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DNA amplification is rare in normal human cells

[drug resistance/methotrexate/*N*-(phosphonacetyl)-L-aspartate/hydroxyurea/cancer]

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ABSTRACT **Three types of normal human cells were selected in tissue culture with three drugs without observing a single amplification event from a total of 5×10^8 cells. No drug-resistant colonies were observed when normal foreskin keratinocytes were selected with *N*-(phosphonacetyl)-L-aspartate or with hydroxyurea or when normal mammary epithelial cells were selected with methotrexate. Some slightly resistant colonies with limited potential for growth were obtained when normal diploid fibroblast cells derived from fetal lung were selected with methotrexate or hydroxyurea but careful copy-number analysis of the dihydrofolate reductase and ribonucleotide reductase genes revealed no evidence of amplification. The rarity of DNA amplification in normal human cells contrasts strongly with the situation in tumors and in established cell lines, where amplification of oncogenes and of genes mediating drug resistance is frequent. The results suggest that tumors and cell lines have acquired the abnormal ability to amplify DNA with high frequency.**

Amplification of many cellular oncogenes is observed commonly in a wide variety of human tumors, and the degree of amplification is often highest in the most malignant tumors (1–4). Although amplification of genes mediating drug resistance has been documented in only a few human tumors (for citations, see refs. 3 and 5), DNA amplification is usually responsible for the high frequency of resistance commonly observed upon *in vitro* selection of many mammalian cell lines with many drugs (for reviews, see refs. 3 and 6).

In contrast to frequent DNA amplification in tumors and cell lines, there are very few reports of amplification in normal human cells and one suspects that such events are quite rare. The genetic instability readily observed in karyotypes of tumor cells and cell lines has not been seen in normal cells (for a discussion and references, see ref. 7) and there is a good correlation between genetic instability and the ability to amplify DNA (8, 9). Furthermore, amplification has not been reported in normal cells of patients undergoing therapy with cytotoxic drugs. For example, many patients have been treated with methotrexate (MTX), a drug that selects for amplification of the dihydrofolate reductase (DHFR) gene in human cell lines and also for other mechanisms of resistance such as impaired drug transport (5, 10, 11). The dose of MTX is limited by its toxicity to bone marrow. Even though some patients have been treated for more than 1 year, we know of no example in which normal bone marrow stem cells have become resistant to MTX. In a recent study, 38 children with acute lymphoblastoid leukemia were treated with MTX plus other drugs for long periods of time. In one child, analysis of resistant leukemic cells showed a 2.5-fold amplification of the DHFR gene but, after remission due to renewed therapy without MTX, there was no DHFR gene amplification in the morphologically normal bone marrow (5). Analysis of several additional remission marrows has also failed to uncover evidence of DHFR gene amplification (Dietrich Niethammer, University of Tubingen, Federal Republic of Germany, personal communication).

We know of three reports of DNA amplification in normal human cells. Srivastava *et al.* (12) found that the *HRAS* gene became amplified up to 4-fold in normal human fibroblasts during senescence in culture. However, more recently (13) only a 2-fold variation in *HRAS* copy number was found in five individual clones and this number did not change during growth. These workers now conclude that succession of clones with high *RAS* copy number occurs during serial passage. In an independent experiment, we failed to observe any difference in *HRAS* gene copy number when a strain of normal senescing fibroblasts was analyzed at passages 23 and 74 (see *Results*).

Turner *et al.* (14) studied preexisting mutations in the hypoxanthine guanine phosphoribosyltransferase gene in normal human lymphocytes selected with 6-thioguanine immediately after isolation. Some of the events that inactivated this gene were accompanied by amplification, as also observed by Nalbantoglu *et al.* (15) for the adenine phosphoribosyltransferase gene in Chinese hamster cells. The probability of DNA amplification in lymphocytes, in which an unusual mechanism for genome rearrangement has been activated, may be different from the probability in most other cell types. Prody *et al.* (16) found that a cholinesterase gene was highly amplified in a farmer and his son, members of a family exposed to organophosphorus insecticides for several generations. The initial amplification event probably occurred and was selected for during spermatogenesis or oogenesis and was then inherited stably. Again, the probability of amplification in germ-line cells may not be typical of most somatic cells.

We know of no case in which amplification has been demonstrated in drug-resistant normal human cells after selection *in vitro*. Amplifications might have been missed previously because selection of normal human cells with cytotoxic drugs *in vitro* is quite difficult. For example, many cell strains have a low plating efficiency at low cell density, the life span of normal cells in culture is limited, and normal cells have the ability to survive exposure to cytotoxic drugs by entering G_0 arrest. We have paid careful attention to considerations such as these.

MATERIALS AND METHODS

Mammary Epithelial Cells. Pure preparations of epithelial organoids were made from reduction mammoplasty speci-

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Abbreviations: PALA, *N*-(phosphonacetyl)-L-aspartate; MTX, methotrexate; HU, hydroxyurea; DHFR, dihydrofolate reductase; DR_{50}, drug concentration that inhibited growth by 50%.

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mens as described by Stampfer *et al.* (17) and Smith *et al.* (18). In MCDB170 medium, epithelial cells migrate from organoid clumps onto tissue culture plates, where they proliferate. After 1 week, large areas of growing cells surround each clump. Upon brief trypsin treatment, only the proliferating cells are removed. For a second passage, suspensions of single cells were plated in modified MCDB170 medium (see *Results*). To determine the dose–response curve, various concentrations of MTX were added 1 day later and cells were selected at 4 × LD_{50} (Table 1). Experiments were also done with selected extended life cultures (see *Results*), for which the LD_{50} was determined independently. Cell line L578T was derived from a primary breast carcinosarcoma; the donor had no prior chemotherapy (19). At passage 24 in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) dialyzed fetal calf serum, the LD_{50} was 10 nM for MTX and the LD_{90} was 30 nM.

Human Keratinocytes. Newborn human foreskin keratinocytes (strains a, c, e, and k; passage numbers 3–8) were used. Such cells usually have a replicative lifespan of 80–100 cell generations prior to senescence (20). Stock cultures were grown in the presence of a feeder layer of mitomycin C-treated 3T3 cells (21, 22), in a 1:3 (vol/vol) mixture of Ham's F12 medium and DMEM containing 1.8×10^{-4} M adenine, 10% fetal calf serum, hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), 10^{-10} M cholera toxin, and epidermal growth factor (10 ng/ml; prepared by C. George-Nascimento and generously donated by Chiron, Emeryville, CA). Experimental cultures were plated without feeder layers in a serum-free medium supplemented with bovine pituitary extract, epidermal growth factor (10 ng/ml), insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml) (complete KGM; Clonetics, San Diego; ref. 23). The concentration of *N*-phosphonacetyl)-L-aspartate (PALA) that inhibited growth by 50% (DR_{50}) in the 3-day assay described by Kempe *et al.* (24) was 22 μM. During selection in PALA, the cultures were fed three times a week for the first 2 weeks and once a week thereafter. The DR_{50} for hydroxyurea (HU), 0.07 mM, was estimated by a modification of the same method.

Human Diploid Fibroblasts. Strain F, obtained from an explant of newborn foreskin tissue, was used at passage 9. Strain L (HSC 172) was obtained from the lung tissue of a 12-week-old female fetus (25, 26). Strain L has a long lifespan *in vitro* of ≈95 population doublings and a normal karyotype (25, 26). The cells were obtained at passage 4 from J. E. Dick (University of Toronto). Both strains were grown in DMEM with 15% fetal calf serum. The sensitivity of these cells to growth inhibition by HU or MTX was determined by the method of Kempe *et al.* (24) but with a growth period of 5 days. Strains F and L both had DR_{50} values for HU of about 0.15 mM and strain L had a DR_{50} for MTX of about 20 nM.

RESULTS

Selection of Normal Mammary Epithelial Cells with MTX. To evaluate MTX resistance one needs a culture medium that supports clonal growth and also allows the toxicity of MTX to be manifest. A well-established system for normal mammary epithelial cells using MM medium (17, 18) is inappropriate because it requires fibroblast feeder cells for efficient clonal growth and indirect effects, such as MTX-induced inhibition of the feeding capacity of the fibroblasts, may be a problem. A serum-free medium that supports efficient clonal growth without feeder cells, MCDB170 (27), contains folinic acid, so that the level of reduced folate is not rate-limiting for growth. We reformulated this medium. Thymidine, adenine, or folinic acid were not required for clonal growth but glycine was. Medium with the normal amount of glycine alone, 10^{-4} M, supported clonal growth equivalently to complete MCDB170 but gave a high (25%) relative plating efficiency in 500 nM MTX. With 10^{-5} M glycine and 10^{-5} M folic acid (instead of folinic acid), we obtained a plating efficiency equivalent to that of MCDB170 medium (range 3–26% with cell isolates from different individuals) and also good toxicity with 500 nM MTX.

Some experiments were done with second-passage cells that have been through fewer than six population doublings and are most similar to normal mammary epithelium *in vivo*. No resistant cells were obtained when 5×10^6 such cells were placed into selection (Table 1, specimen 998EL). When MTX was removed after 4 weeks, the cells in one flask regrew in normal medium but still did not give resistant colonies upon reselection in 800 nM MTX, the concentration of drug used originally.

Human mammary epithelial cells can also grow extensively in culture (27). When organoids are grown in MCDB170 medium, the epithelial cells can be passaged for 10–15 population doublings, when most cells senesce. However, a small epithelial subpopulation continues to grow and overtakes the culture. These selected extended-life cultures, which can grow for 30–60 more population doublings before senescence, offer the opportunity to study MTX resistance in normal epithelial populations. Selections were performed with 150 nM MTX, 3 times the LD_{50}. Specimens from two individuals were tested, with no colonies resulting from either one, by using 2.7×10^7 cells in total (Table 1). MTX was cytostatic, not cytotoxic, for some cells since, after 4 weeks, many flasks contained cells that did regrow in normal medium. However, none of these cells gave colonies upon reselection with 150 nM MTX and their sensitivity to growth inhibition by MTX was, if anything, greater than that of the original cells (Table 1).

Table 1. Selection of normal human mammary epithelial cells with MTX

Specimen	Type of culture	Results of initial selection	Growth in normal medium	Results of reselection
998EL	Passage 2	$0/5.4 \times 10^6$	1/7	$0/1.5 \times 10^5$
437E	Extended life	$0/1.4 \times 10^7$	7/7	$0/24 \times 10^5$
184E	Extended life	$0/1.3 \times 10^7$	3/6	—

Specimen 998EL was from a 50-year-old donor; specimen 437E was from a 17-year-old donor; and specimen 184E was from a 21-year-old donor. Cells were seeded at $2–10 \times 10^3$ per cm^2. LD_{50} and LD_{90} are the MTX concentrations giving 50% or 90% decrease in colonies after 4 weeks, respectively. LD_{50} values were 200 nM for 998EL and 50 nM for 437E and 184E. LD_{90} values were 600 nM for 998EL, 90 nM for 437E, and 140 nM for 184E. For the initial selection, MTX concentrations were 3–4 times LD_{50}: 800 nM for 998EL and 150 nM for 437E and 184E. Results are expressed as the number of resistant colonies/initial number of cells. After 4 weeks, MTX was removed, the cells were cultured in normal MCDB170 medium, and the results are expressed as the number of flasks in which cells regrew/total number of flasks. Reselection was performed in the same concentrations of MTX and at the cell density used originally. LD_{50} values were 28 nM for 437E and 24 nM for 184E. LD_{90} values were 64 nM for 437E and 53 nM for 184E. Results are expressed as the number of resistant colonies/total number of cells retested.

We also examined the breast cancer cell line 578T under conditions similar to those used for the normal cells. Selections were performed at 30–38 nM MTX, 3–4 times the LD_{50}. Resistant colonies were obtained readily, at frequencies 5–40 $\times 10^{-5}$. Several clones from different dishes were analyzed to determine if the DHFR gene had been amplified (Fig. 1*A*). In no case was amplification found, using a method (28) that can detect a 2-fold increase in copy number. This result is not surprising since decreased drug transport and polyglutamation, and not DHFR gene amplification, are the predominant mechanisms of MTX resistance at low drug concentrations for a human mammary tumor cell line (10) and a human squamous cell carcinoma (11). Whatever the mechanism involved, the breast cancer line generates resistance to MTX 100–1000 times more frequently than normal human cells.

Attempts to Isolate PALA- or HU-Resistant Keratinocytes. Keratinocytes were seeded at 1–8 $\times 10^5$ cells per 25-cm^2 flask in complete KGM. Two days later, PALA was added to a final concentration of 55 μM (2.5 $\times$ $DR_{0.5}$) or 88 μM (4 $\times$ $DR_{0.5}$). Six experiments were carried out in which 8 $\times 10^7$ keratinocytes were plated initially. PALA-treated cells were maintained in culture with drug for 1, 1.5, 3, and 4 months, respectively, by using 2 $\times 10^7$ cells in each case. No cell growth was observed.

Flasks maintained with PALA for 11 weeks were transferred to PALA-free complete medium containing 1 mM uridine for 3–17 days, to allow restoration of pyrimidine nucleotide pools, and then to complete medium alone for 3–9 days. The cells were then harvested and replated onto 3T3 feeder cells in the medium used for stock cultures. No keratinocyte growth was observed in any PALA-treated culture, even when the drug was removed and the cells were replated on feeder cells.

Keratinocytes were exposed to HU concentrations that are normally cytotoxic to proliferating cells in culture (26, 32–34). Three separate cultures of 1.4, 2.8, and 2.8 $\times 10^6$ cells each were plated in 75-cm^2 plates in KGM medium and 0.2 mM HU (2.5 $\times$ $DR_{0.5}$) was added 6 hr later. Cells were maintained in drug for 1 week and fresh medium plus 0.2 mM HU was added. Two cultures were incubated for 5 days more, at which time there were many dead cells and also a partial monolayer of attached, dividing cells. The medium was removed and the attached cells were analyzed for gene copy number (Fig. 1*B*, sample K). No increase was observed. The remaining cells were placed in 0.2 mM HU for 30 days more and then transferred to the 24 wells of a multiwell dish. Several days later most of the cells were enlarged and apparently were not able to divide. No colonies were observed upon further incubation without drug. One of the original three cultures was incubated in 0.2 mM HU for 3 days and the cells were removed, washed, and replated in medium containing 0.3 mM HU. After 3 weeks more, many cells were dead and the rest formed a sparse monolayer of enlarged cells. The attached cells were removed, washed, and transferred to a fresh plate without drug. No colonies were observed after several weeks.

Attempts to Isolate HU- or MTX-Resistant Human Diploid Fibroblasts. Two cultures of cell strain F were exposed to increasing concentrations of HU, starting with 0.1 or 0.25 mM and ending with 0.5 mM (Table 2, experiments 1 and 2). After exposure to drug for 12 days, some small colonies were apparent. These were picked and subcultured, either without drug or in 0.5 mM HU. In every case, the cells grew poorly for 1 or 2 weeks and then stopped growing. A similar result was obtained when cell strain L was grown in the presence of HU concentrations from 0.25 to 1.0 mM over a period of 27 days (Table 2, experiment 4). Furthermore, a pool of colonies from experiments 1 and 2 did not grow appreciably after 10 days more in culture with 0.5 mM HU (Table 2, experiment 3). In this case, an analysis for gene copy number revealed no evidence of amplification (Fig. 1*B*).

Attempts were also made to obtain resistant cells by increasing the drug concentrations gradually as the cultures were split. In experiments 5 and 6 of Table 2, cells of strain L were grown in the presence of drug to a partial monolayer, split 1:4 several times, grown without drug to allow recovery, and then grown again in a higher drug concentration, again using 1:4 splits. Thus, the cells were maintained at a high density throughout an experiment in which there was considerable growth, and the total times of exposure to drug were

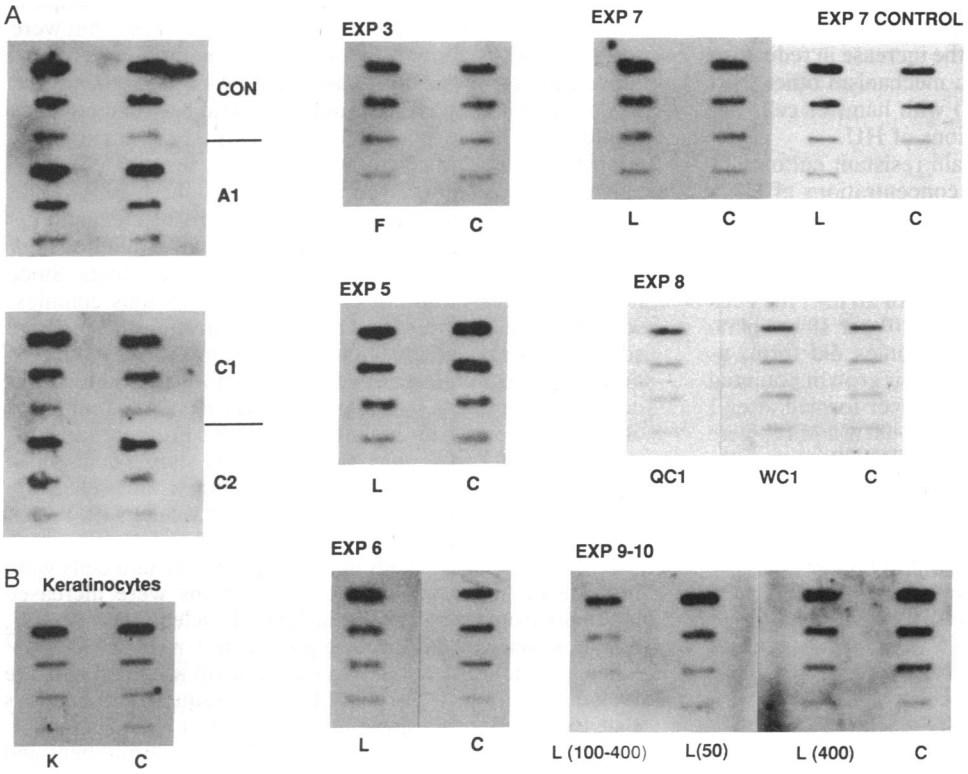

FIG. 1. Slot-blot analyses of relative gene copy numbers. Pellets of 5–20 $\times 10^4$ cells were analyzed (28). A 930-base-pair *Eco*RI–*Pst* I fragment without exons, derived from the 5′ end of the human DHFR gene (from Anna B. Hill, Arizona State University, Tempe) was used to avoid signals due to DHFR pseudogenes (29). The probe for ribonucleotide reductase was the *Pst* I fragment of cDNA clone 10, for the mouse M2 subunit (30). Although there are M2 pseudogenes in human DNA (31, 32), 2-fold amplification of the functional gene would give a 1.5-fold increase in signal in slot-blot analysis. (*A*) MTX-resistant clones from breast carcinosarcoma line L578T. Duplicate analyses of 1:3 dilutions are shown for controls (CON) and three clones (A1, C1, and C2). After normalization for loading (data not shown), there were no differences between the control and experimental samples. (*B*) HU and MTX-resistant normal cells. Experiment numbers correspond to Table 2. Analyses of 1:2 dilutions are shown for test (columns K, F, or L) and control (columns C) cells. Normalizations for loading are illustrated for experiment 7 (control).

Table 2. Selection of human diploid fibroblasts with HU or MTX

Experiment	Cells used, no. or source	Selection protocol	Result(s)
HU selection			
1	2×10^6	0.1 mM (1 day)/0.25 mM (7 days)/0.5 mM (4 days)	19 colonies*
2	2×10^6	0.25 mM (1 day)/0.5 mM (11 days)	14 colonies*
3	Cells from exp. 1 and 2	0.5 mM (10 days)	Many enlarged cells/no growth
4	2×10^6	0.25 mM (6 days)/0.5 mM (14 days)/1.0 mM (7 days)	24 colonies*
5†	4×10^6	0.2 mM/0.3 mM/0.5 mM (total, 30 days)	Partial monolayer
6†	2×10^6	0.1 mM/0.2 mM/0.3 mM/0.4 mM (total, 47 days)	Partial monolayer
7†	Cells from exp. 5	1.0 mM (14 days)	20 colonies
8‡	12×10^7	100 mM (3 days)	150 colonies/reductase elevated
MTX selection			
9†	8×10^6	80 nM/200 nM (total, 80 days)	After replating, 9 colonies* in 50 nM MTX and 4 colonies* in 100 nM MTX
10†	8×10^6	100 nM/400 nM (total, 80 days)	After replating, 1 colony* in 200 nM MTX and 1 colony* in 400 nM MTX

Cells of strain F (experiments 1–3) or strain L (experiments 4–10) were plated initially at 2×10^6 per 10-cm dish, and drug was added several hours later.

*Limited potential for growth (see text).

†Cells grown at a high density in the initial concentration of drug were split 1:4 several times in medium with drug, grown without drug, and then exposed to the next concentration of drug.

‡Selections were as described by Dick and Wright (26).

1 month or more. There was no increase in gene copy number in cells analyzed after the last selection steps of experiments 5 and 6 (Fig. 1*B*). An attempt was made to isolate clones after the last selection step of experiment 5 by culturing 3×10^4 cells in 1 mM HU for 14 days more (Table 2, experiment 7). About 20 colonies were apparent but, as before, none grew well in normal medium. Cells from the nine clones that grew best were pooled and analyzed for increased gene copy number (Fig. 1*B*) with negative results.

In previous work (26), resistant colonies of cell strain L had been selected by exposure to a high concentration of HU (100 mM) for a short time (about 3 days, Table 2, experiment 8). These cells were 2–3 times more resistant to HU than untreated cells and they also had 2–3 times more ribonucleotide reductase activity, alterations in deoxyribonucleotide pools, and reduced replicative potential. An analysis of clones QC1 and WC1 from this experiment revealed no increase in gene copy number (Fig. 1*B*), showing that the increase in reductase activity must have been caused by a mechanism other than DNA amplification, as observed (22) with hamster cells exhibiting resistance to low concentrations of HU.

An attempt was also made to obtain resistant cultures of strain L by gradually increasing the concentrations of MTX (Table 2, experiments 9 and 10), using a protocol very similar to the one used with HU in experiments 5 and 6. At the end of selections in which the cultures were exposed to concentrations of drug far higher than the $DR_{0.5}$ of 20 nM, the cells were replated at a low density of $1–2 \times 10^5$ per 10-cm plate in various concentrations of MTX. Colonies did form, as summarized in Table 2, but in all cases their growth potential was limited and in no case was a monolayer formed after 1 month. Cells from the six colonies in 100–400 nM MTX were pooled and analyzed for an increase in DHFR gene copy number, as were cells from the nine clones resistant to 50 nM drug, with negative results in both cases (Fig. 1*B*). The mixed population of cells from experiment 10 was grown in normal medium after the final exposure to 400 nM MTX and analyzed for DHFR gene copy number as soon as enough cells were available, again with a negative result (Fig. 1*B*).

In view of the results of Srivastava *et al.* (12) suggesting that the *HRAS* gene might be amplified during growth of fibroblasts in culture (see above), cells of strain L were analyzed for the copy number of *HRAS* after 23 and 74 passages, by using the method of McIntyre and Stark (28) in which a Riboprobe representing total genomic DNA is used to normalize each signal. By using the same *HRAS* probe employed by Srivastava *et al.* (12), we obtained normalized densitometer readings for passage 23 and passage 74 cells that were not significantly different, within 20% in duplicate experiments.

DISCUSSION

We failed to observe a single instance of DNA amplification in experiments involving more than 5×10^8 normal human cells selected with three drugs known to select for amplification in permanent cell lines. We thus conclude that the frequency of amplification, as revealed by selection with cytotoxic drugs in cell culture, is very much lower in normal human cells than in human cell lines. Since the result is a negative one, we must consider the possibility that amplifications do exist at a frequency greater than 2×10^{-9} but were not revealed because our selection conditions were not entirely appropriate. In all cases, care was taken to use cell strains at an early passage number, so that resistant colonies would have ample potential for growth. For example, all populations of L fibroblast cells were at less than 30 doublings after selection. Even after cloning, these cells would have still been at less than 40 doublings. Unselected cultures grow vigorously at this stage, since this cell strain does not senesce until after more than 90 population doublings. Since normal fibroblasts do not pile up to give obvious colonies, selections were carried out at cell concentrations low enough so that colonies would have room to grow and could be detected. Furthermore, several attempts were made to recover resistant cells whose growth may have been inhibited at high cell density in the initial selection. For keratinocytes selected with PALA, any resistant cells should have been able to grow even if the surrounding nonresistant cells were arrested but not dead, especially after 11 weeks with PALA and 3 weeks without drug.

In the experiments with fibroblasts, no resistant cells were found even when the drug concentrations were increased gradually from a very low initial level. In selections with HU, colonies were present at a frequency of 4×10^{-6} (6×10^{-7} when only colonies exhibiting slow growth after isolation are included). In selections with MTX the frequency of colonies that exhibited some growth after isolation was 9×10^{-7}. These frequencies are relative to starting cell numbers and

thus are overestimates in selections involving long-term exposure of growing cells to low drug concentrations (Table 2, experiments 5, 6, 9, and 10). In no case was a clone obtained that would grow to more than 10^4 to 10^5 cells. It is possible that the lifespan of the cells was reduced after drug selection, as observed in another study (26). Drugs such as HU may damage DNA, affecting the proliferative capacity of normal cells in a general way. Nevertheless, in many cases enough cells could be obtained to carry out an analysis for gene copy number, with no amplification detected.

Although most previous experiments involving selection of resistant cells with cytotoxic drugs in culture have been done with rodent cell lines, there has been ample success with human cell lines as well, and the frequencies observed, 10^{-4} to 10^{-6}, are roughly similar in cells of different species. Human cell lines have been selected for DHFR gene amplification with MTX (35–39), for amplification of the gene encoding the M2 subunit of ribonucleotide reductase with HU (32), for amplification of the *MDR* gene, encoding the P-glycoprotein responsible for multidrug resistance, with various drugs (40–44), for amplification of the gene encoding the α-subunit of the Na, K-ATPase with oubain (45, 46), for amplification of an adenine deaminase gene with adenine nucleosides (47), and for amplification of the UMP synthase gene with pyrazofurin (48). Conversely, human cell strains can be selected with cytotoxic drugs in culture to give resistant mutants in cases where amplification is not involved. Some examples are selection of fibroblasts with α-amanitin (25), ouabain (49, 50), azaguanine (51), 6-thioguanine (50), and diphtheria toxin (52) and selection of keratinocytes with 6-thioguanine (53).

Since amplification is rare in normal cells and common in tumors and cell lines, what steps in the origin or progression of tumors or in the genesis of cell lines allow amplification to occur at high frequency? The frequency of amplification has been shown to correlate with degree of transformation or tumorigenic potential in two cases (7, 54) but not in one other (55). The propensity to amplify DNA with high frequency may be linked somehow to immortalization of cells and may increase with increasing degree of transformation. We have used simultaneous selection with two drugs to obtain stable cell variants, called amplificator cells, which have an increased ability to amplify DNA (56). Thus, cells with three rates of amplification can be distinguished: normal cells (very low), cell lines (moderate), and amplificator cells (high). The main challenge for the future is to identify the genes whose abnormal expression is responsible for DNA amplification in cell lines and tumors.

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