DEVELOPMENT OF A NONSELECTIVE TECHNIQUE FOR STUDYING 2, 6, DIAMINOPURINE RESISTANCE IN AN ESTABLISHED MURINE CELL LINE[1]

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2,6-DIAMINOPURINE* has been used as a selecting agent in animal cell cultures to obtain variant lines for somatic cell studies (HARRIS and RUDDLE 1960; LIEBERMAN and OVE 1960; BLAIR and HALL 1965). The biochemical basis of DAP resistance appears to be due to a loss of AMP pyrophosphorylase activity needed in the conversion of adenine or its analogue to its phosphorylated derivative (LIEBERMAN and OVE 1960; BLAIR and HALL 1965; KALLE and GOTS 1961). However, in some instances altered specificity of the pyrophosphorylase has been shown to confer resistance to DAP in Salmonella (KALLE and GOTS 1963). In other systems permeability changes have been suggested as the basis of resistance (PATERSON 1962).

Although considerable progress has been made in our understanding of the biochemical basis of drug resistance, the genetic basis of purine analogue resistance is less understood. The application of animal cell hybridization techniques to drug resistance has shown that BUDR and 8AG resistance are recessive. From sensitive hybrids carrying the two markers, it was possible to recover the two parental (BUDR and 8AG resistance) cell types, which is suggestive of a nuclear event underlying drug resistance (LITTLEFIELD 1964a, 1964b). It is known that drug resistant variants occur in discrete independent steps (LITTLEFIELD 1964c, SZYBALSKI, SZYBALSKA and RAGNI 1962), are stable (HARRIS and RUDDLE 1960; SZYBALSKI and SMITH 1959), and fluctuation tests appear to indicate that variants arise independently of the drug (LIEBERMAN and OVE 1959). A direct demonstration of independence has not been made, and it is the objective of this paper to report the development of a nonselective technique for this purpose.

MATERIALS AND METHODS

Cell line and culture techniques: The McCoy cell line originally obtained from DR. GEORGE KENNY of the Department of Preventive Medicine, University of Washington and determined to

* Abbreviations: 2,6-Diaminopurine: DAP, AMP pyrophosphate phosphoribosyl transferase (E.C. 2.4.2.7): AMP pyroprosphorylase; 8 azaguanine: 8AG; 5 bromodeoxyuridine; BUDR; tritiated adenine: H^3A; adenosine 5′ monophosphate: AMP; 5-phosphoribose-1-pyrophosphate: PRPP; poly (ethyleneimine): PEI.

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be murine in origin (MORROW 1964) was employed in these experiments. Cells were grown in monolayer culture using EAGLE's minimal essential medium in EARLE's salt solution (EAGLE 1959), supplemented with 10 percent fetal calf serum, streptomycin (100 μg/ml) and penicillin (250 units/ml). Cultures were harvested and transferred in the usual manner with .05 percent trypsin solution plus .02 percent EDTA.

Cloning was carried out according to PUCK, MARCUS and CIECIURA (1956), as modified for plastic flasks by SIGMAN and GARTLER (1966).

Development of resistant and sensitive lines: Resistant lines were obtained by growing cells in the presence of 100 μg DAP/ml, a level at which there is greater than 99 percent kill of these cells. Resistant colonies appeared within two weeks, were removed, transferred to stock bottles, and then recloned. These lines were kept as stocks and periodically checked for resistance to DAP. All have remained resistant.

Enzyme assays: Adenylic acid pyrophosphorylase activity was assayed by a modification of the method described by LIEBERMAN and OVE (1960); the incubation mixture contained proportionally smaller quantities (total volume—.1 ml including 0.02 ml H^3A 50 μc/ml). After two hrs at 37°C, .02 ml of the incubation mixture was spotted on PEI-cellulose layers for separation by thin layer chromatography with water as the solvent. Under these conditions the phosphorylated derivatives are retained at the origin (RANDERATH 1964). The spots were identified by ultraviolet light, cut out, placed in BRAY's (1960) counting medium, and assayed for radioactivity in a liquid scintillation counter. The amount of radioactivity serves as an assay of the amount of adenylic acid pyrophosphorylase activity.

Studies of uptake of radioactivity: The amount of uptake of H^3A by various cell lines was analyzed by growing the cells in the presence of 2.0 μc/ml of H^3A for 4 hrs. The cells were harvested, centrifuged in the cold, and washed five times with saline. They were then resuspended to appropriate dilutions with distilled water, lysed by freezing and thawing, and aliquots placed in 4.0 ml of BRAY's solution for radioactivity assay in the scintillation counter.

Autoradiographic comparison of resistant and non-resistant cells: An autoradiographic method was developed which permitted a clear distinction between resistant and sensitive cells under nonselective conditions. This was based on the fact that resistant cells isolated at 100 μg DAP/ml incorporate little tritiated adenine compared to sensitive cells. Cells growing on cover slips or in Petri dishes were exposed for 24 to 30 hrs to 2.0 μc/ml of H^3A. Following exposure, the cells were fixed in Carnoy's for 20 min, washed four times with 1M NaCl, several times with H_2O, and then coated with Kodak NTB-2 emulsion. The coated preparations were exposed for three days, then developed and stained with tetrachrome.

The technique was standardized by growing wild type cells in the presence of varying levels of DAP (40, 70, and 100 μg/ml). The clones that developed were labeled on the 12th day and processed for autoradiography. In the nonselective experiments wild-type cells (6×10^4) were inoculated in medium without DAP in 100 mm Petri dishes, grown for five to six days, labeled, and processed for autoradiography. The frequency of resistant clones determined in this nonselective system was compared to the frequency of resistant clones that developed under selective conditions (cells were seeded from the same suspension used in the nonselective experiments).

Study of reversion: Revertants were tested for by seeding $1–2 \times 10^6$ resistant cells (having been previously grown in DAP for 1 month) in coverslip bottles in the absence of the drug. The next day, 2.0 μc/ml H^3A was introduced into the medium for 24 hrs, and then the cells were fixed and processed in the usual manner and examined for sensitive cells.

RESULTS

Biochemical characterization of normal and resistant lines: Sensitive and resistant lines were characterized for both H^3A incorporation and level of AMP pyrophosphorylase activity (Tables 1 and 2). These data show a marked difference between resistant and sensitive lines for both adenine uptake and AMP pyrophosphorylase activity. The resistant lines, except PR-4 exhibit about 1%

TABLE 1

Uptake of radioactive adenine in resistant and sensitive clones

Lines	CPM/100,000 cells	Lines	CPM/100,000 cells
Sensitive clones		Resistant mutants	
MC FC_B	35,922	PR-7	328
MC_K	31,136	PR-16	48
MC_I	44,230	RFC-12	. . .
MC_J	70,242	R-10	100
		R-2	82
		RFC-8	58
		R-14	106
		R-18	82
		PR-4	3,648

of wild-type (sensitive) uptake and activity. The loss of enzyme activity correlated very well with the lack of incorporation of H^3A in intact mutant cells and aided in ruling out permeability mutants in the lines studied. This confirms the work of others in mammalian cells (LIEBERMAN and OVE 1960; BLAIR and HALL 1965). PR-4 though fully resistant, shows about 8% of wild-type uptake and activity. It is possible that this mutant is similar to the DAP resistant mutant described by KALLE and GOTS (1963) in Salmonella that retained AMP pyrophosphorylase activity, but had an altered specificity for DAP.

Identification of resistant clones by autoradiography: When cells from the wild-type stock were grown in the presence of various levels of DAP, exposed to H^3A, and processed for autoradiography, four classes of clones were discernable with respect to incorporation; high (H), low (L), intermediate (I), and mixed

TABLE 2

AMP pyrophosphorylase activity

Lines	μg protein	Total counts	Phosphorylated derivatives CPM/μg protein
Sensitive clones			
MC_I	4.8	11,131	2,085
MC_J	3.2	7,466	2,291
Resistant mutants			
PR-7	7.52	387,677	40*
PR-16	4.0	10,080	64
RFC-12	7.2	14,179	37
R-10	5.4	10,581	44
R-2	4.4	5,352	11
RFC-8	5.6	9,472	29
R-14	3.2	8,700	7
R-18	5.8	6,179	4
PR-4	1.64	10,360	155

* Separate experiment in which ten times more tritiated adenine was used. 95% of the label was recovered in all the experiments.

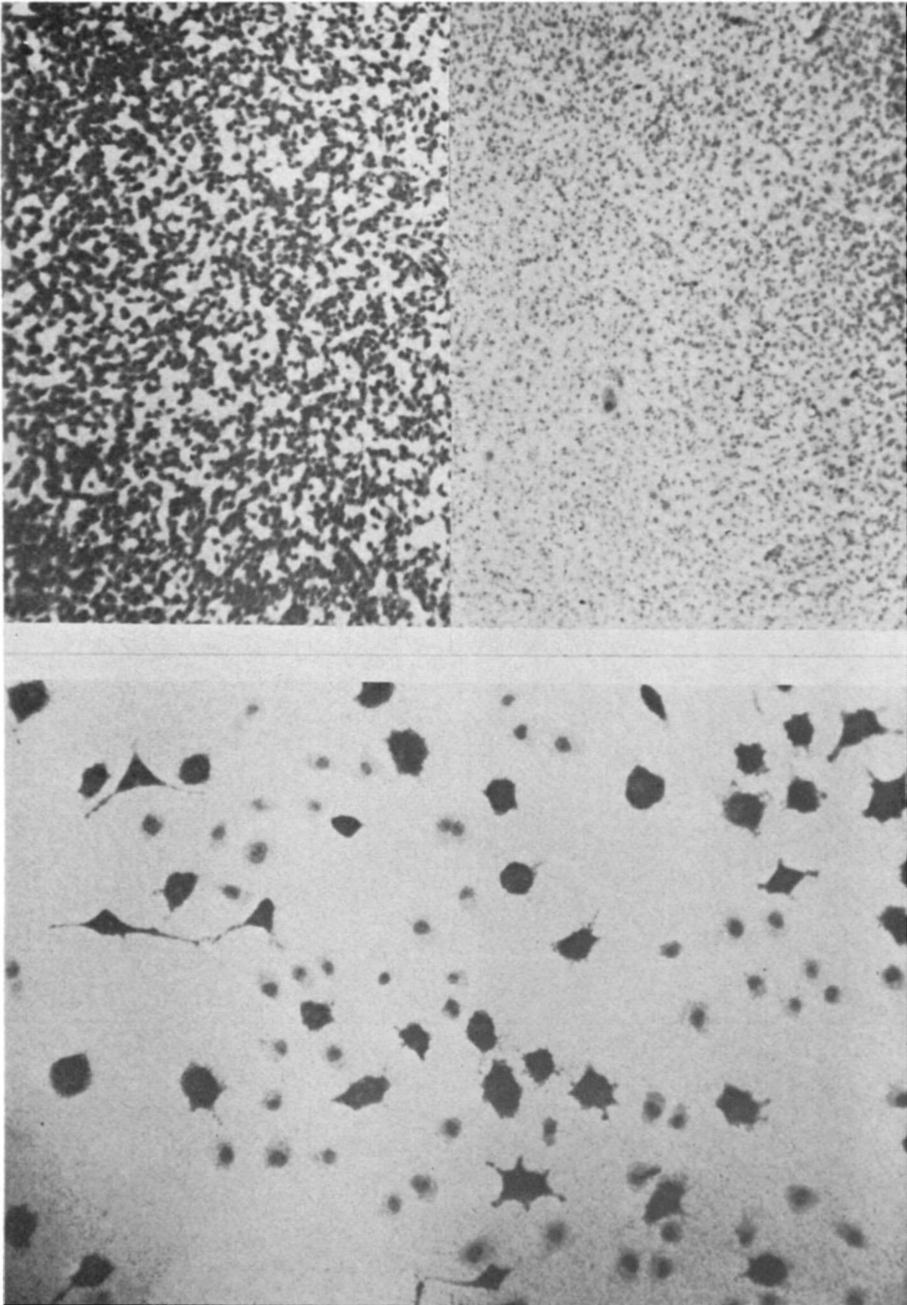

FIGURE 1.—Autoradiographic appearance of sensitive and resistant cells.

A. Sensitive line (MC_K) showing high level (H) of incorporation of tritiated adenine.

B. Resistant line (PR-16) demonstrating low level (L) of incorporation of tritiated adenine.

C. Artificial mixture of sensitive and resistant cells showing the clear distinction between sensitive and resistant cells.

clones. In the H clones, grains were contiguous to one another and consequently the underlying tetrachrome stain was not visible; the low clones had few overlying grains and the nuclear stain was readily apparent; in the I cells the grains, though not contiguous, were sufficiently dense to obscure the underlying stain; the mixed clones were composed of cells showing two distinct classes of incorporation. Known sensitive and resistant lines could clearly be distinguished by the autoradiographic method. All cells of sensitive lines show a consistent autoradiographic pattern and demonstrate high incorporation (H), while resistant cells treated in the same manner show the low (L) incorporation pattern (Figure 1).

Under selection, all clones that developed in the wild type population in the presence of 100 μg DAP/ml showed the L autoradiographic pattern (Figure 2). Single scattered cells showing an H pattern of incorporation were observed and were assumed to represent a nonviable class of cells that did not slough off in the presence of DAP as do most sensitive cells. At 70 μg DAP/ml, the majority of

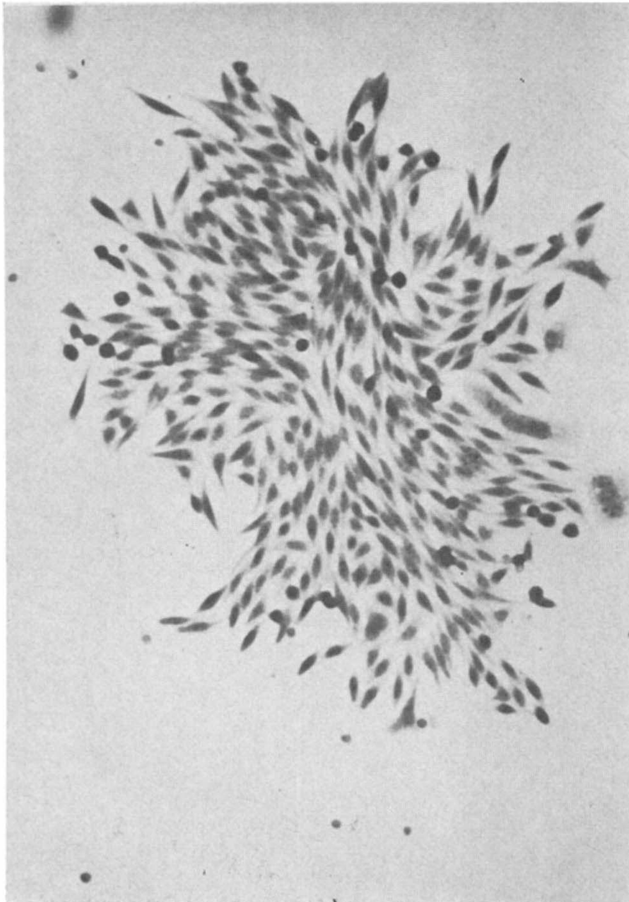

FIGURE 2.—Autoradiographic appearance of an L type clone growing in 100 μg DAP/ml. The black "dots" represent pyknotic sensitive cells.

the clones exhibited the L pattern, but some I clones, one H clone, and one mixed clone were observed. The I and H clones were small (approximately one-tenth of the L clone size). At 40 μg DAP/ml, the majority of clones were those of the low type, but more I type clones and one mixed clone were observed (Figure 3). The I clones were about three times larger here.

It was important to determine if the autoradiographic estimation of resistance correlated completely with the classifications arrived at by the direct selective method. Specifically, were the L pattern clones observed at 40 and 70 μg DAP/ml also resistant to 100 μg DAP/ml? If these L type clones were not resistant to 100 μg DAP/ml, then comparison of resistant frequencies under selective and non-selective conditions would not be possible. The fact that the low type clones at 40 and 70 μg DAP/ml were larger than the I and H clones at the same level suggested that these low type clones may represent faster growing "mutants" resistant to higher levels of the analogue than I and H clones. The difference in

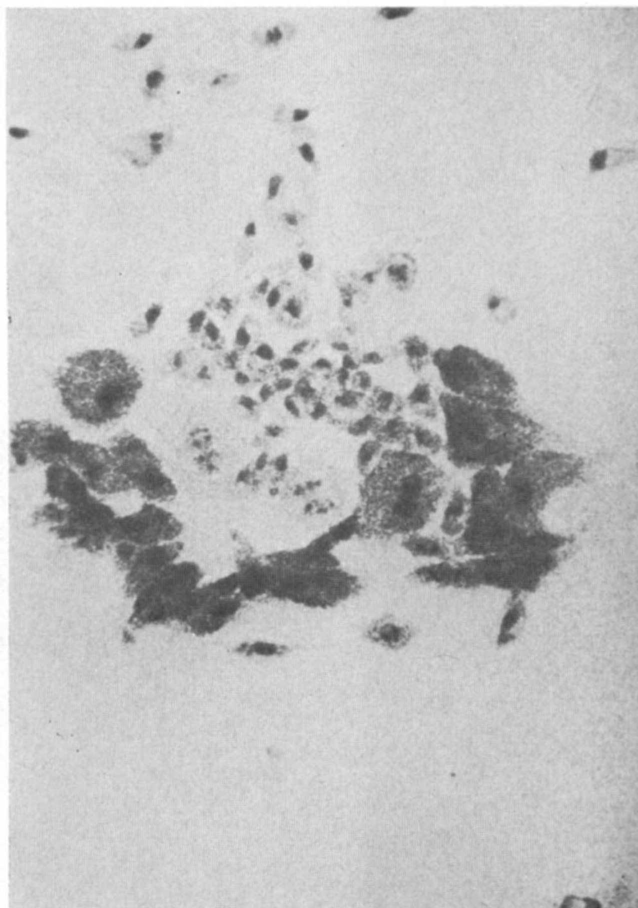

FIGURE 3.—Mixed clone appearing at 40 μg DAP/ml composed of intermediate (I) and low (L) type cells.

TABLE 3

*Number of various classes of clones grown in different concentrations of DAP as determined by autography**

Concentration of drug (μg/ml)	Total number of clones	Numbers of various types of clones observed			
		Low (L)	Intermediate (I)	High (H)	Mixed
100.0	177	177	0	0	0
70.0	173	165	6	1	1
40.0	24	14	10	0	1

* Cell growth occurred for a total of thirteen days and the media and DAP were changed twice during this period.

size of clones suggested a possible way of examining this question. A number (27) of large clones at the lower levels of drug concentration were isolated, and all were resistant to 100 μg DAP/ml and showed L incorporation. No intermediates with respect to levels of resistance were found. This is compatible with the interpretation that the L clones seen at 40 and 70 μg DAP/ml are identical to the L clones seen at 100 μg DAP/ml. Another test to determine if the low type clone is identical to the 100 μg DAP/ml resistant clones was to compare the frequency of mutants growing in 100 μg DAP/ml with the corrected frequency (total number of clones minus I and H clones) for the 100 μg DAP/ml class growing at the lower drug levels. The relative contribution of the I and H clones (ratio to total number of clones) was determined from autoradiographic analysis of clones growing at the lower levels (Table 3). For 40 and 70 μg DAP/ml, the corrected frequencies for the 100 μg DAP/ml class are respectively 1/3300 and 1/4000. The frequency of the 100 μg DAP/ml cells in five selective experiments ranged from 1/2500–1/6800 with the average being 1/4100. From these data, and since only one level of resistance (100 μg/ml) in respect to total lack of adenine incorporation has been found, it was concluded that the L type clone seen under nonselective conditions directly corresponds to the resistant clone isolated at 100 μg DAP/ml.

Estimation of the frequency of variants in the parental population by nonselective and selective methods: About 105,000 clones were studied by the nonselective technique. Of these clones, 31 were pure low type (Figure 4), and 5 were mixed clones composed of H and L cells. These data and the data for comparable clones under selective conditions were compared. The results are given in Table 4. The frequency of resistant mutants occurring under both conditions was not different (χ^2 test P value is about .34).

These results demonstrate directly that variants arise independently of DAP and lend further support to the existence of a one to one relationship of low type clones to 100 μg DAP/ml resistant variants. Variants arising independently of the drug are also shown by comparing the number of variants in newly isolated (4) and older (2) sensitive clones when grown in medium containing 100 μg DAP/ml. No resistant variants in 4.0×10^5 were found in the younger clones (about 21 generations old from isolation) while the older clones (138–161 genera-

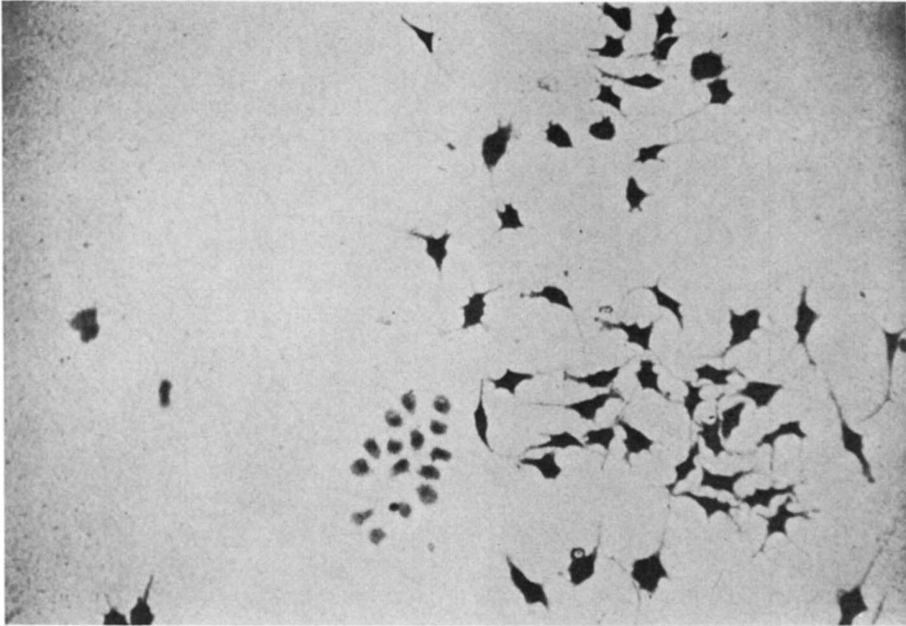

FIGURE 4.—Autoradiographic appearance of an L type clone composed of only L type cells surrounded by H type cells growing under nonselective conditions.

tions) had 7 and 8 resistant clones per 4.0×10^5 cells. If variants did not arise independently, one would expect that all clones would have approximately the same frequency of resistant cells.

TABLE 4

Number of resistant mutants under selective and nonselective conditions

	Experiment	Number of cells seeded	Number of clones counted	Number of resistant clones	Number of mixed clones*	Frequency†
B	Nonselective	12,000	6,000	1	1	1/6000
	Selective	300,000	44	44	.	1/6800
C	Nonselective	42,000	14,240	10	0	1/1400
	Selective	300,000	54	54	.	1/5600
D	Nonselective	36,430	11,379	3	0	1/3800
	Selective	306,000	121	121	.	1/2500
E	Nonselective	60,000	35,782	10	1	1/3600
	Selective	318,000	84	84	.	1/3800
F	Nonselective	94,500	38,022	7	3	1/5400
	Selective	303,750	72	72	.	1/4200
TOTAL						
	Nonselective	244,930	105,423	31	5	1/3400
	Selective	1,527,750	375	375	.	1/4100

* Not able to determine mixed clones in the frequency selection experiments.
† Frequency "rounded off" to nearest hundred.

Frequency of reversion as determined by the nonselective technique: The nonselective technique also permits the direct study of reversion. To determine if it is possible to detect sensitive cells at a low frequency, a mixture of sensitive and resistant cells was seeded in the frequency of one to 6×10^5. After one day's growth, H^3A was introduced; after 24 hrs, the cells were fixed and processed for autoradiography. Sensitive cells were detectable, and were shown to have undergone at least one division. In pilot experiments with resistant lines, only one revertant in one line has been found in about 3.0×10^6 cells. The revertant's autoradiographic pattern demonstrates a lower level than I incorporation (Figure 5). Other resistant lines (7) at the frequency tested (total number about 2.2×10^7) have shown no revertants, which suggests reversion frequency is low.

Nature of mixed clones and their possible significance in estimating mutation rate: Mixed clones could arise through contamination (origin of clone from more than one cell, e.g. one sensitive and one preexisting resistant cell); mutation (forward or reverse) occurring during development of the clone; or other genetic process (somatic recombination and/or segregation of heterozygote, deletion or chromosomal loss etc.).

The question of contamination was investigated by determining directly the frequency with which mixed clones occurred when equal numbers of sensitive and resistant cells were plated together at the same density used in the nonselective experiments. Of 988 clones counted, 394 were L, 594 were H, and 5 (0.5%) were mixed. In the nonselective experiment, the ratio of sensitive to resistant cells is about 3400:1, and the expected frequency of mixed clones due to contamination

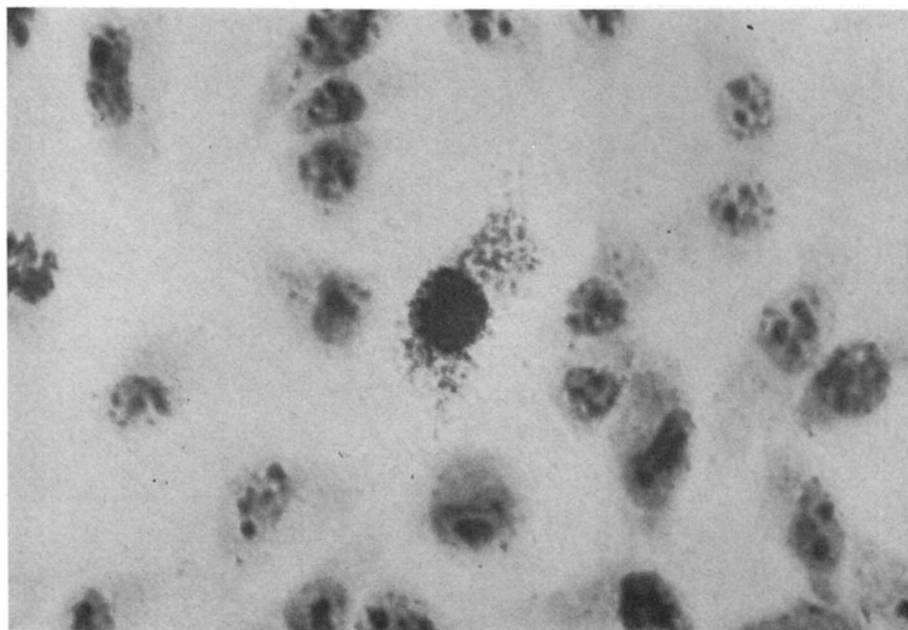

FIGURE 5.—Autoradiographic appearance of the revertant appearing in PR-16 in a background of resistant L type cells. The level of H^3A incorporation is below the intermediate level.

is about 6×10^{-6}; five mixed clones were observed out of 105,000 clones screened in the nonselective experiment (5×10^{-5}). Thus, it seems possible that some of the mixed clones observed may represent genetic changes occurring during clonal development, though the total number observed is too small to warrant statistical analysis.

DISCUSSION

The development of the autoradiographic technique described above has permitted the detection of purine analogue resistance in the absence of selection and thereby provided a direct demonstration of the pre-existence of resistant cells in wild type populations. The nonselective technique also permits the direct study of reversion, though the numbers involved are restrictive at present. Thus far only one partial revertant has been found in over 2×10^{7} cells. This method also opens up the possibility of detection and analysis of mixed clones which should aid in the understanding of the nature of variants occurring in animal cell cultures.

For full utilization of mixed clones, the resolving power of this system must be increased which would make possible the detection of various steps or levels of resistance. The detection and characterization of intermediates is of prime importance in this regard. It was shown that under nonselective conditions only H and L cells were seen, whereas under selective conditions (40 and 70 μg DAP/ml) I cells were also detected. A possible explanation may be that under nonselection the concentration of H^3A is approximately 200 times greater than with selection due to the competing effect of DAP used under selective conditions. It is possible that this relatively high H^3A concentration may mask I cells since there is an upper limit to grain counts due to cell surface area. If this is correct, it should be possible to detect I cells nonselectively by using lower H^3A levels.

Under selective conditions, intermediates with respect to drug levels were also not isolated. This may be due to the high level of analogue used when selecting the resistant lines (100 μg DAP/ml) and the biased selection of only large clones at lower levels of the analogue which by autoradiography were shown to be the L type. Others (LIEBERMAN and OVE 1960; BLAIR and HALL 1965) also have selected for resistant clones at high levels of DAP and have not isolated intermediates. If this is correct, it should be possible to select for intermediates by selecting the smaller clones developing at lower levels of the analogue.

The fact that the frequency of L type cells under nonselection is similar to the frequency of fully resistant cells under selection suggests that DAP does not have a significant mutagenic effect. NOVICK and SZILARD (1951) were also unable to detect any mutagenic action of DAP with *E. coli*; however BIESELE *et al.* (1952) have shown that DAP induces chromosome aberrations in animal cells. It is therefore possible that DAP may have a mutagenic effect in animal cells, but a more sensitive analysis of mutagenesis may be necessary to detect it or the chromosome aberrations induced may not be conducive to cell reproduction or existence and therefore will not be detected.

SUMMARY

DAP was used as a selective agent on an established murine cell line. Lines resistant to 100 μg DAP/ml occurred at a frequency of about 1/4100 cells.—It was found that the resistant lines, in contrast to the sensitive lines, lacked adenylic acid pyrophosphorylase and consequently incorporated very little exogenous adenine. Because of the difference in incorporation of adenine by sensitive and resistant lines, it was possible to develop a nonselective technique utilizing tritiated adenine and autoradiography to study the occurrence and development of resistant variants in the wild-type population.—By the use of the nonselective technique, it could be shown directly that variants arise independently of the drug.—Reversion rate can also be studied by this technique, one partial revertant being found in about 2.2×10^7 cells from 8 different cell lines.

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