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*ISOLATION AND STUDY OF MUTANTS FROM MAMMALIAN CELLS IN CULTURE**

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The observation that a single mammalian cell can be grown to yield a colony of cells[1] has made it possible to search for mutants[2] in a population of cultured cells. Mutants resistant to the antibiotic puromycin (6-dimethylamino-9-[3′-deoxy-3′-(*p*-methoxy-L-phenylalanyl-amino)-β-D-ribofuranosyl]-purine [3, 4]) or the purine analogue 8-azaguanine have now been obtained with a fibroblast-like cell, AMK 2-2.[5] Interestingly, as with bacteria,[6] increased resistance to toxic agents may or may not occur in relatively small steps.

The purpose of this report is to describe the isolation of mutant mammalian cells, to indicate that they can be manipulated in procedures comparable to some of those employed in the study of microbial genetics, and to detail some of the properties of the mutants.

Materials.—The basal medium contained the amino acids, inorganic salts, glucose, and antibiotics suggested by Healy, Fisher, and Parker,[7] and the vitamin mixture of Eagle.[8] The *growth medium* contained in addition 15 per cent beef serum. For cloning, a mixture of human (15 per cent) and horse serum (5 per cent) (*cloning medium*) was substituted for the bovine serum. Since unheated human serum causes rapid lysis of AMK cells, human sera were heated (55°, 30 min) before use.

Puromycin was a gift from Dr. J. P. English, Lederle Laboratories. 8-azaguanine was obtained from the California Foundation for Biochemical Research and from the Nutritional Biochemical Corporation. The samples were spectrophotometrically indistinguishable at various pH levels.

The test tubes used for assay were 16 × 125 mm in size and were fitted with rubber-lined, plastic screwtops; Petri dishes were 60 mm in diameter.

Culture Methods.—Unless otherwise indicated, cells were grown in suspension in 50-ml test tubes fitted with silicone stoppers. To prevent the cells from settling out and adhering to the glass, cultures were incubated horizontally in a drum revolving at 40 rpm.[9]

To prepare a suspended culture from a colony, the colony was encircled with a stainless steel ring and the cells were detached from the Petri dish by aspirating several times with a capillary pipette. They were then transferred to a screwtop test tube containing 2 ml of cloning medium. When confluent, the cells were detached with a rubber spatula and allowed to grow (5 ml of growth medium) on the surface of a 50-ml test tube incubated in an almost horizontal position. Finally, fresh growth medium (10–20 ml) was added, the cells were detached into the medium, and the tube was placed in a revolving drum.

TABLE 1*

EFFECT OF PUROMYCIN ON VIABLE PARENT AND MUTANT CELLS

Puromycin, μg per ml	Culture			
	Pms^{-1}	Pms^{-2}	Pmr^{-1}	Pmr^{-2}
	Cell Number in Thousands			
0	376	384	475	417
2	0	211	...	...
4	...	0	...	...
8	...	...	481	...
12	...	...	435	...
16	...	...	106	336
24	...	...	0	356
40	...	...	...	333
80	...	...	...	31
120	...	...	...	3

* The cells were obtained from exponentially-growing, suspended cultures of clonal descendants. One milliliter aliquots of suspensions in growth medium (10^5 cells per ml) were added to test tubes containing the indicated amounts of puromycin and the tubes were incubated (37°) in an almost horizontal position (the liquid extended about 4 cm from the bottom of the tube). After 72 hr, dead cells were discarded by aspiration of the culture fluid, the attached cells were washed with 1 ml of growth medium, and growth was then estimated by counting the attached cells after detachment into fresh medium.

TABLE 2*

EFFECT OF PUROMYCIN ON NONVIABLE PARENT AND MUTANT CELLS

Puromycin, μg per ml	Culture			
	Pms^{-1}	Pms^{-2}	Pmr^{-1}	Pmr^{-2}
	Cell Number in Thousands			
0	460	520	460	500
4	11	380	...	...
8	3	80	420	...
12	...	2	300	...
16	...	...	240	520
24	...	...	31	380
40	...	...	...	430
80	...	...	...	17

* Each Petri dish received 4 ml of growth medium containing 5 × 10^5 cells obtained from suspended cultures. After 16 hr, the glass-attached cells were washed 3 times with 2-ml aliquots of a NaCl solution (0.15 *M*) and the washed cells were treated with 2 ml of a solution of nitrogen mustard (5 × 10^{-4} *M* in 0.15 *M* NaCl). The alkylating agent was destroyed after 5 min by the addition of growth medium (1 ml), and the cells were washed twice. Puromycin as indicated and growth medium were added to a final volume of 4 ml. The effect of the antibiotic was estimated after incubation (37°, 72 hr, atmosphere = 5 per cent CO_2 — air) by enumerating attached cells after the cells floating in the culture fluid were discarded.

The danger of contamination with mammalian cells from another culture, recently emphasized by the observations of Rothfels *et al.*,[5] was recognized. During these studies, in addition to the considerable care used in handling cultures, no fibroblast-like cell other than AMK 2-2 was maintained, and cultures were often recloned.

Cell counts were made with Levy counting chambers.

Puromycin.—Selection of mutants: To obtain cells resistant to puromycin, cultures of the susceptible parent were prepared on the surfaces of bottles (surface area = 75 cm^2). After heavy, but less than maximal growth was reached (approximately 4×10^6 cells), puromycin (8 μg per ml) was added to the growth medium. When about 95 per cent of the cells had been killed (about 24 hr), the medium was replaced with fresh growth medium lacking puromycin and the culture

TABLE 3*

THE PRESENCE OF Pm^{r-1} CELLS IN Pm^{s-2} CULTURES

Culture			Puromycin, μg per ml	Number of Colonies per Dish
Pm^{s-2}† (Approx. Number of Cells Added)	Pm^{r-1}	Pm^{r-2}		
100	...	...	...	95, 81; 135 ,112
100	...	...	4	90, 92; 139 ,130
100	...	...	8	0, 0; 0, 0
10,000	...	...	12	0, 0; 0, 0
10,000	...	...	40	0, 0; 0, 0
100,000	...	...	12	0, 1; 0, 2
100,000	...	...	40	0, 0; 0, 0
300,000	...	...	12	1, 2; 5, 3
300,000	...	...	40	0, 0; 0, 0
...	100	...	...	95, 130; 124, 111
...	100	...	12	116, 97; 82, 99
100,000	100	...	12	107, 107; 93, 131
300,000	100	...	12	102, 108; 107, 125
...	...	100	...	109, 111; 86, 96
...	...	100	40	98,119; 98, 88
100,000	...	100	40	114, 136; 112, 84
300,000	...	100	40	97, 154; 74, 90

* The culture mixtures in Petri dishes contained cloning medium and the indicated cell number and puromycin concentration in a final volume of 4 ml. On the 4th and again on the 8th day of incubation (37°, atmosphere = 5 per cent CO_2 — air), the medium was replaced with fresh medium containing the same concentration of puromycin as initially. After a total incubation period of 11 or 12 days, the colonies were fixed and stained[9] and enumerated. The semicolons separate the results of experiments with two different recloned cultures.

† The Pm^{s-2} cultures were obtained by cloning the original single clone isolate and the experiment was made as soon as the recloned (suspended) cultures attained sufficient cell density (about 3×10^5 cells per ml).

was grown to its original density. The cyclic process of growth and selection was repeated (6 cycles) until some of the cultures were no longer affected by the toxic agent. The resistant cultures (designated Pm^{r-1}) were capable of growth in the presence of 12, but not 40 μg per ml of the antibiotic.

Cells resistant to higher levels (Pm^{r-2}) were selected from the less resistant mutant cultures with growth medium containing 40 μg of puromycin per ml. In contrast to the less resistant mutants, the Pm^{r-2} cells were obtained with ease, after a single cycle of selection and regrowth. Attempts to obtain cells more resistant than the Pm^{r-2} ones were unsuccessful.

The resistant mutants were cloned and their descendants were maintained in suspended cultures in the absence of puromycin.

Heterogeneity of parent culture: Examination of the descendants of parent (wild type) cells cloned in the absence of puromycin revealed two major classes, a highly sensitive one, designated Pm^{s-1}, and a less sensitive one, designated Pm^{s-2}. The behavior of the parent and mutant clonal isolates in the presence of various con-

centrations of the antibiotic is illustrated in Table 1. The mutant cultures studied had been cultivated in the absence of puromycin for more than 100 generations.

Response of nonviable cells: Parent and mutant cells made nonviable (incapable of cell division) by treatment with nitrogen mustard behaved in a similar manner to the respective growing cells (Table 2). The effect of puromycin was to cause the detachment of the cells from the glass surface. The time of addition of the antibiotic was not important, identical results were obtained when puromycin was added 0, 1, 2, or 3 days after treatment of the cells with the alkylating agent.

Evidence for sequential mutations: The isolation of four heritably different cultures with gradually increasing resistance coupled with the ease with which the $Pm^{r}-^{2}$ cells were obtained from the less resistant ones suggested that the origin of the most resistant cells might involve sequential mutational events rather than a single genetic change. Results consistent with a mechanism involving sequential mutations have been obtained by the quantitative examination of cultures for cells with increased resistance.

Of the ten recloned $Pm^{s}-^{2}$ cultures tested, four were found to contain small numbers of cells with resistance levels similar to the $Pm^{r}-^{1}$ cultures, but none contained detectable $Pm^{r}-^{2}$ cells. The results obtained with two of the cultures are shown in Table 3. With $Pm^{r}-^{1}$ cells, on the other hand, each of the recloned cultures studied yielded relatively large numbers of $Pm^{r}-^{2}$ cells. The results of one of the experiments are shown in Table 4.

TABLE 4*

THE PRESENCE OF $Pm^{r}-^{2}$ CELLS IN $Pm^{r}-^{1}$ CULTURES

Culture ($Pm^{r}-^{1}$ (Approx. Number Cells Added))	Culture ($Pm^{r}-^{2}$)	Puromycin, μg per ml	Number of Colonies per Dish, $Pm^{r}-^{1}$
100	...	...	126, 83
100	...	8	111, 111
100	...	12	84, 97
100	...	16	37, 40
100	...	24	0, 0
100	...	40	0, 0, 0, 0
1,000		40	0, 0, 3, 3
10,000	...	40	15, 30, 16, 11
100,000	...	40	199, 169, 148, 193
...	100	...	91, 109
...	100	40	85, 68
1,000	100	40	95, 112
10,000	100	40	113, 80
100,000	100	40	202, 267

* The culture mixtures were prepared and treated as described for Table 3. The $Pm^{r}-^{1}$ and $Pm^{r}-^{2}$ cultures had been recloned and used as described for the $Pm^{s}-^{2}$ cultures of Table 3.

The possibility was considered that an occasional colony can be formed by genotypically susceptible cells capable, for unknown reasons, of growth for several generations in an otherwise lethal concentration of puromycin. To test this, the cells of some of the colonies considered to be resistant were evenly dispersed in fresh medium containing puromycin. Their resistance was evidenced (after 6 days) by the formation of large numbers of new colonies. In addition, some of the colonies were cultured in the presence of puromycin for an additional period of time (4–8 days) and the cells were then transferred to test tubes. When sufficient growth had occurred, resistance levels were tested in titration experiments as

TABLE 5*

EFFECT OF 8-AZAGUANINE ON THE GROWTH OF PARENT AND MUTANT CELLS

8-Azaguanine, μg per ml	Culture		
	Pm^{s-1}†	Pm^{r-1}	Ag^r
	Cell Number in Thousands		
0	97	61	75
0.10	84	...	...
0.33	0	1	...
1.0	...	...	78
3.3	...	...	51
10	...	...	38
33	...	...	26
100	...	...	13
300	...	...	8

* The cells were from suspended cultures in the exponential phase of growth. One-milliliter aliquots of suspensions in growth medium (5,000 cells per ml) were added to test tubes containing the indicated amounts of 8-azaguanine. After incubation (37°) for 4.5 days, growth was estimated by counting the attached cells after detachment with a rubber spatula.

† The Pm^{s-1} and Pm^{r-2} cultures behaved identically with the susceptible cultures shown.

described for Table 1. The cells derived from Pm^{s-2} and Pm^{r-1} cultures were indistinguishable from the Pm^{r-1} and Pm^{r-2} mutants, respectively.

8-Azaguanine.—Selection of resistant mutant: Resistant cells were selected from susceptible parent cultures by repeated exposure to azaguanine (1 μg per ml) and regrowth in its absence as described for the Pm^{r-1} mutant. The culture (designated Ag^r) has been grown for more than 50 generations in the absence of the purine analogue without detectable loss in resistance.

"One-step" increase in resistance: In striking contrast to the relatively small and graded increases in resistance observed with puromycin, as shown in Table 5, the cells selected in the presence of 1 μg per ml of azaguanine grew, albeit slowly, in a medium containing a 100 times greater concentration of the analogue.

The results of experiments with single cells (from the uncloned culture not previously exposed to concentrations of the analogue greater than 1 μg per ml) also indicated a "one-step" increase in resistance of the Ag^r cells; no evidence for cells with intermediate levels of resistance was found. Thus, when 100 cells were plated (growth medium supplemented with 5 per cent horse serum) with 0, 3, 10, 30, and 100 μg per ml of analogue, the number of colonies formed was 59, 51, 55, 61, and 54, respectively.

Discussion.—While cultured mammalian cells cannot be handled in numbers as large as those conveniently manipulated by the microbe geneticist, it is indeed encouraging to find that a few mutant cells can be selected from at least a relatively large population.

The graded increase in resistance observed with puromycin was a not wholly unexpected result in view of the similar observations made with drug-resistant bacteria[6] and with metabolic analogue-resistant neoplastic cells cultured in mice.[10, 11] Although it seemed possible that a study of the puromycin-resistant cultures would reveal another similarity with the bacterial system,[12, 13] the existence of cells with many degrees of resistance intermediate between the most susceptible and the most resistant ones, this does not appear to be the case.

Cultures derived from single susceptible cells commonly contain small numbers of low resistance mutants but no detectable high resistance ones. Coupled with the presence of large numbers of high resistance mutants in all clonal descendants of lower resistance ones, these observations are consistent with a mechanism involving

sequential mutations to puromycin resistance. The possibility is obviously not excluded that susceptible cells may, in addition, give rise to highly resistant ones by a single mutational event but at a rate too low to be detected by present methods.

Summary.—Cells with heritable resistance to the antibiotic puromycin or the purine analogue 8-azaguanine have been isolated from a culture of fibroblast-like mammalian cells, AMK 2-2. Resistance to puromycin, but not to azaguanine occurs in relatively small steps. Evidence has been presented to show that resistance to the former compound involves sequential mutational events.

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*ESTIMATION OF MUTATION RATES WITH MAMMALIAN CELLS IN CULTURE**

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In a previous report[1] the isolation and some of the properties of cultured mammalian cells heritably resistant to metabolic analogues were described. With one of the toxic agents, puromycin, low resistance (Pm^{r-1}) and higher resistance (Pm^{r-2}) mutants were obtained. Evidence was presented to show that the increase in resistance from the parent (Pm^{s}) to the high resistance mutant involves at least two sequential mutational events.

To determine the rates of these events the classical method of Luria and Delbrück[2]