data suggests that there is no interaction between the damage caused by the drug and that caused by the irradiation, while the fact that the data for the air-breathing irradiation are a good fit to the line for irradiation without previous drug treatment indicates that the ratio of oxygenated to hypoxic cells in the population surviving CY has probably not changed as a result of the drug treatment.

The results in Chart 3b present a different picture, in that although the anoxic data are a good fit to the anoxic line, the data for air-breathing mice lie intermediate between the 2 lines. Statistical analysis of these data for air-breathing mice (irradiated 2 hr after drug treatment) indicates that it is significantly different in terms of its position, but not its slope, from the similar data shown in Chart 3a (p < 0.01) and from the data for air-breathing mice shown in Chart 2 (p < 0.01). The results of the anoxic irradiations again suggest no interaction between the damage caused by the drug with that caused by the irradiation. However, the data for air-breathing mice indicate that the pretreatment with BCNU has resulted in a preferential killing of oxygenated cells, because the surviving population appears to have a higher fraction of hypoxic cells than in untreated tumors.

Treatment with Irradiation followed by Drug. The preferential sparing of the hypoxic cells during BCNU treatment could be due either to an inability of the drug to get to the cells or to an inherent resistance of such cells to BCNU treatment. To investigate this latter possibility, a series of experiments was performed in which the tumors were treated with a single dose of 1000 rads followed 1 hr later by a series of doses of BCNU. The irradiation would be expected to kill virtually all the well-oxygenated cells, leaving only hypoxic cells surviving (see Chart 2), and hence...
any further cell killing by the drug would be of hypoxic cells. For the purpose of comparison, these experiments were also done using CY instead of BCNU.

Groups of tumor-bearing animals were treated with 1000 rads and then, 1 hr later, with a range of concentrations of either CY or BCNU. Two hr after the drug treatment, the tumors were removed and assayed for surviving cells. The results of these experiments are shown in Chart 4. The survival due to irradiation alone was 5% (see Chart 2), but with added CY or BCNU the data define a survival curve that has a distinct shoulder (Line B). The broken lines (Line A) are taken from Chart 1, a and b, indicating the drug response of previously untreated tumors. These lines have been shifted down the charts to an intercept at 5% survival (Line C). It can be seen that for both drugs the data points lie above the broken Line C, indicating that, for a given level of survival, the irradiated tumors require a larger drug dose than do previously untreated tumors. However, the data appear to define survival curves that, beyond the shoulder, have similar slopes to the broken lines. Thus, once an effective drug concentration is reached, cells from untreated tumors and cells from previously irradiated tumors (hypoxic cells) have the same sensitivity to the drug.

DISCUSSION

The results presented have shown that, for treatment with CY, BCNU, and probably CCNU, the fraction of cells surviving in the tumors at 2 hr after drug administration is the same as that at 22 hr after drug administration. Since it is unlikely that more than 1 doubling of the surviving cell population occurs in the 20 hr that is the difference in time between the 2 assays, these results indicate that, within the error of the experimental system, the toxic concentrations of these drugs in the mice have disappeared by 2 hr after injection. For CY or BCNU this is in agreement with other published results, indicating that the antitumor half-lives of these drugs for L1210 leukemia are less than 1 hr (1, 7). Using the same procedure, however, Kline et al. (7) have found the half-life of CCNU to be 94 min, which suggests that large doses of CCNU should still have some activity 2 hr after injection.

The similarity of the survival at 2 and 22 hr after treatment with CY is in contrast to the results of Hahn et al. (4), who found that when the EMT6 mammary sarcoma was assayed at 24 hr after CY treatment, the survival was consistently higher than when the assay was done at 2 hr after treatment. They suggested that this increase in survival was most satisfactorily explained by repair of some potentially lethal lesions in the time interval between the assays. On this basis our results imply that the B16 melanoma does not repair potentially lethal lesions following CY and BCNU treatment. A similar absence of repair of potentially lethal lesions has been observed in the B16 melanoma following irradiation treatment (W. U. Shipley, unpublished observation).

The effectiveness of single doses of the 4 drugs on the B16
melonoma is compared in Table 1, in which the expected surviving fraction of cells (±95% confidence limits derived from the regression analysis) in the tumor for a drug dose equal to the reported lethal dose for 10% of C57BL × DBA/2 F1, mice (10) is shown. In these terms MeCCNU, when assayed at 22 hr, is by far the most effective of the drugs tested, with CCNU slightly more effective than are CY and BCNU. MeCCNU clearly has a longer lifetime in the animal than do the other drugs, and this may be the reason that it is very much more effective against the B16 melanoma than are CY, BCNU, or CCNU.

The results of the combined treatments present something of an anomaly. The irradiation-after-drug experiments indicate that CY treatment kills oxygenated and hypoxic cells to the same degree (Chart 3a), whereas BCNU treatment preferentially spares hypoxic cells (Chart 3b). From the results of the drug-after-irradiation experiments, however, it appears that the cells surviving irradiation (mostly hypoxic) required a higher dose of both drugs to reach a given level of survival than did cells from previously untreated tumors (mostly oxygenated), suggesting that treatment with either drug should preferentially spare hypoxic cells.

A factor that must be considered, however, is the possibility of an interaction between the 2 modalities of treatment. In the irradiation-after-drug experiments, the survival data for anoxic irradiation indicated that the irradiation response was normal after treatment with either of the 2 drugs, making significant interaction between the treatments unlikely. In the drug-after-irradiation experiments no such internal check was possible, so it cannot be ruled out that the initial irradiation treatment affected the drug response of the surviving cells. The irradiation could, for example, have altered vessel or tissue permeability or affected binding of the drug. More specifically, it might have changed the activation of CY or altered the intracellular sulfhydryl levels, thus affecting the response to BCNU. In this regard it is interesting to note that, in the combined treatment involving BCNU, 0.5 mg/mouse, and 1000 rads, the overall survival is approximately the same (6 × 10⁻³), regardless of which is given first. For the combined treatment involving CY, 3 mg/mouse and 1000 rads, the overall survival is a factor of 3 higher if the irradiation is given first (10⁻², as compared to 3 × 10⁻³ for treatment with drug first). Considering the discussion above, this suggests that irradiation is affecting the response to CY but not to BCNU.

Wharam et al. (13) have recently reported some irradiation-after-drug experiments very similar to ours. They treated the EMT6 mammary sarcoma with a dose of 1 of 4 drugs, including CY and BCNU, and followed this 2 hr later by aerobic irradiation. For initial CY treatment their results are very similar to those presented in Chart 3a, and they have interpreted them in the same way, to indicate equal killing of the oxygenated and hypoxic cells in the tumor. In contrast to our results (Chart 3b), however, there also appeared to be equal killing of the oxygenated and hypoxic cells when using BCNU. A possible explanation for this difference is that they were using drug doses that gave higher survivals than did those used here, and this would tend to reduce the effect of any preferential sparing of cells at a distance from blood vessels.

In conclusion, the results suggest that, in the B16 melanoma, CY can kill at least 95% of the cells equally well, regardless of whether they are hypoxic or not. BCNU, on the other hand, probably preferentially spares hypoxic cells. If BCNU action is being limited by nonuniform drug distribution, it must either be more rapidly bound or metabolized by the oxygenated cells than CY or it must have a shorter half-life in the animal than CY. Results with nitrogen mustard in another tumor system (R. P. Hill and R. S. Bush, in preparation) have indicated that this drug, which is known to have a very short half-life in the animal, does not kill the hypoxic cells unless given at high concentrations. Thus, a shorter half-life for BCNU in the animal could well be the reason for the different results with CY and BCNU. However, further studies are necessary before a definitive answer can be given.

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