Loss in Cell Killing Effectiveness of Anticancer Drugs in Human Gastric Cancer Clones Due to Recovery from Potentially Lethal Damage in Vitro

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ABSTRACT

The ability of human gastric cancer clones to recover from potentially lethal damage was studied. Recovery was greatest following treatments with bleomycin or Adriamycin; the recovery ratios (i.e., survival) increased almost 8-fold during a posttreatment incubation period. Recovery was also possible following treatments with actinomycin D, 1,2:5,6-dianhydrogalactitol, and diaziquone; however, the recovery ratios never increased above 2. No recovery was observed following treatment with 5-fluorouracil.

Recovery from potentially lethal damage may be related to the heterogeneity in survival responses observed following treatment with some anticancer drugs. Bleomycin and Adriamycin treatments result in large heterogeneous survival fractions among these human stomach cancer clones, and the potentially lethal damage recovery ratios were larger (and variable). However, actinomycin D, diaziquone, and 1,2:5,6-dianhydrogalactitol produce very uniform killing effects in these cells and the recovery ratios are very much smaller and less variable.

Finally the large amount of recovery observed after bleomycin or Adriamycin treatments resulted in the loss of cell killing effectiveness of the agents. Because the survival fractions increased during the recovery period, the net effect on cell killing was reduced to an amount normally obtained with doses that were up to six times smaller.

INTRODUCTION

Mammalian tumor cells have the capacity to recover from drug- and radiation-induced damage. Recovery has been shown to occur both in vitro and in vivo. Recovery from one type of injury, PLD, is measured by the increase in survival observed when cells are exposed to suboptimal growth conditions after treatment (8). Possibly such conditions prevent the cells from progressing through the cell cycle and thereby allow time for the repair processes to take place. Conversely, when PLD is not repaired the damage becomes lethal and survival decreases. Factors such as inhibition of macromolecular synthesis, incubation in nutritionally depleted growth medium, and altered temperatures may influence the survival of cells after treatment with drugs or radiation (4, 7, 8, 10). The clinical implications of such recovery are of great interest, since cells that recover from damage and survive may express altered drug and radiation sensitivities, and could contribute to the regrowth of the tumor. Eventual treatment failure in human tumors is often attributed to expressed or acquired drug resistance among various subcolonial populations in the tumor. This heterogeneity of drug response within a single tumor (11) could occur because each clone possessed variable DNA indices and growth potentials (12), differential sensitivities to anticancer drugs and irradiation, and altered repair and recovery properties (13-20).

In vitro tumor model systems have proven useful for obtaining pertinent information about the selection of proper drug combinations and schedules for the control of multiclonal tumors (12-15, 21-29). It is imperative, therefore, that the drug sensitivity and recovery studies be performed on tumor models which are important clinically. For example, carcinoma of the stomach ranks seventh as the cause of cancer deaths in America (30, 31). Surgical excision remains the only potentially curative treatment for cancer of the stomach (32); however, for patients who have gastric cancer that cannot be totally removed by surgical excision (regional lymph node metastases and unresectable disease), the prognosis remains dismal; the median duration of survival from diagnosis is 4 months (32). In this paper we have used human gastric cancer clones isolated and characterized in our laboratory (11, 33, 34) and several drugs to: (a) determine which of the clones could recover from PLD during posttreatment nonnutritive growth conditions; and (b) determine whether heterogeneous and uniform drug survival responses were related to the ability to repair and recover from PLD.

MATERIALS AND METHODS

Culture Conditions. Three clones of a human adenocarcinoma of the stomach, AGS-1, AGS-6, and AGS-10, and the parent line (AGS) from which the clones were obtained were grown in Ham's F-10 medium plus 20% fetal calf serum (Grand Island Biological Co., Grand Island, NY). The cells were maintained in exponential growth in a humidified incubator (5% CO₂ and 95% air) at 37°C. Under these conditions the cells had population doubling times which ranged from 24 to 27 h, growth fractions of 100%, and plating efficiencies greater than 50%.

Survival Studies for PLDR. Exponentially growing cells were treated in 60-mm tissue culture dishes with the drugs for 1 h. Following treatment one group of cells was plated immediately at 0 h for colony formation (thus allowing no time for recovery), while other groups were washed and incubated at 37°C in HBSS for 1 to 4 h before plating to allow time for recovery from PLD.

At the time of plating for colony formation monolayers of cells were washed with 0.85% NaCl solution and were removed from the plates with trypsin (0.1%). Cell counts and dilutions were made and known numbers of single cells were plated into triplicate tissue culture dishes and incubated at 37°C for 10-14 days. Colonies were fixed, stained with crystal violet, and counted. A cell was considered to have retained its reproductive potential if it gave rise to a colony of 50 or more cells. Each experiment was performed at least three times. Average survival data
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Recovery ratios (R/R₀) in each experiment were calculated by dividing the survival fraction at 0 h (R₀) into the survival fraction of cells obtained at each hour of posttreatment incubation (R). The recovery ratios were then averaged and are presented in the charts. A recovery ratio of 1.0 means no recovery; less than 1.0 indicates cell death occurred during the posttreatment incubation period; and a ratio greater than 1.0 means recovery from PLD. Drug Solutions. The drug solutions were always prepared immediately before use to prevent loss of activity due to decay. The drugs were first dissolved in the appropriate solvent, and then diluted to final treatment concentrations in medium or HBSS. Solvent controls were always included in each experiment. The PLDR survival responses to the following drugs were tested: DAG, ACT D, BLEO, 5-FUra, ADRIA, and AZQ. Doses chosen were usually those which resulted in 90 to 99.9% kill, and are shown on the charts for each drug.

RESULTS

Recovery from drug-induced PLD was measured as a function of changing survival values during a 1- to 4-h posttreatment incubation at 37°C in HBSS (i.e., suboptimal growth conditions). In most cases the survival values among the clones at 0 h were extremely variable, reflecting the heterogeneous drug responses reported for these cells in an earlier study (11). For this reason recovery ratios (R/R₀) were calculated from the averaged survival data at each hour in order to normalize the data for comparisons among the clones.

Bleomycin (50 µg/ml for 1 h). When the AGS parent line and clones were treated for 1 h with BLEO and plated immediately for colony formation, the 0-h survival fractions ranged from 0.0078 in AGS to 0.035 for the AGS-6 clone (Fig. 1). Recovery began immediately as indicated by the increased survival fraction values seen at 1 h, and continued throughout the 4-h incubation period. Recovery occurred in all four cell lines. Although the AGS parent line was initially the most sensitive to BLEO, it experienced the greatest amount of recovery. It can be seen in Fig. 2 that there was a 3-fold increase in the recovery ratio (survival) by 1 h and this had increased to 7-fold by 4 h. The rate of recovery in AGS-10 was similar to that for AGS at 1 h, but plateaued over the next 2 h before rising to 4.8. AGS-1 showed the smallest amount of recovery and the values for AGS-6 were generally between those for AGS-10 and AGS-1.

Adriamycin (2 µg/ml for 1 h). The 0-h survival values for different clones treated with Adriamycin were heterogeneous (Fig. 3), ranging from 0.00033 (AGS-1) to 0.005 (AGS-6). As with BLEO this heterogeneity was expected (11). Survival increased in all cell lines during the first h of incubation and plateaued by the second h in each clone except AGS-1, where survival continued to rise. The comparisons of recovery among the clones and parent line can be seen better in Fig. 4. All clones exhibited a rapid rise in recovery ratios by 1 h, ranging from 2 to 2.75. Although AGS-1 and AGS-10 were more sensitive to ADRIA, they showed the largest final recovery ratios. The average recovery ratio for AGS-10 plateaued at 3.5, while that for AGS-1 was 7.5, and still rising at the fourth h of posttreatment incubation. The least sensitive line, AGS-6, expressed the lowest PLD.
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ADRIAMYCIN (2 µg/ml x 1 Hr)

Fig. 4. Recovery ratios of AGS clones indicating recovery from PLD after Adriamycin treatment.

DAG (15 µg/ml x 1 Hr)

Fig. 5. Average recovery ratios in AGS clones after DAG treatment.

AZQ (6 µg/ml x 1 Hr)

Fig. 6. Average recovery ratios in AGS clones after treatment with AZQ.

ACT-D (0.2 µg/ml x 1 Hr)

Fig. 7. Variable recovery response exhibited by AGS clones after treatment with Act D.

RECOVERY RATIOS

Both survival fractions and averaged recovery ratio data were presented for BLEO and ADRIA so that relationships between these two parameters could be easily seen. The PLDR data for the rest of the anticancer drugs used in these studies will be presented only as the recovery ratios calculated from the averaged survival fractions (±SE).

DAG (15 µg/ml for 1 h). It can be seen in Fig. 5 that AGS-10 and AGS-1 showed a gradual increase in recovery ratios throughout the 4-h post-DAG-treatment incubation period. However, in AGS and AGS-6 the recovery ratios (and, therefore, survival) decreased below 1.0. Additionally, the maximum recovery ratios at 4 h were very much lower after DAG treatment than after BLEO and ADRIA treatments.

AZQ (6 µg/ml for 1 h). The averaged recovery ratios following treatment with AZQ can be seen in Fig. 6. Recovery from AZQ-induced PLD was observed in all cell lines, with the AGS parent line exhibiting the fastest and highest recovery. Once again all recovery ratios were below 2 and, therefore, were much lower than those observed in BLEO- or ADRIA-treated cells.

ACT D (0.2 µg/ml for 1 h). From Fig. 7 it can be seen that the PLD recovery ratios for ACT D in AGS-1, AGS-6, and AGS-10 increased at the same rate through the second h of posttreatment incubation. The R/R0 for AGS-1 continued to increase until the third h before falling slightly to 1.65. Recovery in AGS-6 plateaued at 2 h at 1.3, while that for AGS-10 began decreasing at 2 h and fell to 1.0 by 4 h, indicating a return to preincubation survival levels. Recovery in AGS was very slight; it did not begin until the second h of incubation; and rose only to a R/R0 of 1.1.

5-FUra (1000 µg/ml for 1 h). Even at 1000 µg/ml for 1 h, 5-FUra killed fewer cells than any of the other drugs used in this study. The survival values at 0 h were: AGS-6, 30%; AGS, 29%; AGS-1, 13%; and AGS-10, 5%. Most of the recovery ratios were below 1.0 throughout the incubation period, and ranged from 0.64 to 0.85 at 4 h (Fig. 8). The values for AGS-10 fell constantly, reaching a ratio of 0.44 before increasing slightly to 0.64 at 4 h.

DISCUSSION

Ten permanent clones from a human adenocarcinoma of the stomach have been isolated, and recently we reported their in vitro growth characteristics and the cellular properties of in vivo growth in athymic mice (34). We also reported the heterogeneous responses of the clones to six anticancer agents, and the more homogeneous or uniform responses to three others (11, 33).
When the doses of each drug lethal to 90% of the cells were used for comparisons between the most and the least sensitive clones, large differences were observed for: 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (100%); chlorambucil (60%); ADRIA (130%); BLEO (180%); 5-FUra (260%); and melphalan (500%). Because of the changing shapes of the survival curves these differences became even larger at higher doses. However, clones exhibited more uniform survival responses to three other agents and the doses lethal to 90% of the least sensitive cells were only 28% greater for ACT D, 25% greater for DAG, and 27% greater for AZQ (11, 33). In the studies reported here we have shown that all of the drugs tested except 5-FUra can produce PLD; and most of the human stomach cancer clones can recover from the drug-induced damage. The recovery ratios observed for each clone after treatment were always different from each other. The largest recovery ratios were observed following treatments with BLEO or ADRIA, two of the agents which produced very large heterogeneous survival responses in the earlier drug studies (11). Their recovery ratios were also heterogeneous and large, ranging from 3 to 7 for BLEO-treated cells, and from 2.4 to 7.7 for those cells treated with ADRIA (Figs. 1-4). It is also possible that the increased survival observed after treatment with anticancer drugs (which is called recovery from PLD) is an artifact of the experimental technique. It has been suggested (35) (but not proven) that trypsinization after drug treatment might alter binding of drug to the cell surface or the efflux of the drug from the cell and thereby change survival during posttreatment incubation. This may be especially true of BLEO and ADRIA. Recovery ratios from PLD following treatments with DAG, AZQ, and ACT D (Figs. 5-7) were very much lower and less variable: their $R/R_0$ ratios were all below 2. These are the three drugs which produced more uniform killing effects among the clones in earlier studies (11, 33). These data suggest that there may be a relationship between heterogeneous survival responses and recovery from drug-induced damage.

All clones exhibited recovery from AZQ, although the $R/R_0$ for AGS-6 was only 1.2. All clones showed recovery from ACT D; however, that for AGS was only 1.1 and the $R/R_0$ for AGS-10 fell to 1.0 at 4 h after a higher $R/R_0$ was indicated at 2 h. Although AGS-10 and AGS-1 showed slight recovery from DAG, AGS-6 and AGS did not. Their $R/R_0$ ratios were below 1.0.

In an earlier study (11) the effects of nine anticancer drugs on survival of AGS parent cells were compared by using the slopes of the survival curves ($D_0$) and the doses lethal to 90% of the cells. 5-FUra was the least effective drug tested, followed by BLEO, DAG, and AZQ. The most cytotoxic agents were ACT D and ADRIA. It is interesting to note that cells treated with the drug which produced the least amount of cell kill, 5-FUra (11), exhibited no recovery from PLD; whereas recovery was greatest following treatment with ADRIA (Fig. 4), but there was only minimal recovery from ACT D-induced PLD. We do not yet have explanations for these apparent inconsistencies. Experiments are under way to determine which form of macromolecular synthesis (DNA, RNA, or protein) is required for PLD for each drug, and to determine whether these requirements differ among the clones. In earlier studies (1, 36, 37) we demonstrated that CHO cells could recover from BLEO-induced PLD in plateau phase and in every phase of the cell cycle except mitosis. Additionally we showed that by inhibiting RNA synthesis with noncytotoxic doses of ACT D, recovery from BLEO-induced PLD could be completely blocked (37).

It is possible that the conditions and requirements for PLD will differ for each gastric cancer clone and for each agent tested. It is important, therefore, that such studies be performed so that ways of inhibiting recovery may be found; especially because of the possible effects that repair and recovery may have on the effective treatment of cancer. This is best illustrated through the use of the recovery data plotted in another way in Figs. 9 and 10. In Fig. 9 it can be seen that survival decreased in AGS cells as the BLEO dose increased (○). Using the PLD inset it can be seen that survival at the 50-μg dose/ml (the 0-h PLD survival point) was 0.0072. The survival (recovery) can be seen to increase upon further incubation (○). After 4 h of recovery time, survival had increased 7-fold to 0.052. The dashed lines indicate that a survival value of 0.052 usually results from a treatment with a dose that is six times lower (8-10 μg/ml). Therefore the cell killing effectiveness of the 50-μg dose/ml was greatly reduced because of PLD recovery. Similarly, in Fig. 10 it can be

![Figure 8](image_url)  
**Fig. 8.** Lack of recovery in AGS clones following 5-FU treatment.

![Figure 9](image_url)  
**Fig. 9.** Survival curve (○) indicating decreasing survival with increasing dose of Bleo. Inset, PLD recovery (○) demonstrating the loss of effectiveness of Bleo due to recovery from PLD.
seen that after a treatment with 2 μg ADRIA/ml, recovery caused survival to rise from 0.0015 at 0 h to 0.0045 at 4 h (a 3-fold increase in survival); and that corresponded to a survival resulting from a treatment with a dose that was 30% lower (1.4 μg/ml). It is known from the work of Hahn et al. (6) that PLDR occurs in in vivo solid tumor systems. It may be inferred from these two studies, therefore, that PLDR also occurs in human tumors. If that is the case and if the toxicity to normal tissue is cumulative (i.e., for BLEO, lung; and for ADRIA, heart) then recovery from drug-induced PLD represents a drastic reduction in therapeutic effectiveness in the tumor while the contribution to the drug-induced toxicity in normal tissues remains high.

Conclusions. We have shown that clones of a human gastric cancer cell line can recover from drug-induced potentially lethal damage. PLD and recovery occurred after treatment with BLEO, ADRIA, DAG, AZQ, and ACT D. However, the data suggest that treatment with 5-FUra does not result in potentially lethal damage; and that although the amount of cell kill produced by 5-FUra in these clones is minimal, the effects are complete. The recovery ratios for each drug were different for each clone. The recovery was not always the greatest for the same clone, nor did any one clone always show the least amount of recovery. In answer to the question of whether heterogeneous and uniform drug survival responses among the clones is related to recovery from PLD, there is a noticeable trend. BLEO and ADRIA produce large heterogeneous survival responses among the clones and the recovery ratios following treatments with these agents were large, ranging up to 7.7. Conversely, AZQ, ACT D, and DAG produced more uniform killing effects and the recovery ratios after treatments with these agents were below 2 and less variable. Therefore there may be a relationship, but additional studies are needed. Other studies are continuing in an effort to find ways of inhibiting recovery from PLD and to determine why initial recovery was sometimes followed by a reduction in survival upon further incubation.

ACKNOWLEDGMENTS

The authors wish to thank B. Wilson and T. Uphoff for their excellent technical assistance, and D. McClure for assistance in preparing the manuscript.

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