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Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (adriamycin) resistance

(dihydrofolate reductase/P-glycoprotein/hypoxia/gene amplification/cancer chemotherapy)

GLENN C. RICE*†, VICTOR LING‡, AND ROBERT T. SCHIMKE§¶

*Cancer Biology Research Laboratory, Stanford University, Stanford, CA 94305; ‡The Ontario Cancer Research Institute, Toronto, ON, Canada M4X 1K9; and §Department of Biological Sciences, Stanford University, Stanford, CA 94305

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ABSTRACT **We have determined the frequency with which Chinese hamster cells become resistant to either methotrexate or doxorubicin (former generic name, adriamycin) alone or to the two drugs simultaneously. We find that the frequency of acquisition of simultaneous resistance is 10–100 times higher than that predicted from the frequency of each resistance selected independently. In ≈50% of cloned resistant variants, resistance is the result of amplification of the dihydrofolate reductase gene (methotrexate) and/or of the multiple-drug-resistance P-glycoprotein gene (doxorubicin). Prior exposure of cells to hypoxia markedly enhances these resistance frequencies. Our results indicate that the simultaneous emergence of resistance to these two cancer chemotherapeutic agents are not independent events, and we interpret them to constitute two consequences of the same basic process occurring at a high frequency.**

A standard textbook presentation of mutational events and the acquisition of drug resistances states that the selection occurs only on preexisting mutants—i.e., the selecting agent is generally not considered "mutagenic" *per se*. In addition, when cells are simultaneously selected for resistance to two different agents whose cytotoxic mechanisms differ, it is generally considered that "mutational events" leading to resistances are independent events; hence, resistance to two agents simultaneously should be the product of the two independent resistance frequencies. Yet it is well known that the spontaneous frequency of resistance to cancer chemotherapeutic agents in tumors can be extremely high and, further, that simultaneous resistances to a variety of cancer chemotherapeutic agents can develop rapidly (1–3).

We, as well as others, have documented in cultured somatic cells a number of instances in which drug resistance results from amplification of specific genes (see refs. 4 and 5 for reviews). In addition, clinical resistance to methotrexate can be associated with gene amplification (6–9). Schimke *et al.* (10) have proposed that amplification occurs as a result of perturbation of DNA replication patterns in S-phase cells, leading to overreplication of DNA in a single cell cycle and, secondarily, to a variety of possible recombinational events that can result in gene amplification and other mutational events. During recovery of DNA synthesis following a variety of perturbing conditions, including exposure of cells to inhibitors of DNA synthesis, to the carcinogen *N*-acetoxy-2-acetylaminofluorene, to UV irradiation, or to transient hypoxia (11–16), a significant proportion of a cell population will have >4*C* DNA content (where *C* is the content of the haploid genome). From this subset of original S-phase cells are derived the cells with increased frequency of methotrexate resistance (14, 16). This suggests that the original event (i.e., DNA overreplication) may involve significant portions of the cellular genome, and hence such "extra" DNA can constitute a substratum for the selection of multiple amplified genes (and drug resistances) in the same cells.

In the study described here, we analyzed the frequencies of resistance to methotrexate and to doxorubicin (former generic name, adriamycin) independently and simultaneously. In addition, we subjected cells to transient hypoxia, a treatment that Rice *et al.* (14) have shown generates marked DNA overreplication and enhances the frequency of methotrexate resistance and dihydrofolate reductase gene amplification. Our results indicate that the emergence of simultaneous resistance is not the result of independent events.

MATERIALS AND METHODS

Cell Culture. All experiments were performed essentially as described previously (14). As few as 200 or as many as 10^5 Chinese hamster ovary (CHO) fibroblast [AA8 subclone (17)] were plated in 60-mm^2 dishes so that the number of viable colonies 2–3 weeks later was 20–500 per dish. Density effects were carefully tested by plating various densities of cells for each drug dose and assessing colony number 2–3 weeks later. All colony counts were normalized to the plating efficiencies of the nontreated culture, either with or without prior hypoxic pretreatment (plating efficiencies were 60–80% for normal cells and 30–50% for cells pretreated with hypoxia). To generate the hypoxic state, 1–2 × 10^6 cells were plated in 60-mm^2 Permanox (Lux Scientific) Petri dishes. After a 2-hr attachment period at 37°C, the medium was removed and 2 ml of fresh medium was added. The plates were placed in specially designed airtight nylon holders, sealed, and gassed for 1 hr at 25°C with certified 95% N_2/5% CO_2. Measured O_2 levels of the effluent gas after 1 hr were less than 5 ppm. After flushing, the nylon holders were sealed and incubated at 37°C. Twenty-four hours later, the plates were removed and the cells were trypsinized, counted, and plated directly into drug-containing medium.

Measurement of Gene Copy Number. Drug-resistant colonies were cloned by use of cloning rings and expanded in drug-containing medium. DNA was isolated, and gene copy number was measured by the slot-blot procedure of Brown *et al.* (11) as modified by Rice *et al.* (14). Plasmid pB13-7 (18) was used as a hybridization probe for the dihydrofolate reductase gene (*DHFR*). Plasmid pCHP1 (19) was used to probe for *MDR1*, the multidrug-resistance gene encoding

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†Current address: Department of Molecular Immunology, Genentech, Inc., 460 Pt. San Bruno Boulevard, South San Francisco, CA 94080.
¶To whom reprint requests should be addressed.

P-glycoprotein. An α-fetoprotein cDNA probe provided a control for the amount of DNA bound to the nitrocellulose filters. In addition, random clones were probed in an analogous manner by blot hybridization of electrophoretically separated restriction fragments (Southern blot analysis), using *Eco*RI digests for *DHFR* and *Hin*dIII digests for *MDR1*, and similar results were obtained. Blots were autoradiographed and hybridization signals were integrated by scanning the autoradiographs with an E-C densitometer (E-C Apparatus, St. Petersburg, FL), taking care to be within the linear range of the film. All hybridizations were done at least in duplicate and the results were averaged. The data shown in Table 2 are derived from the averaged slot-blot data only.

RESULTS

Figs. 1 and 2 show the results of two experiments in which cells were plated in medium containing doxorubicin, methotrexate, or both drugs, with or without a 24-hr period of hypoxia prior to drug selection. We provide the results of two independent and internally complete experiments (Figs. 1 and 2) to indicate the resistance frequencies obtained and their variability from experiment to experiment. The same general conclusions can be drawn from these two experiments, as well as from four additional experiments (data not shown). For the purpose of presentation, we also provide the results of Fig. 1 in tabular form (Table 1). In this table the values in parentheses indicate the expected frequencies as calculated from the values of each separate selection frequency. We make three general conclusions. (*i*) A 24-hr period of hypoxia prior to placement of cells under drug selection increases the frequency of both methotrexate and doxorubicin resistance ≈10-fold. These results extend to doxorubicin the enhancement of drug resistance by hypoxia pretreatment reported previously for methotrexate (14). (*ii*) In all instances studied, the frequency with which cells are resistant simultaneously to doxorubicin and methotrexate is 10–100 times greater than that predicted from the frequencies calculated from single-drug selection frequencies (Table 1, values in parentheses). This disparity is also observed when the frequency of resistance is enhanced by prior treatment with hypoxia. In the experiment of Fig. 1 (Table 1), the observed frequency (2×10^{-3}) is 10 times that predicted from the single-drug selection frequencies of cells subjected to

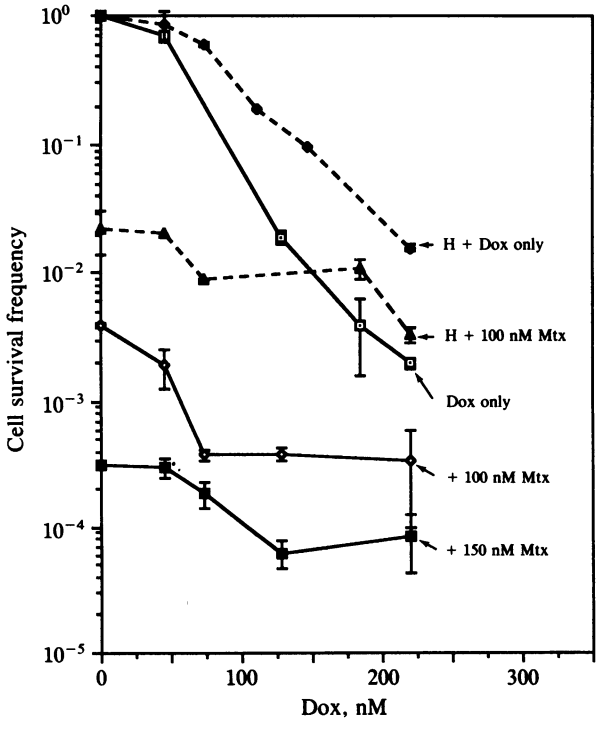

FIG. 1. Survival frequency of Chinese hamster ovary AA8 cells (8) selected with doxorubicin (Dox) alone or plus methotrexate (Mtx), with or without a 24-hr hypoxia (H) pretreatment. Bars represent standard deviations of the means derived from at least triplicate plates per point.

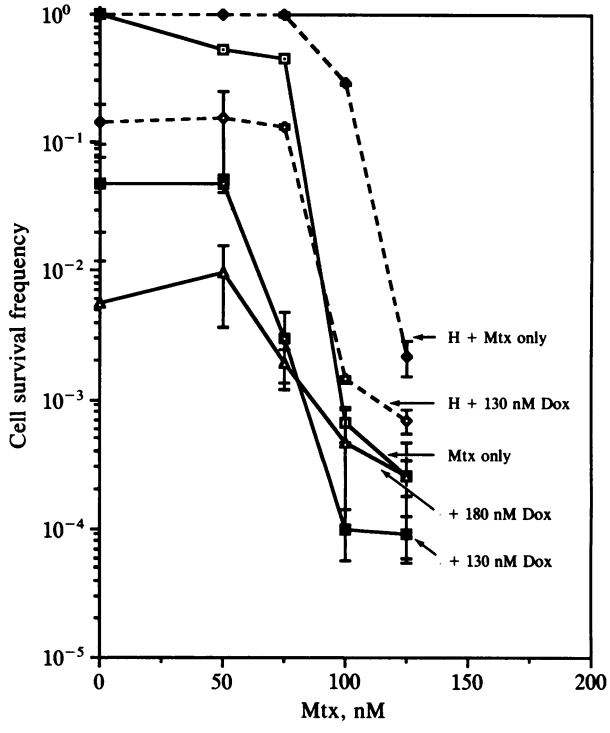

FIG. 2. Clonal survival of Chinese hamster ovary AA8 cells in various concentrations of doxorubicin (Dox) and/or methotrexate (Mtx), with or without a prior 24-hr pretreatment with hypoxia (H). This experiment was performed essentially as that of Fig. 1, except that the concentration of methotrexate rather than doxorubicin was varied.

Table 1. Frequencies of resistance to methotrexate and doxorubicin in AA8 cells subjected to one- or two-drug selection, with or without hypoxic pretreatment

Drug(s)	Cell survival frequency	
	No hypoxia	Hypoxia
Methotrexate		
100 nM	4×10^{-3}	2×10^{-2}
150 nM	3×10^{-4}	—
Doxorubicin		
120 nM	2×10^{-2}	1×10^{-1}
230 nM	2×10^{-3}	1×10^{-2}
Methotrexate plus doxorubicin		
100 nM + 120 nM	4×10^{-4} (8×10^{-5})	—
150 nM + 120 nM	6×10^{-5} (6×10^{-6})	—
100 nM + 230 nM	4×10^{-4} (8×10^{-6})	2×10^{-3} (2×10^{-4})
150 nM + 230 nM	8×10^{-5} (6×10^{-7})	—

Data are from the experiment of Fig. 1. Values in parentheses are the expected frequencies in two-drug selections, calculated from the frequencies in one-drug selections.

hypoxia [$(2 \times 10^{-2}) \times (1 \times 10^{-2}) = 2 \times 10^{-4}$] and 250 times greater than that predicted from the independent frequencies of cells that were not subjected to hypoxia treatment (8×10^{-6}). (*iii*) The disparity between observed and predicted dual resistance frequencies is usually greater at higher drug concentrations. This disparity is minimal in the range of 10% survival of cells (data not shown) and increases with the severity of selection [higher doxorubicin concentration; in Table 1, this disparity is ≤10-fold at low selection conditions (100 nM or 150 nM methotrexate plus 120 nM doxorubicin) but 50- or 130-fold at higher selection conditions (100 nM or 150 nM methotrexate plus 230 nM doxorubicin)]. This pattern was observed in all experiments (data can be calculated from Fig. 2). The same type of result was observed in dual selections for resistance to methotrexate and actinomycin D (H. Diddens, personal communication). Guilotto *et al.* (20) recently reported studies on independent and dual selection for resistance to methotrexate and *N*-phosphonoacetyl-L-aspartate (PALA), an inhibitor of the CAD protein (which constitutes the first three enzymes of *de novo* pyrimidine synthesis), in baby hamster kidney cells and also concluded that the frequency of dual selection (and independent amplification events) occurs at a higher frequency than predicted from the frequency of single resistances.

Table 2 summarizes slot-blot analyses of cloned cells resistant independently or simultaneously to methotrexate and doxorubicin for amplification of the dihydrofolate reductase gene (*DHFR*) and/or the P-glycoprotein gene (*MDR1*). As described by Brown *et al.* (11) and employed in various studies, this technique compares the hybridization of the specific gene relative to the α-fetoprotein gene. We judge cell variants to contain amplified genes when the relative hybridization (compared to sensitive-cell DNA) is greater than 1.5. For methotrexate resistance, we find that the dihydrofolate reductase gene is amplified in ≈50% of the resistant variants, and this percentage does not change when the frequency of resistance is increased by hypoxia. Resistance to doxorubicin, likewise, results from amplification of the P-glycoprotein gene in ≈50% of the cell variants. Since the number of cell clones analyzed is small, we cannot say whether the hypoxia-associated increased frequency of doxorubicin resistance alters the percentage of cells resistant by virtue of P-glycoprotein gene amplification. Inasmuch as selection for the mixed phenotype could possibly result in a low-level resistance to methotrexate, we examined the doubly resistant cells for amplification of both dihydrofolate reductase and P-glycoprotein genes by rehybridization of the same blots with the two probes. In all of the doubly resistant variants in which the dihydrofolate reductase gene was amplified, the P-glycoprotein gene was also amplified. Thus the resistance to methotrexate was not imparted by amplification of the P-glycoprotein gene (i.e., was not the result of the same gene-amplification event).

DISCUSSION

We undertook these studies because of the vexing rapidity with which resistance emerges, either spontaneously or following chemotherapeutic regimens. We have shown that the observed frequency of simultaneous acquisition of resistance to two drugs employed in cancer chemotherapy, methotrexate and doxorubicin (adriamycin), is higher than that predicted from the independent frequencies of each selected alone. Approximately 50% of these resistances can be attributed to amplification of either the dihydrofolate reductase gene or the multiple-drug-resistance (P-glycoprotein) gene. That such amplification events impart resistance is in keeping with the concepts that overproduction of dihydrofolate reductase provides resistance to methotrexate (1–3, 11–15) and that overproduction of the cell-surface-associated P-glycoprotein prevents intracellular accumulation of a variety of drugs with various heterocyclic ring structures (19–25). We propose that the higher-than-expected frequency results from the fact that the initial process leading to these two gene amplification events—i.e., overreplication of a variable amount of the genome—is the same and that subsequent to recombination, selection pressure is imparted on those cells that contain (one or more) amplified genes, thereby generating the selectivity of drug resistance. Because the P-glycoprotein gene (*MDR1*) and the dihydrofolate reductase gene (DHFR) in Chinese hamster ovary cells reside on chromosomes 1 and 2, respectively (26, 38), the two amplification events are necessarily independent events.

In these studies we found that ≈50% of the methotrexate-resistant colonies that were obtained by selection with only methotrexate (low concentration) could be attributed to dihydrofolate reductase gene amplification. When this frequency was enhanced by hypoxia, this proportion was not changed. This result is similar to that described previously for enhancement of methotrexate resistance with hydroxyurea (11) and UV radiation (12). Thus, such treatments enhance gene amplification but also equally increase other classes of mutational events resulting in methotrexate resistance. Analysis of additional resistance mechanisms for the nonamplified variants indicates that a large proportion are a result of defective inward methotrexate transport (27). This proportion of methotrexate resistances is in keeping with the studies of Sirotnak *et al.* (28), who generated resistance to S-180 tumor cells in intact mice. If the process resulting in the methotrexate-transport defect occurs by a deletion mechanism, as is the case for loss of thymidine kinase in an experimental cell culture system (29) and has been reported

Table 2. Amplification of the dihydrofolate reductase (*DHFR*) and P-glycoprotein (*MDR1*) genes in drug-resistant variants

Drug(s)	Hypoxia pretreatment	*DHFR* Amplified,* no./no. tested	*DHFR* Amplification factor	*MDR1* Amplified, no./no. tested	*MDR1* Amplification factor
Methotrexate	−	2/7	1.7		
	+	16/25	6.5		
Doxorubicin	−			12/21	4.0
	+			12/14	2.0
Methotrexate plus doxorubicin	−	11/17	2.2	12/17	3.0

Chinese hamster ovary AA8 cells were selected for resistance to methotrexate (150 nM), doxorubicin (230 nM), or both, with or without 24 hr of incubation under hypoxic conditions prior to transfer to drug-containing medium. Resistant clones were expanded in drug-containing medium, and DNA was isolated for analysis by slot-blot hybridization with *DHFR*- and *MDR1*-specific probes.

*Fraction of independent clones of the total examined that were judged to be amplified for the gene in question (>1.5 control values).

for the hypoxanthine phosphoribosyltransferase gene in Lesch–Nyhan disease (30), then it is possible to account for the enhancement of this type of resistance by the same treatments that enhanced resistance by amplification events, inasmuch as deletions are among the recombination events subsequent to overreplication (10). We note that amplification of the P-glycoprotein gene does not account for all of the doxorubicin-resistant variants. Various investigators have reported overproduction of a protein with a molecular weight different than that of the M_r 170,000 P-glycoprotein (31, 32). Others have reported resistance to doxorubicin as a result of increased levels of a glutathione transferase, which presumably acts by suppressing free radicals generated by doxorubicin metabolism (33, 34). Employing a flow-cytometric technique (35), we have observed that most of our drug-resistant variants have elevated glutathione levels (3.3-fold, compared to sensitive cells, for the doxorubicin-resistant variants and 2.0-fold for the methotrexate-resistant variants). Whether overproduction, either by gene amplification or transcriptional activation, of enzymes affecting intracellular glutathione levels accounts for some of the resistance phenomena observed remains to be determined.

The reduced vascularization of a number of solid tumors may well be an important contributing factor to the difficulty in successful therapy. Not only are chemotherapeutic drugs delivered inadequately to all tumor cells, but the attendant hypoxic state reduces the efficacy of radiation treatments (36, 37). In addition, the hypoxic state, by virtue of its ability to provoke DNA overreplication (14), may also contribute to the high frequency of spontaneous and induced drug resistances to multiple chemotherapeutic agents.

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