PUROMYCIN RESISTANCE IN HAPLOID AND HETEROPLOID FROG CELLS: GENE OR MEMBRANE DETERMINED?

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ABSTRACT

The frequency of colony formation in monolayers of cultured frog cell lines treated with puromycin was compared in (*a*) haploid and heteroploid lines and (*b*) mutagen-treated and nontreated haploid lines. Evidence that resistant colonies result from gene mutation was negative, since the colony frequency is independent of both ploidy and mutagen treatment. A study of five frog cell lines showed that colony formation in puromycin depends on (*a*) the concentration of puromycin, (*b*) preselection of the population with puromycin, and, particularly, (*c*) the capacity of the treated population to survive some exposure to puromycin. One haploid and one heteroploid strain showing stable resistance to puromycin have been isolated; comparison of those variants with sensitive populations has shown that resistance to puromycin is correlated with the cells' capacity to exclude the drug. The evidence for different levels of membrane permeability, combined with evidence for many degrees of resistance among and within cell populations, suggests a model of self-determining membrane units. The evolution of a resistant phenotype may result from changes in the proportion of specific units in the membrane population.

INTRODUCTION

Investigations of mutation in cell culture have been complicated by the uncertainty that a variant arising in culture is the result of gene mutation (changes in DNA base sequence) or of an extragenic event (1, 2). This difficulty can be attributed in part to the absence of effective segregation tests and the spurious colony formation which complicates a Luria-Delbrück analysis. In cases where the variant phenotype has been correlated with a molecular difference, it is still not certain that there has been a genetic change. The nature of these heritable changes is particularly hard to determine in drug-resistant variants, as has been demonstrated by Harris for puromycin resistance in cultures of pig kidney cells (3).

A second difficulty of cell culture mutation experiments has been the absence of a haploid cell line, in which recessive gene mutations could be detected and isolated. In such a line, any phenotypic change resulting from a recessive gene mutation would be expected to occur with many times (1/mutation rate) the frequency observable in a diploid population; the frequency should be further increased by treatment with mutagens. The first such vertebrate lines, derived from embryos of the frog *Rana pipiens* (4), were used in the studies reported here.

A comparison of the development of puromycin-resistant variants in the haploid population with that in heteroploid lines was undertaken to test the hypothesis that such variants were the result

of gene mutation. The experiments reported below indicate that colony production in puromycin is independent of both cell ploidy and pretreatment with mutagens. An analysis of a number of cell populations and resistant variants suggests that the degree of resistance is (*a*) dependent on a large number of units and (*b*) correlated with a decreased permeability to the drug. A model of self-determining membrane units could explain both the heritability of puromycin resistance and the formation of variants; it may also be applicable to some phenotypic changes which occur in differentiation and tumorigenesis.

MATERIALS AND METHODS

The origin and maintenance of the five sensitive ("wild-type") frog cell lines studied has been described elsewhere (4–6). The two lines which have yielded resistant variants are the haploid ICR 2A and the pseudodiploid ICR 132; both were initiated from haploid *Rana pipiens* embryos. They have now been maintained for over 100 subcultures (a subculture, S,[1] corresponds to 3–4 generations); they multiply in 60% L-15 medium with 10% fetal calf serum at 25°C with a doubling time of 40 hr (6).

A typical procedure was as follows: about 2–3 × 10^5 trypsinized cells were inoculated into 12 ml of standard medium in a 75 cm^2 plastic flask. When the cells had formed a monolayer of about 2 × 10^6 cells, freshly prepared medium of the appropriate concentrations of puromycin was added to the drained flasks; the solution was renewed every 10 days. The puromycin dihydrochloride stock solution (50 μg/ml) was kept frozen when not in use. The decrease or increase in cell number was followed by counting the cells *in situ* using an inverted microscope fitted with a net reticule. Flasks were examined weekly for the appearance of colonies visible to the eye. When most of the colonies in a flask contained a minimum of 100 cells, the side of the flask over the colonies was removed with a soldering iron; individual colonies were then harvested by the steel cylinder method and dispersed into 2 ml of standard medium (without puromycin).

For determinations of puromycin uptake, 25 cm^2 flasks containing monolayers of cells were set up; before testing, the cells were allowed to multiply in medium without puromycin for at least a week. Puromycin-^{3}H (SA 1.11 Ci/mmole) in standard medium was added to the drained flasks; after a specified time, the solution was decanted and the flask washed with 5 ml salt solution with 2.5 μg/ml cold puromycin. After the cells were detached by trypsinization, they were poured onto a Millipore filter disc (Millipore Corporation, Bedford, Mass.) and again washed with the salt and cold puromycin solution. After drying was completed, the radioactivity was determined by scintillation spectroscopy. For calculations of internal puromycin concentration, cell diameters were determined with a Vickers image-splitting eyepiece (Vickers Instruments, Inc., Malden, Mass.).

[1] *Abbreviations:* C, clone; EMS, ethylmethanesulfonate; I, impermeable; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; P, permeable; S, subculture.

RESULTS

Reaction of Cell Populations to Puromycin

A typical curve for the relation of survival to concentration of drug is shown in Fig. 1; all of the frog lines tested have given such a biphasic curve, with the break between 2 and 4 μg/ml puromycin. Each cell line shows a different and typical level of response, although the level may change over large numbers of cell generations. The reaction to puromycin is independent of ploidy (and therefore cell size) since one haploid line, ICR 2A, and a diploid line, ICR 132, have high survival rates while the haploid line ICR 205 and a pseudotriploid line, ICR 134C_4, fare relatively badly in the drug (Fig. 2 and Table I). Cell lines initiated from sibling embryos can differ in their survival in puromycin, as in the case of ICR 132 and 134C_4.

The relationship between cell population and duration of drug treatment also gives a biphasic

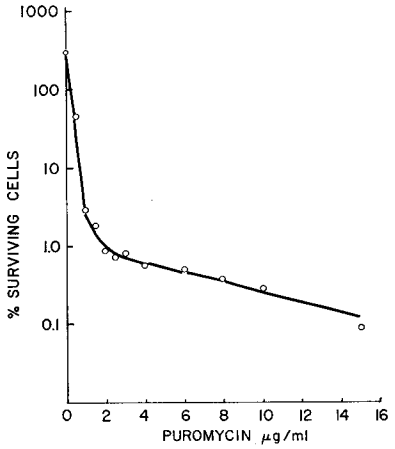

FIGURE 1 Survival of cultured frog cells from line ICR 132 (S_{24}) as a function of puromycin concentration. The ordinate is the number of attached cells remaining after a 72 hr exposure to puromycin as a per cent of the number present at zero time.

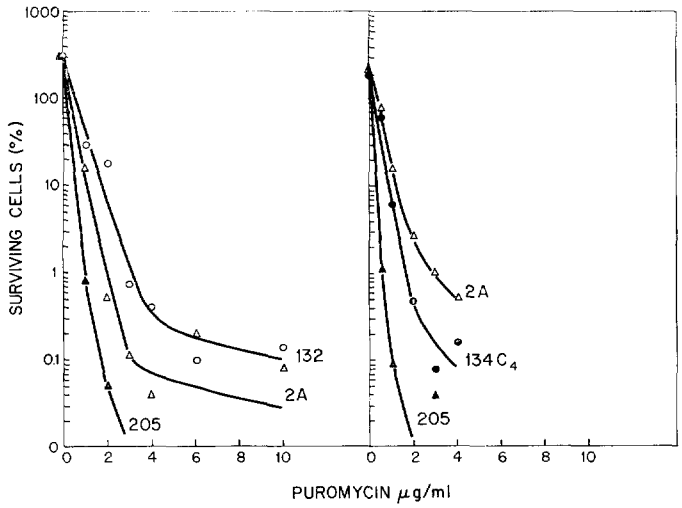

FIGURE 2 Comparison of the survival of cultured frog cells of different ploidy values as a function of puromycin concentration. The cell lines are: Fig. 2 *a*, ICR 2A S_{26}, haploid; ICR 205 S_{31}, haploid; ICR 132 S_{57}, pseudodiploid; Fig. 2 *b*, ICR 2A S_{39}, haploid; ICR 205 S_{41}, haploid; ICR 134C_4 S_{55}, pseudotriploid. Fig. 2 *a* shows survival after 6 days, Fig. 2 *b* after 7 days of puromycin treatment.

TABLE I

Cell Survival and Colony Formation of Haploid, Heteroploid, and Puromycin-Selected Frog Lines in Puromycin

	Colony formation			Survival in 1 μg/ml	
	μg/ml 1	2	3	1 wk	2 wk
	*No. of colonies**			%	
Haploid					
2A(S_{26})	11	0	0		
	16	0	0	16	9
205(S_{31})	0	0	0		
	0	0	0	1	0.1
Pseudodiploid					
132(S_{40})	39	1	0		
	45	0	0	26	8
132 puro(S_{19})	54	3	1		
	—	5	2	22	20
132 puroC_{14}(S_6)	Many	4	0		
	Many	4	1	23	18
Pseudotriploid					
134C_4	0	0	0		
	0	0	0	6	4

* Each datum represents the colony count in one flask containing 10^6 cells at the time of puromycin application. No colonies were found at 4, 6, and 10 μg/ml puromycin in these experiments.

curve (Fig. 3). There is, therefore, in a "sensitive population" a group of cells which is able to withstand longer periods as well as higher doses of puromycin. It is evident from Fig. 4 that after 3 or 4 wk, at least some of these cells not only survive but multiply for a time in concentrations up to 2 μg/ml of puromycin. If the slopes of the lines showing the resulting increase in cell number are extrapolated back to zero, a figure is obtained for the fraction of cells present at that time which were able to multiply in puromycin at the concentration in the flask (see Fig. 5). The fraction of cells able to multiply is correlated with the concentration of puromycin. A sensitive population consists, therefore, of cells differing in their capacities to synthesize protein and to multiply in the presence of puromycin.

A more resistant portion of such a sensitive population can be selected by treating with low levels of puromycin. If cells which survive 1 μg/ml puromycin for 7 wk are allowed to multiply in drug-free medium and are treated again, the observed resistance is higher than that of the parent line (Fig. 3). However, if propagation in drug-free medium is longer continued (e.g., 50 generations; see Fig. 3), the resistance is lower. The character of resistance is not completely stable although it remained higher than that of the parent line for many generations. Therefore, one of the results of cell multiplication in medium

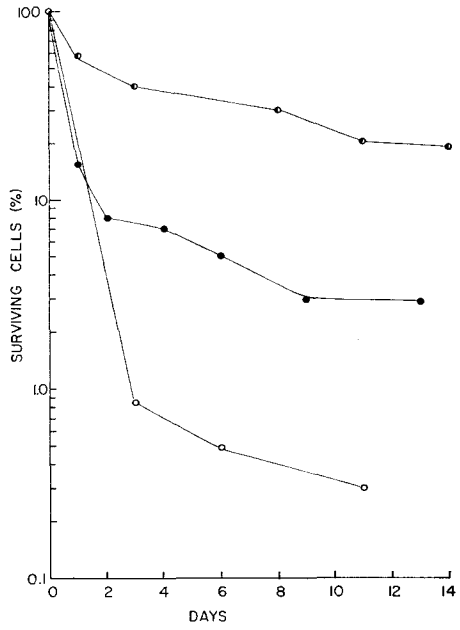

FIGURE 3 Comparison of survival in puromycin (2 μg/ml) of a sensitive cell line ICR 132 S_{24} (○) and a cell strain derived from ICR 132 S_{24} by treatment with 1 μg/ml puromycin. The cell strain was tested several generations after it was removed from puromycin (◐) and after 6 months of multiplying in the absence of puromycin (●).

without puromycin is the production of a series of less resistant cells. Evidence that cells with higher resistance are also produced under these circumstances is the fact that these populations yield colonies at concentrations of puromycin in which none had previously been found, as discussed in the next section.

Colony Formation

Colonies (64 or more cells) become visible from 3 to 7 wk after continuous puromycin treatment, indicating that there is a lag or delay in cell division upon exposure to the drug. The frequency of colonies is dependent on (*a*) the drug concentration, (*b*) the particular cell line exposed to the drug, and (*c*) whether or not preselection by puromycin treatment has occurred. Their appearance is not sporadic as would be expected with a single mutation; colonies at a given concentration are always accompanied by more frequent colonies at a lower concentration. For example, in the experiment depicted in Fig. 4, the average number of colonies at the 2.5 μg/ml concentration was 2;

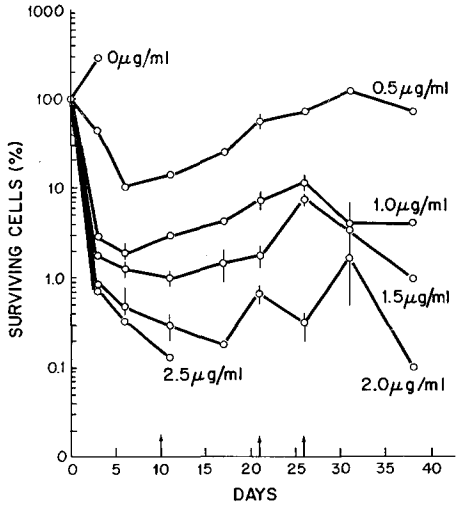

FIGURE 4 Survival and multiplication of cultures of ICR 132 S_{24} cells in puromycin for 38 days. The arrows indicate the times at which the medium was replaced with fresh puromycin medium.

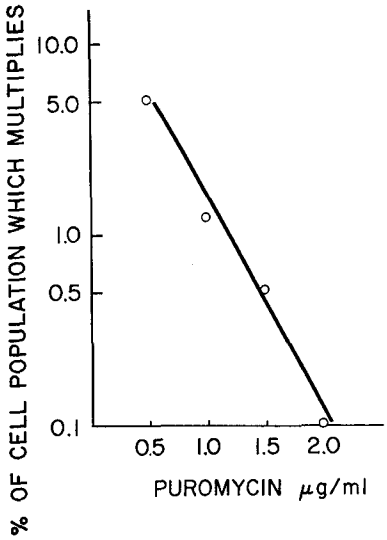

FIGURE 5 The relation of the fraction of the sensitive cell population able to multiply in puromycin to the concentration of puromycin. The values for the ordinate were obtained by graphically extrapolating to zero in Fig. 4.

at 2.0 μg/ml, 5 colonies; at 1.5 μg/ml, 40 colonies; and at 1.0 μg/ml, 60 colonies. The data in Table I also illustrate this point and furthermore show that the ploidy of a cell line does not determine the yield of colonies. The one property of a cell line which is correlated with the formation

of colonies is the capacity of the culture to survive the first week or two of puromycin treatment as shown in Table I.

To determine the extent and stability of the resistance to puromycin, 37 individual colonies were harvested from 1 μg/ml puromycin. When the progeny cultures were reexposed to puromycin, they showed a wide range of capacities to survive in puromycin. The average cell survival for all the isolates was 10 times that of the parent line but was still not adequate for a population increase in 1 μg/ml puromycin. The increase in the proportion of cells able to survive some puromycin treatment is accompanied upon reexposure to puromycin by the eventual appearance of more colonies at higher concentrations of drug (Table I, 132 puro and 132 puroC_{14}), thus confirming the relationship between survival of the monolayers and frequency of colonies. Colonies appear in 3 μg/ml only after a second exposure to puromycin, indicating that selection and subsequent cell multiplication results in more highly resistant cells.

Mutagenesis

A further test for the possible genetic origin of colonies was made by treating the haploid cells with mutagens before exposure to puromycin. Two compounds which produce mutants in bacteria and which had resulted in auxotrophic variants in Chinese hamster cells were tried; these are ethylmethanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (7–9).

The results of mutagen treatments yielding 25–50% cell survival are given in Table II. In 1 μg/ml puromycin, colonies from mutagen-treated populations appear earlier and are therefore larger than those in normal populations, but the final number of colonies is about the same. In 2–5 μg/ml puromycin, out of a total of 92 flasks, 2 MNNG-treated flasks had colonies; these may be the result of a single event since the populations came from the same pool before drug application. The only other experiment which produced colonies in such high concentrations of puromycin was in ICR 132(S_{57}), a pseudodiploid, nonmutagen-treated population. There is therefore no significant evidence that mutagen treatment of haploid cells increases colony formation in puromycin.

Resistant Strains

From several hundred colonies harvested from cultures treated with puromycin, two highly resistant strains have been obtained; these multiply in medium containing concentrations of puromycin up to 10 μg/ml. Isolation of the haploid variant, ICR 2A puro 374, began with exposure to 1 μg/ml puromycin of ICR 2A cells previously treated with EMS (300 μg/ml). In this experiment, 11 colonies were tested for resistance; only No. 37 was able to multiply in 2 μg/ml puromycin. The level of resistance decreased when the cells grew in the absence of the drug; after 20 generations, reexposure to a 2 μg/ml concentration killed the cells, leaving only four colonies. The progeny of one of the colonies was exposed to 5 μg/ml puromycin; the colony which resulted, No. 374, produced the haploid strain ICR 2A puro 374. The cells multiply in 10 μg/ml puromycin but resistance decreases if they are cultivated in the absence of the drug. The plating efficiency is zero at 10 μg/ml and 2% in 2 μg/ml. In appearance, the cells are like fibroblasts with some tendency toward parallel orientation which increases with exposure to puromycin. By contrast, the

TABLE II

Colony Production by the Haploid Cell Line ICR 2A before and after Treatment with Mutagens

Puromycin conc.	Mutagen treatment		
	None	EMS (300 μg/ml)	MNNG (1.5 μg/ml)
μg/ml	*colonies/10^6 cells*	*colonies/10^6 cells*	*colonies/10^6 cells*
1	85 (5 × 10^6)*	95 (5 × 10^6)	92 (5 × 10^6)
2		0 (2 × 10^6)	0 (2 × 10^6)
3	0 (2 × 10^6)	0 (2 × 10^6)	0 (2 × 10^6)
5	0 (10 × 10^6)	0 (30 × 10^6)	0.1 (40 × 10^6)

* Numbers in parentheses are total number of cells treated.

sensitive parent ICR 2A cells are spindle shaped and do not show contact inhibition of movement.

A second, more resistant variant was obtained from the pseudodiploid line ICR 132 without the kind of cycling procedure used for puro 374. ICR 132 puro 46 started as a large colony which grew in a flask of cells treated with 10 μg/ml puromycin. In this particular experiment, colonies arose in all of the flasks set up from a pool of ICR 132(S_{57}) cells, including those with concentrations of puromycin at which colonies have not before or since been found (6–10 μg/ml). However, in this experiment there was no significant increase in the survival of the cell monolayer as a whole. These observations are consistent with the presence of a small group of resistant cells in the inoculum before puromycin was applied. Such a reservoir is not characteristic of the line itself, as experiments at passages 24, 30, 40, 102, and 110 have shown. In order to obtain this highly resistant heteroploid variant, no further exposure to puromycin was necessary. From its isolation, it maintained the capacity to multiply in 10 μg/ml, even after 18 passages in the absence of the drug. There is evidence that a small, less resistant population accumulates in the absence of selective pressure from puromycin: when cultures are returned to the drug, there is a short lag in multiplication, a lag which is more severe at higher drug concentrations. The remaining cells then multiply at the same rate as in the absence of the drug. However, even after 150 generations in the absence of puromycin, most of the population multiplies when returned to 10 μg/ml puromycin. By exposure to higher concentration of puromycin, cells have been selected which grow in 20 μg/ml but not 50 μg/ml of the drug. The plating efficiency of ICR 132 puro 46 in 2 μg/ml (11%) is almost as high as in the absence of drug (15%) and is the same as that of the parent line in normal medium. In 10 μg/ml puromycin, only 2% of the cells produce colonies under plating conditions. The puromycin resistant variant of ICR 132 is fibroblast-like in appearance as is the parent line; they are both partially contact inhibited. However, the variant has larger cells than the parent strain; it is essentially hypotetraploid with a modal chromosome number of 45 (n = 13).

Puromycin Uptake

We have been unable to find in the literature any explanation of how resistant cultured cells avoid the effect of puromycin. The first question we posed was that of entry. It was found that ICR 132 puro 46 takes up only about 10% as much puromycin per cell as its parent line; for ICR 2A puro 374, the uptake is one-third as much as the sensitive parent line (Table III).

TABLE III

Uptake of Tritiated Puromycin by Frog Cell Strains

Exp.	Date	ICR 132		ICR 132 puro 46		ICR 2A		ICR 2A puro 374	
		cpm /10^6 cell	%	*cpm /10^6 cell*	%	*cpm /10^6 cell*	%	*cpm /10^6 cell*	%
I	10/31/69	4400	100	380	9				
II	3/23/70	16,500	100	180	1	3800	100	0	0
		23,000		370		2650		0	
III	4/3/70	13,500	100	1500	11	2250	100	990	34
		11,000		1390		4800		1360	
IV	4/23/70	17,000	100	1700	9	2300	100	1800	53
		21,000		1600		5000		2000	
V	5/1/70	21,000	100	2100	13				
		29,500		4300					
VI	5/7/70	21,190	100	1192	8	1910	100	835	43
		18,240		1757		2040		835	
				Average	8			Average	32

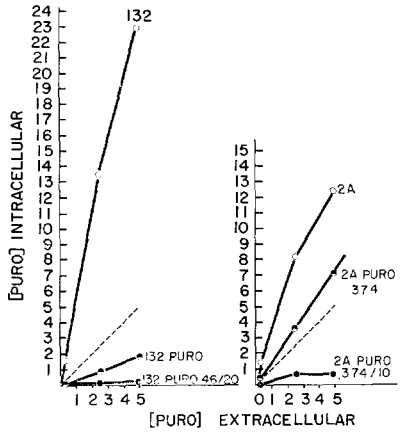

FIGURE 6 Uptake of puromycin in 150 min by sensitive and resistant frog cells as a function of the puromycin concentration of the medium. The dotted line represents equality of the concentration of puromycin in the medium and intracellularly (including puromycin derivatives). The volume of the ICR 2A cells ($1.22 \times 10^3\ \mu^3$) was calculated from measurements of the diameter of the trypsinized (spherical) cells using the Vickers image-splitting eyepiece. The volume of the other cells was derived from the observation that the volume of the cultured frog cells is a function of their chromosome number (4).

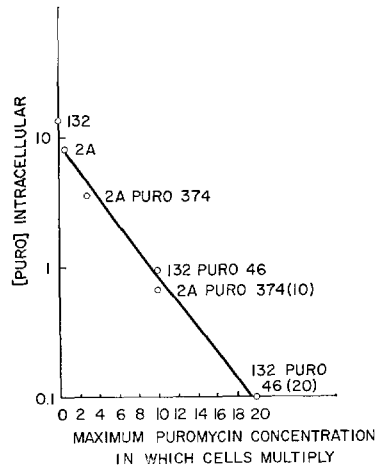

FIGURE 7 Puromycin uptake of six strains of frog cells as a function of the maximum concentration at which they multiply. The values for uptake were taken from Fig. 6.

An opportunity to investigate in more detail the correlation of puromycin resistance and uptake was afforded by the existence of variants of ICR 132 puro 46 and ICR 2A puro 374 whose resistance was increased by maintenance in puromycin. Thus, ICR 132 puro 46/20 multiplies in 20 μg/ml, whereas ICR 132 puro 46 multiplies in 10 μg/ml. ICR 2A puro 374 grows in 2.5 μg/ml and not in 5 μg/ml, whereas ICR 2A puro 374/10 grows in 10 μg/ml puromycin. Comparison of cell-associated radioactivity with that in the medium (Fig. 6), shows a correlation between resistance and puromycin exclusion. Furthermore, if the intracellular radioactivity resulting from 150 min exposure to 2.5 μg/ml puromycin is plotted against the maximum concentration (approximate) in which each strain can multiply (Fig. 7), the variants with least resistance have the greatest uptake of drug.

There are a number of considerations which support the view that puromycin resistance is determined by the permeability of the cell surface. It is evident from Fig. 6 that the puromycin molecule is concentrated in the sensitive cell lines, probably in the form of puromycin-peptides or proteins (10). When these puromycin-containing molecules are formed in a cell, they retain the tritium of the labeled compound; they are counted in the uptake experiments as intracellular puromycin. If resistance were the result of interference with such a coupling reaction inside the cell, the drug would perhaps not be concentrated; however, it is unlikely that the puromycin present in the cell would be as far below the concentration of external puromycin as in puro 46 cells (Fig. 6). The same data indicate that exit of labeled compounds from the cell would have to take place at a higher rate than entrance of puromycin if resistance were ascribed to the massive formation of diffusible puromycin derivatives.

DISCUSSION

The two strains of frog cells able to multiply in puromycin have a number of similarities: the capacity to retain a level of resistance greater than the parent line while multiplying in the absence of the drug; an increase under these conditions of a part of the population less resistant to puromycin; the emergence of a population with a higher degree of resistance after alternate exposure to medium with and without puromycin. However, the dissimilarities between the two strains are more numerous: ICR 2A puro 374 is haploid, euploid, of a size and chromosome number the same as that of the parent line; compared to the heteroploid variant, it is smaller in size, less resistant, less stable, and was obtained only after

repeated exposure to increasing concentrations of puromycin. The ICR 132 variant has four times the volume and chromosome number of the haploid, twice that of its parent line; it is aneuploid, resistant to higher concentrations of puromycin and more stable. The cells were highly resistant during, and probably before, a first exposure to puromycin.

In spite of the differences described above, there is apparently a common mechanism which enables the resistant variants of ICR 2A and ICR 132 to multiply in medium containing puromycin. Resistance is correlated with the degree of uptake in each case. Such an observation suggests that the surface membrane of the resistant cell differs from that of its sensitive progenitor.

The reaction of frog lines of puromycin can be interpreted as follows: a population never before exposed to puromycin consists of cells with many degrees of resistance to the drug in a continuous distribution. Since resistance to puromycin has no selective value in normal medium, the average sensitivity of the population is stable. However, in puromycin, some cells die, starting with those at the less resistant end of the distribution. Populations with a high average resistance will decrease slowly; the range is more likely to include a few cells resistant enough to form colonies. If colonies arise at 2 μg/ml puromycin, more will be found at 1 μg/ml, since there is a continuous range of resistance (Table I). It also follows that the colonies isolated from a cell population will show a variety of degrees of resistance, as they do.

One has now to consider the question of whether puromycin resistance arises as a result of gene mutation or of something quite different. There are probably a number of sites at which a mutation could decrease the transfer of a molecule: a change in binding protein, transfer protein or the shape of a "hole" through which the drug may pass. It is assumed that one or more of these entities exist, although little is known about membrane transport in eucaryotic cells for molecules of this size. Since transport systems show specificity, one would argue that the majority of mutations would decrease rather than increase permeability and so yield resistant cells. However, mutagen treatment of haploid cells has thus far been ineffective in increasing colony incidence. In the absence of puromycin, resistant cell strains gradually become less resistant although mutations would tend to decrease permeability.

There are other observations difficult to reconcile with a mutation hypothesis. The frequency of colony formation in various cell lines is different and independent of ploidy. If a small part only of the population is unusually resistant, the intermediate stages continue to exist, judging by colony formation at various drug concentrations and/or by survival curves. If a change in resistance resulted from one gene mutation, intermediate stages would be absent at the time the mutation was finally expressed. If polygenes are invoked for the spectrum of resistance, it is necessary to assume either a high mutation rate or a mechanism for extensive segregation.

Puromycin resistance in frog cells is behaving as though determined by a large number of units. Since resistance is the result of a change in the surface membrane, we should consider the possibility that these units are part of the membrane and that they are self-determining.

The following model is proposed as a possible explanation of how the cultured frog cells may acquire resistance to puromycin. Let us assume that the degree of uptake is determined by the percentage of units of an "impermeable" type which are present in a membrane. The simplest membrane would consist of two types of units: I (impermeable) and P (permeable). As the cells grow in size, the surface membrane is continually expanding by the addition of more of these units. Which one of several types, or perhaps configurations, is incorporated into the membrane may be dependent on the type, or types, already present at the point of insertion. The new unit inserted next to an I unit would be more likely to be an I unit than a P unit. Therefore, a second assumption for the model is that the units composing a cell membrane are self-determining and so it is possible to treat the membrane as a dynamic population of units. If the self-determining ability, and thus the adaptive value, of each kind of unit were equal, the proportion of I and P units in a membrane would tend to remain the same. The properties of a cell and its progeny, and therefore of a cell population, would be stable. The population would actually be self-determining at two levels: the unit of membrane structure (molecular) and the unit of the cell (cellular). It follows from this that a shift in the drug resistance of a cell population could occur as a result of environmental changes affecting the self-determining potentials of either the mem-

brane units or the cellular units, or both. If the environmental change (addition of puromycin) acts only at the cellular level, it would be termed a selective agent. If, however, puromycin acted in such a way that I membrane units would have a better chance to determine incoming units than P units, or even by directly affecting the structure of a membrane unit, it would act as an inducer. Although these experiments make it clear that puromycin acts as a selective agent, they do not reveal whether or not it also has a direct effect on membrane structure.

Assuming the model to be applicable, resistant variants could be selected by exposing a cell population to cycles of increasing concentrations of puromycin, interspersed with normal medium to allow for expansion and diversification of the cell population. After each cycle, the cell population would have a higher proportion of impermeable membrane units and thus a greater probability of containing resistant cells. Such a cycling procedure yielded the haploid ICR 2A puro 374 strain. For the heteroploid strain derived without selection by puromycin, it is postulated that the shift in proportion of membrane units occurred in a small part of the population by a process of "genetic drift." The absence of selection against resistant cells in normal medium combined with the continued random halving of the membrane units in a cell by division would make such a result possible. The presence of more frequent colonies at all the lower concentrations of puromycin in this experiment points to the survival of the intermediate stages in the process.

Although the self-determining aspect of membrane units has been theoretically analyzed by Changeux et al. (11) among others, the evidence for it in membranes is sparse. However, for the cortex of *Paramecium*, where patterns can be microscopically distinguished, Beisson and Sonneborn (12) have shown that "cortical differentiations are essential for their own reproduction."

In eucaryotic cells, phenomena such as differentiation, immunity, and transformation share with drug resistance a number of characteristics in which self-determined membrane units could play a role. All exhibit stable phenotypes, cell surface membrane changes, a requirement for cell division, a frequency affected by environmental molecules. It should also be noted that cell surface changes could act like repressors; i.e., a decreased permeability to a specific substrate molecule would result in a deficiency of an enzyme stabilized by this substrate.

Although present knowledge of membrane structure may limit the approaches which can be used to test the model for eucaryotic cells, the puromycin system in frog cells has a number of advantages for such an investigation. The amount of resistance can be assayed, the degree of selective pressure can be chosen, cell strains which vary in resistance exist, resistant cell strains with various chromosome constitutions, including the haploid, can be compared. Possible correlations with tumorigenicity can be tested by placing the cells into tadpole tail tips. Finally, the nature of puromycin resistance can be explored by substituting the nucleus of a resistant cell for one in the activated egg using the Briggs and King technique (13).

The production of stable phenotypes without changes in the genome is considered to be a talent of somatic cells. Perhaps one of the mechanisms is based on the existence and properties of self-determining membrane units.

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