Proliferation-dependent Cytotoxicity of Anticancer Agents: A Review

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Summary

One of the factors of importance in determining the killing of mammalian cells following exposure to a variety of anticancer agents is the proliferative state of the cell population. Generally, proliferating cells are much more sensitive to anticancer agents than are nonproliferating cells. In this review, the cellular aspects of this differential sensitivity are discussed with the hemopoietic stem cell population and tissue culture cells as the focus for the analysis. This phenomenon is not only of concern to the cell biologist but also has implications with regard to scheduling of anticancer agents against human tumors.

Introduction

A scientific approach to anticancer therapy requires that the investigator appreciate and understand the host of factors involved in optimal therapeutic design (71). The biochemistry of the drug and of the tumor, the cell population kinetics of host and tumor tissue, and the pharmacokinetics of the drug are only a few of the parameters to be considered prior to therapy and, indeed, to be reconsidered during the course of therapy. In this review, we have decided to restrict our attention to a small but important facet of this subject, the relationship between the cellular lethal effect of anticancer agents and the proliferative state of the exposed cell population. Because there have been few reviews of this subject (42, 50, 76, 81), we will attempt to present here the literature available, suggest mechanisms for the proliferation-dependent cytotoxicity of anticancer agents, and indicate the clinical importance of this phenomenon. Our review emphasizes the results obtained by study of the hemopoietic system and evaluates them in retrospect against newer information, which indicates that these results are due both to direct interactions between drugs and cells and to indirect effects that are a consequence of the complex self-renewal system involved in hemopoiesis. Finally, we discuss briefly the semantics of the cell kinetic designation "resting cells" and "plateau-phase cells" in tumors and in tissue culture as they relate to drug sensitivity.

Initial Studies Reflecting Proliferation-dependent Cytotoxicity

The fact that a tumor-bearing host can contain malignant cells in excess of 10^{12}, all of which must be destroyed, while at the same time the therapist must not destroy more than about 99.9% of the normal stem cells of the hemopoietic system and gastrointestinal tract, implies that a simple approach to chemotherapy would be to use anticancer agents with large differential sensitivities (16). This consideration was the basis for the examination of the cellular effects of anticancer agents on HCFU and a transplanted leukemia done by Bruce et al. (15) in the 1960's. They found that there were 2 classes of agents: one, which gave exponential dose-survival curves and was termed either nonspecific or cycle specific, and a 2nd class for which the surviving fraction reached a plateau with increasing dose and was termed phase specific. Nearly all agents showed some differential killing between the malignant and normal cell populations. For both classes of agents, it was postulated that the difference in cell killing was due to 2 major factors. First, the phase-specific agents killed cells in one phase of the cell cycle and had little effect on cells in other phases at the doses used, while cycle-specific agents killed cells throughout the cell cycle and demonstrated less cytotoxicity for cells in a nonproliferating state. Second, there was a difference in the proliferative state of the 2 cell populations; the transplanted leukemia cells were all in cell cycle, whereas a significant fraction of the HCFU was out of cell cycle and in a resting G state (16).

Although Berry (7) had already drawn attention to differences in dose-effect curves observed after different agents and given an explanation based on cell kinetics; the extensive systematic studies of the Toronto group have had much more impact on our understanding of the relation between cell kinetics and drug sensitivity. The greater impact of their work probably results from their having studied both a malignant and a normal cell population in vivo and from their having given an explanation and a model for successful use of chemotherapy in eradicating a much larger fraction of malignant cells than of normal bone marrow cells.

A major criticism against defining agents as being either phase or cycle specific, in terms of their differential effect on...
tumor and normal cell populations was simply that the 2 cell populations were different, and the results might reflect some physiological differences between the populations rather than any cycle-dependent effects of the agent. This criticism is of obvious importance if one intends to make use of these results in a therapeutic attack against human tumors. A good example is shown in Chart 1, where dose-survival curves for leukemic (73) and plasmacytoma cells (55) are compared to that for hemopoietic stem cells after exposure to melphalan. While the clonogenic cells are rapidly proliferating for both malignant cell populations, their differential sensitivity compared to the HCFU is obviously not the same. There seems little doubt that this difference reflects a difference in the intrinsic biochemistry of these malignant cell populations. Similar differences might be expected for other anticancer agents and other tumor populations, reemphasizing the caution required of the investigator in directly translating chemotherapy results not simply to human tumors but, possibly more important, to human tumors categorically different from the experimental tumors.

**Hemopoietic Stem Cells as a Model System**

One approach to the possibility of proliferation-dependent cytotoxicity is to examine the cytotoxic effect of anticancer agents on a single-cell population in both proliferating and nonproliferating states. One of the 1st populations so studied was the stem cells of the hemopoietic system, since they could be forced into a proliferative state after their transplantation into heavily irradiated recipients. Of equal importance was the existence of a quantitative cellular assay (the spleen colony assay) for these cells. After supralethal doses of radiation, the vast majority of endogenous stem cells in mice are destroyed. If syngeneic bone marrow is injected i.v. into these mice, there is a lag period that is followed by an exponential increase in the number of stem cells assayed as colony-forming units (Chart 2). It has been shown that, during this proliferative phase, the majority of the HCFU are in cell cycle (5). When the hemopoietic tissue has been nearly repopulated, HCFU proliferation becomes inhibited, presumably because the stem cells are forced out of cell cycle and into the G0 state. A similar kinetic response has been demonstrated in rats (22). Valeriote and Bruce (72) examined vinblastine and 5-FU (13) with this system and found that the proliferating HCFU were much more sensitive to the lethal effect of both these agents than were HCFU in a nonproliferating state. The effect of 5-FU is shown in Chart 3 where the repopulating HCFU appear as sensitive as the cells of a transplanted lymphoma.

Studies with cycle-specific agents have been pursued further by van Putten et al. (77, 78, 80) and by Evenaar et al. (26), who demonstrated differential killing of proliferating HCFU for a wide variety of alkylating agents, as well as for a few nonalkylating agents. Of the 19 agents that they examined, all showed an increased cytotoxic effect on proliferating HCFU when compared to resting HCFU (Table 1). More recently, bleomycin has been found to have a pronounced differential effect (12). In contrast, Mitomycin C showed no difference in cell killing between proliferating and nonproliferating HCFU (41).

We know that the response of different tumors to a given drug can vary tremendously while at the same time there may be little difference in the response of normal HCFU in the hosts (A. Razek, F. Valeriote, T. Vietti, and H. Lin. Quantitation of the Effect of Daunorubicin on Different Murine Tumors, submitted for publication). We might expect that the differential response for normal and proliferating HCFU would remain the same for a given drug in the tumor-bearing hosts examined in spite of the large variation in tumor response.
Proliferation-dependent Cytotoxicity of Anticancer Agents

actinomycin D have a significantly greater effect on the more rapidly proliferating cells. With a similar assay, Blackett and Adams (8) demonstrated little proliferation-dependent cytotoxicity for CY and a small difference for nitrogen mustard, 5-FU, and (±)-1,2-bis(3,5-dioxopiperazin-1-yl)propane. It should be remembered, however, that erythrocytic repopulation ability measures the functional activity of a population of cells rather than the stem cells alone; it probably includes the stem cells, the committed erythrocytic stem cells, and possibly other early cell types in the erythrocytic series that are transferred into the recipient. However, the time interval between transfer and assay most probably assures that, at most, only the 1st 2 cell types indicated above are the major determinants. Further, a close association between HCFU and erythrocytic repopulation ability might be expected since the macroscopic colonies enumerated in the spleen colony assay are erythroid (or mixed erythroid). More recently, the effect of cytotoxic agents on the erythrocytic series has

One difficulty in extrapolating the effects of drugs on murine HCFU to human HCFU results from the differential drug effect on normal and proliferating cells. It is likely that the fraction of HCFU in a Go state varies among different species, and while about 80% or more of murine HCFU are in a Go state, it is possible that 80% of human HCFU are proliferating. Also, the kinetics of recruitment after depletion of these stem cells or destruction of their progeny might vary so much that extrapolation of drug schedules from murine models based on these kinetics would become invalid.

A 2nd method that has been used to study HCFU in different proliferative states is to force the population to proliferate in the normal host, thereby obviating the need for studies in irradiated hosts. Reissmann et al. (61) and Eaves and Bruce (24, 25) used endotoxin to stimulate HCFU proliferation and showed a concomitant increase in their sensitivity to a number of anticancer agents. The latter investigators also showed that a presumed progenitor cell in the granulocytic pathway, the CFU-C, became more sensitive to 5-FU and vinblastine after endotoxin stimulation.

The erythrocytic repopulation ability of marrow (68) is another hemopoietic model system which can be used to assess the drug sensitivity of proliferating and nonproliferating cells. Femoral marrow is prepared from drug-treated mice and injected into supralethally irradiated mice. Seven days later the in vivo incorporation of radioactive iron is assayed. The donor marrow can be put into a more rapidly proliferating state by treatments such as chronic irradiation. While X-radiation and dimethyl Myleran have little differential effect on the erythrocytic repopulation ability of bone marrow whether or not it is in a more rapidly proliferating state, methotrexate, vinblastine, and

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Resting colony-forming units (A)</th>
<th>Rapidly proliferating colony-forming units (B)</th>
<th>Ratio A/B</th>
</tr>
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<tr>
<td>1-(2-Chloroethyl)-3-(trans-4-methylcyclo-hexyl)-1-nitrosourea</td>
<td>29.0</td>
<td>22.6</td>
<td>1.28</td>
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<tr>
<td>Trilophosphamide</td>
<td>47.1</td>
<td>34.7</td>
<td>1.35</td>
</tr>
<tr>
<td>BCNU (given s.c.)</td>
<td>13.4</td>
<td>8.98</td>
<td>1.50</td>
</tr>
<tr>
<td>Dimethyl Myleran</td>
<td>3.08</td>
<td>2.07</td>
<td>1.50</td>
</tr>
<tr>
<td>Dianhydromanninol</td>
<td>4.12</td>
<td>2.65</td>
<td>1.55</td>
</tr>
<tr>
<td>1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea</td>
<td>24.5</td>
<td>15.5</td>
<td>1.58</td>
</tr>
<tr>
<td>Isophosphamide</td>
<td>151</td>
<td>96</td>
<td>1.58</td>
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<tr>
<td>5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide</td>
<td>380</td>
<td>240</td>
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<tr>
<td>Mechlorethamine (given s.c.)</td>
<td>0.79</td>
<td>0.48</td>
<td>1.63</td>
</tr>
<tr>
<td>Busulfan</td>
<td>20</td>
<td>11.7</td>
<td>1.71</td>
</tr>
<tr>
<td>Chloramphicol</td>
<td>17.6</td>
<td>9.7</td>
<td>1.80</td>
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<tr>
<td>CY</td>
<td>126</td>
<td>54</td>
<td>2.28</td>
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<tr>
<td>Nor-nitrogen mustard</td>
<td>21.6</td>
<td>9.4</td>
<td>2.32</td>
</tr>
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<td>Triethylenemelamine</td>
<td>0.58</td>
<td>0.25</td>
<td>2.32</td>
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<td>Melphalan</td>
<td>4.06</td>
<td>1.65</td>
<td>2.45</td>
</tr>
<tr>
<td>Aminochlorambucil</td>
<td>7.77</td>
<td>3.12</td>
<td>2.49</td>
</tr>
<tr>
<td>Mechlorethamine (given i.p.)</td>
<td>2.26</td>
<td>1.24</td>
<td>2.70</td>
</tr>
<tr>
<td>BCNU (given i.p.)</td>
<td>40.2</td>
<td>12.2</td>
<td>3.31</td>
</tr>
<tr>
<td>5-FU</td>
<td>49.4</td>
<td>12.3</td>
<td>4.00</td>
</tr>
<tr>
<td>LEO 1031</td>
<td>125</td>
<td>22.2</td>
<td>4.13</td>
</tr>
</tbody>
</table>

* For exact chemical nomenclature, see list of compounds in Refs. 26 and 80.

Dₘ, dose increment that will lead to a reduction of cell survival by a factor of 0.37 on the linear part of the curve.
been examined in further detail (54). The response of erythrocytic repopulation ability, mature RBC production, and the erythropoietin-sensitive cell population in general support the findings of proliferation-dependent cytotoxicity if one assumes the latter cell population to be more rapidly proliferating than the erythrocytic repopulation ability but similar to those assayed for mature RBC production. However, the results for Myleran indicate a greater effect on the slowly proliferating cells.

A similar GRA assay has been used in the study of anticancer agents. However, significant differences following a number of drugs have been demonstrated between the GRA and erythrocytic repopulation ability (43). Possibly, the GRA would more closely reflect changes observed with CFU-C, which might be a more relevant assay considering that granulocyte depletion following anticancer agents is the usual factor responsible for limitation of drug dose.

Ogawa et al. (56) assayed the survival of HCFU and CFU-C after chemotherapy *in vivo* and *in vitro* and were able to demonstrate an increased sensitivity of proliferating HCFU and CFU-C to CY, BCNU, and 5-FU. The possibility that CFU-C could become more sensitive to anticancer agents was a consequence of the finding that an increased fraction of this cell population could be forced into cell cycle (38). Recently, Blackett et al. (10) have cautioned investigators about the interpretation of *in vitro* and *in vivo* data obtained on the proliferative status of HCFU by tritiated thymidine suicide. They claim that the *in vitro* finding of a low thymidine suicide rate of normal mouse bone marrow stem cells may be a technical artifact since it can be at variance with the data on *in vivo* killing. Although a critical appraisal of methods that are taken for granted is always useful, it should be stressed that an alternative interpretation of the *in vivo* results on the basis of early recruitment as a consequence of irradiation by tritiated thymidine cannot be excluded, since earlier assay indicates less killing (48).

After the demonstration of this kinetic-related cytotoxicity, it was thought that all proliferating malignant cells in a given host might show a dose-response relationship similar to the proliferating clonogenic HCFU. However, an overriding importance of the biochemistry of the cell was elegantly demonstrated by Ogawa et al. (55). They showed that whereas there was no difference in the killing of normal cells and proliferating HCFU by melphalan, there was a large difference in cell killing when proliferating tumor cells were compared to normal cells. That is, the malignant cells were much more sensitive than the proliferating HCFU.

**Phase- and Cycle-specific Agents**

By definition, as well as from experimental evidence (53), phase-specific agents kill cells in 1 or more phases (G1, S, G2, or M) of the cell cycle. Cells obviously have to be in cycle to be killed by such agents, and cells that are out of cycle are not killed. The phase-specific agent vinblastine is known to exert its lethal action on cells in a specific phase or phases of the cell cycle, and thus it is to be expected that proliferating cells would be much more sensitive than nonproliferating cells. However, there is evidence that, by increasing the dose of a number of phase-specific agents, cells in other phases of the cell cycle as well as nonproliferating cells can be destroyed (74).

A further problem that might arise in attempting to distinguish phase- and cycle-specific agents is that any agent that is stored in a cell system can be released later and will then exert its action. It might then yield a dose-effect curve without a typical plateau, since the drug taken up while cells are either not proliferating or in the "nonsensitive" phase might become an effective killing agent when the cells are later brought into cell cycle for assay of clonogenicity or when they pass through the sensitive phase. This might explain why 5-FU, which interferes with nucleic acid synthesis, is not a phase-specific agent against the AKR leukemia, whereas it has been demonstrated to be phase-specific for another tumor (46). However, to the present, ambiguities in the classification of drugs have been exceptions rather than the rule, and the classification is essentially unchallenged. One important consequence of this classification has been the insight that for the phase-specific drugs an increase in the dose per unit time might not increase toxicity whereas increased duration of treatment will markedly do so. An increased dose of such a drug (e.g., hydroxyurea) might thus permit effective treatment of resistant tumors without increased toxicity.

**Effect of Assay Time on Experimental Results**

Other experiments have not always led to a similar conclusion concerning proliferation-dependent cytotoxicity. Blackett and Millard (9) demonstrated no difference in sensitivity for Myleran between proliferating and nonproliferating HCFU. However, it is questionable whether these results are at all suitable for comparison. One experimental parameter that has varied among studies assessing the effects of anticancer agents on HCFU is the time between administration of the drug and the assay. Whereas Bruce and his colleagues assayed the effects at 1 or 24 hr, van Putten carried out the assay at 16 hr after drug administration. Since HCFU can differentiate into other cells as well as proliferate to produce more stem cells, the number assessed may depend upon the time of assay. This was illustrated by the studies of Dunn (23), who examined the cytotoxic effect of melphalan on proliferating and nonproliferating hematopoietic stem cells. Differential killing of proliferating cells was observed when the assay was carried out at 4 hr following drug administration, while no differential killing was demonstrated if the assay was done at 24 hr or later. Van Putten and Lelieveld (77) and Hellman and Grate (34) found differences in the form of the survival curve depending upon the time interval between exposure to CY and HCFU assay (Table 2).

The ideal time to assay for proliferation dependency of agents on a cell population would be when all cell killing is complete and before the remaining cell number changes due to proliferation, differentiation, and/or other causes. However, the actual duration of cell exposure *in vivo* to cytostatic agents after a single drug injection is uncertain. If a delay in assay causes decreased HCFU survival, it may be
difficult to decide whether this is due to cell death by recruitment of resting HCFU into cell cycle and their subsequent passage through the sensitive phase of the cycle at a time when there are still cytotoxic drug levels or by drug-induced stem cell differentiation associated with a loss of capacity to form a visible spleen colony. This problem can be partly overcome if we refer to radiation as an alternative agent since the duration of radiation exposure is clearly defined. If, after irradiation of a donor mouse, the assay of surviving HCFU is delayed, the survival curve is found to be displaced downward. The slope remains essentially unchanged ($D_0 = 69$ rads instead of $73$ rads), but the extrapolation number is significantly reduced from 2.4 to 1.1 (79). An analysis of the time curve of this effect has shown that a minimum is reached at about 24 hr after exposure. After this, HCFU survival increases, presumably as a consequence of multiplication of surviving cells. It has been postulated that the decrease is due to loss of HCFU, which differentiate to a type of precursor cell that has lost the capacity to form a visible spleen colony (79).

Exposure of HCFU to cell cycle phase-specific agents yields dose-effect curves that show a plateau after higher drug doses. If drug levels are maintained for at least 1 full cell cycle, the plateau level of survival should be similar for each drug and reflect only the destruction of the proliferating HCFU. However, this is not the case (75). Apparently, the simultaneous killing of cells in the proliferating and maturating compartments in bone marrow causes a disturbance in the cell kinetics of the hemopoietic system, and resting stem cells are triggered to proliferate or differentiate, or both, in order to compensate for the cell killing. Thus, while generalizations can be made concerning both the extent of killing and the form of the dose-survival curve, exact quantitation probably requires that we be knowledgeable about the effect of drugs on the many other hemopoietic cell populations and that we know the effect of their depletion on the proliferation or differentiation of the stem cells. At present, this is an area of limited study.

From the data on irradiation cited above and the data on cell cycle phase-specific agents, it appears that the above phenomenon occurs relatively independent of dose. For the radiation survival curve, the slope does not change with increasing time, while the extrapolation number does; for dose-survival curves of phase-specific agents, the slope is unaffected by the time between exposure and assay, while the level of the plateau decreases with time. This latter effect has been demonstrated if drug levels are maintained constantly (14), but for a limited period of time this decrease may also be observed after a single exposure, just as after irradiation. A similar effect on extrapolation number for a cycle-specific agent, CY, is shown in Table 2. The results do not contradict the supposition that the extrapolation number decreases with increasing time interval between injection and assay, although the data are not very convincing. Extrapolation numbers have a notoriously low degree of accuracy, but the supposition may find some support from the large number of data on dose-effect curves in which extrapolation numbers lower than unity are found. However this may be, it is clear from the above reasoning that one should not trust colony-forming unit survival curves to express only the degree of accumulation of sublethal damage in the value of the extrapolation number.

Another point evident in Table 2 is that with an increasing time interval between exposure an assay there is also a change of slope ($D_0$). This has been ascribed to repair of potentially lethal damage (77). It has been demonstrated that repair of alkylation occurs in mammalian cells (17). There is also evidence that a delay in proliferation will permit more repair in cells exposed to alkylating agents (62), a phenomenon that has long been known in microorganisms (57). It has been speculated that duplication of DNA during S phase might fix the unrepaired damage irreversibly. Thus, a delayed assay of resting cells will allow for a delayed entry into the cell cycle leading to more repair of damage in the resting cells. A similar finding has been reported by Hahn et al. (33) for EMT6 tumor cells. Assay of the clonogenic cells at 24 hr following exposure to CY or 5-FU showed a large increase over the 2-hr determination. The authors suggested the difference for these 2 drugs was a consequence of repair of potentially lethal damage.

This variability in response to assay time implies that a single quantity denoting sensitivity of HCFU in absolute terms cannot be obtained. The quantitative results expressed by the slope and extrapolation number obviously are only of relative value, reproducible only under fixed conditions of administration and assay. Valid conclusions can be drawn only from comparison of drugs studied by uniform assay techniques. Even so, while the conclusions derived above resulted from data on spleen colony-forming stem cells, it is possible that they also apply to studies on other cell renewal systems.

### Increased Drug Sensitivity of Proliferating Cells and Its Implication for Repeated Drug Administration

The clinical importance of an enhanced cytotoxicity in proliferating cells, and especially in hemopoietic stem cells, results from the possibility that, during therapy in which multiple courses of anticancer agents are given, an increased fraction of the stem cells of normal cell renewal populations of the host would enter the proliferative state, thereby becoming more sensitive to continued treatment. This

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**Table 2**

<table>
<thead>
<tr>
<th>Parameters of survival curves for HCFU after CY treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_0$ (mg/kg)</td>
</tr>
<tr>
<td>Normal bone marrow</td>
</tr>
<tr>
<td>Assay 16-20 hr after 1 single injection</td>
</tr>
<tr>
<td>Drug administered to assay recipients</td>
</tr>
<tr>
<td>Regenerating bone marrow</td>
</tr>
<tr>
<td>Assay 16-20 hr after 1 single injection</td>
</tr>
<tr>
<td>Drug administered to assay recipients</td>
</tr>
<tr>
<td>Assay 3 hr after last of 4 doses at 2.5 hr intervals</td>
</tr>
</tbody>
</table>

* n, extrapolation number.
* Not significantly different from 1.0 ($p < 0.05$).
possibility has been verified for repetitive doses of CY by DeWys et al. (19), who examined the cytotoxic effect on stem cells of a 2nd dose of CY given at different times following a 1st dose (Chart 4). The 1st dose was chosen so as to reduce the stem cell population to approximately 10% of control. It was found that the stem cells were most sensitive to CY during their proliferative phase. The increased sensitivity of the stem cells was found also to correlate with increased toxicity of the 2nd dose of CY. This effect has been demonstrated clinically for CY (6) where proper spacing of large doses greatly minimizes hematological toxicity. It is likely that large intermittent doses of cycle-specific agents will prove to be the optimal clinical schedule. Appropriate timing, probably 2 to 3 weeks in humans, will then be required to minimize destruction of stem cells in the normal cell renewal systems.

The recruitment of nonproliferating normal stem cells discussed above for CY is obviously an important consideration in combination therapy schedules. A schedule-dependent toxicity has been demonstrated for the combination of radiation and actinomycin D by Smith and Wilson (66). When the drug followed the radiation, decreases in animal survival, granulocyte count, and endogenous spleen colonies were greater than expected. The change in the latter parameter was found to depend on the time after radiation that the drug was given, suggesting that the stem cells recruited into cell cycle following the radiation were more sensitive to the lethal effect of actinomycin D. Eaves and Bruce (25) have shown that, following moderate doses of either radiation or CY, the remaining HCFU reach a maximum sensitivity to 5-FU within 12 to 24 hr and require a number of days to return to the normal 5-FU sensitivity.

Thus, since stem cells in a cell renewal system can enter the cell cycle to repopulate their radiation- or drug-depleted compartment as well as that of their differentiated descendants and then return to their normal out-of-cycle condition, subsequent courses of anticancer agents, especially those that demonstrate significant proliferation dependence on cell survival, should be scheduled at a time after which the proliferating stem cells have moved back into the resting state.

Proliferation-dependent Cytotoxicity of Cells in Vitro

The proliferation-dependent cytotoxicity of anticancer agents has been studied extensively in vitro and, at present, represents a major focus of investigations of this phenomenon. Initially, bacteria in either log-phase growth or at rest (after depletion of nutrients) were used as a model system to examine proliferation-dependent effects (58, 59). Proliferating bacteria were found to be more sensitive to a variety of anticancer agents than were the nonproliferating bacteria. Pursuing a similar system with mammalian cells in vitro, Schabel et al. (64) demonstrated, as might be expected, that the phase-specific antimetabolites had no effect on nonproliferating L1210 cells although they were quite effective on proliferating L1210 cells.

As considered previously, phase-specific agents should be much more effective on proliferating cells in culture than on nonproliferating cells. This has been shown nearly invariably to be true (3, 11, 27, 47, 64) and need not be considered further, except for anomalous results such as those shown recently for hydroxyurea (52). While the expected results were obtained for short exposure periods, nonproliferating cells were found to be very sensitive to hydroxyurea following 6 to 8 hr of exposure. The authors cannot explain their results in cellular terms and are left with the unlikely hypothesis that an additional cytotoxic mechanism for hydroxyurea occurs and that it appears to be restricted to nonproliferating cells.

For the agents that kill nonproliferating as well as proliferating cells, the results and their analysis become more complex. Many of the early studies considered 2 distinct types of cells in cultures: exponentially proliferating cells and nonproliferating stationary or plateau-phase cells. Madoc-Jones and Bruce (49) demonstrated that stationary-phase L-cells were much less sensitive to the lethal effect of 5-FU than were proliferating L-cells. Wilkoff et al. (82) expanded these studies to show that a number of antitumor antibiotics were also less cytotoxic to nonproliferating L1210 cells. Similarly, phleomycin has recently been shown to be much more effective on proliferating L-cells than those in a stationary state (65). In an extensive series of in vitro experiments on L-cells, Bacchetti and Whitmore (1) demonstrated that L-cells in the logarithmic phase of growth were much more sensitive to actinomycin D than were stationary-phase cells. In contrast, Thatcher and Walker (67) have shown that the anticancer agents actinomycin D, BCNU, and sulfur mustard are equally effective on proliferative and stationary hamster embryo cells in vitro and attributed the difference seen by Madoc-Jones and Bruce (49) for L-cells to 5-FU as resulting from the L-cells being in G1 in stationary phase whereas the hamster embryo cells are in G2 in stationary phase. Thus, some differences seen with the anticancer agents might simply reflect differential effects on cells in G1 and G2 and not reflect the fact that they are in a stationary versus a proliferating state. A 2nd possibility that these authors entertain is that cells in the stationary phase...
may have a greater permeability to some of the anticancer agents than do those in the proliferating state. Such a possibility should be easily examined through the use of radioactive anticancer agents. In fact, for actinomycin D, such an increased incorporation has been shown for proliferating cells compared to their nonproliferating controls (1). Also, it has been demonstrated that transport mechanisms carrying amino acids and other substances into the cells are more active in rapidly proliferating cells, and it is known that cytotoxic drugs are transported into the cell by such carriers. Such transport seems to occur at an increased rate during rapid proliferation (28).

In line with the 1st argument above for the differences found for 5-FU, it has been demonstrated that results derived in plateau-phase cells not only may differ from one investigator to another but also may indicate a very complex and dynamic system. A most interesting and important question that has not been answered at this time relates to the response to anticancer agents of nonproliferating cells that are in different physiological states. For example, mammalian cells can become nonproliferative as a consequence of their response to a number of factors. They can be “contact-inhibited”; they can be hypoxic; their nutrient source(s) may be depleted; their environment may be depleted of specific “nonnutrient” metabolites such as isoleucine; they may be at a low temperature; or they may be stopped in cell cycle by a number of different blocking agents. It might be expected that anticancer agents would vary greatly in their lethal effects on these different nonproliferating cells.

For example, while proliferating cells are simply those in the exponential portion of the growth phase, “nonproliferating” or plateau-phase cells differ in their growth kinetics and radiation response when “fed” and “unfed” conditions are compared (32). The fed condition probably represents “density inhibition,” most probably caused by lack of a slowly diffusible nutrient; in this condition, different fractions of the population may be proliferating depending upon the cell type (32, 51) while the whole population is in steady state due to different degrees of cell loss. In the unfed situation, generally few cells are in cycle, and a decrease in the number of cells with time is likely as a consequence of cell death without renewal in the nutrient-depleted population. It has been shown that both monolayer and suspension cells can be in a plateau phase of growth in which the nonproliferating cells contain the G₁ content of DNA (27, 51). In cases where medium exhaustion occurs, nonproliferating cells may also be found to occur with a G₂ content of DNA. While we indicated earlier that phasespecific agents would logically be expected to have no effect on nonproliferating cells, it is quite possible that agents with an age response demonstrating selective killing for proliferating cells in G₁ might also demonstrate some degree of killing for nonproliferating cells in this state. A recent study examined this question for fibroblasts under hypoxic conditions and found, however, that while such cells accumulated in G₁, their sensitivity to radiation was unlike a synchronized, proliferating G₁ population (40).

The complex nature of the interaction of functionally different cell populations during the late “plateau” phase of growth has recently been demonstrated (51, 83) and a model illustrating this is shown in Chart 5. Not only are there attached and floating cells (primary tumor and metastasis) but also the attached cells contain a nonproliferating, and probably a proliferating, clonogenic subpopulation. Further, both the nonproliferating and the proliferating cells in the plateau-phase culture give rise to nonclonogenic cells as assayed by plating efficiency following trypsinization of the parent culture.

There are presently data that indicate that in some situations nonproliferating cells are more sensitive than proliferating cells. Both bleomycin and BCNU are more...
effective in killing nonproliferating Chinese hamster ovarian cells in stationary growth phase than proliferating Chinese hamster ovarian cells (4). For both drugs, the authors attribute the differential response to a decreased capacity of nonproliferating cells to repair drug damage. However, neither van Putten et al. (80), comparing proliferating and nonproliferating hemopoietic stem cells; nor Lin (H. Lin, personal communication), using antigen-sensitive cells; nor Twentyman and Bleehen (69), using hemopoietic colony-forming cells; nor Mauro et al. (52), using V79 Chinese hamster cells, have demonstrated this increased activity of either BCNU or bleomycin on nonproliferating cells. On the other hand, the observation was confirmed in other systems by Twentyman and Bleehen (70) for confluent EMT6 mammary tumor cells in vitro exposed to BCNU and also, when late plateau-phase cells were studied, for bleomycin. One explanation for differences found between investigators using plateau-phase cultures has been demonstrated recently (P. R. Twentyman, personal communication) in which the plateau-phase cells were either more or less sensitive than exponential-phase cells depending on the length of time the cells had been in the plateau phase when the studies were done. Also, recent experiments by Hahn et al. (31) demonstrate the difficulty of interpreting in vitro experiments involving BCNU because of serum binding of this agent and the variability of serum components in the media of metabolizing cells.

Proliferation-dependent Cytotoxicity to Tumors

If we examine the effect of anticancer agents on tumors in vivo, we find results that conflict with in vitro studies. For example, Hagemann et al. (29) examined the effect of BCNU on P815X2 mastocytoma cells in culture and found the plateau-phase cells (0.7% labeling index) to be more sensitive than the the exponential-phase cells (53.6% labeling index); the former had the same $D_0$ but no shoulder. When this tumor was examined in vivo as an ascites and as a solid tumor, the ascites demonstrated dose-survival curves similar to those in the in vitro experiment, cells in the early solid tumor were more sensitive than those in the late tumor, while those in intermediate tumors were least sensitive. However, in vitro, the cells were much more sensitive to the drug in any stage of growth than were similar cells in vivo. A major component in this finding is likely the difference in local drug concentration after i.p. administration of drugs to an animal carrying an ascites tumor versus an animal carrying a s.c. solid tumor. Nevertheless, there is a clear implication of increased or at least intermediate drug sensitivity of cells under the condition of maximal crowding. The late-stationary-phase tumor cells were always more sensitive than the early-stationary-phase cells.

DeWys (18) demonstrated that an experimental solid tumor was much more sensitive to CY in its early stage of growth than in the late stage. While this was probably due to a difference in the proliferative state of the tumor at these different times, with fewer malignant cells being in cell cycle at the later time, the author does not discount the possibility of a poorer drug supply to the larger tumor. Recent results (R. Hill, unpublished observations) indicate that this possibility is unlikely for CY, since hypoxic and oxygenated clonogenic cells appear to be exposed to similar concentrations of drugs.

There are a few examples of the action of anticancer agents either on tumor cells in vivo during proliferating and nonproliferating stages or on different populations within a tumor. For L1210 cells, there is exponential growth initially following inoculation as an ascites, then a decrease in tumor growth rate. This correlates with the rate of DNA synthesis, in that the fraction of cells in S phase decreases in the late tumor. Hryniuk and Bertino (36) used both the L1210 and the L5178Y leukemias to show that amethopterin is effective on proliferating cells but had a much diminished lethal effect on resting cells. It has been commonly found that the growth rate of solid tumors decreases with increasing size and that the proliferating population decreases concomitantly. It has been considered likely, although open to speculation, that the nonproliferating cells contain a clonogenic fraction.

Recently, solid tumors have been divided into proliferating (P-cells) and nonproliferating (Q-cells), and populations of both have been examined in terms of clonogenicity following treatment with anticancer agents (2, 35). It has been demonstrated that a fraction of the Q-cells can be recruited into the P-cell compartment following treatment with anticancer agents (39) and that recruitment occurs from cells with both the G1 and G2 content of DNA (21).

Clinical Implications for Administration of Proliferation-dependent Agents

One clinical implication of these results is that it is quite difficult to treat the tumor-bearing host effectively during the late stage of tumor growth since a large fraction of the malignant cells may be out-of-cycle and thus insensitive to the majority of the chemotherapeutic agents. Optimal therapy with agents that are more effective on proliferating cells should be to administer low doses to destroy the proliferating cells and thereby recruit the nonproliferating cells into cycle. Following this recruitment, chemotherapy might then be effective. Such scheduling has been successfully attempted recently by Schabel (63) for a solid tumor.

The action of these agents becomes very important when one considers the generation time distribution of human tumor cells. The distribution variance is very large, probably as a result of a large distribution in the $G_1$ transit times. Whether cells in a long $G_1$ respond to these agents in the same way as cells in a short $G_1$, or whether the former respond in a manner similar to nonproliferating cells remains unknown. Further, the clonogenic fraction of cells that are out of cycle and their kinetics of recruitment during therapy must be taken into consideration in scheduling anticancer agents as must the facts that normal stem cells are also moving in and out of cycle during the therapy and that their destruction must be minimized.

Practical Importance of Anomalous Sensitivity of Resting Tumor Cells

The anomalous sensitivity of resting tumor cells has 2 important aspects. First is the fundamental mechanism by
which out-of-cycle cells in specific circumstances are observed to be highly sensitive to drug actions. A discussion of this is given below, and a further study of the mechanisms involved may well give important clues for the development of better drugs. Second are the practical consequences. So far, no evidence is available to indicate that the high sensitivity of a subpopulation of resting cells has a clear effect on the curability of tumors. The only experimental system that permits cell survival analysis, in which these problems were studied, is the system described by Hahn et al. (33) in which an increased sensitivity could be observed only after early assay, but not if there was a 24-hr interval between exposure and assay. Furthermore, it is clear that in any mixed population the single-dose cell survival is determined by the most resistant subpopulation and the sensitivity of other subpopulations is rarely relevant. The terminal slope of the dose-effect curve in Chart 6 is not affected by the degree of sensitivity of Population C although this is markedly different between the 2 theoretical populations. The main consequence is a difference in the optimal dose per fraction for the 2 populations. However, this presupposes fractionated treatment of a tumor in which sensitive resting cells reappear after being eliminated by a 1st dose. Further studies are necessary to evaluate this possible advantage.

In more general terms, the idea of higher sensitivity in rapidly proliferating tumor cells seems to be confirmed by clinical experience. Zubrod (84) first drew attention to the better clinical results of chemotherapy on rapidly proliferating cancers in man, and this tendency has not been reversed by the availability of new drugs.

Other Proliferating and Nonproliferating Populations

Other cell systems which have the capacity to move from the proliferating to the nonproliferating state and vice versa, can be analyzed, and some have been. These include lymphocytes, hepatocytes, epidermal cells, and mucosal cells. Recently, Lin (45) demonstrated that a variety of anticancer agents, both phase- and cycle-specific, were much more effective in killing proliferating B-cells than in killing nonproliferating B-cells. The cells were recruited into cell cycle by administering an antigen, sheep RBC in this case, and the effect of the drug treatment was assayed in terms of the number of plaque-forming cells.

One importance of studying different cell populations in different proliferative states is that a single system might not be a good model for both the resting normal stem cell or the resting tumor cell. For example, the resting tumor cell could be out-of-cycle because of nutrient depletion while the resting stem cell could be out-of-cycle because of a regulatory factor in the environment. At this time, many experimental and clinical oncologists consider that the physiology and drug sensitivity of all out-of-cycle cells are the same and base much of their conceptualizing on the resting hematopoietic stem cell. It must be remembered, however, that the tumor cell presumably has a decreasing ability, if not a total inability, to respond to normal regulatory control mechanisms and therefore, from a physiological as well as drug-response viewpoint, is quite different from the hematopoietic G0 stem cell. It seems imperative that the effect of the anticancer agents on cell populations which are out-of-cycle as a consequence of different mechanisms be examined and further that the physiological basis for any differences be determined.

Although it seems clear that there is a degree of uniformity in the response of normal tissue cells in vivo, thereby confirming the original observation on hematopoietic stem cells, this uniformity is lacking when we try to extrapolate these results to tissue culture cells in plateau phase or to nonproliferating cells in tumors. We should remember that our terminology and classification of cells on the basis of their cell kinetic status is a shorthand notation for an apparently common status in which different cells exist. If we find that some resting tumor cells respond differently from resting stem cells, this does not imply that cellular proliferation has a variable effect on drug sensitivity, but rather that our cell kinetic classification of cells as nonproliferating fails short. The classification, being a shorthand notation, fails to give an adequate description of the physiological state of the cell. Apparently, it should include some other biochemical aspects of the cell such as the activity of the DNA repair enzyme system or possibly the avidity with which certain drugs are taken up by a common carrier system in the absence or decreased concentration of the normal nutrient. This aspect indicates that the cell kinetic status alone, giving insufficient correlation with drug sensitivity, is not an absolute parameter for resting tumor and tissue culture cells.

Proliferation-dependent Cytotoxicity of Anticancer Agents

Cellular and Biochemical Mechanisms for Differential Sensitivity

In assaying lethal damage to cells, the possible effects of sublethal and potentially lethal damage must be considered. Nonproliferating cells might demonstrate a greater drug sensitivity as a result of a lesser capability for repair of sublethal damage. This has been shown following X-irradiation of Chinese hamster cells in exponential and plateau phases of growth (30) and shown to be likely for the P815X2 mastocytoma following BCNU exposure (29). Thus, one mechanism proposed for the difference in sensitivity is a consequence of repair mechanisms. Alternatively, nonproliferating cells may have time to repair potentially lethal damage [as has also been observed with X-radiation (32)], while proliferating cells continue DNA synthesis, thereby expressing the lethality. That repair of alkylation does occur has been demonstrated in mammalian cells (17, 20). A test of this possibility would be to force cells into the proliferative state shortly after exposure to a given agent and assay cellular survival.

The expression of potentially lethal damage depends strongly upon the time of assay following drug treatment. As previously indicated, Hahn et al. (33) examined the effects of a number of antinecancer agents on the EMT6 tumor and obtained biphasic dose-survival curves which they interpreted in terms of a greater cell killing of the proliferating, clonogenic cells. When they assayed for survival at 2 hr after injection of the drugs, they found more cell killing, especially after exposure to 5-FU or bleomycin, than when they delayed the assay for surviving cells until 24 hr after drug injection. These data were interpreted to show that repair of potentially lethal damage occurs if the cells are left in situ, whereas such repair is circumvented if the cells are induced to proliferate early in the assay system. Similar results were found for nitrogen mustard and 5-FU (60). In this respect there is a parallelism between in vivo survival of the nonproliferating fraction of the tumor cells and in vitro survival of plateau-phase cells.

Finally, we must consider possible biochemical mechanisms by which proliferating cells become more sensitive to anticancer agents. Mechanisms dealing with differential transport or repair processes have been discussed above. For alkylating agents, the difference might result because the DNA is in a more exposed state in the proliferating than in the nonproliferating cells. As cells progress through the cell cycle, mRNA must be made, and this process probably exposes the DNA to critical alkylation. In the nonproliferating state, the DNA is probably transcribing little RNA and is thereby protected from alkylation by its surrounding proteins. Also, since protein, RNA, and lipid turnover is much more rapid in nonproliferating than in proliferating cells, it has been suggested (44) that proliferating cells are less able to renew the damaged macromolecules critical to their survival. Whatever the mechanism, it seems clear that this area of investigation is an important one with a potential to effect drastically and positively therapeutic scheduling of chemotherapeutic agents.

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