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Chromosomal Variation and the Origin of Drug-Resistant Mutants in Mammalian Cell Lines

(polymorphism/bromodeoxyuridine/thioguanine)

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ABSTRACT **Drug-resistant mutants of somatic cell lines fall into two classes: one seems to correspond to classical gene mutation, the other is characterized, albeit transiently, by karyotypic instability, high reversion frequency, and low plating efficiency. An interpretation of the origin of these drug-resistant mutants is offered on the basis of chromosomal variation, which generates variation of the number of copies of each individual chromosome and, consequently, of gene dosage.**

The behavior of somatic cell mutants presents a number of unresolved paradoxes (for general reference see ref. 1). In particular, although the Luria–Delbrück fluctuation analysis, when applied, indicates that variants arise at random and independently of the selective agent, the lack of effect of mutagenic agents and of the degree of ploidy on the frequency of mutation, has led some authors to postulate epigenetic changes as the basis of drug-resistant phenotypes (2, 3). Recently, however, evidence for a conventional mutational event was found in structural alteration of the gene product (4–7). Considerable evidence, recently confirmed by the application of high-resolution techniques for chromosome identification (8–10) shows that established cell lines are to be seen as highly heterogeneous populations. Their cytogenetical polymorphism is determined by at least two factors: fitness of overall metabolic balance, and fitness of the chromosomal number relative to certain restrictions posed by the mitotic apparatus (11). Even when the cells have the same total number of chromosomes, they usually possess a variable number of homologues (10, 12, 13).

The variation in gene dosage resulting from the variation in the number of homologues (chromosomes or chromosome fragments) may not only have an effect on the frequency of mutants in polyploid populations (14) but may also have a *direct* effect on the cell phenotype (15–19). The direct approach for investigating the phenotypic effects of variations in gene dosage would be to analyze karyotypes. However, since the chromosomal location of the relevant structural and regulatory genes is unknown, an alternative approach is possible if the cells that survive a selective treatment owing to an exceptional gene dosage, are also exceptional in overall chromosomal number. As the mitotic apparatus of these cells tends to adjust to some preferred chromosomal number (11), they would remodel their karyotype. In this process, genetic instability would be revealed by many lethals (causing a low efficiency of plating) and high frequency of reversion.

I will show that by analyzing the behavior of drug-resistant mutants historically, day by day, classical stable mutants can be clearly distinguished from mutants associated with chromosomal variation.

EXPERIMENTAL RESULTS

1. Syrian Hamster Line B1. B1 is a bromodeoxyuridine (BrdUrd)-resistant derivative of BHK, isolated by Littlefield and Basilico who used a step-wise selection procedure (20). It grows well in E4 medium (11) containing up to 30 μg/ml of BrdUrd, and its thymidine kinase activity (ATP: thymidine 5′-phosphotransferase, EC 2.7.1.75) is only 3% that of the parental strain BHK. Littlefield and Basilico also reported that a 20-fold increase in thymidine kinase activity was associated with reversion.

From a clone of this B1 line, I isolated 24 spontaneous revertants (R) capable of growing in HAT medium (20) and designated them R1 to R24. They occurred at a frequency of 5×10^{-6} which was not changed by treatment with 0.5 μg/ml of nitrosoguanidine for 3 hr (21). As soon as the revertant clones had reached the size of a few thousand cells some of their properties were analyzed; similar measurements were repeated at later times.

The efficiency of plating of these revertants measured in HAT soon after isolation was between 1% and 5%; in E4 medium it was not much higher but 1 month later it went back to 30%, which is characteristic of B1.

These revertants, if plated *en masse*, seem to grow both in HAT and BrdUrd. However, this is not a physiological property possessed by all revertant cells, as suggested in other systems (22, 23), but rather a manifestation of genetic instability. In fact, as shown in Table 1, the revertant clones, although isolated in HAT, did not necessarily maintain HAT-resistance in a stable way. R4 and R20 grew better in BrdUrd than in HAT; R8 and R3 were at the other extreme; the other clones were in between. Moreover the different growth in 5 and 30 μg/ml of BrdUrd rules out, as a possible explanation, contamination of the revertant clones by B1 cells.

A direct measure of the reversion frequency of the HAT-resistant phenotype was obtained by plating some of the revertants in BrdUrd-containing medium. This was done in Linbro trays (Linbro 15-FB-96 TC 0.2 ml per cup) at low cell density (0.1–0.2 cells per cup) in order to minimize the possible effects of metabolic cooperation (24) or, more generally, any

Abbreviations: HAT, E4 medium + hypoxanthine, aminopterin and thymidine; eop, efficiency of plating.

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TABLE 1. *Relative growth performance in different media*

Clones	Growth (relative to E4)			Clones	Growth (relative to HAT)		
	HAT	BrdUrd5	BrdUrd30		HT	BrdUrd5	BrdUrd30
Control (B1)	<0.002	1.1	1	R6		0.05	
R1	1	0.11	0.02	R8		0.005	
R2	0.02	0.7	0.6	R10	1.2	0.2	
R3	0.7	0.12	0.01	R11	1.7	0.1	
R4	0.002	0.7	0.4	R12	6	1	
R5	0.9	0.7	0.5	R14	5	0.8	
				R15		0.11	0.02
				R18		0.2	0.01
				R20		20	3.4
				R24		1	0.6

Cells of the various revertant clones, after isolation in HAT, were subcultured for 1 week in HT. They were then trypsinized and seeded at 10^3 cells per 50-mm dish in different media. They were allowed to grow for 4 days and were then trypsinized and counted. The ratios of these counts in different media, relative to E4 or HAT, are reported in the table. BrdUrd5 and BrdUrd30 were media containing 5 and 30 μg/ml of BrdUrd, respectively. HT is E4 plus hypoxanthine and thymidine.

TABLE 2. *Chromosome counts*

Strain	Time (in days)	Range				
		Haploid	Diploid	Triploid	Tetra-ploid	Higher ploidy
B1	0		50			
	120	1	48	1		
R2	0	1	39		1	
R2R1	35		35		14	1
	84		25	1	22	2
	146		28	18	5	
R3	2	1	24	2	20	
R3R2	30	5	41	2	2	
	84		41		9	
R3R3	84		48		2	
R3R4	84		43		6	1
R3R5	84		21	3	26	
	146	1	31	13	4	1
R4	0		24	12	13	1
	7	1	30		17	2
R4R1	35		31	1	17	1
	84		41		9	
R5	7	1	29	4	18	1
	84		48		2	
R6	7		23	2	24	2
	84		47		3	
R7	7		5	26	16	3
	84		4	8	38	
	146		10	15	22	3
R9	7	3	17	12	18	
R1R1	35		32		17	1
	84		48		2	
R21R2	35		40		9	2
	84		45	1	5	
R21R2R	147	1	37	2	10	
R21R5	35		14		36	
	84		43	2	5	
	146		41	4	5	
R21R5R	147		31	10	13	1

Chromosome counts of approximately 50 mitoses of different clones at different times as indicated. Time zero is when the first revertants were isolated. Haploid range corresponds to chromosomal counts of 22 $\pm$ 11, diploid to 44 $\pm$ 11, triploid to 66 $\pm$ 11, tetraploid to 88 $\pm$ 11, and higher ploidy to numbers greater than 100.

effect due to the presence of different cell types in the same environment. The number of colonies counted in BrdUrd compared to those growing in HAT was 0.05, 0.13, and 0.15 for the three clones analyzed (R1, R3, R21, respectively). One or 2 weeks later these frequencies were down to 10^{-3} to 5×10^{-5}.

Among the colonies that developed in BrdUrd-containing Linbro trays inoculated with R21, we isolated four clones (RR) called R21R1 to R21R4. They were again replated at 0.1 cells per well in Linbro trays in order to measure the number of cells capable of growing in HAT (RRR) compared to those growing in BrdUrd. This frequency was 0.25 and decreased to 10^{-3} and 2.10^{-5} when plating was repeated 1 or 2 weeks later.

The chromosomal distribution of the various revertant clones was analyzed as soon after isolation as possible and at different times, thereafter. The results, presented in Table 2, refer to the HAT-resistant revertant clones (R series), to their revertants isolated in BrdUrd (RR series) and to their further revertants re-isolated in HAT (RRR series).

Whereas more than 95% of the cells of our B1 clone are in the diploid range, and in particular, more than 80% show chromosomal numbers between 40 and 44, all the revertant clones show dramatic changes in chromosomal distribution. Data are not presented in detail because they would be of little significance since the chromosomes were only counted and no attempt was made to identify them. Even so, the chromosomal counts grouped in classes (Table 2) demonstrate a tendency of the revertants towards polyploidy and a greater dispersion of chromosomal counts. In addition there is also some indication that, with time, the karyotypes tend to stabilize around the diploid number and dispersion is reduced (see e.g., R4, R5, R6; R3 and its various derivatives; R21R5, etc.).

2. Chinese Hamster Line DON. These studies test whether the transient genetic instability of the B1 revertants applies to forward mutations as well. The Chinese hamster cell line DON was selected because it is fast growing and has a low chromosome number with a fairly narrow distribution in the diploid region. Starting from a clone, I measured the frequency of mutation towards drug resistance to BrdUrd or thioguanine with and without mutagen treatment. Thio-

guanine was used instead of the more common analogue azaguanine in that it kills the cells at lower concentration and it requires hypoxanthine phosphoribosyltransferase (IMP: pyrophosphate phosphoribosyltransferase EC 2.4.2.8) activity for cellular toxicity (25). Revertants from either drug-resistant type, can be selected in HAT medium (1).

In 30 μg/ml of BrdUrd, no mutants were observed when plating 5×10^5 cells but in 5 μg/ml of BrdUrd, clones were observed at a frequency of the order of 10^{-4} (treatment with nitrosoguanidine does not seem to affect this frequency). These results are in line with those of Littlefield (26) in mouse fibroblasts, since he observed a frequency of 10^{-10} for the occurrence of mutants resistant to 30 μg/ml of BrdUrd and 10^{-8} at 5 μg/ml of BrdUrd. The mutation rate reported by Breslow and Goldsby (27) in the DON line for deficiency of thymidine incorporation was also of the same order of magnitude (2.6×10^{-4}). These authors also found the mutation rate unaffected by mutagen treatment. BrdUrd-resistance in this line may be due to a deficiency in thymidine transport rather than to the activity of thymidine kinase (27).

Among the clones obtained by plating DON in 5 μg/ml of BrdUrd, I isolated 10 resistant colonies, designated BU1 to 10. They are characterized by low efficiency of plating in E4 ± BrdUrd, strongly dependent on the concentration of the drug, by high reversion rate, and by instability of the karyotype. Not all tests were done on all clones.

The chromosomal distribution of BU1, followed at different times, shows a decrease in variability and a stabilization in the diploid range. These and other histograms (BU6 and BU9) are presented in Fig. 1 together with the chromosome distribution of DON.

Clone BU8 plated with an efficiency of 3.7% in E4, 1.66% in 5 μg/ml BrdUrd, 0.28% in 15 μg/ml of BrdUrd, and 0.01% in 30 μg of BrdUrd/ml. In HAT, the efficiency was 1.83%. From the efficiencies in HAT and E4, the reversion frequency would be calculated as 0.5.

BU6 was plated in Linbro trays at 0.2 cells per well (ten trays were used for each medium). Six clones were recovered in E4, five in HAT and only one in 5 μg of BrdUrd. All these clones were replated in E4 medium plus hypoxanthine and thymidine (HT), HAT, and BrdUrd (see Table 3). A high reversion rate was found for the BU6 mutation and for its revertants as well. In general there was a tendency to evolve towards the wild-type constitution, in the sense that without selection, the HAT-resistant type becomes much more frequent than the BrdUrd-resistant type.

No spontaneous mutants resistant to 3 μg/ml of thioguanine were obtained from 5×10^5 cells. After mutagenic treatment (21) instead, mutants (designated tg 1 to 5) were obtained at a frequency of 10^{-4}. No revertants in HAT were obtained out of 5×10^6 cells; the efficiency of plating of mutant clone tg5 was 25% up to a concentration of 20 μg of thioguanine per ml, equal to that in the absence of the drug [relative eop (efficiency of plating) = 1]; tg3 grew well in 10 μg of thioguanine per ml, and at 20 μg the relative eop was still 0.6 (see Fig. 2). The karyotype of these resistant clones was similar to DON (Fig. 1). In brief these variants looked like classical stable mutations.

When, instead of a screening concentration of 3 μg/ml, a lower concentration of thioguanine such as 0.5 or 1 μg/ml was used, spontaneous mutants were obtained. At 0.5 μg of thioguanine per ml, resistant clones were found spontaneously at a frequency of 19×10^{-4} and at 1 μg/ml at a frequency of 2×10^{-4}. In a mutagen-treated population the frequency of these resistant clones was within a factor of two (13 and 2.3×10^{-4} respectively) that of the untreated sample.

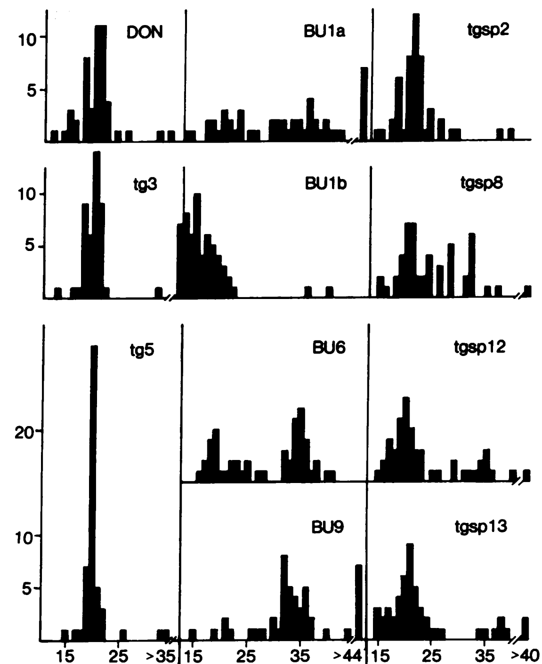

FIG. 1. Frequency distribution of chromosomal numbers in DON and some of its derivatives: tgsp are spontaneous thioguanine resistant, BU are spontaneous BrdUrd-resistant, and tg are nitrosoguanidine induced. (BU1 was analyzed at two different times: BU1a soon after isolation and BU1b 1 week later. The vertical scales indicate the frequency with which the chromosomal numbers, indicated in the horizontal scale, were found.

The spontaneous mutants, designated tgsp, were characterized in terms of karyotype (Fig. 1), efficiency of plating (Fig. 2) and reversion rate (Table 4).

Whereas BrdUrd-resistance may have various causes, i.e., thymidine kinase deficiency in B1 (20) and a deficiency in

TABLE 3. *Plating efficiency in different media*

Sub clones		Colonies in HT	BrdUrd	HAT
BrdUrd	1	30	25	22
HAT	1	207	51	201
	2	170	30	159
	3	96	23	91
	4	143	61	93
	5	56	18	38
E4	1	72	19	54
	2	203	60	195
	3	44	2	28
	4	103	20	61
	5	60	12	32
	6	26	0	22

BrdUrd-resistant clone BU6 was plated in different media. One clone, BrdUrd-1, grew in BrdUrd, five clones (HAT 1–5) grew in HAT and six clones (E4 1–6) in E4. These clones were picked and replated in HT, HAT, BrdUrd (5 μg/ml). The number of colonies obtained after plating 5×10^2 cells into a 90-mm dish are reported in the table.

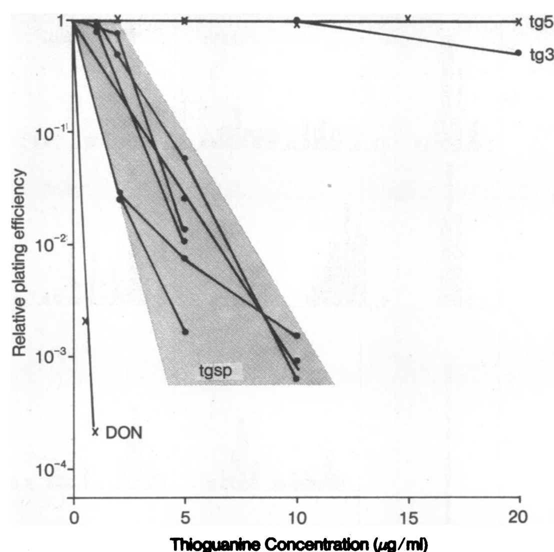

FIG. 2. Relative efficiency of plating at different thioguanine concentrations (semilog plotting) of DON and some of its thioguanine-resistant derivatives.

thymidine transport in DON (27), it seems that the basis for thioguanine (azaguanine) resistance is in the reduced activity of hypoxanthine phosphoribosyltransferase (25, 28). In order to determine whether in our thioguanine-resistant strains or their revertants, the phosphoribosyltransferase presented a qualitative alteration, Cellogel electrophoresis was performed according to Shin *et al.* (29). The results showed that the stable thioguanine-resistant mutants of DON, tg3, and tg5 had no measurable activity whereas tgsp2 and tgsp8 had a reduced activity without any change in electrophoretic mobility.

3. Mouse Line 3T6H. A thioguanine-resistant mutant of the mouse line 3T6 originally obtained from Dr. H. Green (designated 3T6H) was plated in HAT medium to score for revertants. Out of 4×10^6 3T6H cells, one revertant clone was obtained. This clone, 3T6R1, grew equally well in E4 ± HAT. In E4 + thioguanine (thioguanine medium), the plating efficiency relative to E4 (reversion frequency) was 0.06.

3T6R1 was subcultured and maintained both in HAT and E4. Ten days later, the relative plating efficiency in thioguanine-medium (reversion frequency) was found to be a few percent for cells grown in E4 but 10^{-3} if they had grown in HAT; 2 months later the reversion frequency was 10^{-5} irrespective of the conditions of growth.

No differences in electrophoretic mobility were found between the hypoxanthine phosphoribosyltransferase of 3T6R1 and of 3T6H cells; there were only differences in the amount.

TABLE 4. *Efficiency of plating*

	E4	HAT
tg3	0.25	$<2.10^{-7}$
tg5	0.25	$<2.10^{-7}$
tgsp12	0.027	0.009
13	0.031	0.017
16	0.041	0.028
17	0.049	0.016
18	0.017	0.004

Plating efficiency of thioguanine-resistant derivatives of DON in E4 and HAT media.

TABLE 5. *Karyotype analysis*

Strain	Chromosome type							Total number of chromosomes	No. of metaphases
	I	II	III	IV	V	VI	?		
PTK1	2	2	2	2	2	1		11	19
	2	1	1	2	2	1	1	10	1
	2	2	2	2	1	2		11	1
	2	1	2	1	2	1	1	10	1
	2	1	2	2	2	1	1	11	2
PG	2	2	2	2	2	1		11	12
	2	2	1	2	2	1	2	12	1
	2	2	2	2	1	1	1	11	1
PG-HAT	2	2	2	2	2	1		11	4
	2	2	2	2	2	2		12	1
	1	2	2	2	2	1		10	2
	1	2	2	2	2	—		9	1
	1	3	2	2	2	2		12	1
	2	2	1	2	1	1	1	10	1
	2	2	1	2	4	1		12	1
	2	2	2	1	1	—	2	10	1
	2	2	2	2	1	1		10	1
	2	2	1	3	2	—		10	1
	2	2	1	3	3	1		12	1
	2	2	1	3	2	1		11	1
	2	3	2	2	2	—		11	1
	2	2	2	3	2	1		11	4
	2	1	2	2	2	1		10	1
	2	2	2	2	3	1		12	1
	2	2	3	1	2	—		10	1

Analysis of karyotypes and frequency with which they occurred in populations of PTK1, PG, and PG growing in HAT. The chromosomes are designated in order of decreasing size. Those involving some form of rearrangement were simply put in a separate column marked ? without attempting an identification of the segments involved. The sample is not random in the sense that hyperdiploid cells are not represented in this analysis.

Four revertants of 3T6R1 were isolated in thioguanine-medium. Their frequency of re-reversion to thioguanine sensitivity, measured as capability to grow in HAT, was 10^{-3} one week after isolation and 10^{-5} after 2 months of growth in E4.

4. Kangaroo-Rat Line PTK1. The modal class in this line has only 11 chromosomes, all easily identifiable (30). These cells are, therefore, very suitable for cytogenetical analysis. From this line, the thioguanine-resistant mutant PG was obtained after mutagenesis. PG cells are resistant up to 20 μg/ml of thioguanine and their karyotype is not substantially different from PTK1.

Revertants could be obtained by plating PG in HAT medium. They were capable of growing both in thioguanine and HAT medium. Since no cloning was done, it is not known whether each cell is physiologically capable of growing both in HAT and thioguanine, or if the population is mixed because of chromosomal variation. The karyotypes of PG cells surviving selection in HAT are much more heterogeneous. The results, presented in Table 5 do not allow any definite conclusion concerning which chromosomes are to be present, in one or more copies, or about the frequency of chromosomal rearrangement; they seem sufficient, however, to substantiate the hypothesis that the capacity of PG cells to survive the HAT selection was paralleled by some sort of karyotype remodeling.

DISCUSSION

Among our drug resistant mutants two classes can be identified. The first class probably is made up of classical mutations, i.e., alterations in the base sequence of a structural or regulatory gene. Examples of this class in our experiments are the thioguanine-resistant mutants of the DON line, like tg3 and tg5 (and perhaps PG), in which the gene product is inactive or absent, the frequency of mutation is enhanced by mutagenic treatment, reversion is rare, no karyotype alteration is demonstrable, and resistance is acquired in one step over a fairly wide range of drug concentrations.

The second class (which includes all other mutants studied in this work) is characterized, albeit transiently, by low plating efficiency, high reversion rate, and karyotypic disturbances.

The fact that the frequency of mutants in this class is not increased by treatment with nitrosoguanidine and the fact that no qualitative alteration was found in the relevant gene product could be an indication that these mutant phenotypes are caused, as suggested by Harris (2) by a "shift in phenotypic expression." However, since there is a correlation with chromosomal variation, there may be behind these shifts, a genetic phenomenon, namely, variations in gene dosage. Furthermore, the higher rate of occurrence of these mutants in permanent cell lines when compared to diploid cultures need not be based on an impaired control in established cell lines since the difference can be attributed to higher variability of gene dosage in these lines.

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