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Hypersensitivity to low level cytotoxic stress in mouse cells with high levels of DHFR gene amplification

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Low level cytotoxic stress greatly accelerates the loss of unstably amplified dihydrofolate reductase (*dhfr*) genes from methotrexate-resistant mouse cell lines. To understand this drug-induced loss of amplified genes, the highly methotrexate-resistant mouse R500 cell line was flow sorted into two subpopulations with higher and lower average *dhfr* gene dosage respectively. The subpopulation with higher levels of gene amplification was much more sensitive to low level cytotoxic stress as judged by both cloning efficiency and growth in the presence of low concentrations of cytotoxic drugs. These results suggest that high levels of gene amplification can confer hypersensitivity to cytotoxic stressors such as anticancer drugs.

Key words: Methotrexate, gene amplification, dihydrofolate reductase, double minute chromosomes.

Introduction

Gene amplification is closely associated with the problem of cancer. There is evidence that facile gene amplification is one aspect of the genetic instability associated with the transformed phenotype.[1,2] However, the relationship between the transformed phenotype and gene amplification is likely to be complex.[3] Gene amplification is a common mechanism of cancer cell resistance to antineoplastic drugs[4] and activation of oncogenes by amplification can occur during tumor progression.[4,5]

Cytotoxic stress and DNA damage increase the frequency of gene amplification and drug resistance in cultured cell populations.[6,7] This is thought to be due to the generation of small extrachromosomal DNA fragments when cells replicate DNA under unfavorable or stressful conditions.[8–12] Some of these DNA fragments stabilize and become autonomously replicating episomes. The submicroscopic episomes can increase in size and eventually give rise to visible double minute chromosomes (DM).[13,14] Unstably amplified genes are carried on DM and episomes.[15–17]

DM are autonomously replicating, acentric extrachromosomal DNAs which distribute randomly between daughter cells at mitosis. This random distribution and the tendency of DM to be lost at mitosis[17–20] combine to generate substantial heterogeneity within the cell population. There can be great cell-to-cell variation in amplified gene dosage,[20,21] and in numbers and sizes of DM.[18,19,22,23] When cells owing their drug resistance to unstable gene amplification are grown in drug-free medium, the population progressively decreases in average amplified gene copy number and regains drug sensitivity.[4,17,24] This is due in part to a replication disadvantage associated with unstably amplified genes.[14,20]

DM can integrate into cellular chromosomes to give rise to homogeneously staining regions (HSR).[13,14] These genes are stably amplified and are not lost when the cells are grown in the absence of the selecting drug.[4,17,24]

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Treatments which can selectively act against amplified genes or cells which carry amplified genes are of special interest because of the involvement of gene amplification in tumor progression and cancer cell resistance to antineoplastic drugs. The loss of unstably amplified genes can be greatly accelerated by very low level cytotoxic stress from a variety of mechanistically unrelated stressors including antimetabolites and topoisomerase inhibitors.[25,26] The effect is often seen at stress levels that only slightly slow the growth of the cell population and do not cause increases in cell death as judged by plating efficiency and trypan blue exclusion. To better understand this phenomenon, we have separated the highly methotrexate-resistant R500 cell line into subpopulations on the basis of *dhfr* gene dosage. Comparison of these subpopulations for growth and cloning efficiency under conditions of low level cytotoxic stress has shown that those cells with the highest levels of *dhfr* gene amplification are supersensitive to such stress.

Materials and methods

Cell culture

Cells were grown in Dulbecco's modified Eagle's medium (Gibco #430-2100), pH 7.4, supplemented with sodium bicarbonate (3.7 g/l), 10% fetal bovine serum, and 0.5% penicillin-streptomycin (Gibco). The cultures were maintained under a 5% CO_2 atmosphere at 37°C in a humidified, water-jacketed incubator. R500 cells, a gift of R Schimke, were maintained in medium with dialyzed calf serum and 250 μM methotrexate (MTX).

Cell sorting and flow cytometry

A fluorescent derivative of methotrexate, fluorescein methotrexate triammonium salt (f-MTX), was purchased from Molecular Probes. For uptake of f-MTX, cells were grown to 90% confluence, rinsed well with MTX-free medium, and placed in MTX-free media for 24 h, with three media changes. The cells were then incubated for an additional 24 h in medium containing 25 μM f-MTX. Just before cell sorting or flow cytometry, the cells were removed by gentle trypsin digestion, and suspended in fresh medium. A cytofluorograph 50H flow cytometer was used to separate the R500 population into two subpopulations, one composed of cells falling below 16 green fluorescence units ($R500^-$) and one composed of cells above 16 fluorescence units ($R500^+$) (refer to Figure 1). The success of the sorting was confirmed by performing flow cytometry on samples of these sorted populations. Flow sorted populations were placed in 35 mm tissue culture plates and grown to confluence. The low fluorescence subpopulation was grown to confluence in MTX-free medium while the high fluorescence subpopulation was maintained in 250 μM MTX. After reaching confluence, the cells in each of the 35 mm plates were passed to one 10 cm tissue culture plate. When these plates reached confluence, the cells were used for experiments.

Cloning efficiency

Cells were seeded in 100 mm plastic tissue culture plates (3×10^3 cells/plate) in the presence or absence of test drugs. The plates were then placed in the incubator and left undisturbed for 2 weeks. To fix the colonies, the plates were drained of medium, rinsed gently with phosphate buffered saline, and filled with 7% formaldehyde in isotonic saline. The fixed colonies were visualized by staining with 1% crystal violet. Only colonies containing 40 or more cells were scored.

Growth curves

Growth curves were initiated by seeding 5×10^4 cells per plate in 60 mm tissue culture plates. After allowing 24 h for attachment, plates were removed in groups of four at regular intervals and the cells from each plate were harvested by trypsin digestion.

Quantitation of gene dosage

DNA was isolated as described, and duplicate DNA samples were immobilized on nitrocellulose sheets with the use of a slot blot apparatus.[26] The duplicate DNA samples were probed separately with cloned, ^{32}P-labeled DNA probes for the *dhfr* and the α-globin genes. Cloned probe sequences were separated from vector sequences by restriction digestion and agarose gel electrophoresis prior to labeling by the random primer method.[27] The probes used were pSV2-*dhfr* (American Type Culture Collection) and P1.3 (α-globin, a gift of Elio F Vanin[26]). Autoradiographic exposures

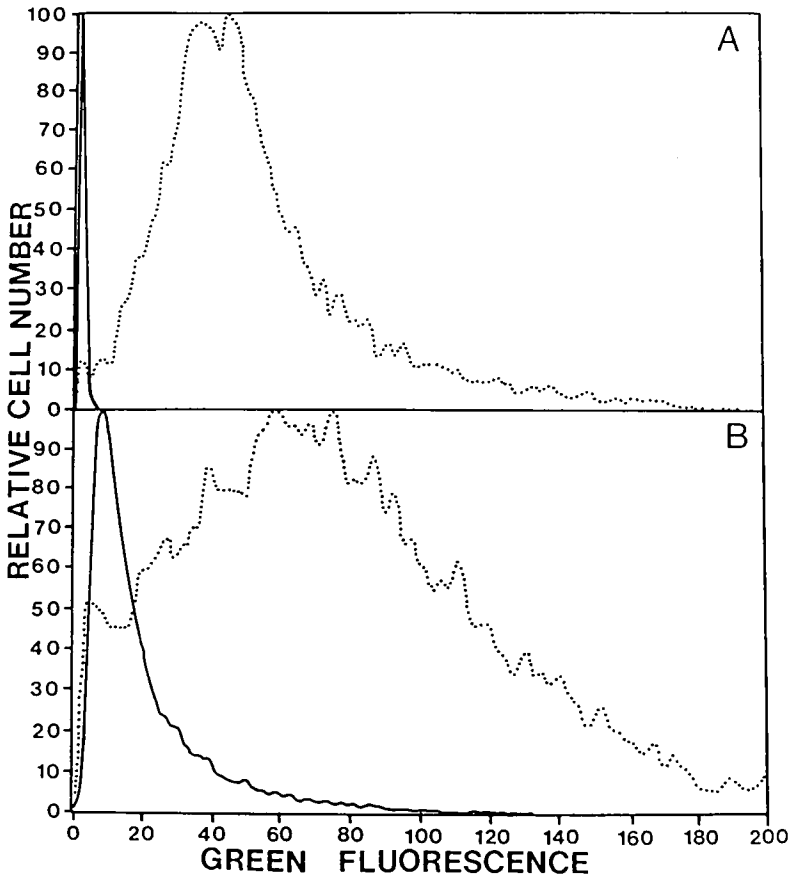

Figure 1. Distribution of f-MTX in R500 and related cell lines. (A) Retention of f-MTX by NIH3T3 cells (solid line) and R500 cells (dashed line). (B) Retention of f-MTX by $R500^-$ (solid line) and $R500^+$ (dashed line) subpopulations derived from R500 cells. The $R500^-$ and $R500^+$ populations were obtained by flow sorting the R500 population into cells with less than or more than 16 green fluorescence units respectively.

were made on Kodak X-AR5 film, and the autoradiograms were quantitated with an LKB 2222-010 Ultroscan XL soft laser densitometer. Peaks were integrated electronically with the LKB 2400 GelScan XL software package and an IBM AT computer.

Results

Sorting of the R500 cell population

Methotrexate-resistant cells with amplified dihydrofolate reductase genes overproduce dihydrofolate reductase.[28,29] Since methotrexate and methotrexate derivatives bind very tightly to dihydrofolate reductase, cellular retention of these drugs is proportional to the intracellular enzyme levels.[21] Thus, for cells with amplified *dhfr* genes, retention of f-MTX is an indirect measure of gene amplification.[30,31] Fluorescent derivatives of methotrexate have been used to evaluate the distribution of dihydrofolate reductase gene dosages in cells of stably and unstably methotrexate resistant cell lines.[16,17]

R500 cells have an average *dhfr* gene dosage of about 200 copies per cell.[22] Flow cytometry (Figure 1A) shows that cells of the highly MTX-resistant R500 cell line retain on average much higher levels of f-MTX than do cells of the MTX-sensitive NIH3T3 line. It is also clear that the cells of the R500 line are very heterogeneous with respect to f-MTX retention. At the low end of the distribution, a few cells retain no more f-MTX than NIH3T3 cells. The distribution also has a long tail at the high end, suggesting a small population of cells with very high levels of intracellular dihydrofolate reductase. These results are in

agreement with earlier cytometric studies on similar MTX-resistant cell lines.[16,17]

Cells of the R500 population were separated into high and low f-MTX retention subpopulations by flow sorting. Flow cytometry of these populations immediately after sorting (Figure 1B) showed that the average f-MTX retention of the low f-MTX population $R500^-$ was lower than that of unsorted R500 cells, but higher than that of MTX-sensitive NIH3T3 cells. The average f-MTX retention of the high f-MTX subpopulation $R500^+$ was higher than that of the unsorted R500 cells. Slot blot analysis of *dhfr* gene dosage relative to the mouse α-globin gene showed that the $R500^-$population had an average of 132 copies of the *dhfr* gene per cell, and the $R500^+$ population had an average of 240 copies of the *dhfr* gene per cell relative to the R500 level of 200.

Growth curves

The three MTX-resistant populations (R500, $R500^-$ and $R500^+$) were compared for growth in 50 μM hydroxyurea. This concentration of hydroxyurea slightly slows the growth of unstably MTX-resistant mouse cell lines and causes a very rapid drop in the *dhfr* gene dosage of the population.[25,26] The slowed growth is due in part to increased cell size and early cell–cell contact in the presence of the drug.[25] Under these conditions, there is no significant increase in cell death as determined by trypan blue exclusion and plating efficiency.[25,26] Growth in 50 μM hydroxyurea slows the R500 population and leads to confluence at a lower cell density (Figure 2, top). The same growth conditions slowed the growth of the $R500^+$ population significantly, but had no effect on the $R500^-$ population (Figure 2, bottom and middle). This result is suggestive of selective action by hydroxyurea against those cells in the R500 cell population with the highest levels of *dhfr* gene amplification.

Cloning efficiency

Although 50 μM hydroxyurea does not significantly decrease plating efficiency (number of cells attached 24 h after plating at high cell density) or trypan blue exclusion in R500 cells, it is possible that these methods might miss selective killing of a small subpopulation. The long tail in the distribution of f-MTX retention by R500 and $R500^+$ cells suggests that there might in fact be small subpopulations of cells with very high levels of *dhfr* gene amplification within the larger population. In addition, the complex kinetics of drug-induced *dhfr* gene loss in R500 cells could be a manifestation of differential targeting of subpopulations by different drugs.[26] These observations, together with the apparent selective growth inhibition of R500 and $R500^+$ by hydroxyurea, led us to test these cell lines for cloning efficiency in the presence of low levels of cytotoxic drugs known to accelerate the loss of unstably amplified *dhfr* genes from MTX-resistant mouse cell populations.

When R500 cells were compared to an NIH3T3 cell population for cloning efficiency in 50 μM hydroxyurea, there was only a small difference in reduction of cloning efficiency (40% reduction for R500 and 30% reduction for NIH3T3 when plated at 3 × 103 cells/plate in 50 μM hydroxyurea). While this might suggest that gene amplification has little effect on sensitivity to cytotoxic agents, the interpretation of such a result is complex. The R500 line is highly heterogeneous with respect to numbers of DM per cell,[22] and numbers of *dhfr* genes per cell. These cells are also likely to show clonal variation in a number of characteristics not associated with *dhfr* gene amplification. NIH3T3 cells also become heterogeneous during growth in culture, and this heterogeneity might include sensitivity to cytotoxic agents. The R500 line was isolated from NIH3T3 cells approximately 10 years ago,[16] and it is possible that substantial population drift has taken place over the years. Thus, a more rigorous way of conducting the experiment would involve comparison of a large number of clonal isolates of NIH3T3 cells with a large number of clonal isolates of R500 cells for cloning efficiency in low level cytotoxic drugs. Our feeling is that a comparison of at least 100 such isolates of each line would be required for such a study.

As an alternative, we compared the sorted $R500^+$ and $R500^-$ populations for cloning efficiency under a variety of cytotoxic conditions. Any variations in the R500 population not associated with *dhfr* gene amplification should be distributed randomly between the two subpopulations during the flow sorting.

As shown in Figure 3, the cloning efficiency of the $R500^-$ and $R500^+$ populations was the same in the absence of any drugs. In the presence of 250 μM MTX, the $R500^-$ population showed a slightly reduced cloning efficiency relative to the $R500^+$ population. In low levels of hydroxyurea and camptothecin, the situation was dramatically

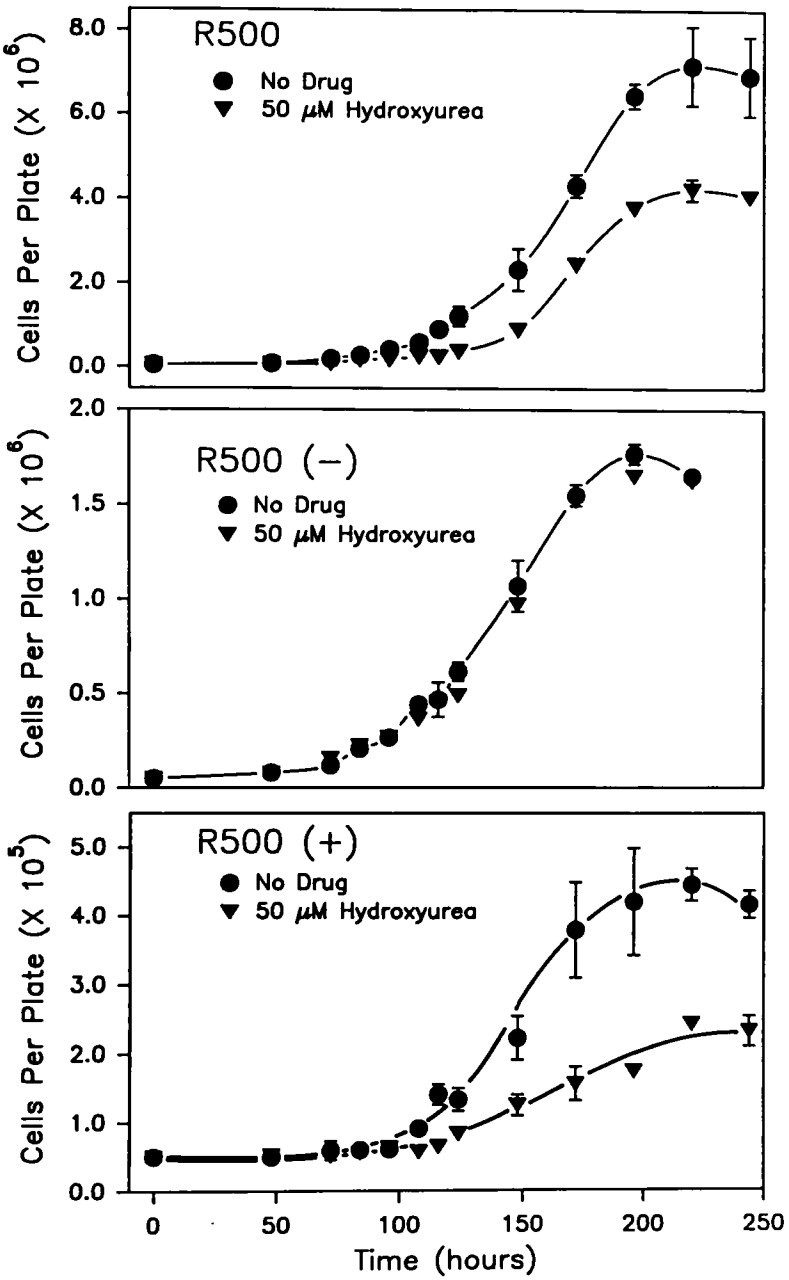

Figure 2. Growth of R500 cells and subpopulations with lower ($R500^-$) and higher ($R500^+$) average *dhfr* gene dosage in 50 μM hydroxyurea.

reversed. Both of these drugs reduced the cloning efficiency of $R500^+$ cells to a much greater extent than that of the $R500^-$ cells. For both cell populations and both drugs, the reduction in cloning efficiency was concentration dependent.

The effect of these drugs in combination with MTX was also examined in cloning efficiency experiments (Figure 4). Even in the presence of 250 μM MTX, the $R500^+$ cells are much more sensitive to low level cytotoxic stress. Thus, cytotoxic stress can act selectively against cells with the highest levels of *dhfr* gene amplification even in the presence of the selecting drug, MTX.

Discussion

Two R500 cell subpopulations with different average *dhfr* gene copy numbers were found to differ significantly in their sensitivity to low level

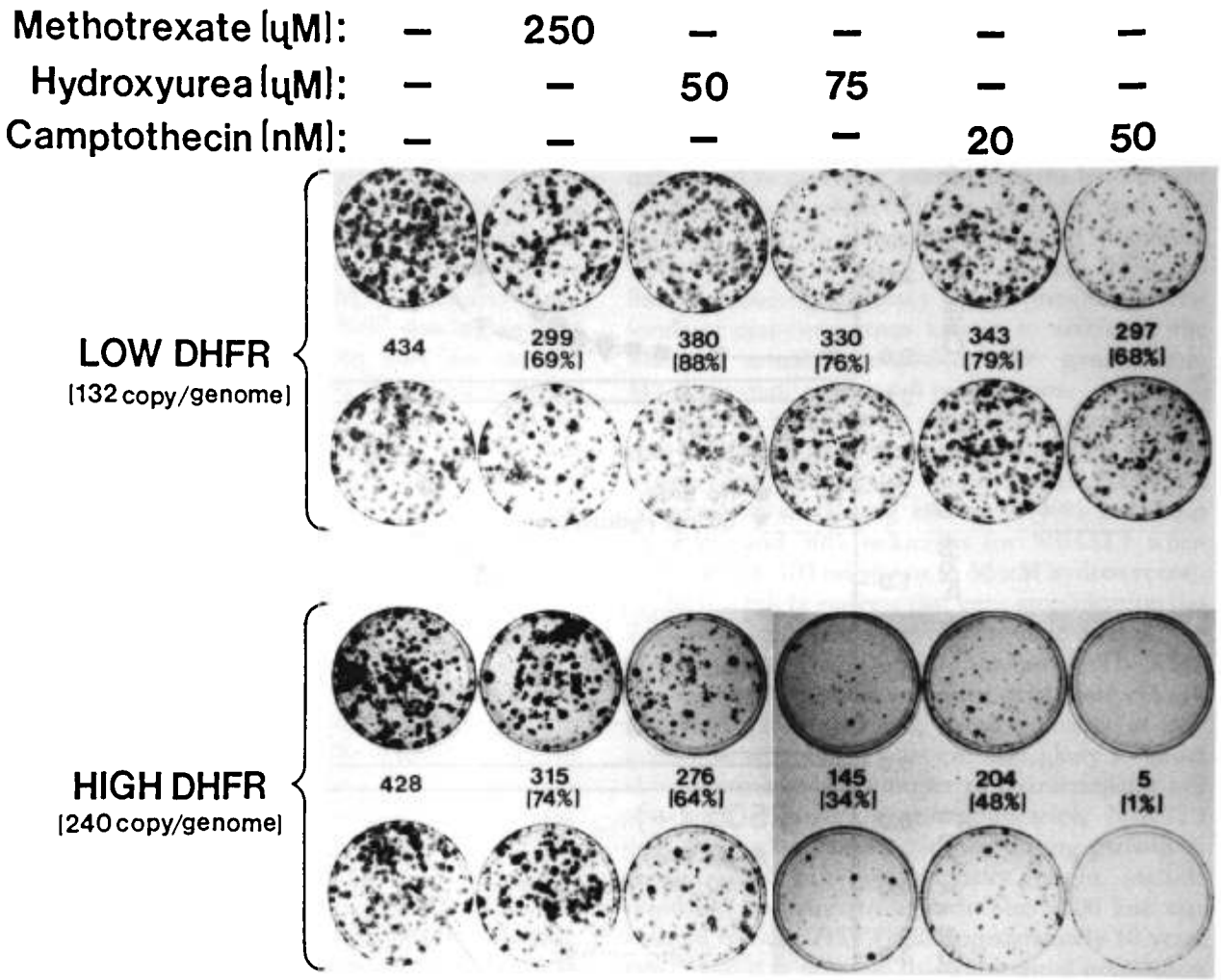

Figure 3. Cloning efficiency of the R500$^+$ and R500$^-$ subpopulations in media containing MTX, hydroxyurea or camptothecin. For each pair of duplicate plates, the average number of colonies is shown with the percentage of the 'no drug' control being given in parentheses.

cytotoxic stress as judged by cloning efficiency and growth. These findings suggest that within a population of cells with unstably amplified genes, there may be differences in sensitivity to cytotoxic stress which are associated with the level of gene amplification. This difference in sensitivity to stressors is likely to be a key factor in drug-induced loss of unstably amplified genes. The differential sensitivity was also seen in the presence of MTX.

Low level cytotoxic stress does not accelerate loss of stably amplified genes.[25] This is predictable if the induced gene loss effect is based on differences in sensitivity to stressors that are correlated with the amplified gene dosages within the population. Since stably amplified genes are integrated into cellular chromosomes, they are evenly partitioned between daughter cells at mitosis. In such a population, the distribution of amplified gene copy number is very narrow relative to that seen in a cell population with unstably amplified genes.[17]

We would like to note that the cell sorting procedure used in this study is itself stressful, and thus the subpopulations obtained, while differing in *dhfr* gene copy number, may not completely represent the corresponding subpopulations of the parental R500 population. That this may be so is suggested by the observation that both sorted populations have maximum cell densities significantly lower than that of the unsorted population. Several observations suggest that the lowered maximum cell density may be related to *dhfr* gene amplification and the ability of stressors to accelerate loss of amplified genes in populations of

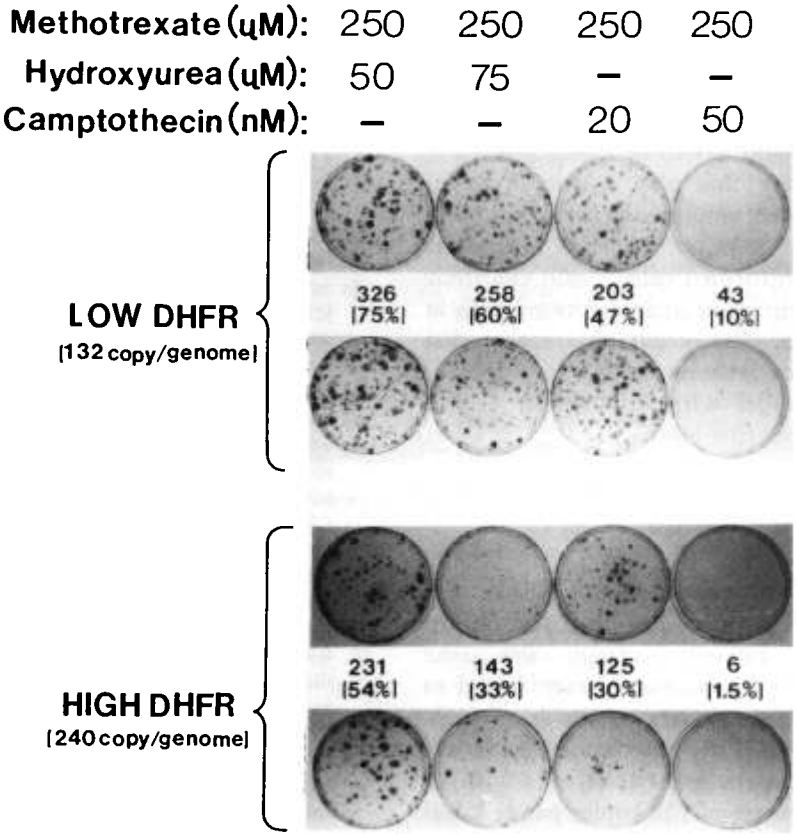

Figure 4. Cloning efficiency of the R500$^+$ and R500$^-$ subpopulations in media containing MTX with either hydroxyurea or camptothecin. Average number of colonies for each pair of duplicate plates is shown, with the percentage of the 'no drug' control (see Figure 1) being given in parentheses.

unstably MTX-resistant cells. First, the slowing of growth by 50 μM hydroxyurea for R500 cells[25] and for R500$^+$ cells (this report) appears to be mainly due to lowered maximum cell density and early contact inhibition. Second, the maximum cell density of the R500$^+$ population is much lower than that of the R500$^-$ population. Third, forward blue light scattering during flow cytometry (proportional to cell size) is greater in the R500$^+$ population than in the R500$^-$ population (data not shown).

Normal loss of unstably amplified genes from drug-resistant cell populations has been studied by flow cytometry and by *in situ* hybridization.[20,32,33] These studies all show that loss of unstably amplified genes is very heterogeneous as expected for a process that is based on the random distribution of DM at each mitosis. Since the cells with fewer DM have a replication advantage, they quickly take over the population.[20] However, cells losing unstably amplified genes in the absence of the selecting drug tend to generate a subpopulation with an amplification level significantly higher than any seen in the original resistant population.[20,32] Although the population as a whole is losing gene copy number, these cells undergo a transient gain in gene copy number. If these highly amplified cells were selectively killed or inhibited, the rate of gene loss for the population would be sharply accelerated. Our data suggest that within a heterogeneous population of cells with unstably amplified genes, those cells with the highest gene dosage may be selectively targeted by low level cytotoxic stress.

This selective sensitivity might be due to the total stress level experienced by the cells. Stressors typically slow cell growth and increase cell size.[34,35] It may be that maintenance of amplified genes is stressful to cells. The observation that gene amplification is inversely related to cell growth rate[20] is consistent with this idea, as is the observation that R500$^+$ cells appear to be larger

than R500$^-$ cells. The additional stress from low concentrations of cytotoxic drugs might raise those cells with the highest levels of gene amplification above a threshold beyond which survival or cell division would be compromised.

Differentiation may also play a role in drug-induced loss of unstably amplified genes. The ability to amplify genes appears to be a unique characteristic of transformed cells,[1,2] and cytotoxic drugs induce differentiation in transformed cells at low concentrations.[36] The recent observation that human promyelocytic leukemia cells lose amplified c-*myc* genes in parallel with differentiation into granulocytes supports this model.[37]

Conclusion

A highly MTX-resistant mouse cell population with unstably amplified *dhfr* genes has been sorted into subpopulations of different average *dhfr* gene dosage. These two subpopulations were found to differ significantly in sensitivity to low level cytotoxic drug treatments. Fifty micromolar hydroxyurea had no effect on the growth of cells with an average *dhfr* gene dosage of 132 copies per cell, but strongly suppressed the growth of cells with an average *dhfr* gene dosage of 240 copies per cell. As in the case of the unsorted population, this growth inhibition by hydroxyurea appeared to be mainly due to increased cell size and lowered cell density at confluence. Low level cytotoxic stress also decreased cloning efficiency to a much greater extent in the high gene dosage subpopulation than in the low gene dosage subpopulation. Selective action by cytotoxic drugs against cells with high levels of *dhfr* gene amplification is the probable basis for the phenomenon of drug-induced loss of unstably amplified genes.

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