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THE COMPARISON OF RADIATION AND ANTIMETABOLITE RESPONSES OF GENETICALLY ALTERED MURINE LEUKEMIC CELLS*

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Abstract—By clonal isolation from diploid P815Y cells, two hyperdiploid lines and one hypertetraploid line were selected for study.

Cell-reproduction studies demonstrated that the hypertetraploid line was sensitive to chronic γ-irradiation and to cytosine arabinoside, but was unaltered in sensitivity to all other agents investigated, when compared with the diploid and hyperdiploid lines.

Different leukemic cell lines (P815Y, L5178Y, and MLSY) could be characterized in culture by differences in sensitivity to antimetabolites.

Drug-resistant clones isolated from sensitive populations of L5178Y cells, with but one exception, were unaltered in sensitivity to other antimetabolites.

THIS study was undertaken in order to determine whether mammalian cell lines of different genetic constitutions could be characterized in culture by sensitivity to agents of biochemical and therapeutic interest. Several studies show that the cells of many human and experimental animal neoplasms are heteroploid in chromosomal number.[1–5] The possible relationship of ploidy to therapeutic response is of clinical interest and may also be important in elucidating more basic genetic information.

During exposure of a murine mastocytoma cell line (P815Y) to suboptimal growth conditions designed to induce heteroploidy,[6] marked changes were noted in the chromosomal constitution of many cells. Single-cell isolation yielded several clones of cells with differing ploidies. The responses of these clones to antimetabolites and radiation were then studied and compared with an isologous cell line (L5178Y) and a homologous cell line (MLSY). A hypertetraploid line showed increased sensitivity to one antimetabolite and to irradiation.

MATERIALS AND METHODS

Cell lines

The origin of the mast tumor cell line P815Y has been described previously;[7] it has been maintained in continuous culture for more than 5 years. This line (also called X-2) has a modal chromosome number of 40. The subline, X-1c, derived from X-1, has at present a modal chromosome number of 74 (Figs. 1 and 2). The P815Y cells were used for experiments devised to induce chromosomal changes.

P815Y cells at an initial concentration of $2{\cdot}6 \times 10^4$ per ml were exposed to 4°

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during 4-hr sessions at daily intervals. Between sessions they were returned to normal incubation temperatures of 37°. By the fourth day about 25% of the metaphases showed high chromosome numbers.* Three days later, single-cell isolations were performed.[8] Of the 36 cells isolated and inoculated, 11 developed into clones. Karyotype analyses demonstrated that 8 clones had a modal number of 40, 1 a modal number of 41, and 2 a modal number of 80. Selected for further experiments were clone 23, representing a modal number of 40, clone 27, representing 41, and clone 30, representing 80.

The culture techniques used for the study of the murine mastocytoma cells (P815Y), leukemia cells (L5178Y), and the lymphosarcoma cells (MLSY) have been described.[9] The origins of the line of murine lymphoblasts (L5178Y) resistant to methotrexate by virtue of an increase of folic acid reductase (mx^{e17}) and the line with resistance to methotrexate that is attributable to an altered transport mechanism for the drug (mx^{t}) have been described.[10] A clone (mx^{e200}) with a 180-fold increase in folic acid reductase activity was selected by incubation with methotrexate in a manner indicating that five to seven mutational steps had occurred during the selection process, each of which resulted in higher levels of folic acid reductase activity.[11] A clone (ca^{k}) resistant to 1-β-D-arabinofuranosylcytosine (cytosine arabinoside), selected from L5178Y cells as a single-step mutant, proved to be deficient in enzymatic capacity to convert the cytidine analog to the 5'-phospho-derivative.[12] Chromosome counts of 40 to 60 metaphase figures in line L5178Y and each of its mutant derivatives disclosed modal numbers of 40.

Chromosome analysis

Cells in log phase were exposed for 4 hr to diacetylmethyl colchicine, 1·2 μg/ml of medium, then to 1% sodium citrate in water for 30 to 45 min, fixed in a fresh mixture of 3 parts absolute methanol to 1 part glacial acetic acid; subsequently "air dry" or "blaze"[6, 13] preparations were made. At least 50 metaphase figures were counted from each of the mastocytoma cell lines. During 4 months of continuous propagation a shift of karyotype was noted in two clones. Line 23, with an original modal number of 40, developed one additional intermediate size chromosome with terminal centromeres; this subline appeared to represent, at least originally, a perfect doubling of chromosomes in respect to line X-2. No alterations in the marker chromosomes were noted in any line (Figs. 1 and 2). Volume determinations by means of direct micrometer measurement of diameters were performed. Calculation from the mean diameters of these spherical cells showed the following volumes: line 23, 1,080 $\pm$ 290 μ^3; line 27, 1,080 $\pm$ 290 μ^3; line X-lc, 1,950 $\pm$ 535 μ^3; and line 30, 1,880 $\pm$ 516μ^3.

Radiation experiments

An irradiation device was designed to give exposure rates of approximately 5 r and 3 r/hr. Concentric rings of culture tube holders were arranged around a central source containing 50 mg of radium suitably shielded to eliminate α and β particles. The radium-tube distance for the 4·8 dose-rate circle was 9·6 cm; the circle receiving a dose rate of 2·8 r/hr was 13 cm from the source. The entire facility was shielded and maintained in a large incubator room at a temperature of 37° $\pm$ 1·5. For chronic

* Attempts to repeat this effect have not been successful, indicating that conditions other than temporary shock contributed to this very high frequency of aneuploidy.

irradiation studies the cells were maintained in an exponential phase by dilution in the incubator room with warm medium. The final volume of medium was 5·0 ml in a screw-cap tube (16 × 125 mm) fitted with a rubber liner. The interval of removal of cells from the radiation device never exceeded 10 min every 2 days. Cell numbers were determined with a Coulter counter, model A. Radiation measurements were made with a Victoreen condenser dosimeter (model 5) inserted into the culture tube positions in the irradiation rack.

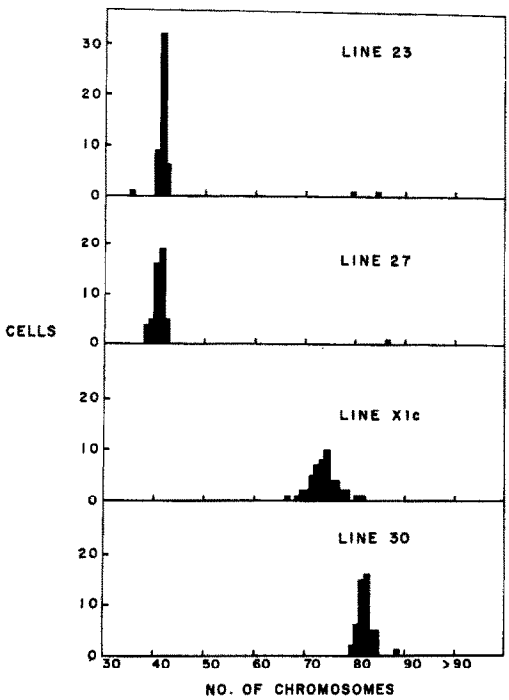

FIG. 1. Frequency distribution of chromosome number in four sublines of P815Y.

Antimetabolite experiments

Two methods were used to evaluate antimetabolite sensitivity. In the first, the inhibition of the rate of cell reproduction by an antimetabolite was determined. A level of drug was selected that would significantly prolong the generation time of the cells.

In the second method, the level of drug (Gd_{50}) was determined that would have limited cell growth to one half the generations attained in controls incubated without drug. Cells at a concentration of 2×10^3/ml were incubated at 37° with a range of concentrations of antimetabolite (i.e. for methotrexate, 3·0, 4·0, 5·3, 7·1, 9·8, 13, 17, 23, and 31×10^{-9} M) for a period of 86 to 96 hr. The logarithm of the cell number was plotted as a function of the drug level, and the level of drug that would have provided one half the logarithm of the cell number of the uninhibited controls was determined (Gd_{50} in Fig. 3). With L5178Y cells, the value for cytosine arabinoside was found to be $1{\cdot}35 \times 10^{-7}$ M $\pm$ 0·23 for a total of 15 experiments done over a period of one year; the variability of the other antimetabolites was no greater. In every instance, the data presented were obtained from simultaneous incubations

with antimetabolites of the cell lines to be compared (e.g. L5178Y, MLSY, and P815Y, Table 3), a procedure that reduces variability. It is possible that different preparations of horse serum vary in ability to inactivate or to bind a particular antimetabolite.

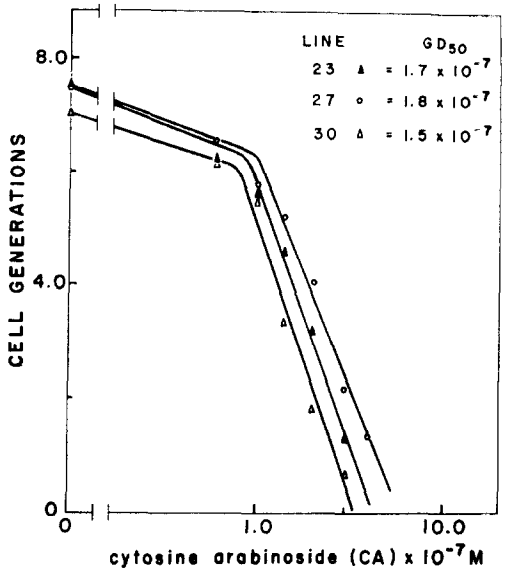

FIG. 3. Sensitivity of three mastocytoma cell lines to cytosine arabinoside. Cells at an initial concentration of 2×10^3/ml were incubated at 37° with levels of cytosine arabinoside (in duplicate) for a period of 96 h. The level of cytosine arabinoside that would have provided only one half the generations undergone by the control (Gd_{50}) was determined. The results were obtained in a single experiment.

The agents studied were methotrexate (amethopterin); 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (pyrimethamine); 1-β-D-arabinofuranosylcytosine (cytosine arabinoside); 2-β-D-ribofuranosyl-*as*-triazine-3,5-(2H,4H)-dione (6-azauridine); 1-β-D-2′-deoxyribofuranosyl-5-fluorouracil (5-fluorodeoxyuridine); and 1-β-D-2′-deoxyribofuranosyl-5-iodouracil (5-iododeoxyuridine).

RESULTS

In those experiments using the parent strain and three sublines of P815Y (i.e. 23, 27, and 30), no difference in sensitivity to methotrexate, pyrimethamine, 6-azauridine, 5-fluorodeoxyuridine, or 5-iododeoxyuridine was seen (Table 1). Cell line 30, however, in comparison with its hyperdiploid counterparts, line 23 and line 27, was much more sensitive to both cytosine arabinoside (Fig. 4) and chronic irradiation (Fig. 5).

It was found, however (Table 2), that L5178Y lymphoblasts and clones resistant either to methotrexate, mx^e and mx^t, or to cytosine arabinoside, ca^k, were unaltered in their sensitivity to other antimetabolites, with one exception: line mx^t demonstrated an increased sensitivity to 5-fluorodeoxyuridine. Line L5178Y and its derivatives differed uniformly from line P815Y and its derivatives with respect to sensitivity to methotrexate, pyrimethamine, and 5-fluorodeoxyuridine (Table 3). Similarly, the responses of line MLSY to all agents studied differed from those of P815Y cells;

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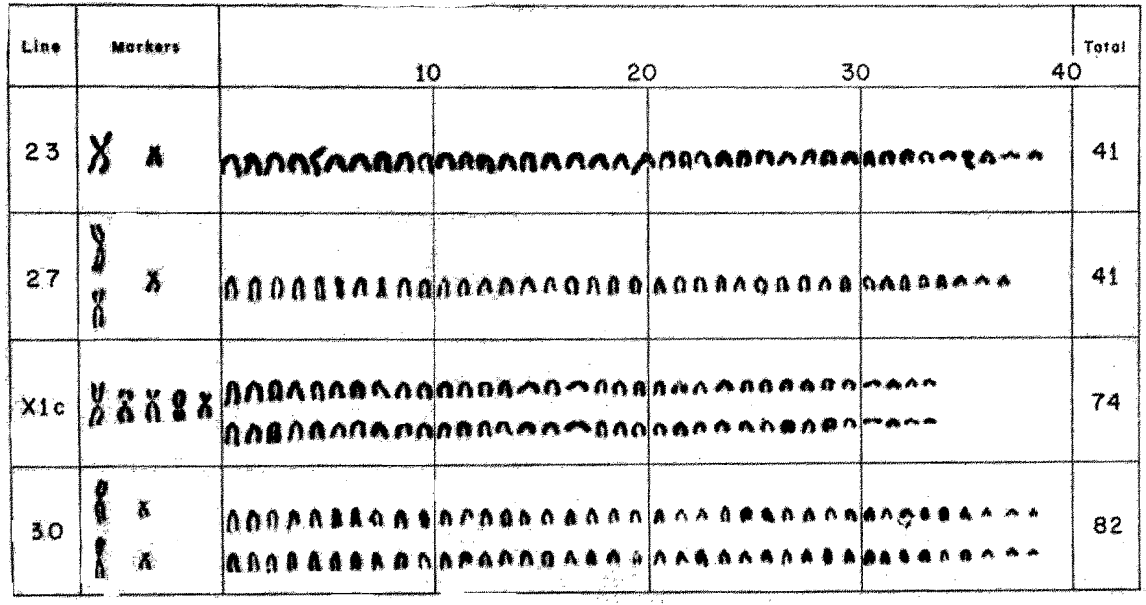


FIG. 2. Modal karyotypes of four sublines of P815Y. The marker chromosomes of lines 23, 27, and 30 are similar in morphology. The chromosomes of line 30 appear to be a doubling of these in line 23. Line X-Ic has several unusual markers and a hypotetraploid number.

TABLE 1. UNALTERED SENSITIVITY OF MASTOCYTOMA CELL LINES OF DIFFERING PLOIDY TO FIVE ANTIMETABOLITES*

	Gd_{50} of Antimetabolites			
Cell line	P815Y	23	27	30
Agent (molarity) Mode	40	41	41	82
Methotrexate (10^{-9})	1·8	2·0	1·9	1·7
Pyrimethamine (10^{-7})	6·1	6·2	7·0	5·8
6-Azauridine (10^{-6})	2·1	1·9	1·7	1·7
5-Fluorodeoxyuridine (10^{-9})	1·4	1·4	1·6	1·4
5-Iododeoxyuridine (10^{-5})		1·2	1·1	1·2

* Cells at a concentration of 2×10^5/ml were incubated with different levels of drug (see Fig. 3). After a period of 86 to 96 h, the cells were counted in a Coulter particle counter, model A, and the level of drug (Gd_{50}) was determined that would have limited cell growth to one half the generations attained in controls incubated without drug. The values represent the average of 4 determinations

TABLE 2. UNALTERED SENSITIVITY TO ANTIMETABOLITES OF DRUG-RESISTANT LINES DERIVED FROM L5178Y*

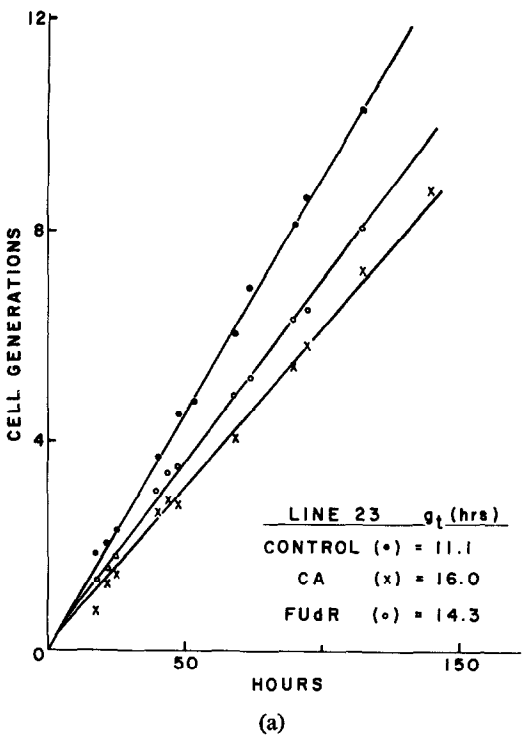
	Gd_{50} of Antimetabolites				
	Cell line				
Agent (molarity)	L5178Y	mx^{e17}	mx^{e200}	mx^{t}	ca^{k}
Methotrexate (10^{-8})	0·8	13	140	54	1·0
Pyrimethamine (10^{-7})	1·3	21	86	1·5	1·5
Cytosine arabinoside (10^{-7})	1·3		1·5	1·2	47
6-Azauridine (10^{-6})	1·3	1·4	1·1	1·3	1·2
5-Fluorodeoxyuridine (10^{-10})	4·5		4·4	1·1	3·4

* See footnote to Table 1.

TABLE 3. DIFFERENT SENSITIVITIES OF ONE HOMOLOGOUS (MLSY) AND TWO ISOLOGOUS LINES OF MURINE LEUKEMIA CELLS*

	Gd_{50} of Antimetabolites		
	Cell line		
Agent (molarity)	L5178Y	P815Y	MLSY
Cytosine arabinoside (10^{-7})	1·4	1·7	0·8
Methotrexate (10^{-9})	8·0	20·0	8·0
Pyrimethamine (10^{-7})	1·3	6·7	
6-Azauridine (10^{-6})	1·3	1·9	0·9
5-Fluorodeoxyuridine (10^{-10})	3·8	14·0	4·2

* See footnote to Table 1.



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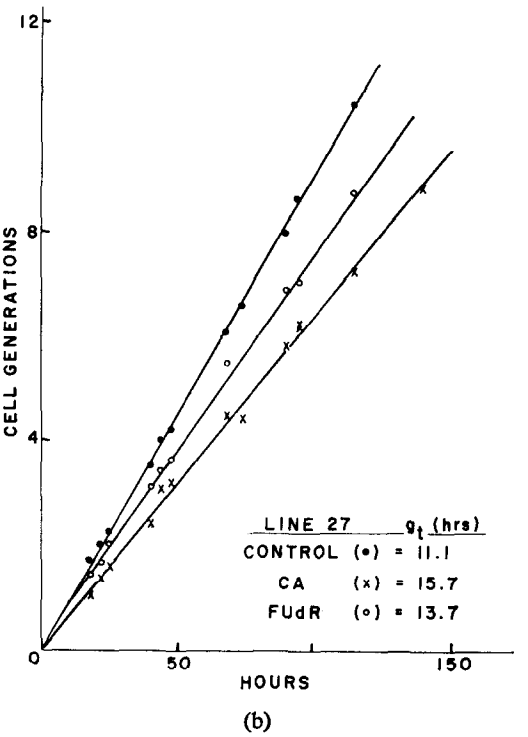

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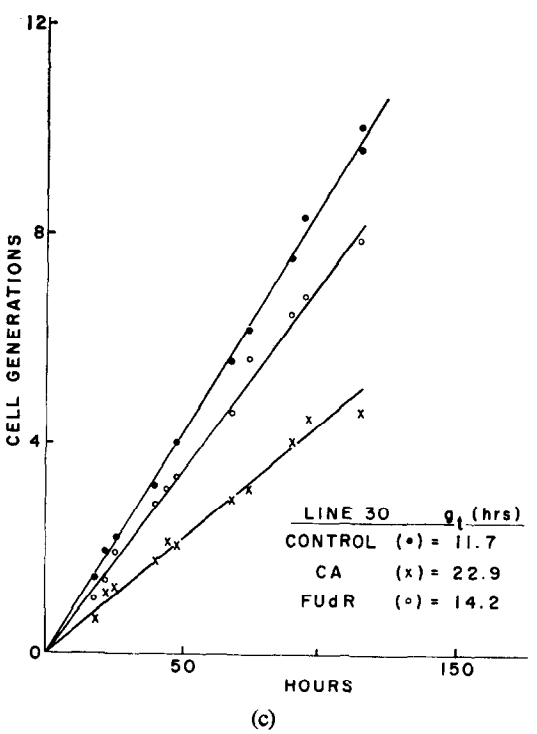

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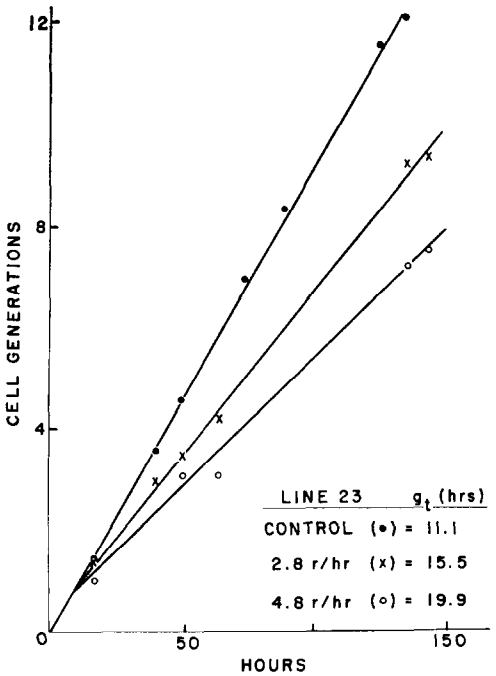
FIG. 4. Growth rates of sublines 23, 27 and 30, incubated in media containing antimetabolites. The results were obtained in one of three similar experiments. The greater sensitivity of line 30 (mode 82) to cytosine arabinoside is noted.



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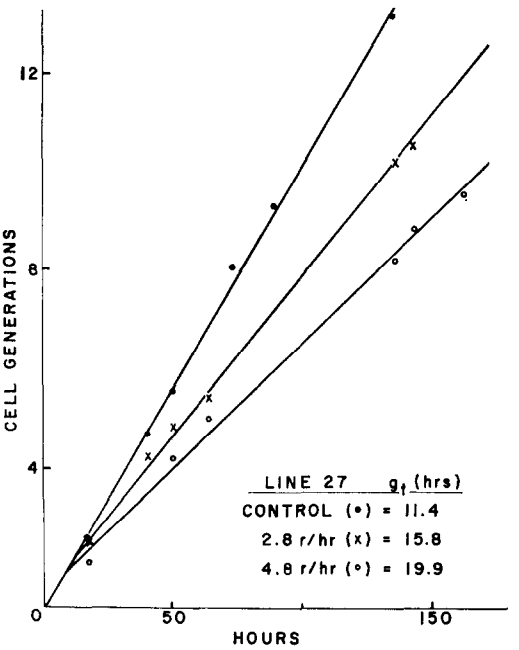

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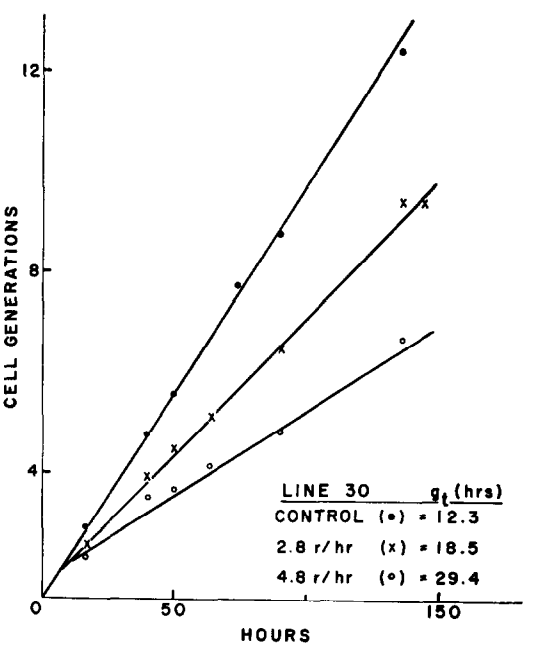

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FIG. 5. Growth curves of sublines 23, 27, and 30 to γ-irradiation. The results presented were obtained in one of four similar experiments. The greater sensitivity of line 30 (mode 82) to chronic irradiation is noted.

furthermore, MLSY cells exhibited a greater sensitivity to cytosine arabinoside, and possibly to 6-azauridine than did L5178Y cells (Table 3).

In an attempt to isolate a variant relatively resistant to radiation, cultures of line 23 were exposed to 2·8 r/hr and 4·8 r/hr, respectively, for a period of nearly 1,000 hr (Fig. 6). During this time the only change seen in growth rates occurred after 590 hr

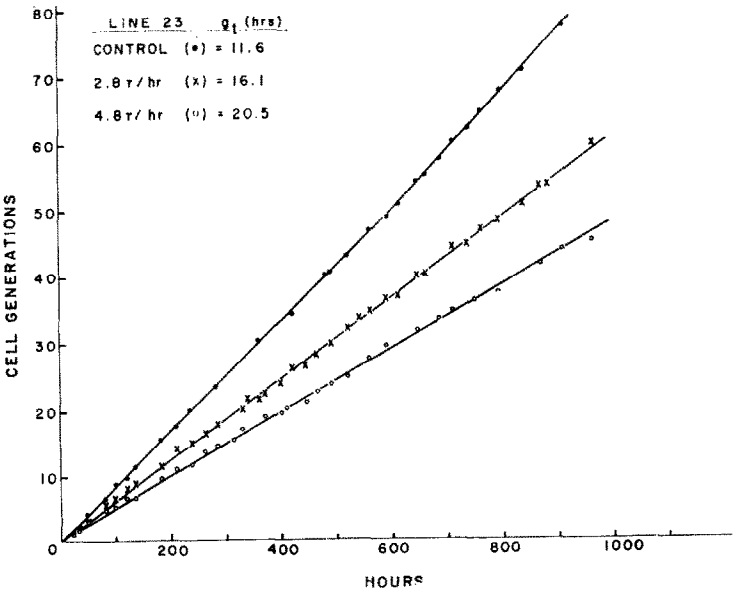

FIG. 6. Constant inhibition by chronic irradiation during an extended period of cell reproduction of mastocytoma cell line 23.

when the untreated controls changed from generation times 11·9 to 11·5 hr, possibly because of a change in the serum used in the medium. The inhibited cells that were removed from irradiation after approximately 800-hr incubation grew at the rate of the untreated controls after a lag period of 5 to 12 hr, thus indicating that the viability of the population was high. Because rapidly growing cell lines were not recovered during this extensive period of selection pressure, it is assumed that such a mutation to resistance to irradiation, if it does occur, is a very rare event.

DISCUSSION

A clone of L1578Y cells, ca^k, whose resistance has been attributed to a deficiency of deoxcytidine kinase, was not altered in sensitivity to the agents studied (Table 2). In addition, clones of L5178Y origin, resistant to methotrexate by virtue of increased cellular levels of folic acid reductase, mx^{e17} and mx^{e200}, also were unaffected in sensitivity to metabolites whose inhibition of cellular reproduction is presumed to result from activity involving different biochemical sites within the cells, It has been demonstrated, by appropriate studies of cell-reproduction and enzyme activities, that pyrimethamine limits or prevents cell division by inhibition of folic acid reductase within the cell,[14] and cross-resistance between this agent and methotrexate in mx^e

was predicted. A clone of lymphoblasts with an altered transport system for methotrexate, mx^t, retained full sensitivity to pyrimethamine, a result that would be predicted if it were assumed that the entry of pyrimethamine into the cell depends upon a mechanism other than that utilized by methotrexate, since folic acid reductase did not appear to be quantitatively or qualitatively altered in this mutant.[10] Thus, in L5178Y cells, single mutagenic events appear to have affected but one biochemical capacity of the cell. A correlated change (e.g. the increased sensitivity of mx^t to 5-fluorodeoxyuridine) might indicate a biochemical or genetic relationship between the two effects (the transport of methotrexate and sensitivity to 5-fluorodeoxyuridine), although direct evidence is not at hand to permit the conclusion that the mutant clones derived from L5178Y are genetic in origin.[15]

Mastocytoma cell sublines, the genetic constitutions of which differed from that of P815Y, were investigated. One of these, with a possible chromosomal duplication (line 30) of line 27, demonstrated an increased sensitivity to chronic irradiation and to cytosine arabinoside (Figs. 4 and 5), while in other respects line 27, line 30, and P815Y cells exhibited an entirely similar sensitivity to the agents studied. The possibility that radiation and cytosine arabinoside share a common sensitive site in the cell must be explored by similar studies in other genetically altered clones of cells.

Such an attempt was made in experiments which demonstrated that low dose-rate γ-irradiation (2·8 and 4·8 r/hr) produced a constant inhibition of the growth rate similar to that obtained by incubation with appropriate levels of an antimetabolite. The constancy of the level of inhibition suggested, for the dose-rate levels studied, that cumulative injury did not occur, at least none that was detected by further prolongation of cell generation time. A similar interpretation, relating to injury effects of low dose rates, has been reported by other workers.[16–18]

Further evidence of the lack of cumulative injury was found in the last experiment (Fig. 6), in which nearly 1,000 hr of radiation exposure at either 2·8 or 4·8 r/hr failed to alter the generation time of the recovered cells.

The relationship of ploidy to radiation sensitivity of mammalian cells appears to be inconstant in one recent study,[19] although others have reported[20, 21] predictable enhanced radiation tolerances in higher ploidy lines. The findings of relative sensitivity of the one polyploid line studied in these experiments appears to support the first viewpoint. However, the variables of dose rate, and cell survival versus growth inhibition, must be studied in relationship to these and other clones of differing ploidy before valid conclusions are justified.

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