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Characterization of Resistance to Amethopterin, 8-Azaguanine and Several Fluorinated Pyrimidines in the Murine Lymphocytic Neoplasm, P388

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The development of methods for cultivating mammalian somatic cells (Eagle '55 and Puck et al. '56) in a fashion analogous to the methods employed with microorganisms has suggested the possibility of studying the genetics of these cells. For such an investigation it is necessary to have clearly defined, heritable characteristics which can be used as genetic markers. Experiments were initiated with a population of mammalian cells to determine the types and the stability of variants resistant to several purine, pyrimidine and folic acid analogs.

A murine lymphocytic neoplasm, capable of growth both in cell culture and in the histocompatible mouse was selected as the cell strain. In selecting genetic markers the techniques of Law ('50), which had been used to obtain resistant sublines *in vivo*, were modified. Investigated were the effects of several cytotoxic analogs upon the growth of single cells to colonies which was considered to be the period when maximum sensitivity occurred. At each increased level of analog a new subline was cloned, the cells established as a resistant step and their characteristics studied, both in culture and in mice.

This report describes the techniques used to select drug-resistant sublines. It further characterizes the resistance of these sublines to the various analogs, their cross-resistance and collateral sensitivities to the other analogs used in this study, and to related compounds. The stability and other characteristics of the markers in cells maintained in culture and in serial passages in animals have also been investigated.

A report of the data for a few of these variant sublines has already been presented (Roosa and Herzenberg, '59). Several other reports Fisher ('59); Aronow ('59); Lieberman and Ove ('59); Szybalski and Smith ('59); and Harris and Ruddle ('60) have also described the development and the use of resistant sublines as markers in various mammalian cells in culture. The present study serves to summarize our characterization of these markers, and presents a baseline for studies (1) by Bradley et al. ('62), concerned with the transfer of genetic material by means of extracts rich in DNA and (2) by Brockman et al. ('62), and Davidson et al. ('62) concerned with elucidating the mechanism responsible for the resistance to 8-azaguanine, one of the inhibitors used.

MATERIALS AND METHODS

These studies were initiated with the continuous cell line, designated P388, which was established *in vitro* by Dawe and Potter ('57). A cloned line of cells, P388/P, sensitive to all the analogs in this study, has been considered as the parental population.

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Herzenberg and Roosa ('60) reported the nutritional requirements and the cultural conditions required for the growth of dispersed cell suspensions into colonies. Eagle's basal medium supplemented with 5% whole calf serum, 1 mM Sodium Pyruvate, and 0.2 mM L –serine was used. The medium was changed three times weekly, the cultures usually split once a week and pyrex and soda glass bottles were used interchangeably.

Resistant cultures were obtained by exposing a bottle containing a full sheet of cells to medium containing a molar level of inhibitor[7] which was slightly more than sufficient to inhibit growth. The cell sheet most often degenerated and after a period of time a few cells, whose appearance was considered as "healthy," slowly grew to colonies. The number of colonies, the time of appearance, and growth characteristics varied with each compound used. Cells from these colonies were isolated and maintained on the same level of inhibitor until they could be cloned by a technique similar to that described by Puck et al. ('56). This was done by plating a few cells in a Petri dish in a CO_2 incubator (37°C with a humid atmosphere of 5% CO_2 and 95% air). When the cells grew into small colonies, they were removed from the dish either with a platinum loop or by isolating a colony within a glass cylinder and removing them with a Pasteur pipette. Subsequently, the cells were replated and reisolated using either of the above techniques several times.

When the cells had grown to a sizeable population, they were split into three flasks by scraping the glass surface with a rubber policeman, pipetted to new flasks, additional medium added, and the pH corrected to 7.0 with CO_2. One flask of cells was maintained on the same level of inhibitor as described previously, the second in the absence of inhibitor, and the third exposed to a higher level of the inhibitor. Generally, the next higher concentration of analog used was one in which a preliminary screening showed the molar concentration to be growth inhibitory for all the cells resistant to the previous lower concentration. The above procedure was repeated each time cells grew rapidly at the next higher concentration of analog. At these times the cells were cloned and the populations were considered as "resistant steps." At each increase in drug concentration, therefore, a new subline or "resistant step" was obtained.

The levels of resistance for the various sublines were determined as follows: Graded molar concentrations of the inhibitor were pipetted into duplicate dry 60 mm pyrex Petri dishes. Four to eight control dishes, void of inhibitor, were included. Fresh medium was then added to a rapidly growing culture (whose medium had been changed the day before), the cells scraped from the glass wall with a rubber policeman, and counted in a hemocytometer. Using a Cornwall Automatic Syringe, 5 mls of a suspension of 100 cells/ml, in the previously described medium were withdrawn from the flask in

[7] *Inhibitors used, their abbreviations and sources*

Amethopterin (Methotrexate, sodium)	AMT	Lederle Laboratories
5-Fluorouracil	FU	Cancer Chemotherapy National Service Center
5-Fluorouridine	FUR	Cancer Chemotherapy National Service Center
5-Fluro-2′-deoxyuridine	FUDR	Cancer Chemotherapy National Service Center
8-Azaguanine	AZG	Cancer Chemotherapy National Service Center
8-Azaguanylic acid	AzaGMP	Cancer Chemotherapy National Service Center
8-Azaguanosine	AzaGuR	Cancer Chemotherapy National Service Center
6-Mercaptopurine	6-MP	Cancer Chemotherapy National Service Center

which the cells were kept dispersed with a revolving teflon-coated magnetic bar, and dispensed to each dish. The tray of dishes was placed in the CO_2 incubator for seven days. At the end of the incubation period the colonies were stained by adding 2 ml of 0.5% crystal violet for 30 minutes, carefully washed in tap water, and then inverted to dry. The colonies were counted on a grid at 20 × magnification with a dissecting microscope. In various experiments counts were duplicated, usually by at least two investigators. In addition to these precautions, many of the experiments were repeated after a year or more, some in two different laboratories using different medium, incubators, and etc. Each figure represented in the tables, is an average value for a minimum of two dishes from a single experiment.

The cloning efficiency for each experiment was computed; the values ranged from 10–75% with an average of 27.7%.

Growth of cells in suspension

Experiments concerned with transferring genetic characteristics (Bradley et al., '62) necessitated the collection of massive numbers of cells in order to extract DNA from both the sensitive and some of the resistant populations. When cells attach to glass, one is, of course, limited by the area of the glass surface provided; if on the other hand they will grow in a cell suspension, large quantities of cells can be readily obtained. Cultures of both the parental population and of the most resistant 8-azaguanine cells ($P388/AZG^{r-8}$) were adapted to grow in medium in which the cells were kept in suspension with a rapidly rotating teflon-coated magnetic bar. Eagle's basal medium in double concentration was used; Earle's salts were modified to give 10 × mono-sodium phosphate, and the calcium chloride omitted. Supplements included 10% whole calf serum, 1 mM sodium pyruvate, 0.2 mM L-serine.

Preservation of cell lines

Preservation and maintenance of the selected sublines at −70°C for extended periods (to date the longest test time has been two years) did not appear to alter their degree of resistance or growth characteristics.

Animal studies

P388 cells grown *in vitro* produced progressively growing neoplasms when injected into histogenetically compatible mice. Cells were most often injected intraperitoneally in order to obtain ascitic tumors; or by the subcutaneous route in order to secure solid tumors. The mice used in all cases were the $(BALB/c \times DBA/2)F_1$ animals from the NIH animal production unit.

The tumor material was tested for its level of resistance to inhibitor by injecting the compound intraperitoneally and either comparing the mean survival time when the tumor was in the ascitic form or by comparing the weights of the excised tumors (a localized mass in the subcutaneous connective tissues) when grown in the solid form. Further comparisons were also often made by removing ascitic cells and re-establishing them in cell culture, where experiments could then be carried on in Petri dishes.

RESULTS

Amethopterin (AMT) at a concentration of 4×10^{-8} M was added to the medium of a fully grown culture of cells in a Blake bottle. In a short period of time the cells showed degenerative changes and most of them fell off the glass. The medium was replaced at irregular intervals depending upon changes of pH and the general appearance of the culture, but was not allowed to remain longer than one week without being substituted. Weeks later a few healthy cells, which eventually grew to form colonies, were observed on the surface of the bottles. Some of the colonies were isolated by picking up cells with a small platinum loop and transferring them to a Petri dish or small flask for testing and cloning.

The response of the parental population to graded concentrations of AMT is presented in the first column of table 1. A concentration of 2×10^{-9} M AMT does not inhibit the growth of these cells; the 100% value in this column refers to the fact that as many cells will form colonies at this concentration of AMT as will be

TABLE 1

Per cent of cells forming colonies in vitro in the presence of amethopterin (AMT) as compared with growth in the absence of the inhibitor

	Subline	388/P[1]	388/AMT^{r-1}[2]		388/AMT^{r-2}		388/AMT^{r-3}		388/AMT^{r-4}		388/AMT^{r-5}		388/FU^{r-2}	388/FUR^{r-2}	388/FUDR^{r-1}	388/FUDR^{r-2}	388/AZG^{r-3}
	Subline number	22	1	1	3	3	7	7	9	9	51	51	46	19	21	47	55
	Molar conc. AMT maintained on		4×10^{-8}		1×10^{-7}		2×10^{-7}		8×10^{-7}		8×10^{-6}						
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Molar concentration of AMT	1×10^{-9}													100	100	100	100
	2×10^{-9}	100[3]												74	59	89	71
	5×10^{-9}	1											100	45			
	1×10^{-8}	0											48	0	0	0	0
	2×10^{-8}		100	100									0				
	5×10^{-8}		40	36													
	1×10^{-7}		0	4	100	100											
	2×10^{-7}			0	41	80	100	100									
	5×10^{-7}				0	0	30	15		100							
	1×10^{-6}						0	0	100	87							
	2×10^{-6}								44	67							
	5×10^{-6}								0	0	100	100					
	1×10^{-5}										39	45					
	2×10^{-5}										0	0					

[1] The parental population.

[2] Indicates the analog to which the cells are resistant and the degree of resistance.

[3] $\frac{\text{No. of cells forming colonies in presence of drug}}{\text{No. of cells forming colonies in absence of drug}} \times 100$.

formed in the absence of the inhibitor. At 1×10^{-8} M none of the cells is able to divide rapidly enough to form colonies in the seven days incubation.

Cells from the first subline resistant to AMT are denoted as "step r-l," these cells were selected and maintained at 4×10^{-8} M AMT and as was the characteristic of all the AMT resistant steps, growth of the cells when first isolated was irregular. Continued growth on the inhibitor, however, soon gave a subline whose multiplication rate was similar to the cells comprising the parental population. Once the "r-l step" had been isolated, it was split and one of the cultures grown in the absence of the inhibitor for a period of several months, in order to determine whether growth in the presence of the analog was due to a temporary adaptive change rather than a true heritable change. The next column in the table tabulates the sensitivity of these cells to AMT after an extended period of growth in the absence of the analog and shows that the marker was retained. The minimum test period throughout this study for growth in the absence of analog has been three months. However, many of the experiments were completed after six months to one year of growth in the absence of inhibitor.

Five steps resistant to AMT are shown in table 1, each step representing rather small increases in resistance to AMT. Thus, it can be said that resistance to AMT is characterized by many small steps, and in every case, the selection period for each step takes many months. Once obtained, however resistant populations were very stable as can be noted by comparing the response to AMT of each step maintained both in the presence of and in the absence of the analog. Although the steps are relatively small, there is a good spread between the first and the last and more than a 1,000-fold increase separates the parental cells from the "r-5" resistant subline.

Similar studies were also made from representative populations of sublines resistant to other inhibitors (described later) to determine whether any of them were cross-resistant to amethopterin. These data are also presented in table 1 and show that there was no significant cross-resistance as none of these sublines form colonies at concentrations suitable for the AMT^{r-1} subline.

Previous studies (Roosa and Herzenberg, '59) showed that cross-resistance to aminopterin and to mono- and dichloroamethopterin does occur with the AMT-resistant lines.

5-Fluorouracil (FU)

Two sublines resistant to FU were selected; both represented relatively slight increases in resistance to inhibitor. This compound was the most difficult to work with from the point of securing resistant cells. The parental cells and the cells from the first resistant step were incubated and were about to be discarded when suddenly they appeared healthy and began growing rapidly in the presence of FU. On numerous occasions during this period of trial the cells looked so poor that the medium was replaced with growth medium void of inhibitor. After recovery and growth, the inhibitor was again added. The parental cells were unaffected by 5×10^{-8} M FU but were completely inhibited from forming colonies at 2×10^{-7} FU. The search for resistant cells began by incubating sensitive cells in complete medium containing 5×10^{-7} M FU. Once cells were cloned at this level, they were split, one group maintained at the same level, one in the absence of FU, and in the other the concentration was doubled. Eventually, a second resistant subline was isolated at 1×10^{-6} M FU. Both resistant lines grew well and appeared to be capable of indefinite maintenance in either the presence or absence of FU.

Cells from each of the two resistant sublines were titrated with graded molar concentrations of FU, and as can be seen in table 2, were stable, heritable markers.

Selected sublines from each of the other resistant populations were tested for cross resistance to FU and only the FUR^{r-2} subline (table 2) appeared to have an increased number of cells capable of growth on inhibitory levels of FU.

5-Fluorouridine (FUR)

Results of attempts to secure resistant sublines to FUR were similar to those with FU in that the resistant steps were small

TABLE 2

Per cent of cells forming colonies in vitro in the presence of 5-fluorouracil (FU) as compared with growth in the absence of the inhibitor

Subline	388/P	388/FU^{r-1}		388/FU^{r-2}		388FUR^{r-2}	388/FUDR^{r-1}	388/FUDR^{r-2}	388/AMT^{r-4}	388/AMT^{r-5}	388/AZG^{r-3}
Subline number	22	15	15	46	46	19	21	47	9	51	55
Molar conc. FU maintained on		5×10^{-7}		1×10^{-6}							
Molar concentration of FU	%	%	%	%	%	%	%	%	%	%	%
5×10^{-8}	100					100	100	100	100	100	100
1×10^{-7}	79					89	64	78	58	85	87
2×10^{-7}	0		100			42	33		15	31	18
5×10^{-7}		100	66			37	0	3	0	0	0
1×10^{-6}		13	9	100	100	3		0			
2×10^{-6}		0	0	54	63	0					
5×10^{-6}				0	0						

and extremely difficult to obtain (table 3). Here, again, it was possible to obtain only two steps, but both appeared stable and once established grew well.

Cells from the parental population were uninhibited at 2×10^{-9} M FUR but were unable to form colonies at 1×10^{-8} M FUR. The first resistant subline was selected at 1×10^{-8} M FUR, these cells were subsequently incubated at 8×10^{-8} M FUR in order to obtain the second resistant step. Titers indicated in table 3 show that both resistant sublines, after growth in the absence of FUR were stable, heritable, genetic markers.

Although the steps are few and small in the FU and FUR resistant sublines, growth in the presence of the analogs was good and these cells could be readily distinguished from the parental population. In reconstruction experiments in which 1 or 10% resistant cells were mixed with parental cells and then plated in the presence of the analog, a proportional number of colonies was recovered, thus indicating that these cells could be readily recovered from a mixed population.

Cross-resistance experiments with the other sublines show some variability in the response to graded concentrations of FUR by both the FU and FUDR resistant sublines. Although they could not be considered cross-resistant in the degree that aminopterin is to amethopterin, there certainly is a greater proportion of cells in these populations which is capable of colony formation at levels inhibitory to cells in the parental populations. Sublines from the AMT and AZG resistant cells did not exhibit this ability. Both the low and high resistant steps to FUDR were compared regarding their cross-resistance to FUR, as they had been compared with FU (table 2), and in both cases the increase of FUDR resistance did not appear to increase cross resistance to either of the other fluorinated analogs.

5-Fluorodeoxyuridine (FUDR)

To develop a subline resistant to FUDR a concentration of 5×10^{-9} M FUDR was added to a culture of parental cells. The isolation of the first resistant subline with this analog was obtained in a fashion similar to those previously described and

TABLE 3

Per cent of cells forming colonies in vitro in the presence of 5-Fluorouridine (FUR) as compared with growth in the absence of the inhibitor

Molar concentration of FUR	Subline	388/P	388/FUR^{r-1}		388/FUR^{r-2}		388/FU^{r-2}	388/FUDR^{r-1}	388/FUDR^{r-2}	388/AMT^{r-2}	388/AMT^{r-4}	388/AMT^{r-5}	388/AZG^{r-3}
	Subline number	22	6	6	19	19	46	21	47	R-46	9	51	55
	Molar conc. FUR maintained on		1×10^{-8}		8×10^{-8}								
		%	%	%	%	%	%	%	%	%	%	%	%
2×10^{-9}		100					93	100	100	100	100	100	100
5×10^{-9}		17						72		15	29	71	95
1×10^{-8}		0	100	100			65	56	34	0	1	0	14
2×10^{-8}			47	43				30	17		0		0
5×10^{-8}			37	4	100	100	5	8					
1×10^{-7}			0	0	32	51		0	0				
2×10^{-7}					5	3							
5×10^{-7}					0	0							

was also stable. The isolation of the second subline contrasted with the other lines in that it was clearly a large step to resistance. When the "r-l" cells had been cloned and split, one group was placed at 5×10^{-8} M FUDR; these cells appeared unhealthy at first but quite rapidly established themselves at this level of inhibitor. After cloning, the cells were tested and it was found that all were capable of forming colonies at concentrations of FUDR far exceeding that to which they had been exposed. In fact, it took more than a one log increase in the molar concentration to completely inhibit growth. These results are shown in table 4.

It should also be pointed out that when these cells are maintained for extended periods in the absence of FUDR, they are no longer capable of growth at the high level established by cells grown continuously in the presence of inhibitor. However, the cells certainly have not lost their resistance marker as they are still capable of growth at approximately the highest level of FUDR to which they were previously exposed. They rapidly reacquire the ability for growth at higher levels when incubated continuously at 5×10^{-8} M FUDR.

Cross-resistant studies demonstrated that the second step FU resistant subline grew as well or better on FUDR as did the first step FUDR line. Unfortunately, we were unable to secure a higher resistant step to FU in order to determine if they would be cross resistant to higher levels of FUDR. None of the other sublines was found to be cross-resistant to this inhibitor.

8-Azaguanine (AZG)

The first resistant step to AZG, as shown in table 5, is very small when compared with the sensitive population, and it appears to be less than what has been previously considered as a "resistant step." Selection of these cells was accomplished at 1×10^{-6} M AZG. When using inhibitors which yield large steps as a result of exposing the cells to excessive amounts of the inhibitor, there could be a by-passing of numerous small steps at the lower concentrations of inhibitor, each one a stable subline such as this. After months of growth in the absence of inhibitor, this

TABLE 4

Per cent of cells forming colonies in vitro in the presence of 5-Fluorodeoxyuridine (FUDR) as compared with growth in the absence of the inhibitor

	Subline	388/P	388/FUDR^{r-1}		388/FUDR^{r-2}		388/FU^{r-2}	388/FUR^{r-2}	388/AMT^{r-2}	388/AMT^{r-4}	388/AMT^{r-5}	388/AZG^{r-3}
	Subline number	22	21	21	47	47	1/6	19	R-46	9	51	55
	Molar conc. FUDR maintained on		5×10^{-9}		5×10^{-8}							
Molar concentration of FUDR		%	%	%	%	%	%	%	%	%	%	%
	1×10^{-10}	100						100	100			
	2×10^{-10}	93						50	80	100	100	100
	5×10^{-10}	26	100	100			100	38	50	60	64	57
	1×10^{-9}	2	17	57			95	6	3	14	3	42
	2×10^{-9}	0					78	0	0	0	0	23
	5×10^{-9}		0	0			2					0
	1×10^{-8}						0					
	2×10^{-8}											
	5×10^{-8}											
	1×10^{-7}											
	2×10^{-7}											
	5×10^{-7}											
	1×10^{-6}				100	100						
	2×10^{-6}				69							
	5×10^{-6}				39							
	1×10^{-5}				0							

TABLE 5

Per cent of cells forming colonies in vitro in the presence of 8-azaguanine (AZG) as compared with growth in the absence of inhibitor

	Subline	388/P	388/AZG^{r-1}		388/AZG^{r-2}		388/AZG^{r-3}		388/FU^{r-2}	388/FUR^{r-2}	388/FUDR^{r-1}	388/FUDR^{r-2}	388/AMT^{r-2}	388/AMT^{r-4}
	Subline number	22	RB	RB	44	R–58	55	550Sp	46	19	21	47	R–46	9
	Molar conc. AZG maintained on		1×10^{-6}		2×10^{-5}		1×10^{-4}							
Molar concentration of AZG		%	%	%	%	%	%	%	%	%	%	%	%	%
	5×10^{-8}	100												
	1×10^{-7}	90	100						100	100	100	100	91	86
	2×10^{-7}	79	—	100						89	46	70	79	17
	5×10^{-7}	15	79	68					0	0	1	22	15	1
	1×10^{-6}	0	60	76							0	0	0	0
	2×10^{-6}		48	50	100	100								
	5×10^{-6}		13	9	58	65								
	1×10^{-5}				40	52								
	2×10^{-5}				25	30								
	5×10^{-5}				0	0								
	1×10^{-4}							100						
	2×10^{-4}							48						
	5×10^{-4}													
	1×10^{-3}						100							
	2×10^{-3}						92							
	5×10^{-3}						24							
	1×10^{-2}						0							

"r-l" population was titered against graded concentrations of AZG, and the results shown in column 3 attest to its stability.

This first step is also of importance as a reference point for the studies of Bradley et al. ('62) concerned with transfer of genetic markers with preparations rich in DNA.

Cells were also isolated after incubation of P388/P in 2×10^{-5} AZG. These cells represent the second resistant step and table 5 presents the data to show that they could be clearly distinguished from the parental cells, even after lengthy growth in the absence of AZG.

The "r-3" subline, derived from a population representing the second (r-2), was isolated as a large step and is noteworthy in that these cells were isolated after growth on 1×10^{-4} M AZG; yet 100% of the cells is capable of colony formation at a one long molar higher concentration. In order to eliminate cell multiplication, the AZG was added to a concentration of 1×10^{-2} M, an increase of four logs over that required to inhibit the parental population, thus clearly establishing this subline as an excellent genetic marker. When these cells are grown in the absence of analog for an extended period and then retitered, it should be noted that they will not clone at the same high concentrations as cells continuously maintained on AZG. However, it must be pointed out that 100% of these cells is capable of growth at 1×10^{-4} M AZG which is the highest level of drug to which they have been exposed. The cells referred to (column 7, table 5) were grown in a spinner culture and titrated against inhibitor after six months of logarithmic growth in the absence of AZG. Cells from this subline growing on glass in the absence of AZG were also tested and showed a similar response to the analog.

Table 5 indicates that when representative sublines resistant to other inhibitors were tested against AZG, none was found to be cross-resistant although a level of 5×10^{-7} M AZG was required to clearly distinguish them from the first step population.

Additional cross-resistant experiments with the AZG^{r-3} resistant line are shown in table 6. These show a complete cross-resistance to 6-mercaptopurine (6-MP); in comparing the differences between the

TABLE 6

A comparison of the ability of cells from the 8-azaguanine sensitive and resistant sublines of P388 to form colonies in vitro in the presence of various inhibitors

Inhibitor	8-Azaguanylic		8-Azaguanosine		6-Mercaptopurine	
Subline	388/P	388/AZG^{r-3}	388/P	388/AZG^{r-3}	388/P	388/AZG^{r-3}
Subline number	22	55	22	55	22	55
Molar conc. AZG maintained on		1×10^{-4}		1×10^{-4}		1×10^{-4}
Molar concentration of inhibitor	%	%	%	%	%	%
2×10^{-9}					100	
5×10^{-9}					62	
1×10^{-8}						
2×10^{-8}					15	
5×10^{-8}					0	
1×10^{-7}	100		100			
2×10^{-7}	98		84			
5×10^{-7}	57		75			
1×10^{-6}	0		17			
2×10^{-6}			0			
5×10^{-6}						
1×10^{-5}						
2×10^{-5}				100		
5×10^{-5}		100				
1×10^{-4}		51		94		
2×10^{-4}		0		0		100
5×10^{-4}						51
1×10^{-3}						0

sensitive and resistant populations when using either AZG or MP there is a one log increase in the spread of values when using 6-MP.

Studies with 8-azaguanylic acid and 8-azaguanosine show a graded cross resistance. The AZG resistant cells, although capable of growth at levels of both 8-azaguanylic acid and 8-azaguanosine which would be lethal (200 fold) to the parental cells, do, however, exhibit a degree of sensitivity to both compounds. By comparing tables 5 and 6 it can be determined that the AZG resistant cells are more sensitive to both compounds than to 8-azaguanine.

Animal studies

It was previously mentioned that when the proper strain of mice is injected intraperitoneally or subcutaneously with P388 lymphoblasts that have been maintained *in vitro* tumors will arise and cell suspensions from these tumors may at any time be re-established back in cell culture. The establishment of these tumors is, however, considerably slower than animal passaged transplants. The cells when replaced in culture generally grow with a low cloning efficiency, in some of the sublines the cells had to be maintained in culture for extended periods before attempting experiments. In addition to this low cloning efficiency the addition of growth inhibitors to the media further impeded the cells. Therefore it was difficult to compare the following studies with the previous experiments. The results are that most of the resistant lines retained a resistance which was at a somewhat lower level than when they were originally injected into the animals. With the parental cells this was, however, not the case, a line was selected which grew well *in vivo*, re-established rapidly *in vitro*, and whose drug response was consistently similar to the original population.

Most animal passaged lines were discontinued after experiments were completed to determine their titer in analog, but several were retained in culture in growth media void of inhibitors. One culture, P388/AMT^{r-2}, has recently been retested after growth in the *absence of analog* during its transfer in animals (12 months) and for a period (18 months) in cell culture. These cells cloned as well as any of our *in vitro* lines and when retested with the original drug, amethopterin, were capable of growth at the same levels of the inhibitor as originally observed. These data are not included in the table since only one subline was so tested; just those lines tested within a short time after their several passages *in vivo* are included.

The same subline, R-63, which originated from P388/P cells, was used as the base-line test for parental cells in all the studies concerned with cell responses to analogs after growth *in vivo*. The first column of table 7 presents the response of this subline to amethopterin and as expected, is almost identical to the original parental population which has been maintained continuously in cell culture (table 1). On the other hand, the two AMT resistant sublines tested, "r-2" and "r-3," have both shifted to a position of being slightly more sensitive than when they were originally injected into mice.

Table 7 indicates that there was no cross-resistance of the other resistant sublines to AMT. Here again there is a slight shift towards sensitivity to AMT.

After four passage generations in mice the AMT^{r-2} line was compared with a parental population by injecting an equivalent number of cells and treating the mice IP with 2.0 mg/kg/day of AMT. The results in table 8 show that this dosage is capable of extending the survival of mice injected with the parental cells by 21.6% over the controls (non-treated animals) in the same group, whereas the treatment did not extend the survival of mice bearing AMT^{r-2} cells after treatment. This experiment was repeated with a different tumor line, started from representative *in vitro* cells from the same "r-2 subline," and gave identical results.

When the same tumors were treated intraperitoneally with 3 mg/kg/day of AMT, the survival of the parental population was increased by 51%, whereas two experiments using the second step resistant cells and the same dosage of AMT were increased by only 5.3%.

At the eighth passage generation the effects of AMT on the weight of solid tumors were studied. Data, shown in table 9 point out that while the parental tumor

TABLE 7

Per cent of cells after transplantation in (BALB/c × DBA/2)F1 mice capable of forming colonies in vitro in the presence of amethopterin (AMT) as compared with growth in the absence of the inhibitor

	Subline	388/P	388/AMT^{r-2}	388/AMT^{r-3}	388/FU^{r-2}	388/FUR^{r-1}	388/FUR^{r-2}	388/AZG^{r-2}	388/AZG^{r-3}
	Subline number	R–63	R–46	R–37	R–49	R–53	R–61	R–58	R–68
		%	%	%	%	%	%	%	%
Molar concentration of AMT	1×10^{-9}				100			97	86
	2×10^{-9}	100			65	100	100	65	72
	5×10^{-9}	5	90		48	71	46	4	26
	1×10^{-8}	0	58		0	23	4	0	9
	2×10^{-8}		51			0	0		0
	5×10^{-8}		26	86					
	1×10^{-7}		0	44					
	2×10^{-7}			20					
	5×10^{-7}			1					
	1×10^{-6}			0					
	2×10^{-6}								
	5×10^{-6}								

TABLE 8

Per cent increase in survival time after treatment with amethopterin (AMT) of mice [(BALB/c × DBA/2)F1] carrying amethopterin resistant and sensitive populations of P388

Cell line	Subline number	Tran. gen.	CMPD	mg/kg[1]	Survival of animals		
					Span in days	MST[2]	Increase in survival
							%
			—	—	24–29	25.5	—
P388P	R-26	4	AMT	2.0	24–36	31.0	21.6
			AMT	3.0	32–46	38.5	51.0
			—	—	18–22	19.0	—
P388/AMT^{r-2}	R-21	4	AMT	2.0	17–21	18.8	0.0
			AMT	3.0	17–22	20.0	5.3
			—	—	20–24	22.8	—
P388/AMT^{r-2}	R-25	4	AMT	2.0	20–24	22.8	0.0
			AMT	3.0	22–25	24.0	5.3

[1] Treatment on alternate days, a total of seven times.
[2] Eight animals per group, 1×10^6 cells/animal.

TABLE 9

Sensitivity to amethopterin (AMT) of parental (P388/P) and resistant (P388/AMT^{r-5}) lymphocytic neoplasms after 5 transfer generations in mice (BALB/c × DBA/2)F1

Cell line	No. mice 1×10^6 cells	CMPD	Dose mg/kg x days[1]	Tumor weight (mgm) at 25 days
P388/P	8	—	—	519.0(218–991)
	8	AMT	3×9	231.4(75–597)
P388/AMT^{r-5}	8	—	—	202.0(60–486)
	8	AMT	3×9	272.8(32–648)

[1] Treatment on alternate days.

is sensitive to this dosage of inhibitor, the resistant tumor, (8 passages in the absence of AMT), having retained its trait, is able to grow well in the presence of AMT.

Both FU resistant sublines were passaged in animals, it was found that when these cells were returned to cell culture they also became more sensitive to FU. As shown in table 10, the parental line remained essentially unchanged in its response to FU while two FU resistant sublines are now less resistant than before animal passage. Cross-resistant studies were completed with only two sublines, both AZG resistant, and they were now slightly more sensitive than the parental lines.

Similar studies were also completed with parental and FUR resistant sublines, and these data are shown in table 11. Here there is a wide spread in the response of

TABLE 10

Per cent of cells after transplantation in (BALB/c×DBA/2)F1 mice capable of forming colonies in vitro in the presence of 5-Fluorouracil (FU) as compared with growth in the absence of the inhibitor

	Subline	388/P	388/FU^{r-1}	388/FU^{r-2}	388/AZG^{r-2}	388/AZG^{r-3}
	Subline number	R-63	R-36	R-49	R-58	R-68
	FU originally on		5×10^{-7} M	1×10^{-6} M		
Molar concentration of FU		%	%	%	%	%
	1×10^{-8}				100	94
	2×10^{-8}	100			90	8
	5×10^{-8}	95	100		17	0
	1×10^{-7}	71	65	100	0	
	2×10^{-7}	18	8	74		
	5×10^{-7}	0	0	57		
	1×10^{-6}			22		
	2×10^{-6}			0		
	5×10^{-6}					

TABLE 11

Per cent of cells after transplantation in (BALB/c×DBA/2)F1 mice capable of forming colonies in vitro in the presence of 5-Fluorouridine (FUR) as compared with growth in the absence of the inhibitor

	Subline	388/P	388/FUR^{r-1}	388/FUR^{r-1}	388/FUR^{r-2}	388/AMT^{r-2}	388/AZG^{r-3}
	Subline number	R-63	R-39	R-53	R-42	R-46	R-68
	FUR originally on		1×10^{-8} M	1×10^{-8} M	8×10^{-8} M		
Molar concentration of FUR		%	%	%	%	%	%
	1×10^{-9}	100				100	83
	2×10^{-9}	72				87	61
	5×10^{-9}	59				52	
	1×10^{-8}	28	100	100	100	0	53
	2×10^{-8}	8	50	90	86.5		
	5×10^{-8}	0		40	4.4		0
	1×10^{-7}		0	0	0		
	2×10^{-7}						
	5×10^{-7}						

the parental cells to the analog, but two different first resistant step sublines behaved similarly to the original populations response to FUR. On the other hand, the second step resistant cells appear to have reverted to give a response similar to the "r-1" cells. Neither the AMT nor the AZG resistant sublines was cross-resistant to FUR.

The response of the parental and FUR^{r-1} cells to treatment with FUR *in vivo* is presented in table 12. Although this shows that there is not a complete resistance to the comopund, a comparison at the FUR^{r-1} subline with the parental line certainly depicts a considerable degree of resistance. Treatment with FUR increased the survival of the parental population by 201% over untreated animals while similar treatment to the resistant cells increased survival by only 66%. Cross-resistant experiments with the AMT and AZG resistant sublines correlated with the *in vitro* maintained sublines.

Table 13 shows the response of *in vitro* adapted cells to AZG after passage through animals. The response of the parental cells is similar to the cells before they were injected into mice and two different "r-2" sublines also show stability in their degree of resistance. Two sublines resistant to either amethopterin or 5-fluorouracil were tested after passage through animals and both gave a response similar to the parental line.

The response of the $P388/AZG^{r-3}$ step was only tested *in vivo* as indicated in table 14. It this case, cells from the fifth passage generation were injected subcutaneously into mice which were then treated

TABLE 12

Sensitivity to 5-Fluorouridine (FUR) of parental (P388/P) and resistant ($P388/FUR^{r-1}$) sublines in mice (BALB/c × DBA/2)F1

Cell line	Subline no.	Tran. gen.	No. mice 1×10^{-6} cells	Mg/kg x Days[1] IP	Survival of animals		
					Range	MST	Inc. in survival
							%
P388/P	R-26	54	8	—	17–18	17.8	—
			8	5×18	75–119	106.4	497
$P388/FUR^{r-1}$	R-53	15	8	—	21–22	21.3	—
			8	5×19	35–65	53.6	151

[1] Daily treatment

TABLE 13

Per cent of cells after transplantation in (BALB/c × DBA/2)F1 mice capable of forming colonies in vitro in the presence of azaguanine (AZG) as compared with growth in the absence of the inhibitor

Subline	388/P	$388/AZG^{r-2}$	$388/AZG^{r-2}$	$388/AMT^{r-2}$	$388/FU^{r-2}$
Subline number	R-63	R-54	R-43	R-46	R-49
AZG originally on		2×10^{-5} M	2×10^{-5} M		
Molar concentration of AZG	%	%	%	%	%
1×10^{-7}				85	100
2×10^{-7}	100			70	74
5×10^{-7}	7			37	57
1×10^{-6}	4			13	22
2×10^{-6}	0			0	0
5×10^{-6}					
1×10^{-5}		93	100		
2×10^{-5}		43	69		
5×10^{-5}		7			
1×10^{-4}		0	0		
2×10^{-4}					
5×10^{-4}					

TABLE 14

Sensitivity to 8-Azaguanine (AZG) of parental (P388/P) and resistant (P388/AZG^{r-3}) lymphocytic neoplasms in (BALB/c×DBA/2)F1 mice after 5 transfer generations

Cell line	No. mice	No. of cells inj. $\times 10^6$	CMPD	Dose mg/kg x days[1]	Tumor weight (mgm) at 25 days
P388/P	8	1.2	—		519.3(218–991)
	8	1.2	AZG	75×17	137.3(0–323)[2]
P388/AZG^{r-3}	8	1.3	—		514.0(221–1207)
	8	1.3	AZG	75×20	479.0(75–935)

[1] Daily treatment.
[2] Three mice survived, tumor free.

intraperitoneally with daily injections of 75 mg/kg of 8-azaguanine for the number of injections they would tolerate. The localized tumor mass was excised on the twenty-fifth day and weighed. The last column of table 14 shows both the average weights and the weight variations for the group. A large difference exists between the average weights of the parental cells with and without treatment while there is little difference between the weights of the treated and non-treated tumors of the resistant group, indicating that the highest step to azaguanine resistance was retained after animal passage.

DISCUSSION

A test system employing mouse leukemia lymphoblasts was established whereby the capability of isolated single cells to divide and form colonies in the presence of inhibitors was tested. Thus the experiment measured the growth ability of single cells, not the combined efforts of many living and dying cells.

When amethopterin was used as a growth inhibitor, resistance was secured by isolating many successive small steps, each slightly more resistant than the previous one. Five such resistant sublines were developed and when compared with the sensitive population, cells from the most resistant line required approximately a 2,000-fold increase in the drug concentration to eliminate colony formation. The sensitivity of the parental cells compares closely with the results of both Fisher ('59) and Aronow ('59). When the AMT resistant populations, after passage in animals, were tested back in culture a slight loss in the degree of resistance in two of the lower steps occurred. These findings are comparable to those of Fisher ('59) with a different mouse lymphoma. When one of the lower steps was checked for its degree of resistance *in vivo* by treating mice bearing the lymphocytic neoplasm, it was found to be quite resistant to amethopterin. These cells were not cross-resistant nor did they show collateral sensitivity to any of the other compounds in these studies. Their excelent growth both in culture and in animals along with their stability in the absence of inhibitor make them suitable for use as genetic markers or for investigations concerned with the biochemical mechanisms responsible for the resistance.

Three of the fluorinated pyrimidines were selected for consideration. The sensitivity of the parental cells for the compounds is in the order FUDR>FUR>FU. Resistant sublines were selected through the use of each of the these compounds. Both the FU and FUR sublines were selected by isolating only small steps to resistance, whereas the FUDR subline was characterized by a small step at the lower concentration and a large step at the higher levels of FUDR.

Cross-resistance was exhibited to FU by the FUR resistant subline but not by the FUDR resistant subline. A substantial number of FU resistant cells was capable of growth in concentrations of FUDR which would not allow the growth of the parental cells. Both the FU and FUDR resistant cells were cross-resistant to FUR. Thus the FU subline exhibits cross-resistance to FUR and FUDR, as was reported for the Ehrlich ascites carcinoma by Heidelberger et al. ('60) while the FUR resistant cells are only cross-resistant to FU, and the FUDR resistant cells are only

cross-resistant to FUR. The highest FUDR step could, of course, be mixed with any cells in this study and be reisolated since the cells grew rapidly at a concentration of FUDR well above that which would inhibit the growth of any of the other sublines.

Resistance was retained by all sublines when maintained in the absence of the test compound in cell culture. Stability when reintroduced into the animal has varied. In the study of the FU resistant line there was a slight loss of resistance. Studies with the FUR line demonstrated that the first resistant step retained its level of resistance whereas the second, whose growth characteristics were not optimal, was not retained after animal passage in the absence of the drug; suggesting that these cells probably did not constitute an established resistant subline. *In vivo* studies supported the findings of the stability of the first step since the ascites tumor, transplanted in the absence of the compound, retained its resistance.

The FUDR sublines were stable in the absence of this compound in culture, although there was a slight decrease in the response of the highest step. They were, however, still capable of cloning at the highest concentration of FUDR to which they had previously been exposed.

Populations representing three increasingly resistant steps were isolated after incubation in the presence of AZG and although the first two were small steps, the last was certainly a large or "single step" to resistance. These cells, like the high resistant step to FUDR, were capable of cloning at levels of 8-azaguanine in excess of previous exposure levels. All the cells from this subline maintained logarithmically in a spinner culture in the absence of AZG for six months would, on the other hand, clone only to the level of analog to which they had previously been exposed. There was no evidence that any of the other resistant sublines would be cross-resistant to 8-azaguanine and either of these three lines could be identified in mixtures of the other sublines. There also was no evidence that these cells were more resistant to either of the fluorinated pyrimidines as has been observed in another mammalian line in cell culture, Detroit-98, by Szybalski and Smith ('59).

When the parental and second step AZG resistant cells were compared by injecting them SC in mice and then treating the animals IP with AZG it was found that they had retained a high level of resistance to this compound after many animal passages in the absence of the analog. When these cells were recultured *in vitro* they also retained a clear resistant step to AZG. Similar to the findings of Law ('51) no cross resistance was exhibited by this line to FU or AMT after animal passage.

The studies presented here show that these various resistant sublines possess stable, heritable characteristics suitable for genetic studies such as those of Bradley et al. ('62) concerned with transfer of genetic markers by extracts rich in desoxyribonucleic acid. These cells are also suitable for studies concerned with the mechanism of resistance to these various inhibitors, and studies with P388/AZG$^{r-1\&3}$ by Roosa et al. ('61) and Brockman et al. ('62) and with Davidson et al. ('62) employing all three AZG resistant sublines, have shown that resistance to AZG is accompanied by a loss of IMP and GMP pyrophosphorylase activity with a resultant loss of enzyme capacity for synthesis of 8-azaguanylic acid and 6-MP ribonucleotide.

The most resistant AZG marker was accompanied by a complete cross resistance to 6-MP, as has been shown in L1210 ascites tumor by Brockman et al. ('59) and by Lieberman et al. ('60) with mammalian cells (AMK 2-2) in culture. Brockman et al. ('62) using 6-MP-S^{35} in P388 cells showed that the parental cells but not the resistant cells extensively metabolized the analog to a nucleotide yet the nucleic acids of both are weakly radioactive; these findings are discussed by Brockman ('62). P388/AZG^{r-3} showed a partial cross resistance to both azaguanylic acid and azaguanosine; Szybalski and Smith ('59) have also shown a similar incomplete cross-resistance between AZG resistant cells and azaguanosine in cell culture. In P388 cells azaguanylic acid completely inhibited the growth of the AZG resistant cells at a lower concentration than was

required by azaguanine. Brockman et al. ('62) speculate that azaguanylic acid may be first cleaved to azaguanosine.

SUMMARY

P388/P, a cloned line of leukemic lymphoblasts of murine origin capable of rapid growth in cell culture and in animals was selected to be used for a study of somatic cell genetics. These studies required, therefore, that the cells be identified with a series of heritable markers. We chose to select for resistance to growth inhibitors as phenotypic markers and this study characterizes these markers as a baseline for studies already in progress relating genetic and biochemical investigations of drug resistance.

Resistance to the folic acid analog, amethopterin, was characterized by many small steps. Cells from each subline could be isolated that were capable of growth on higher concentrations of AMT. Each of the isolated sublines retained its resistance in the absence of AMT while in cell culture; however, after animal passage some lines appeared to be slightly less resistant. These same cells, after lengthy growth back in cell culture, in the absence of AMT, were again able to be cloned at the original levels of the inhibitor.

Two sublines resistant to FU were also selected and these remained stable after *in vitro* growth in the absence of the inhibitor. However, when retested in cell culture after several passages in animals these cells were more sensitive to FU. FU resistant cells displayed a slight cross-resistance to FUR and FUDR.

Resistance to FUR was characterized by small steps which were difficult to isolate but once obtained grew well and were stable when maintained in the absence of the compound. These cells exhibited some cross resistance to FU but not FUDR. When the two steps were returned from animal passage and tested in culture the first step retained its level of resistance whereas the second did not, suggesting that the latter was not a true second step.

The isolation of resistant steps to FUDR differed from the other fluorinated pyrimidines in that the second step was a very large step. These cells were stable in the absence of the compound but unfortunately animal studies were not completed; cross resistance was exhibited to FUR.

Three resistant steps were isolated after incubation in the presence of AZG and although the first two were small the last step was certainly a large or "single step" to resistance. Each of these steps was stable in the absence of AZG in cell culture as they also were after passage in animals. These sublines were cross-resistant to 6-MP, and partially cross-resistant to azaguanosine and azaguanylic acid. They were not cross-resistant nor did they show collateral sensitivity to the other compounds in this study.

The experiments reported here centrally test the sensitivity of individual cells of the various selected lines to the drugs studied. The stable heritable nature of the changes in sensitivity (resistant steps) may make some of them suitable for use as genetic markers in attempting to display genetic transfer in mammalian somatic cells.

The sublines selected here through the use of three types of growth inhibitors were not associated with any modifications in morphology nor changes in cultural characteristics.

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