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MUTATION TO 6-THIOGUANINE RESISTANCE IN HUMAN LYMPHOCYTE LINES

by

Sandra Anita Streeter Powell

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Human Genetics)
in The University of Michigan
1975

Doctoral Committee:

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To my husband, Isaac, and my

parents, Cecil and Anita

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TABLE OF CONTENTS

	PAGE
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF ILLUSTRATIONS	vii
LIST OF ABBREVIATIONS	viii
CHAPTER I: INTRODUCTION	1
Detection of Mutations in Human Somatic Cell Cultures	1
A. Hypoxanthine-Guanine Phosphoribosyltransferase Deficiency and Guanine Analog Resistance	
B. Selection for Purine and Pyrimidine Analog Resistance in Mammalian Fibroblast Cultures	
C. Selection for Purine and Pyrimidine Analog Resistance in Long-Term Lymphocyte Cultures	
The Mutational Origin of Guanine Analog Resistance in Cultured Cells	16
The Proposed Research	22
CHAPTER II: THE EXPRESSION OF HGPRT IN ESTABLISHED LYMPHOCYTE LINES	25
Introduction	25
Materials and Methods	26
A. Uptake of ^{3}H-hypoxanthine	
B. HGPRT Direct Assay	
C. Growth of Cells in Selective Medium	
Discussion	32

PAGE

CHAPTER III: RECONSTRUCTION EXPERIMENTS 42

Introduction 42

Materials and Methods 48

Results 50
A. Growth of Cells in 6-TG Medium
B. Cytogenetic Analysis of Cultures

Discussion
A. Recovery Efficiency and Metabolic Cooperation
B. Presence of XX Cells
C. Presence of Tetraploidy

Summary 61

CHAPTER IV: ESTIMATION OF MUTATION RATE TO 6-TG RESISTANCE IN ESTABLISHED HUMAN LYMPHOCYTE LINES 62

Introduction 62

Materials and Methods 65
A. Cell Lines
B. Cloning on Soft Agarose
C. Fluctuation Analyses
D. Enzyme Assays
E. Chemical Mutagenesis

Results 71
A. Cloning on Soft Agarose
B. Fluctuation Analyses
C. Calculation of Mutation Rate Estimate
D. Enzyme Assays
E. Induction of Mutation with EMS and MNNG

Discussion 89

Summary . . . . .

REFERENCES 104

LIST OF TABLES

TABLE		PAGE
I.	Estimates of Spontaneous Mutation Rates in Somatic Cell Cultures.	11
II.	Specific HGPRT Activities in Lymphocyte Lines.	31
III.	Composition of Mixed Cultures in Reconstruction Experiments.	49
IV.	Cytogenetic Analysis of the Cultures in Reconstruction Experiments.	52
V.	Fluctuation Tests Estimating the Spontaneous Rate of Mutation to 6-TG Resistance in Lymphocyte Lines.	74-78
VI.	Summary of Mutation Rate Estimates Derived from Fluctuation Analyses	81
VII.	HGPRT Assays of Normal and 6-TG Resistant Lymphocyte Lines	83

LIST OF ILLUSTRATIONS

FIGURE		PAGE
1.	Purine biosynthesis and metabolism.	6
2.	Blocks in de novo purine and pyrimidine biosynthesis produced by the aminopterin present in "HAT" medium	10
3.	Cultured normal (UM-39) and Lesch-Nyhan (UM-10) lymphocytes incubated with ^{3}H-hypoxanthine.	30
4.	Growth curves of an HGPRT-deficient lymphocyte line (UM-10) in varying concentrations of 6-thioguanine	34
5.	Growth curves of a normal male lymphocyte line (UM-61) in varying concentrations of 6-thioguanine	36
6.	Growth curves of a normal femlae lymphocyte line (UM-56) in varying concentrations of 6-thioguanine	38
7.	The effect of varying concentrations of ethyl methanesulfonate (EMS) on the logarithmic growth of a lymphocyte line (UM-61) derived from a normal male.	86
8.	The effect of varying concentrations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on the logarithmic growth of a lymphocyte line (UM-61) derived from a normal male.	88

LIST OF ABBREVIATIONS

8-AG	8-Azaguanine
AMP	Adenylic acid, Adenosine monophosphate
BUdR	5-Bromodeoxyuridine
EMS	Ethyl methanesulfonate
GMP	Guanylic acid, Guanosine monophosphate
HAT	Culture medium containing hypoxanthine, aminopterin, and thymidine
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
$HGPRT^-$	Hypoxanthine-guanine phosphoribosyltransferase deficient
$HGPRT^+$	Hypoxanthine-guanine phosphoribosyltransferase normal
IMP	Iosinic acid, Inosine monophosphate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
6-MP	6-Mercaptopurine
PRPP	5-phosphoribosyl-1-pyrophosphate
6-TG	6-Thioguanine
TK	Thymidine kinase
TK^-	Thymidine kinase deficient
TK^+	Thymidine kinase normal
XMP	Xanthinylic acid, Xanthine monophosphate

CHAPTER I

INTRODUCTION

Muller's discovery of the mutagenic effects of X-rays in 1927 began an era of study on the induction of mutations and the development of techniques for their detection and methods to quantitate their rates of formation. Current interest in human gene mutation has been stimulated by concern over the contamination of the environment by various agents, particularly chemicals, that have the capabilities of producing heritable changes in DNA.

Detection of Mutations in Human Somatic Cell Cultures

The development of the techniques for the *in vitro* cultivation of human somatic cells in the 1950's added a new dimension to the indirect study of mutation induction in man. The use of such cultures is relevant to man in that direct effects of mutagenic agents on human genes can be assayed with no intervening animal models.

In general, human somatic cell cultures have several characteristics that would seem to be useful in mutation induction studies. First, they can be easily derived from several different tissues. Second, large populations of cells can be obtained from a single tissue specimen. Third, the generation time of cultured cells

is hours or days which reduces the time in which mutagenic effects can be detected. Finally, in most instances, the cells are karyotypically and genetically stable over several generations in vitro.

The feasibility of a cell culture approach to the study of spontaneous and induced mutations has been previously established, and both chromosomal mutations and gene mutations have been used as indicators of mutagenic insult. Chromosomal mutations are visible alterations in the structure of chromosomes detected by light microscopic examination. Gene mutations on the other hand, occur at the nucleotide level of the genetic code and cannot be observed by microscopic examination.

Nutritional requirements and drug resistance have proved successful as indicators of mutagenic changes in cultured mammalian somatic cells. Based on the growth properties of Chinese hamster fibroblasts, Puck and colleagues have selected for auxotrophs which require glycine, hypoxanthine, adenine, thymidine or proline (Puck, 1971). Although cell populations were treated with mutagens to obtain some of the nutritional auxotrophs, spontaneous mutations in mammalian cell cultures under the conditions of Puck's laboratory occurred with a frequency of 1×10^{-8}.

Resistance to purine and pyrimidine analogs has provided a means for selecting single gene mutants in cultures of mammalian cells. Insensitivity to the guanine analogs 8-azaguanine (8-AG) and 6-thioguanine (6-TG) and the thymidine analogs 5-bromodeoxyuridine (BUdR) and 5-fluorodeoxyuridine (FUdR) has been found in mammalian cells. Mutations at the hypoxanthine-guanine phosphoribosyl

transferase locus cause resistance to both hypoxanthine and guanine analogs. Resistance to thymidine analogs has been found to result from mutations at the thymidine kinase locus, although the same phenotype may result from mutations in thymidine transport (Breslow and Goldsby, 1969).

A. Hypoxanthine-Guanine Phosphoribosyltransferase Deficiency and Guanine Analog Resistance

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in man is coded for by a locus on the X-chromosome (Seegmiller, Rosenbloom, and Kelley, 1967). This transferase catalyzes the conversion of three purines, hypoxanthine, guanine, and xanthine, to their respective 5'-mononucleotides, inosinic acid (IMP), guanylic acid (GMP), and xanthinylic acid (XMP) in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) and magnesium (Kelley and Arnold, 1973). Naturally occurring mutations at the HGPRT locus have been found in man. In 1964 Lesch and Nyhan described two brothers with a familial disorder of uric acid metabolism and central nervous system function. Now known as the Lesch-Nyhan syndrome, this disorder is clinically characterized by self-mutilation, mental retardation, choreoathetosis, and hyperuricemia caused by excessive purine biosynthesis (Kelly and Wyngaarden, 1972). The syndrome is rare in that there are only about eighty known cases (Crawhall, Henderson, and Kelley, 1972). Seegmiller *et al*. (1967) studied two children with clinical signs of the syndrome and demonstrated a complete absence of HGPRT in erythrocyte hemolysates and fibroblast extracts. HGPRT is found in many tissues of man, but its activity is greatest in those of

the central nervous system, followed by ovary, leucocytes and erythrocytes (Kelley and Wyngaarden, 1972).

Studies by Arnold and Kelley (1971) on the biochemical and physical properties of HGPRT purified from normal erythrocytes revealed that the enzyme is composed of two subunits with identical molecular weight and net charge. Arnold and Kelley also found three electrophoretically distinct forms of the native enzyme (isozymes), the origin of which they attributed to post-transcriptional, nongenetic alterations. Although separable electrophoretically, all three isozymes have the same two subunit structure, and molecular weight. Furthermore, the three isozymes can neither be distinguished catalytically nor immunologically, and there seems to be an interconversion between two of the forms under certain conditions such that one isozyme assumes the electrophoretic mobility of a second. Several experiments failed to shed any light on the nature of this interconversion, nor on the need for the interconversion. Physical studies by Kelley, Rosenbloom, Henderson, and Seegmiller (1967) led them to conclude that enzyme activity for both hypoxanthine and guanine resides in the same molecule, and it was later found that a single binding site exists for both purines (Henderson, 1968b).

The pathways of purine biosynthesis and metabolism are seen in Figure 1. The nucleotide GMP and the precursor substance IMP can both be syntehsized <u>de novo</u> from PRPP and glutamine via several consecutive enzymatic reactions. Alternatively, the free bases guanine and hypoxanthine are phosphorylated to GMP and IMP, respectively, by HGPRT.

Figure 1. Purine biosynthesis and metabolism.

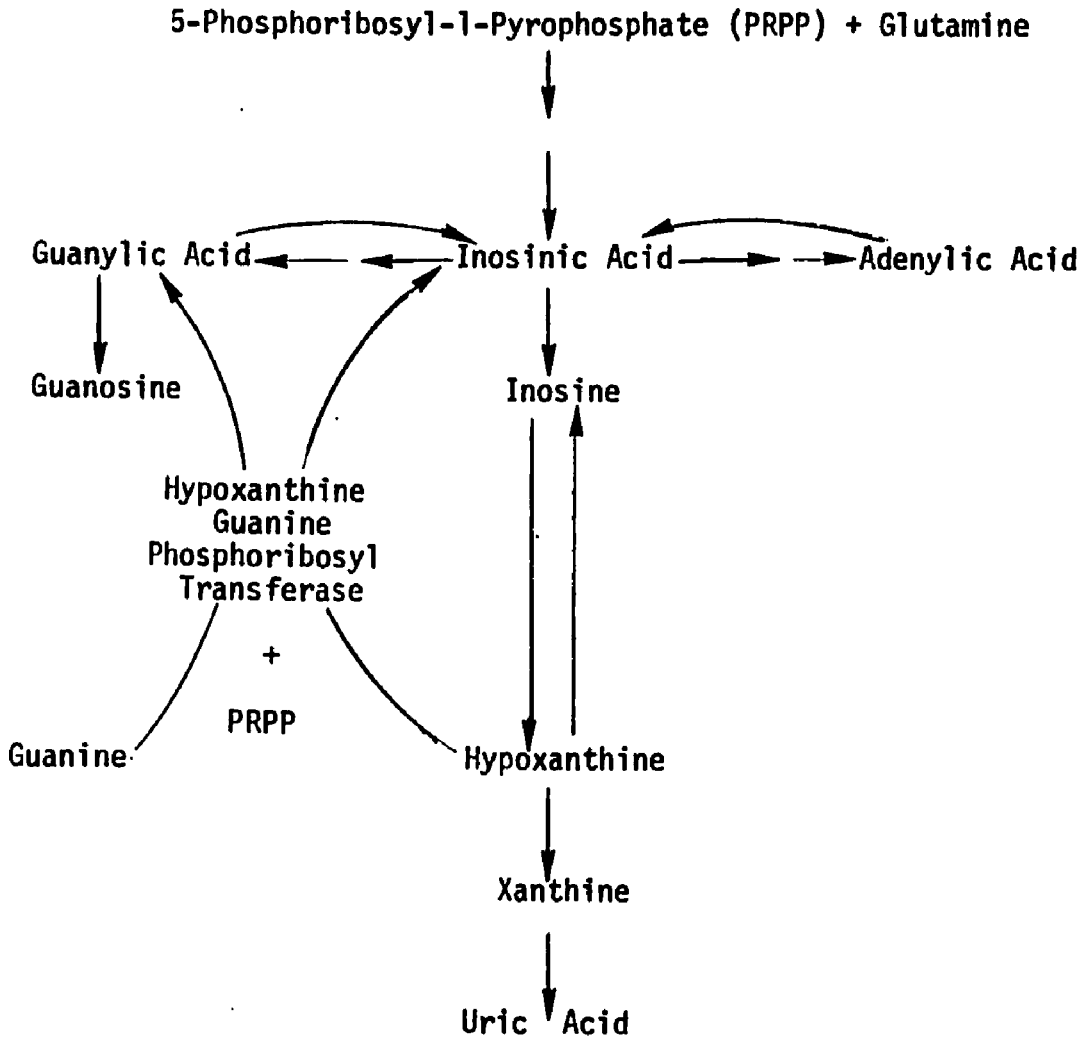

5-Phosphoribosyl-1-Pyrophosphate (PRPP) + Glutamine
Guanylic Acid
Inosinic Acid
Adenylic Acid
Guanosine
Inosine
Hypoxanthine
Guanine
Phosphoribosyl
Transferase
+
PRPP
Guanine
Hypoxanthine
Xanthine
Uric Acid

Figure 1

In addition to the conversion of natural purine bases to the 5'-monophosphates, HGPRT will also catalyze the formation of the 5'-monophosphates of allopurinol and 6-mercaptopurine, which are hypoxanthine analogs, and the guanine analogs, 8-AG and 6-TG (Kelley and Arnold, 1973). The inability of the normal enzyme to distinguish the substituted purines from the natural bases forms the foundation of an *in vitro* assay for the presence or absence of enzyme activity. 8-AG and 6-TG are cytotoxic, and cells with normal levels of HGPRT are inhibited when cultured in medium containing these analogs. On the other hand, the growth of HGPRT deficient cells is uninhibited by the presence of the analogs in the culture medium.

It is also possible to select for the growth of normal cells rather than those of HGPRT defiicient ($HGPRT^-$) cell growth. Based on the findings of Hakala (1957), Szybalski, Szybalska, and Ragni (1962) developed a growth medium that would allow for the growth of normal cells, but kill rare mutant cells that showed a resistance to 8-AG or 8-azahypoxanthine. The medium contained hypoxanthine, aminopterin, and thymidine and was called "HAT" medium. Siniscalco, Klinger, Eagle, Koprowski, Fujimoto, and Seegmiller (1969) later demonstrated that cells from $HGPRT^-$ Lesch-Nyhan males failed to grow in HAT medium, while the growth of cells with normal levels of HGPRT was unaffected.

The basis of the selection for growth in HAT medium is shown in Figure 2. Aminopterin is a folic acid antagonist and, when present in the culture medium, blocks the *de novo* synthesis of

both purines and pyrimidines (Szybalski et al., 1962). Cells with HGPRT activity can convert the exogenous hypoxanthine to IMP and both GMP and AMP are formed. The thymidine is added to alleviate the effects of aminopterin on pyrimidine biosynthesis. Major pyrimidine biosynthesis is performed de novo, and like purine synthesis, it is folate-dependent and blocked by folic acid antagonists. The exogenous thymidine is converted to thymidylic acid by thymidine kinase (TK) in the presence of ATP. TK-deficient (TK^-) cells are unable to utilize the thymidine, suffer pyrimidine starvation, and die in the presence of aminopterin. Thus with hypoxanthine and thymidine as the sole purine and pyrimidine sources, respectively, cells with normal levels of HGPRT and TK can grow in the presence of aminopterin. Deficiencies in the levels of either HGPRT or TK cause cell death in HAT medium.

B. Selection for Purine and Pyrimidine Analog Resistance in Mammalian Fibroblast Cultures

The findings of several investigators who selected rare drug resistant cells from mass cultures of mammalian fibroblasts are summarized in Table 1. Among the earliest reports of selection of purine analog resistant cells are those of Szybalski (1959) and Szybalski and Smith (1959). Using a clonal subline of the heteroploid strain Detroit-98 (D-98), a minor population of cells resistant to the guanine analog 8-AG was selected. The parental D-98 line was derived from human sternal bone marrow (Berman and Stulberg, 1956), and the major cell population was found to be sensitive to 8-AG at a minimum concentration of 0.1 microgram

Figure 2. Blocks in de novo purine and pyrimidine biosynthesis produced by the aminopterin present in HAT medium. Alternate enzymatic steps in the salvage pathways allow the utilization of exogenously supplied hypoxanthine and thymidine.

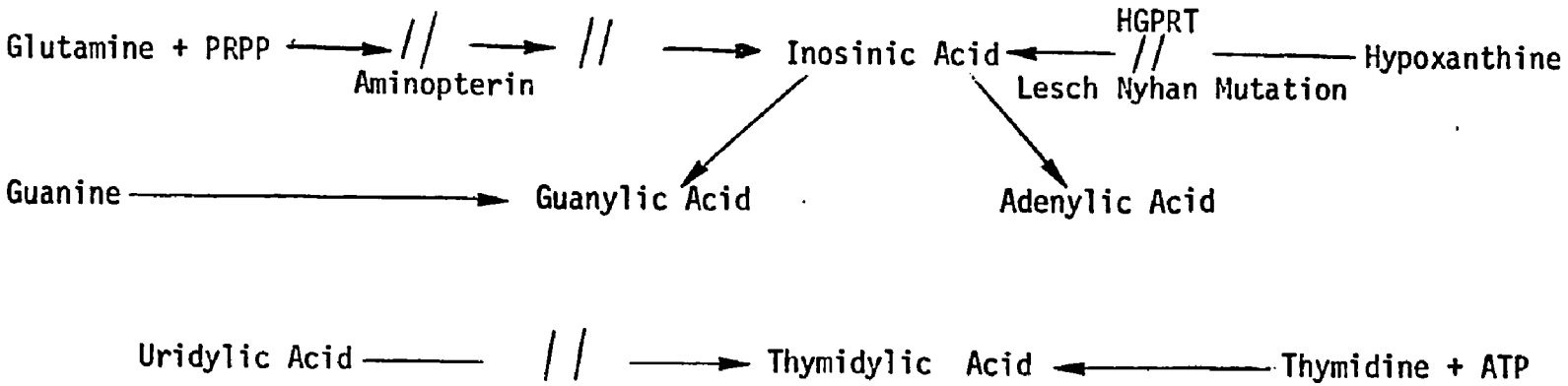

Glutamine + PRPP
Aminopterin
Inosinic Acid
HGPRT
Hypoxanthine
Lesch Nyhan Mutation
Guanine
Guanylic Acid
Adenylic Acid
Uridylic Acid
Thymidylic Acid
Thymidine + ATP

Figure 2

TABLE I

ESTIMATES OF SPONTANEOUS MUTATION RATES IN SOMATIC CELL CULTURES

Investigator	Cell Line	Mutation	Mutation Rate Per Cell Generation
Puck and Kao (1967)	Chinese hamster fibroblasts	$pro^- \longrightarrow pro^+$	10^{-6}
Chu et al. (1969)	Chinese hamster fibroblasts	$gln^- \longrightarrow gln^+$	1.4×10^{-7}
		$8\text{-}AG^s \longrightarrow 8\text{-}AG^r$	1.5×10^{-8}
		$8\text{-}AG^r \longrightarrow 8\text{-}AG^s$	3.0×10^{-6}
DeMars and Held (1972)	Human diploid fibroblasts	$8\text{-}AG^s \longrightarrow 8\text{-}AG^r$	0.45×10^{-6} to 1.8×10^{-6}
Szybalski (1959)	Human heteroploid fibroblasts	$8\text{-}AG^s \longrightarrow 8\text{-}AG^r$	4.0×10^{-4}
Shapiro et al.	Chinese hamster fibroblasts	$6\text{-}MP^s \longrightarrow 6\text{-}MP^r$	1.5×10^{-5}
Clive et al.	Mouse Lymphoma	$BUdR^s \longrightarrow BUdR^r$	0.6×10^{-7}

per ml of culture medium. Szybalski (1959) observed that the mutation rate from 8-AG sensitivity to 8-AG resistance was on the average 4.9×10^{-4} per cell generation. The 8-AG resistant phenotype was stable over several culture generations in the absence of the drug.

The rate of mutation found by Szybalski is higher than those found by other investigators who used guanine analog resistance. Furthermore, in a later report (Szybalski et al., 1962) ultra-violet light, alkylating agents, nitrogen mustard, and several other known mutagens failed to increase the frequency of mutation to 8-AG resistance. Perhaps these findings may be accounted for by presently known characteristics of the D-98 line. Gartler (1967) has presented evidence to the effect that the D-98 line is probably of HeLa cell origin. Chu and Giles (1958) found that the HeLa line and some of its mutant clonal derivatives are subject to changes in chromosome constitution, particularly changes in chromosome number. They suggest that these changes may be responsible for mutant phenotypes in heteroploid cell lines. Thus it seems reasonable to question whether or not changes in chromosomal constitution as well as gene alteration may be responsible for the high frequency of mutation to 8-AG resistance and a lack of response to mutagens observed by Szybalski.

Chu, Brimer, Jacobson, and Merriam (1969) were successful in obtaining 8-AG resistant cells and L-glutamine auxotrophs from Chinese hamster cells. Their studies showed that the rate of

mutation from 8-AG sensitivity to resistance was 1.5×10^{-8} per cell generation. The rate of spontaneous reversion from L-glutamine auxotrophy to prototrophy was estimated to be 1.4×10^{-7} per cell generation. When the cultures were treated with ethyl methanesulfonate (EMS) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) the frequency of the resistant phenotype was increased several fold over the spontaneous frequency (Chu and Malling, 1968).

Shapiro, Khalizev, Luss, Marshak, Petrova, and Varshaver, (1972a) using human lung and Chinese hamster fibroblasts were also able to select for spontaneous mutants resistant to 8-AG. In the human lung fibroblasts, these resistant mutants occurred at a rate of 7.1×10^{-5} per cell generation, wile the Chinese hamster cells mutated to resistance at a rate of 1.5×10^{-5}. In addition to 8-AG resistance, resistance to 6-mercaptopurine (6-MP) was found to occur at a rate of 0.45×10^{-5} per cell generation in the Chinese hamster cells. In a subsequent report Shapiro et al. (1972b) induced mutation to 6-MP resistance in the Chinese hamster fibroblasts. When the cells were treated with N-nitrosomethylurea, a highly mutagenic compound believed to act via methylation and deamination of purines and pyrimidines (Loveless and Hampton, 1969), the mutation rate to 6-MP resistance was also increased to 0.6×10^{-3} by BUdR, to 0.65×10^{-3} by X-irradiation, and to 1×10^{-4} by ultra-violet irradiation.

Albertini and DeMars (1970) and DeMars and Held (1972) have reported the isolation of spontaneous 8-AG resistant cells from cultures of normal human diploid fibroblasts. The frequency of the

resistant colonies in fibroblast cultures derived from sixteen unrelated individuals ranged from 0.4×10^{-6} to 19×10^{-6}. Their data showed that the spontaneous rate of mutation to 8-AG resistance was between 0.45×10^{-6} and 1.8×10^{-6} per cell generation. X-irradiation caused an increase in the rate of mutation to resistance, and the observed increases were dose dependent (Albertini and DeMars, 1973).

C. Selection for Purine and Pyrimidine Analog Resistance in Long-Term Lymphocyte Cultures

The experience with lymphocyte lines for use in mutation studies is not as extensive as that with fibroblasts. Clive, Flamm, Machesko, and Bernheim (1972) investigated mutations at the thymidine kinase locus in a mouse lymphoma cell line, L5178Y. This lymphoma cell line was derived by Fischer (1958), grows in suspension culture, and has a cloning efficiency of 80 to 100% on soft agar. The thymidine analog BUdR was used to select for thymidine kinase deficient (TK^-) cells. Clive et al. defined three TK phenotypes on the basis of BUdR sensitivity ($BUdR^s$) or resistance ($BUdR^r$), HAT medium sensitivity (HAT^s) or resistance (HAT^r), and thymidine kinase activity. Assuming two allelic forms of the TK locus in L5178Y cells, these three phenotypes may be represented as follows: (1) homozygous $TK^{+/+}$, $BUdR^s$, HAT^r, normal levels of TK activity, (2) heterozygous $TK^{+/-}$, $BUdR^s$, HAT^r, half normal levels of TK activity, and (3) homozygous $TK^{-/-}$, $BUdR^r$, HAT^s, low to undetectable levels of TK activity. When $TK^{+/+}$ cells were cultured in BUdR, the spontaneous rate of mutation to $TK^{-/-}$ was estimated to be 5×10^{-11}

per locus per generation, based on a single mutant. Cells from this BUdR resistant clone when cultured in HAT medium showed a rate of mutation from $TK^{-/-}$ to $TK^{+/-}$ or 6.0×10^{-9}. Single locus secondary forward mutation from $TK^{+/-}$ to $TK^{-/-}$ was estimated to be 1.2×10^{-7} per locus per cell generation. When $TK^{+/-}$ cells were treated with X-irradiation, $TK^{-/-}$ cells occurred at a rate of 3×10^{-4} per locus per generation. Depending upon dose EMS gave induced forward mutation rates of 9×10^{-5} per locus per generation.

Human lymphocytes have only recently been successfully established in long-term culture (Moore, Gerner, and Franklin, 1967). Sato, Slesinski, and Littlefield (1972) have reported their results from mutagenesis studies with a human lymphocyte line. PGLC33H was derived from the peripheral blood of a patient with infectious mononucleosis. A subline, derived by cloning in soft agarose, was chosen for mutation studies. This clonal line, L33-6-1, had a doubling time of 18 to 22 hours, and initially showed a normal female karyotype. However, at the time of their studies, a bimodal distribution of cells with two karyotypes, 46, XX and 47, XX+C, was observed. The uncloned parental line also contained the two karyotypically different cell populations. The cloning efficiency on soft agarose was 30% for the L-33-6-1 subline. 6-thioguanine was used to test for mutations at the HGPRT locus. Spontaneous mutations to 6-TG resistance occurred at a frequency of about 1×10^{-5} when 10^4 to 10^7 cells were plated on soft agarose containing growth medium with 6-TG. EMS treatment of the cell population increased

the mutation frequency of 6-TG resistance 109-fold over that of the spontaneous frequency. When MNNG was used, a 19-fold increase in the resistant phenotype was observed.

The Mutational Origin of Guanine Analog Resistance In Cultured Cells

Several indirect lines of evidence support the mutational origin of the observed 8-AG or 6-TG resistant phenotypes. First, all investigators found that the phenotype was stable over numerous cell generations in the absence of analog selection. Second, the frequency of mutational events was increased by the use of known mutagens. Third, the variability of numbers of mutant cells in fluctuation analyses (Luria and Delbruck, 1943), support the random occurrence expected of mutational events. However, it could be argued that physiological events, independent of mutational change, also exhibit random occurrence. Finally, biochemical and physical characterizations of the mutant enzymes suggest that they are structurally different from the wild-type enzyme.

On the other hand, Harris (1971) suggested that the 8-AG resistant phenotype is epigenetic, i.e., the phenotype is independent of alterations in cellular DNA. He observed that changes in cell ploidy had no significant effect on the frequency with which the 8-AG resistant phenotype occurred. Using the Chinese hamster cell line V70-122D_1, Harris used colchicine to induce tetraploid (4N) and octaploid (8N) clonal derivatives. The average rates of mutation to 8-AG resistance in the diploid (2N), 4N and 8N sublines were

2.2×10^{-6}, 4.7×10^{-6}, and 1.3×10^{-6} per cell generation, respectively. Under the mutation hypothesis this is unexpected for an X-linked recessive mutation. As the number of copies of active wild-type alleles increases, as in ploidy increases, in order to insure survival of the cell in the presence of 8-AG, the extra copies of wild-type alleles must be reduced or eliminated. This can occur either by chromosomal segregation in subsequent cell generations or by mutations to 8-AG resistance in each additional allele. The probabilities of either or both of these events is rare, and the probability of recovering 8-AG resistant cells diminishes accordingly. The constancy of the mutation rate over the different levels of ploidy led Harris to conclude that 8-AG resistance in his cultures was not due to mutation at the HGPRT locus. He suggested that stable shifts in phenotypic expression arise de novo in somatic cell cultures, and are not necessarily dependent on gene mutation.

In reviewing the report of Harris, however, information pertinent to the interpretation of his findings is not presented. First, the levels of HGPRT activity in the 8-AG resistant cells isolated are not reported. Thus it is not known whether there is HGPRT deficiency in the cells that exhibit growth in 8-AG, or whether some colonies were able to escape selection. Sharp, Capecchi, and Capecchi (1973) found that several mouse L-cells selected with 8-AG had high levels of HGPRT activity and reverted to the wild-type phenotype (growth in HAT medium) with frequencies as high as 10^2.

They suggest that 8-AG is a less stringent selective agent, and that many mechanisms (mutational or nonmutational) may exist whereby cells can bypass the killing effect of 8-AG. The possibility of this event in the experiments by Harris may well be evoked to explain the constancy of mutation rate he observed, and his characterization of isolated mutants would have helped to resolve this matter.

Second, the chromosome constitution of the 8-AG resistant cells at the different ploidy levels is not known. The necessity to establish the number of copies of HGPRT alleles is important because of fluctuations in the number of chromosomes in pseudodiploid lines. Conceivably, loss of chromosomes might result in a comparable number of HGPRT alleles despite a maintenance of the diploid, tetraploid, or octaploid chromosome mode.

Finally, metabolic cooperation has been found to occur at the HGPRT locus in fibroblasts. A discussion of this phenomenon and its effect on the selection of HGPRT deficient cells is presented in Chapter III. Suffice it to say at present that metabolic cooperation between $HGPRT^-$ and $HGPRT^+$ cells in fibrobrlast cultures decreases the frequency at which guanine analog resistant cells are observed. It is possible that metabolic cooperation in the experiments of Harris masked the true frequency of $HGPRT^-$ cells. This in turn would affect the rates of mutation to 8-AG resistance found.

On the other hand, 8-AG resistance may not be a true indicator of HGPRT deficiency. Gillin, Beaudet, and Caskey (1972) characterized thirty-five 8-AG resistant clones derived from V-79 Chinese hamster fibroblasts after EMS or MNNG treatment. Gillin et al. argue that sixteen of the mutants showed no detectable HGPRT activity and represent mutations at the HGPRT locus. Five clones showed low, detectable HGPRT activity (2%) and were considered to represent mutations in the HGPRT gene or genes which govern its regulation. The remaining fourteen mutants possessed significant HGPRT activity (20-150%), grow in HAT medium, but were resistant to 8-AG activity. Gillin et al. concluded that the basis of this phenotype is unclear, although purine transport defects were considered. An antiserum against Chinese hamster HGPRT showed that three out of fourteen $HGPRT^-$ clones had a cross-reacting material to HGPRT, indicating the presence of structurally abnormal enzyme (Beaudet, Roufa, and Caskey, 1973). Overall, their results indicate that 40% of the mutants selected with 8-AG or 6-TG represent true mutations at the HGPRT locus, and do not appear to owe their drug resistant phenotype to epigenetic alterations.

DeMars and Held (1972) also observed considerable variation in the levels of HGPRT activity in approximately 90% of their human diploid fibroblast mutants. The enzyme activities ranged from normal amounts to the levels observed in fibroblasts from Lesch-Nyhan males. These mutants resembled those described by

Gillin et al. (1972) in that they were only partially sensitive to 8-AG and were still able to utilize hypoxanthine.

Further studies are necessary before conclusions concerning the genetic or epigenetic origin of the observed 8-AG resistant phenotype can be made. These should include repetition of the Harris experiments with the use of mutagens to test their effects at the different ploidy levels, precise characterization of the colonies isolated with respect to their enzyme levels, their biochemical, physical, and immunological properties, and finally, the stability of the phenotypic change with time. It is apparent, however, that careful study and characterization of the HGPRT in mutant cells are necessary before conclusions as to the mutational origin of the phenotype may be made.

In summary, the detection of rare mutants and revertants at the HGPRT locus can be accomplished by the use of selective medium. With one exception, most investigators have concluded that the cells obtained under the selective growth conditions arise from true mutational events rather than epigenetic phenomena based on the following evidence: (a) the occurrence of the observed phenotype is rare; (b) the acquired phenotype is permanent despite numerous cellular generations in the absence of selective agents; (c) the rate of mutant formation is increased by use of known mutagens; and (d) the biochemical and physical properties of the enzyme are altered in ways that are consistent with changes in the stucture of the protein.

The major piece of evidence inconsistent with the mutational hypothesis is the failure to observe a decrease in the frequency of mutant formation with an increase in cell ploidy, which is to be expected for a supposedly X-linked recessive gene (Harris, 1971). It is clear that 8-AG resistance is not solely attributable to gene mutation at the HGPRT locus and that caution must be exercised in the interpretations of the origins of this phenotype found in cultured mammalian cells. However, consideration must be given to the following: (1) the HGPRT locus, HAT medium, and guanine analog resistance or sensitivity is a selective system currently available for use in human cell culture that allows for the selection of a rare variant phenotype from mass culture, and that is not complicated by the diploid state of human cells (Krooth, Darlington, and Velazquez, 1968); (2) HGPRT is ubiquitous and expressed in several different tissues and in cultured cells at levels that can be assayed with considerable accuracy (Kelley and Wyngarten, 1972); (3) Fluctuation analyses have so far shown that the occurrence of guanine analog resistant cells is random and is independent of the presence of the drug in the culture medium (Harris, 1971; Szybalski et al., 1962; Shapiro et al., 1972a; DeMars and Held, 1972; Chu et al., 1969). Finally, it has been demonstrated that the frequency of guanine analog resistant cells in diploid or pseudo-diploid mammalian cell cultures is a reliable indicator of mutagenic activity of several agents (Albertini and DeMars, 1973; Shapiro et al., 1972b; Chu and Malling, 1968; Gillen et al., 1972;

Sharp et al., 1973; Sato et al., 1972; Chasin, 1974.) Thus until a more efficient selective system becomes available, it seems feasible to use guanine analogs and HAT medium to study mutation at the HGPRT locus.

The Proposed Research

Although there have been a few reports of the selection of guanine analog resistant cells from human lymphocyte lines, these lines have not been used in experiments to detect their rates of spontaneous mutation at the HGPRT locus. Lymphocyte lines have several characteristics that appear to be advantageous for their use in detecting spontaneous and induced mutations. First, their growth in suspension to high densities is somewhat analogous to bacterial cultures, and it should be possible to adapt lymphocyte lines to techniques that have been successful for the detection of mutations in microorganisms. Second, the lymphocyte cultures can be cloned on soft agar, albeit with varying efficiency. Third, from several years experience in our laboratory, the lymphocyte lines from normal donors generally remain karyotypically stable over numerous cell generations in continuous culture as contrasted to fibroblasts. Finally, two lines derived from a male with the Lesch-Nyhan syndrome, have been in culture for over four years and the cells of the line retain the mutant phenotype. Thus with respect to the HGPRT locus, the cells are genotypically stable.

I propose to demonstrate that small numbers of HGPRT$^-$ cells can be detected in large populations of normal lymphocytes, and that the rate of occurrence of the HGPRT$^-$ phenotype can therefore be estimated. This goal will be accomplished in the following manner: The guanine analog 6-TG will be used to select cells that are resistant to its effects by virtue of mutations at the HGPRT locus. 6-TG was chosen as the selective guanine analog because resistant cells selected with it possess lower levels of HGPRT activity more often than those selected with 8-AG (Sharp et al., 1973; Chasin, 1974; Sato et al., 1972), and are thus more likely to be true mutants. In addition Simons et al. (1974) have demonstrated that 6-TG is more stable than 8-AG in culture medium containing fetal calf serum. The data from a Luria-Delbruck fluctuation analysis will be used to estimate the rate of formation of the mutant phenotype. Mutant cells obtained will be assayed for HGPRT activity to prove the deficient state. Further evidence as to the mutational origin of the 6-TG resistant phenotype will be obtained through the demonstration that known mutagens increase the frequency of 6-TG resistant cells. If these experiments prove successful, then perhaps lymphocyte cell lines and 6-TG resistance, in addition to fibroblast cultures, may be used as an indicator system for in vitro testing of the effects of suspected mutagens on human DNA.

The research presented here is divided into four major sections:

1. Preliminary experiments were performed to characterize the expression of HGPRT-normal and HGPRT-deficient cells with respect to their growth in the selective medium, uptake of the labelled substrate, hypoxanthine, and by direct enzyme assay on mutant and normal cells.

2. The efficiency with which small numbers of mutant HGPRT-deficient cells can be selected from wild-type populations was estimated from reconstruction experiments.

3. Twenty-two fluctuation analyses were performed on uncloned cell populations and clonal sublines. The mutation rate to 6-TG resistance was estimated.

4. In the final set of experiments, the effects of ethyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine treatment of the lymphocyte populations are described.

CHAPTER II

THE EXPRESSION OF HGPRT IN ESTABLISHED LYMPHOCYTE LINES

Introduction

The first lymphocyte lines derived from a patient with the Lesch-Nyhan syndrome were established in our laboratory in 1969 (Choi and Bloom, 1970). The patient was a two-year-old male with the clinical characteristics of the syndrome. The lines, derived from peripheral blood lymphocytes, were designated UM-10 and UM-11. Cytogenetic studies revealed a normal male karyotype, and the cultured cells produced immunoglobulins G and M of the heavy chain class and k and λ light chains. Preliminary evidence obtained by Choi suggested that the cells of both lines were deficient in HGPRT activity. First, the cells of UM-10 and UM-11 failed to incorporate ^{3}H-hypoxanthine as determined by autoradiography. Secondly, no growth of cells was observed in HAT medium. Littlefield assayed UM-10 for HGPRT activity and found the line to have 1.4% of the HGPRT activity found in a lymphocyte line derived from a normal donor (personal communication).

In view of the continuous in vitro propagation of the UM-10 line during the three year span between the observations of Choi and the present study, initial experimentation was designed to

determine whether or not the lymphocytes of UM-10 still expressed the HGPRT deficiency. Cytogenetic analysis confirmed the presence of a 46,XY karyotype. A qualitative measurement of enzyme activity was obtained by autoradiography after incubation of the cells with ^{3}H-hypoxanthine. Direct HGPRT assays were performed on cells from UM-10, and the level of sensitivity to varying concentrations of 6-TG was determined during logarithmic cell growth.

Materials and Methods

A. Uptake of ^{3}H-Hypoxanthine

Approximately 5 x 10^{6} cells were inoculated into a T-25 tissue culture flask (Falcon Plastics) containing 10 ml of Roswell Park Memorial Institute (RPMI) 1640 growth medium with 20% fetal calf serum, and cultured at 37°C for twelve hours. Cells from UM-39, which was derived from a normal female, were used as a positive control. ^{3}H-hypoxanthine (New England Nuclear Corp.; specific activity 3.1 Ci-mmole) was added to give a final concentration of 5 μc/ml, and culturing was continued for an additional twelve hours. Cells were washed twice with 0.9% saline and fixed with 3 methanol: 1 acetic acid. Slides were prepared, stained with carbol fuchsin, and dipped in NTB-2 liquid emulsion (Eastman Kodak). After exposure in the dark for seven days, they were developed in D-19 solution (Eastman Kodak).

B. HGPRT Direct Assay

This assay measures the amount of ^{14}C-hypoxanthine substrate used in the presence of phosphoribosyl pyrophosphate and magnesium. The procedure followed that of Sato et al. (1972) except that cells were lysed by three rounds of freeze-thawing instead of sonication. Protein determinations were made by the Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951). In addition to the UM-10 cell line, four other lines were tested simultaneously. UM-61 and UM-56 were derived from normal male and female donors, respectively. UM-21-5 is a clonal subline derived from a male with citrullinemia, but the line is presumably normal for HGPRT activity (Spector and Bloom, 1973). UM-1-TG was derived from a previously established, HGPRT positive, normal male line by culturing the cells in the presence of 10 micrograms of 6-TG per ml of growth medium. All assays were carried out in triplicate.

C. Growth of HGPRT-Deficient and Normal Cells in 6-TG Selective Medium

The effect of 6-TG was tested on UM-10 as well as several lines from normal male and female donors. Cultures containing 2.5×10^5 to 3.0×10^5 cells per ml were initiated in RPMI-1640 growth medium containing 1, 5, 10, 20, or 30 micrograms of 6-TG per ml. Samples were taken from triplicate cultures at 24 hour intervals. Counts were made with a hemacytometer, and viability was determined by trypan blue dye exclusion (Tennant, 1964). Population doubling times for the UM-10 line were calcuated according to the method of Aoki and Moore (1970).

Results

A. Autoradiography

The results of ^{3}H-hypoxanthine labelling of the lymphocytes are seen in Figure 3. UM-39 showed heavy labelling of a majority of the cell population. UM-10, on the other hand, showed sparse, but detectable uptake of the label by most of the cells. Since background labelling was low, the observed labelling presumably indicates that the lymphocytes of UM-10 are not completely devoid of HGPRT activity, but are able to utilize small amounts of exogenous hypoxanthine. Confirmation of this observation was obtained in subsequent direct enzyme assays.

B. Direct HGPRT Assays

The results of the HGPRT assay are seen in Table 11. The UM-10 and the UM-1-TG lines were clearly deficient in HGPRT, but formed small amounts of IMP. Both lines have approximately 1% of the HGPRT activity found in the normal UM-61 line.

C. Growth of Cells in Selective Medium

By virtue of a deficiency in HGPRT, the UM-10 cells should be immune to the cytotoxic effects of guanine analogs, and thus be able to grow in medium containing varying concentrations of substitued purines. As seen in Figure 4, UM-10 was uninhibited over the entire range of concentrations of 6-TG tested. At 10 micrograms of 6-TG per ml, the population doubled in 31.8 hours, and in non-selective medium the doubling time was 32.8 hours. All normal lines

Figure 3. Cultured normal (UM-39) and Lesch-Nyhan (UM-10) lymphocytes incubated with ^{3}H-hypoxanthine.

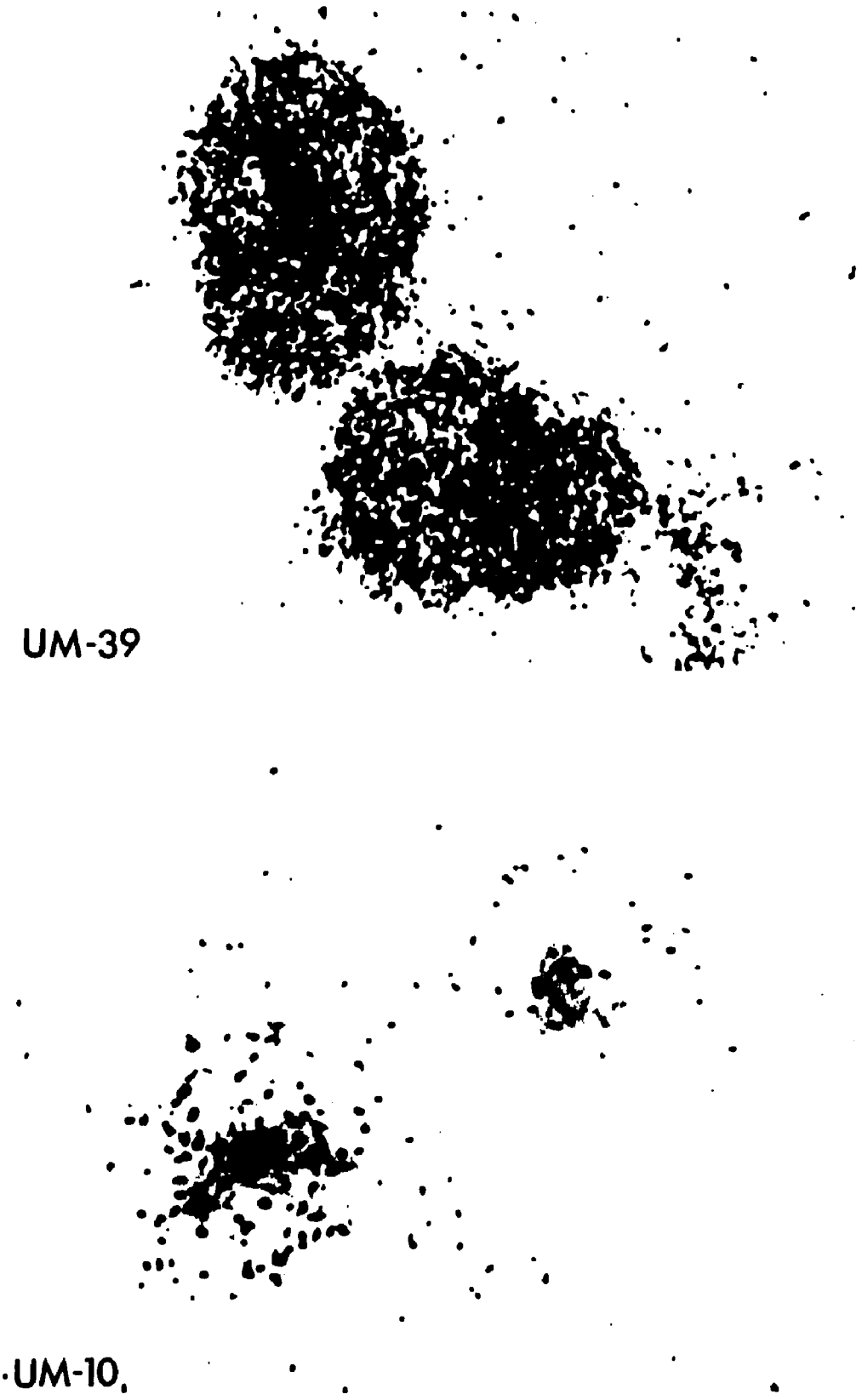


Figure 3

TABLE II

SPECIFIC HGPRT ACTIVITIES IN LYMPHOCYTE LINES

Cell Line	Donor	Nanomoles ^{14}C-Hypoxanthine used/ mg protein/hour
UM-61	Normal Male	41.69
UM-56	Normal Female	52.50
UM-21-5	Citrullinemic Male	57.51
UM-10	Lesch-Nyhan Male	0.38
UM-1-TG*	Normal Male	0.68

*This subline was derived from a previously established, HGPRT positive, normal male line by culturing the cells in medium containing 10 micrograms of 6-TG per milliliter.

were uninhibited only at the lowest concentration of 1 microgram of 6-TG per ml, and no cultures exhibited logarithmic growth at any of the other concentrations (Figures 5 and 6). Counting was stopped at 96 hours because of low cell densities and viability. The cultures were maintained for two weeks, but there was no resumption of growth. Ten micrograms of 6-TG per ml was chosen as the concentration to be used in all subsequent experiments. As seen in Figures 5 and 6 this concentration assures complete inhibition of a population of normal cells, while it had no effect on the growth potential of the UM-10 cells (Figure 4).

Discussion

The HGPRT locus is clearly expressed by establishing lymphocyte cell lines as determined by uptake of ^{3}H-hypoxanthine, direct enzyme assay, and failure of normal cells to grow in the presence of the guanine analog, 6-TG. The UM-10 lymphocytes of our Lesch-Nyhan patient, like fibroblasts from other reported patients (Crawhall et al., 1972), do not appear to be completely devoid of HGPRT activity, but show a low, detectable level of ^{3}H-hypoxanthine uptake and form IMP in small amounts. The low level of enzyme activity detected, however, is apparently below that which renders the lymphocytes sensitive to the cytotoxic effects of 6-TG.

The fact that these observations were made two to four years after the establishment of the normal and mutant cell lines used attests to the genetic stability of the HGPRT locus in lymphocyte

Figure 4. Growth curves of an HGPRT-deficient lymphocyte line (UM-10) in varying concentrations of 6-thioguanine. ——— 0 µg/ml, ---- 1 µg/ml, ... 5 µg/ml, x--x 10 µg/ml, 0--0 20 µg/ml.

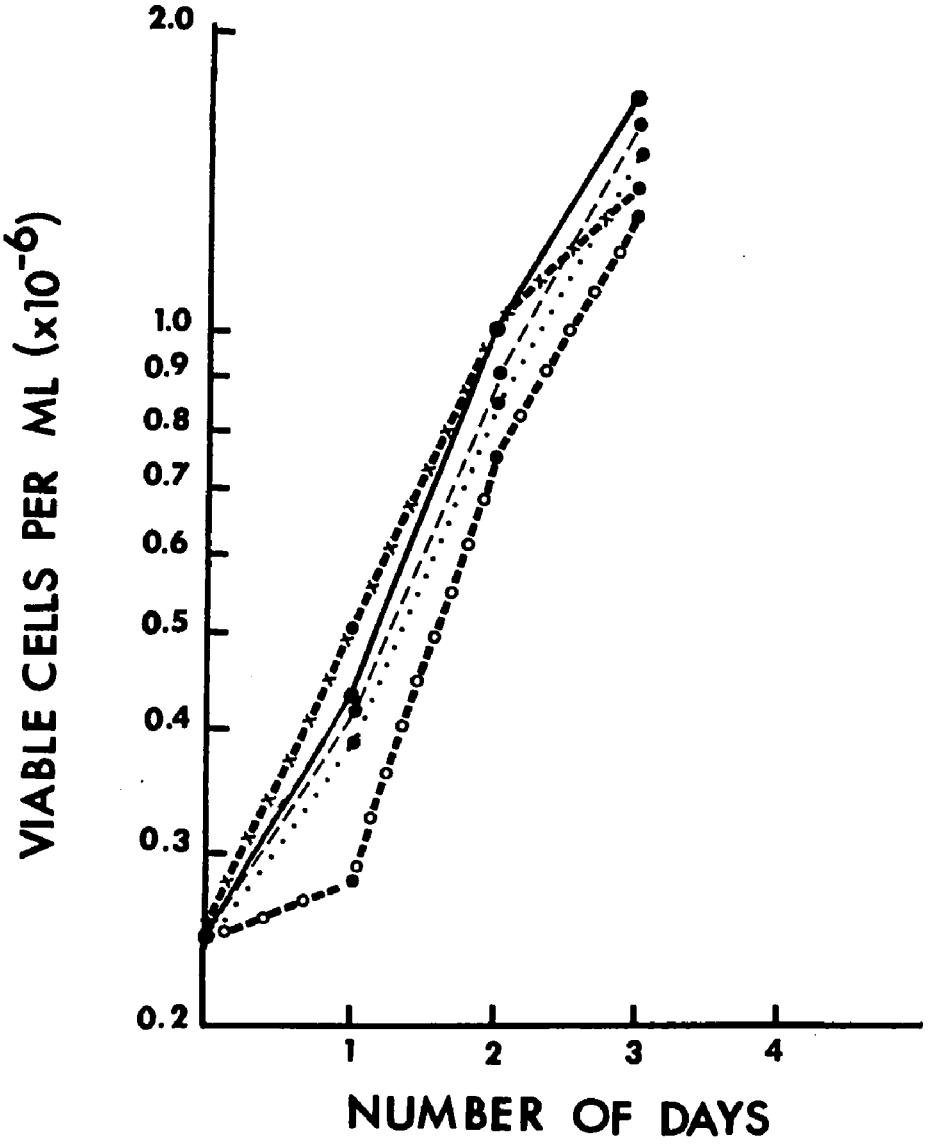

2.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
VIABLE CELLS PER ML (x10⁻⁶)
1
2
3
4
NUMBER OF DAYS

Figure 4

Figure 5. Growth curves of a normal male lymphocyte line (UM-61) in varying concentrations of 6-thioguanine. ———0 µg/ml, ---- 1 µg/ml, ...5 µg/ml, x--x 10 µg/ml, 0--0 20 µg/ml.

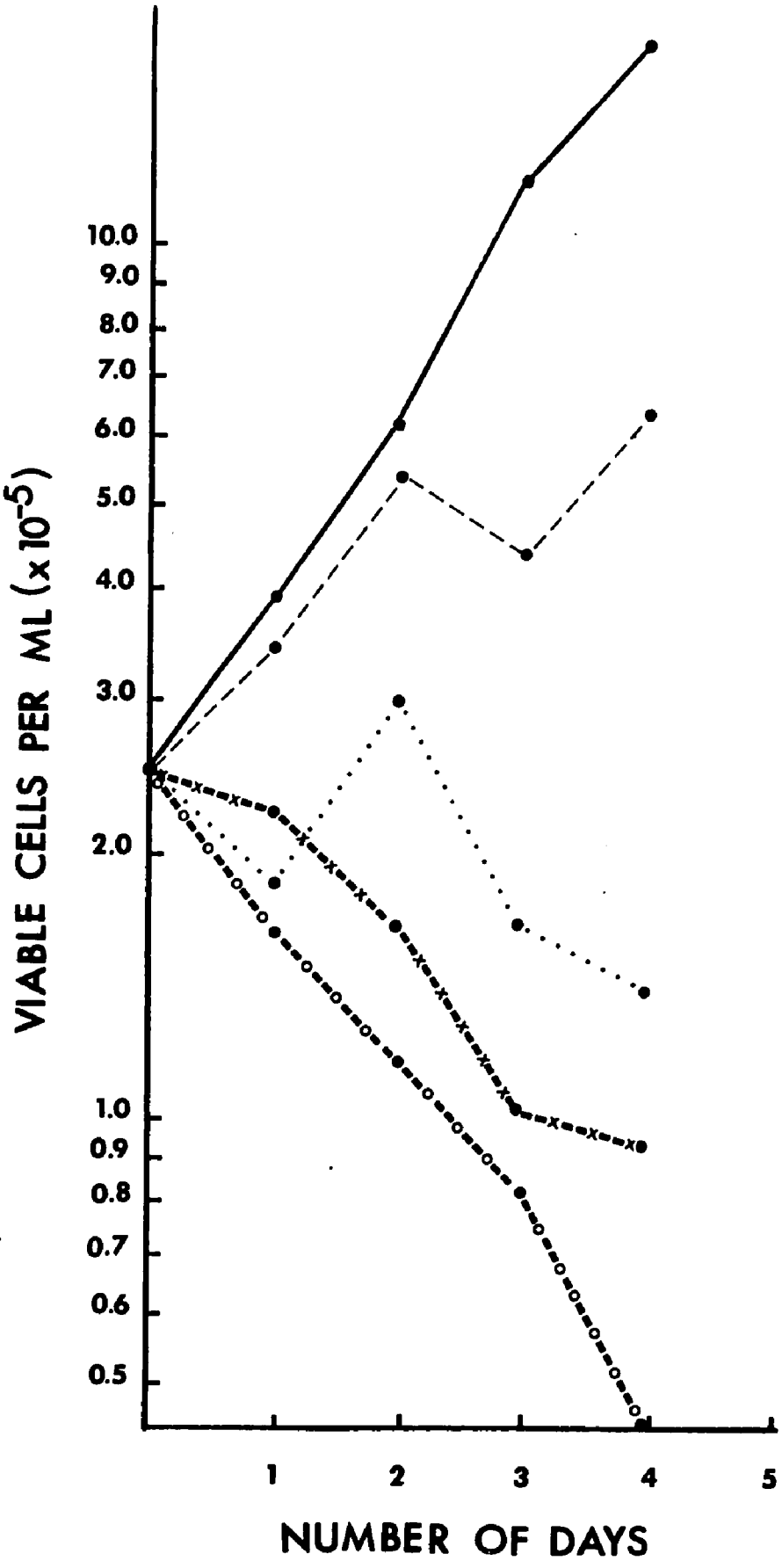

VIABLE CELLS PER ML (x 10⁻⁵)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.9
0.8
0.7
0.6
0.5
1
2
3
4
5
NUMBER OF DAYS

Figure 5

Figure 6. Growth curves of a normal female lymphocyte line (UM-56) in varying concentrations of 6-thioguanine. ———0 µg/ml, ---- 1 µg/ml, ... 5 µg/ml, x--x 10 µg/ml, 0--0 20 µg/ml.

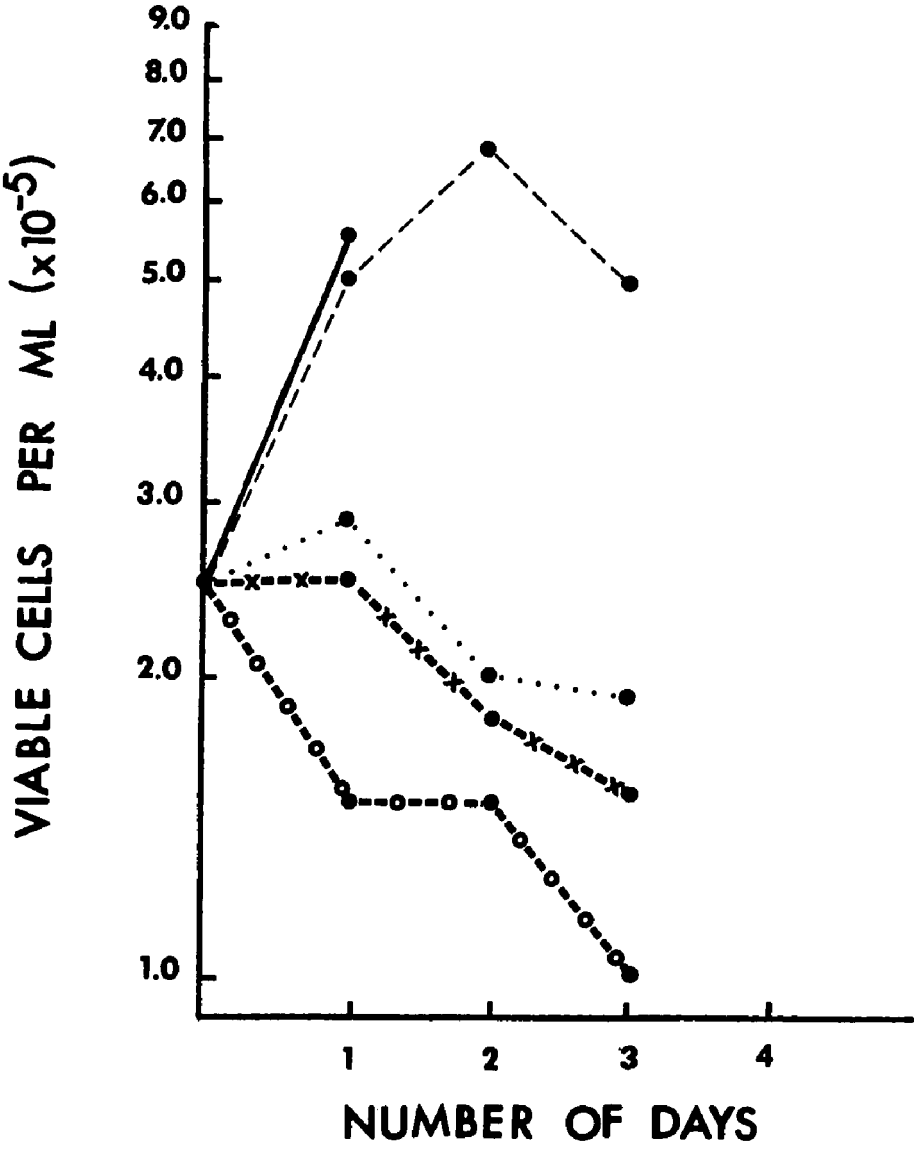

VIABLE CELLS PER ML (x10⁻⁵)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1
2
3
4
NUMBER OF DAYS

Figure 6

lines, despite continuous in vitro propagation. Some human heteroploid fibroblast lines over prolonged periods of in vitro cultivation manifest variable degrees of genetic instability in the form of chromosomal changes (Krooth et al., 1968; Chu and Giles, 1958). A specific effect of this instability may have been observed in the early experiments of somatic cell mutation rates at the HGPRT locus. Using 8-AG resistance, Szybalski (1959) showed an unusually high rate of mutation (5×10^{-4} per cell per generation) at the HGPRT locus (Table I). He used a clonal subline of the human epitheloid-like cell line #98, believed to be derived from sternal bone marrow, but probably of HeLa origin (Gartler, 1967). Use of ultra-violet light, alkylating agents, nitrogen mustard, and several other known mutagens failed to increase the frequency of mutation to 8-AG resistance (Szybalski et al., 1962). Changes in chromosome constitution, particularly changes in chromosome number as observed in heteroploid lines, may be responsible for mutant phenotypes (Chu and Giles, 1958), and the heteroploid state of the D98 line used by Szybalski may have contributed to the high frequency of 8-AG resistance via chromosome loss.

With the exception of the UM-1-TG line, all lines used in the present experiments show a normal diploid chromosome complement corresponding to that obtained from short-term leucocyte cultures of the original donor. Thus, they do not manifest the changes in chromosome constitution observed in heteroploid fibroblasts under

serial propagation. This chromosomal stability of lymphocyte lines has been confirmed by serial observations of several lines in our laboratory. The UM-1-TG subline and the parental UM-1 line contain a translocation between chromosomes B_5 and C_6 of the human karyotype (Bloom and Nakamura, 1974). The fact that the HGPRT deficient subline was derived from a cytogenetically normal line is felt to be fortuitous rather than a specific indication of genetic instability of the HGPRT locus in the UM-1 line. First, the parental UM-1 line retains the HGPRT positive phenotype in a majority of the cells as determined by its ability to growth in HAT medium. Secondly, another $HGPRT^-$ subline has been derived from the UM-61 line, and both this subline and the parent line contain a normal diploid karyotype. And finally, HGPRT deficient cells have been detected in the karyotypically normal female diploid UM-39 line used in the reconstruction experiments discussed in the following section.

Sato et al. (1972) did not find that the frequency of 6-TG resistant lymphocytes in their line was greatly different from that observed by Albertini and DeMars (1970) and DeMars and Held (1972) for human diploid fibroblasts. This finding is particularly significant in view of the fact that their lymphocyte line was chromosomally abnormal, and their suggestion was that the culture was genetically unstable at the HGPRT locus, X-chromosome, or all chromosomes. The frequency of 6-TG resistant cells was approximately 1×10^{-5}, and could be increased several fold by the use of mutagens.

In summary, the HGPRT locus is expressed in established lymphocyte cell lines, and like diploid fibroblasts, mutant cell lines show low detectable activity. The use of growth medium containing 6-TG allows for the normal growth of cells from the HGPRT-deficient line while completely inhibiting the growth of cells with normal levels of HGPRT activity. Finally, the finding that the UM-10 cell line retains the HGPRT-deficient phenotype three years after the original observations of Choi and Bloom (1970) indicates that the phenotype is stable in lymphocyte cell lines in the absence of selection and during continuous propagation. This stability of the phenotype is important for any cell system and locus to be used for mutation rate estimates.

CHAPTER III

RECONSTRUCTION EXPERIMENTS

Introduction

When the Lesch-Nyhan syndrome was recognized as an inborn error of purine metabolism, several studies in large families failed to detect the heterozygous state in mothers and female relatives of affected males on the basis of HGPRT assays on erythrocyte hemolysates (Henderson, 1968a). Autoradiography with ^{3}H-hypoxanthine failed to show two populations of erythrocytes (Nyhan, Bakay, Connor, Marks, and Keele, 1970) and leucocytes (Dancis, Berman, Janse, and Balis, 1968). Proof that the Lyon hypothesis held for the HGPRT locus came from studies by Migeon, Derkaloustian, Nyhan, Young, and Childs (1968). Using clonal subpopulations of fibroblasts from two heterozygous females these investigators demonstrated that some clones failed to incorporate ^{3}H-guanine while others had levels of incorporation of the substrate in the range of normal controls. Uncloned populations from these same women incorporated the label to the same degree as a control.

Shortly thereafter, Dancis, Cox, Berman, Jansen, and Balis (1969), using artificial mixtures of normal and HGPRT-deficient ($HGPRT^{-}$) fibroblasts and natural heterozygote fibroblasts, found

that the detection of the heterozygous state depended upon the density of the fibroblast monolayer. At low cell density, twenty-four hours post subculture, two distinct and roughly equal populations of cells were noted on the basis of ^{3}H-hypoxanthine uptake. Half of the cells were heavily labelled, while the remainder were unlabelled. By ninety-six hours of culture, however, over 95% of the population showed uptake of the label in normal range. They attributed the observed density effect to metabolic cooperation between HGPRT^{-} and normal (HGPRT^{+}) cells in culture.

Metabolic cooperation was the term proposed by Subak-Sharpe, Furk, and Pitts (1969) to describe the relationship between HGPRT^{-} and HGPRT^{+} cells in direct contact with each other. Using baby hamster kidney fibroblasts, they observed that in mixed culture, when there is no contact between HGPRT^{+} and HGPRT^{-} cells, each behaves independently of the other. Therefore, HGPRT^{-} cells do not incorporate ^{3}H-hypoxanthine into their nucleic acid, while HGPRT^{+} cells become heavily labelled. However, once the two types of cells establish contact, the HGPRT^{-} cells show evidence of incorporation of the label. Subak-Sharpe et al. proposed that this cooperation between HGPRT^{-} and HGPRT^{+} cells involved the transfer of substances from HGPRT^{+} to HGPRT^{-} cells, and that these substances could be: (a) preformed nucleotides, (b) preformed polynucleotides, (c) informational polynucleotides, such as messenger RNA or episomal DNA, (d) the GHPRT enzyme, or (e) regulator substance.

Subsequent experiments by others led to the conclusion that either performed nucleotides or the HGPRT enzyme itself is crossfed to the $HGPRT^-$ cells by $HGPRT^+$ cells. Ashkenazi and Gartler (1970) studied fibroblasts from a Lesch-Nyhan male and a HGPRT normal donor. The normal cells exposed to ^{3}H-hypoxanthine for four hours showed greater than 100 grain counts per cell, while similarly treated $HGPRT^-$ fibroblasts showed 0-10 grain counts per cell. The sonicate from normal cells was then incubated with the $HGPRT^-$ cells for twenty-four hours. After removal of the sonicate, ^{3}H-hypoxanthine was added, and autoradiographs showed that the majority of the $HGPRT^-$ cells were labelled with grain counts of 50-100 grains per cell. The label was primarily localized in the cytoplasm. There was a linear correlation between sonicate concentration and enzyme activity up to 0.1 ml of sonicate, and labelling could still be observed up to 24 hours after removal of the sonicate. The addition of cycloheximide to the fibroblast-sonicate mixture did not reduce the corrective effect of the sonicate. On the other hand, activity of the sonicate was destroyed both by preheating the sonicate and pronase digestion. Ashkenazi and Gartler suggested that the corrective factor is proteinaceous. HGPRT showed a half life of 15 to 18 hours under their experimental conditions.

Cox, Krauss, Balis, and Dancis (1970) considered three possible corrective factors that could be cross-fed between $HGPRT^+$ and

HGPRT$^-$ cells; (a) substance(s) such as episomal DNA or mRNA that enable HGPRT$^-$ cells to synthesize a functional HGPRT, (b) preformed functional HGPRT, and (c) the nucleotide or product of the nucleotide. They reasoned that if the first two products, i.e., episomal DNA or mRNA and intact HGPRT, were cross-fed, incorporation of ^{3}H-hypoxanthine would continue in well dispersed mutant cells as long as functional enzyme persisted. The latter mechanism, nucleotide transfer, would predict that incorporation of the tritium label would cease in mutant cells immediately after they are separated. One:one mixtures of HGPRT$^+$ and HGPRT$^-$ cells were harvested just prior to confluency, and exposed to ^{3}H-hypoxanthine for two hours. Autoradiographs showed that 110 out of 200 cells had less than 10 grains per cell, the remainder showing more than 10 counts per cell. Control cultures consisting of HGPRT$^+$ and HGPRT$^-$ cells in intimate contact on cover slips showed only 10 cells with less than 10 grains per cell. Ten or more grains per cell were found in the remaining 190 cells from the control cultures. Thus, two hours after dispersion of the mixed cultures, approximately half of the cells had reverted to the mutant phenotype of less than 10 grains per cell. Cox et al. determined that HGPRT is stable under their experimental conditions for up to twelve hours, thus ruling out instability of the enzyme as a cause of the reduction in labelling.

Present knowledge of the process of metabolic cooperation as defined by Subak-Sharp et al. (1969) may shed some light on the

conflicting results of Ashkenazi and Gartler and Cox et al. First, intimate cell contact is required for cross-feeding to occur (Cox et al., 1972). Conditioned medium from normal cultures does not increase nuclear labelling of mutant cells. Physical separation of the cells prevents cooperation between $HGPRT^+$ and $HGPRT^-$ cells. Second, electronmicrographs show that $HGPRT^+$ cells and $HGPRT^-$ cells participating in metabolic cooperation have gap junctions of the cell membrane (Gilula, Reeves, and Steinbach, 1972). These gap junctions serve as pathways that allow the passage of small molecules and are formed by ionic coupling of the membranes of adjacent cells (Lowenstein, 1966). Gilula et al. showed that $HGPRT^+$ Chinese hamster cells (Don) and an 8-AG resistant clonal subline DA exhibit metabolic cooperation. Electronmicrographs of intimately associated Don and Da cells showed the presence of gap junctions. $HGPRT^-$ A9 mouse fibroblasts could not be corrected by cross-feeding from $HGPRT^+$ cells (Cox et al., 1972), and Gilula et al. observed no gap junction between Don:A9 or A9:A9 cells. This suggests that in the absence of gap junctions, cross-feeding between $HGPRT^+$ and $HGPRT^-$cells does not occur. Third, the distribution of ^3H-hypoxanthine label in crossfeeding cells is primarily nuclear or nucleolar (Cox et al., 1972) rather than cytoplasmic as observed by Ashkenazi and Gartler.

On the basis of these findings it seems reasonable to ask whether or not the experiments of Ashkenazi and Gartler and Cox et al. represent the same phenomenon. If the gap junction is the

site of transfer of the HGPRT corrective factor in fibroblasts, then it should be possible to determine the maximum size molecules able to be exchanged. On the other hand, metabolic cooperation may involve more than one mechanism such that in the presence of free HGPRT molecules, as in the sonicates used by Ashkenazi and Gartler, cell uptake of preformed enzyme becomes possible, while intact cell populations exchange preformed enzyme products through membrane gap junctions.

As can be expected, metabolic cooperation during growth of cells in selective medium influences the frequency with which rare mutant $HGPRT^-$ cells can be recovered. Van Zeeland, van Diggelen, and Simons (1972) using human diploid fibroblasts, showed a significant decrease in the frequency of 8-AG resistant cells as the number of wild-type cells exposed to the drug was increased. The failure to rescue mutant cells can be due to either the cross-feeding of active HGPRT to the deficient cells allowing them to form the 5'-monophosphate of 8-AG, or the cross-feeding of the preformed product from wild type cells. Either alternative precludes the growth of $HGPRT^-$ cells in the selective medium.

Sato et al. (1972) using human lymphocyte lines observed a decrease in the frequency of 6-TG resistant cells only when the wild-type population was 10^7 cells or greater per culture. Although lymphocyte lines are suspension cultures and do not form monolayers, multi-cell aggregates occur, providing contact and the opportunity

for cross-feeding. Sato et al. considered a metabolic cooperation effect for the decline in ability to recover HGPRT$^-$ cells in the presence of 10^7 wild-type cells, or the release of toxic products by the dying cells could also account for their observations.

In anticipation of a density effect in my experiments, reconstruction experiments were designed to determine the efficiency with which HGPRT$^-$ cells could be recovered under the experimental conditions to be used, and to establish the sensitivity of the selection with 6-TG medium. These experiments involved the co-cultivation of small numbers of UM-10 Lesch-Nyhan cells (from a male donor) with increasing numbers of normal HGPRT$^+$ cells (from a female donor) in medium containing 6-TG. The UM-10 cells could be identified by their growth in selective medium and, by the presence of a Y-chromosome.

Materials and Methods

Four separate reconstruction experiments were performed. The number and composition of the cultures in each can be seen in Table III. Cells from the normal female line UM-39, in logarithmic growth, were counted, and the appropriate dilutions with RPMI-1640 medium were made to give the final desired viable cell concentration in one ml. Serial ten-fold dilutions of the UM-10 cells were made in RPMI-1640 medium containing twenty micrograms of 6-TG per ml until the required number of cells per ml was reached. Individual cultures in tissue culture plates (Linbro Chemical Co., New Haven, Conn.) were initiated

TABLE III

COMPOSITION OF MIXED CULTURES IN RECONSTRUCTION EXPERIMENTS

Experiment Number	Number of Cultures Initiated*	Number of Female Cells per Culture**	Number of Male Cells per Culture***
1	12	10^4	100
2	12 (1)	10^5	10
3	82 (9)	10^5	10
4	48 (8)	10^6	10

* Number in paraenthesis euqals cultures lost to contamination.
** UM-39, HGPRT+, chromosomes 46, XX.
*** UM-10, HGPRT-, chromosomes 46, XY.

with one ml of UM-39 cells and one ml of UM-10 cells to give a final culture volume of two ml, and a final concentration of 10 micrograms of 6-TG per ml. Cultures were harvested for chromosome analysis by standard procedures. At least ten metaphases were analyzed from each culture. The number of chromosomes and the presence or absence of a Y-chromosome in each cell examined was determined.

Results

A. Growth of Cells in 6-TG Medium

By the end of eighteen days of culturing, cell growth was evident in most of the cultures in all four experiments. Small, but macroscopic, clumps of cells were visible on the bottom of the culture wells. Experiment No. 1, which contained fewer UM-39 cells and ten times as many UM-10 cells as in the other experiments (Table III) was terminated at this time, since the cell population had reached 10^6 cells. In the remaining three experiments six to eight weeks of culturing were required before 10^6 cells were present. Sample aliquots from a few cultures in each experiment at this time showed an average density of 10^6 cells per culture with a range of 0.69×10^6 to 1.7×10^6 cells per culture.

As seen in Table III, a total of 18 cultures were lost to contamination during the growth period and were discarded. On the other hand, twelve cultures showed no visible signs of growth at the termination of selection. No viable cells, as determined

by trypan blue dye exclusion tests, were detected in samples removed for observation. Contamination was not evident in these cultures, and they were discarded. Excluding contaminated cultures, 85% of the cultures initiated in these experiments yielded significant numbers of cells after approximately four weeks of incubation.

B. Cytogenetic Analysis

Fifty metaphases per culture were examined in Experiment No. 2. Ten metaphases per culture were karyotyped. Of 136 total cultures, 113 showed the presence of cells with a male karyotype (Table IV). Female cells were detected in a few cultures in all experiments. However, they did not comprise the major cell population, as only one or two cells per culture with the XX karyotype could be seen on analysis. When the UM-39 population was increased to 10^6 cells, more cultures and more cells per culture showed the female sex chromosome constitution. No male cells were detected in one culture of Experiment No. 3 and in four cultures of Experiment No. 4. Cell growth in these cultures was uninhibited despite the continuous presence of 6-TG in the culture medium.

Six cultures in Experiment No. 3 consisted primarily of tetraploid cells (Table IV) and the presence of a Y-chromosome could not be concusively determined by direct visualization or karyotyping. No cultures in any of the other experiments showed the presence of tetraploidy over that normally found in lymphocyte lines in our laboratory (2-5%). Over all, the UM-10, $HGPRT^-$ cells were recovered

TABLE IV

CYTOGENETIC ANALYSES OF THE CULTURES IN THE RECONSTRUCTION EXPERIMENTS

Experiment Number	Number Cultures Analyzed*	Number Cultures With Male Cells Recovered	Number Cultures With No Male Cells Recovered	Recovery Efficiency**
1	12	10	2	83.33
2	11	8	3	72.73
3	73***	63	4	86.30
4	40	32	8	80.00
TOTALS	136	113	17	

* Number cultures analyzed = Number cultures initiated - contaminated cultures.

** Recovery Efficiency = $\frac{\text{Total Number of Cultures with Male Cells Recovered}}{\text{Number of Cultures Analyzed}}$ x 100.

*** Six cultures in this experiment contained predominantly tetraploid cells. These cultures are included in the total number of cultures analyzed, but excluded from the number of cultures with male cells.

in approximately 80% of the cultures analyzed. The remaining 20% takes into account the cultures in which no male cells were detected among the cells analyzed, and those cultures yielding no growth of any cells.

Discussion

A. The Recovery Efficiency and Metabolic Cooperation

The results from these experiments indicate that up to 10^6 $HGPRT^+$ lymphocytes did not decrease the ability to recover the UM-10 $HGPRT^-$ cells, even though the initial mutant population consisted of ten cells. These data are in agreement with that of Sato et al., (1972) who found no effect on the recovery of $HGPRT^-$ cells when co-cultivated with fewer than 10^7 $HGPRT^+$ cells. Furthermore, the sensitivity of 6-TG selection, as determined in these experiments, is such that one mutant cell per 10^5 normal cells can be detected 80% of the time (Table IV, Experiment No. 4). Since this is the frequency at which $HGPRT^-$ cells were found in human diploid fibroblast cultures (Albertini and Demars, 1960; Demars and Held, 1972), selection for $HGPRT^-$ cells in lymphocyte lines should be successful.

The failure to recover mutants in several of the cultures in the experiments may reflect technical errors in dilution and inoculation or a population density effect, neither of which may be mutually exclusive. Since serial ten-fold dilutions of the UM-10 cell line were made from densities of 2×10^6 cells per ml

down to 10 cells per ml, some UM-10 cells may have been excluded from some inocula at this low cell density. However, sampling error was tested in initial experiments by culturing several of the one ml inocula used for the reconstruction experiments. Twenty-four consecutive inocula showed the presence of cells 24 hours post-initiation, making it unlikely that cultures of the test groups received no cells. In addition the UM-10 inoculum of Experiment No. 1 consisted of 100 cells per ml instead of 10 cells per ml (Table III), and two cultures still failed to yield growth of cells. The higher cell number used in this instance makes it unlikely that these two cultures received no cells. This indicates that technical error alone could not account for the failure to observe growth in some cultures. If the failure to recover $HGPRT^-$ cells is due to their low numbers in the mixed cultures, this effect could be mediated through any of the mechanisms postulated by Sato et al. These are (a) metabolic cooperation, (b) rapid depletion of the medium by the wild-type, $HGPRT^+$ cells, or (c) the release of toxic products by the dying UM-39 cells.

The efficiency with which $HGPRT^-$ lymphocytes can be recovered from $HGPRT^+$ populations is much greater than that observed in similar experiments with fibroblasts. Albertini and DeMars (1973) found that $HGPRT^-$ fibroblasts achieved 80% of their basic cloning efficiency in the presence of 10^4 normal fibroblasts. When the population of normal cells was increased to 10^5 cells, mutant cell recovery was 5% of the cloning efficiency, and at 10^6 $HGPRT^+$ cells,

none of 200 $HGPRT^-$ cells were recovered. Likewise, van Zeeland et al. (1972) recovered none of 750 $HGPRT^-$ mouse cells from a population of 10^5 $HGPRT^+$ cells even with the use of a feeder layer to enhance colony formation. Their results showed that with a feeder layer, 10^4 $HGPRT^+$ cells per 10 mm petri dish was the maximum number of cells that did not greatly interfere with the recovery of $HGPRT^-$ cells. Therefore, there is a significant metabolic cooperation effect in fibroblast cultures that is not manifested at the same $HGPRT^+$:$HGPRT^-$ cell ratios in lymphocyte lines.

The failure to observe decreases in recovery efficiency of $HGPRT^-$ cells in lymphocyte lines similar to that found in equivalent experiments with fibroblasts lines raises the question of whether or not metabolic cooperation at the HGPRT locus occurs in lymphocyte lines. Hemapoietic cells, like fibroblasts, generally do not manifest the heterozygous state of HGPRT deficiency, either on the basis of direct enzyme assay or ^3H-pruine uptake (Henderson, 1968). Dancis et al. (1968) demonstrated that metabolic cooperation does not occur in short term cultures of human peripheral blood leucocytes. When lymphocytes from four presumed heterozygotes were incubated with ^3H-hypoxanthine and autoradiographs prepared, Dancis et al. observed that grain counts were similar to those of normal donors. They did not observe two populations of labelled and non-labelled cells. The artificial mixture of equal numbers of lymphocytes from a congenital hyperuricosuric patient and from normal donors gave the expected 1:1 ratio of labelled to non-labelled

cells, indicating the effectiveness of the technique. These results indicate that peripheral blood lymphocytes do not exhibit metabolic cooperation at the HGPRT locus. Based on the data of Dancis et al., Sato et al., and the present reconstruction experiments, it is reasonable to suggest that metabolic cooperation at the HGPRT locus does not occur in lymphocyte lines, and possibly not in vivo in lymphoid elements.

The failure to observe metabolic cooperation in lymphocyte lines should not be completely unexpected based on our knowledge of the phenomenon in fibroblast cultures. Intimate cell contact is necessary for cross-feeding to occur. Metabolic cooperation is not seen when $HGPRT^+$ and $HGPRT^-$ cells are separated by physical barriers, nor can conditioned medium from normal cells correct deficient cells (Cox et al., 1972). Gilula et al. (1972) found that those cells undergoing cross-feeding in Chinese hamster fibroblast cultures exhibit ionic coupling and gap junctions of the cell membrane. Mouse L cells which are incapable of serving as donors or recipients of HGPRT corrective factor(s) (Cox et al., 1972), show neither ionic coupling nor gap junctions, despite close membrane associations, (Gilula et al., 1972). Gilula et al. suggested that cross-feeding is a membrane associated function, and that physical alterations of the membrane are necessary for it to occur. The membranes of the majority of cells in lymphocyte lines are coated by immunoglobulins which may physically prohibit

the close associations required for cross-feeding of HGPRT corrective factor(s). Alternatively, Gilula et al. suggest that a specific cell membrane structure may be necessary for cross-feeding. By virtue of the specialized functions of lymphocyte membranes, the differentiation processes may have precluded the presence of this membrane structure, and hence, the ability of lymphocytes to participate in the exchange of HGPRT corrective factor(s).

The question of whether or not lymphocytes can participate in cross-feeding of HGPRT corrective factor(s) might be resolved by mixing experiments similar to those of fibroblasts studies. To determine whether or not lymphocytes are capable of serving as donors of the HGPRT corrective factor(s), a monolyaer of $HGPRT^-$ fibroblasts could be overlaid with lymphocytes from a normal donor in medium containing ^3H-hypoxanthine. After a period of time during which cross-feeding may be expected to occur, the lymphocytes could be washed off and the uptake of the label assayed in the fibroblasts. The reverse of this may be done using $HGPRT^-$ lymphocytes and $HGPRT^+$ fibroblasts as donors to determine the ability of the lymphocytes to receive the corrective factor(s). Since cells of different established lymphocyte lines are indistinguishable morphologically and grow in suspension, one is not able to identify single $HGPRT^+$ and $HGPRT^-$ cells in mixed populations. If it is possible to specifically label one population of lymphocytes, e.g. carbon or carmine particles (Stoker, 1964) such that donor and

recipient cells are easily recognized, then mixing experiments may be done to study cross-feeding between lymphocytes.

B. The Presence of XX Cells in the Cultures of the Reconstruction Experiments

The presence of XX cells in some cultures of the experiments may be explained in either of two ways. First, the XX cells could be an indication that 6-TG selection is inadequate. However, if this were the case, one would expect a higher frequency of cultures showing female cells than was observed in these experiments. This would be especially true in Experiment No. 4 where 10^6 UM-39 cells were present in each culture. Surely if 6-TG selection were inadequate and cytotoxic levels of the drug were exhausted prior to a cytotoxic effect, Experiment No. 4 would have shown more than four out of forty-eight cultures with female cells only. In addition, the UM-39 line had sensitivities to 6-TG similar to those of the normal lines shown in Figures 5 and 6. Thus it is felt that the concentration of 6-TG used in these experiments was adequate to inhibit the growth of $HGPRT^+$ cells.

More likely, the XX cells observed in these cultures represent spontaneously occurring $HGPRT^-$ cells that were present in the original UM-39 inoculum. When eight cultures containing 10^5 UM-39 cells alone were inoculated into 6-TG medium, two cultures gave rise to sublines designated UM-39-TG-1 and UM-39-TG-2. These two

sublines have been maintained for approximately two years in the absence of 6-TG selection. 6-TG was recently added to the medium of UM-39-TG-2 and had no evident effect on cell growth. HGPRT assays showed that the enzyme activity of the UM-39 parent was 63.0 nanomoles/mg protein/hour. UM-39-TG-1 and UM-39-TG-2 had specific activities of 0.07 and 10.80 nanomoles/mg protein/hour, respectively (Table VII). Therefore, the UM-39 line contains spontaneous $HGPRT^-$ cells, and this lends credence to the hypothesis that those present in the cultures of the reconstruction experiments could have been present in the original inoculum of UM-39 cells. Overgrowth of these $HGPRT^-$ UM-39 cells could have prevented the detection of the UM-10 cells in view of the small number of cells analyzed from each culture.

C. Tetraploidy

Tetraploidy is normally found at a level of 2-5% in many lymphocyte lines, and occasionally in short-term phytohemagglutinin-stimulated, peripheral blood leucocyte cultures. In addition some lines maintained in our laboratory have developed as high as 80% tetraploid cells, only to lose them with continuous cultivation. The reason(s) for the mitotic failure is not understood.

The tetraploid cells in Experiment No. 3 may have arisen in any of three ways which are non mutually exclusive. First, the tetraploids may represent 4N cells present in the original inocula of parental UM-39 or UM-10 cells, and these cells might have persisted through the selection period. Secondly, they could have arisen during the selection process by virtue of the failure of the nuclear membrane to breakdown. Finally, they may represent a fusion product of UM-39 and UM-10 cells.

It seems reasonable at this time, however, to exclude cell fusion as a major factor contributing to tetraploidy. In our laboratory proven incidences of lymphocyte-lymphocyte fusion have only occurred in the presence of inactivated Sendai virus, thermal shock, and at cell population densities of 10^7 cells for each parent (Bloom and Nakamura, 1974). No hybrids have been observed under other conditions, which is, however, not to say that they don't occur. In addition, if spontaneous cell fusion did occur, cell numbers would indicate that the most likely fusions would occur between an $HGPRT^+$ cell and an $HGPRT^-$ cell or between two $HGPRT^+$ cells. The latter fusion product would presumably contain two active HGPRT alleles and would not be expected to survive in 6-TG medium. The former fusion product in controlled experiments has been shown to contain levels of HGPRT activity intermediate between normal and $HGPRT^-$ levels, and the $HGPRT^+$-$HGPRT^-$ hybrids survive in HAT medium. Thus the continuous presence of 6-TG in

the culture medium argues against survival of this fusion product, and against the idea that the tetraploids represent rare hybridization events.

Summary

The reconstruction experiments have shown that as many as 10^6 $HGPRT^+$ cells do not interfere with our ability to detect $HGPRT^-$ cells even though only 10 mutant cells were present in the initial mixed culture. Comparison of the results from these experiments with those of other investigators who have used human and non-human fibroblast cultures raises the question as to whether or not the cells of lymphocyte lines engage in metabolic cooperation. Recovery of mutant cells, however, was not 100% under the methods used to detect their presence, and some cultures yielded no growth of mutant cells. If metabolic cooperation is not responsible for incomplete recovery of mutants, then other phenomena, such as nutrient deprivation, cytotoxic factors, or technical error may be responsible. The 6-TG medium is able to suppress the growth of $HGPRT^+$ cells; those cells which survive in this medium are $HGPRT^-$, as evidenced by stability of the $HGPRT^-$ phenotype in the absence of selection and HGPRT assays on the surviving cells. Thus selection for mutants at the HGPRT locus in human lymphocyte lines is feasible under the conditions described in these experiments.

CHAPTER IV

ESTIMATION OF THE MUTATION RATE AT THE HGPRT LOCUS IN ESTABLISHED HUMAN LYMPHOCYTE LINES

Introduction

The estimation of the mutation rate at the HGPRT locus in lymphocyte lines was calculated from the data derived from Luria-Delbruck fluctuation analyses. Luria and Delbruck (1943) used this test to provide evidence for the mutational origin of resistance to bacteriophage infection in bacterial populations. This test makes use of the fluctuations in the numbers of mutant cells in N number of identical cultures. The fluctuations in mutant numbers per culture is ascribed to the randomness of mutation events, the heritability of mutations, and the clonal growth of mutant cells, as well as the time at which mutation occurs.

A fluctuation analysis involves the initiation of a series of identical cultures from a small population of cells presumed to contain no or very few pre-existing mutants. At the same time, a single large culture is initiated such that the cell density is equivalent to that in the series of identical cultures. The series of identical cultures and the single large culture are allowed to proliferate for several generations in the absence of selection.

When a sufficiently large population of cells that will allow accurate detection of mutants is obtained in all cultures, selection is imposed such that wild-type cells are eliminated, and the mutant cell can be identified. The series of identical cultures are exposed simultaneously to the selective agent. Replicate samples of equal volume and cell density are removed from the single large flask and exposed to selection in the same manner.

The principles of the fluctuation analysis are as follows: Under a mutation hypothesis it is assumed that the variant phenotype, be it phage resistance or drug resistance, arose by mutation in the series of identical cultures prior to the addition of the selective agent, and not in response to it. Under this assumption, then, the independent cultures may vary in the number of mutations that occurred in each during the absence of selection. They will also vary in the time at which mutation occurred. These two variables, the number of mutational events and the time of their occurrence, will contribute to large fluctuations in the number of variant cells among the independent cultures. It follows that the distribution of variant cells, among the independent cultures will have a variance many times larger than the mean.

On the other hand, if the variant phenotype was not present in the population prior to the addition of the selective agent, but was induced by interaction with the agent, the distribution of variant cells among the identical cultures would be the same as that

obtained by replicate sampling from the single large culture. This distribution would have a small variance that is equivalent to its mean.

Provided that the expected distributions of variant cells is obtained, the rate of formation of the mutant phenotype can be calculated from the data obtained in the fluctuation analysis. The mutation rate may be defined as the rate of occurrence of new mutational events per cell generation and may be represented mathematically by the formula:

$$\mu = m/g \qquad \text{(Equation 1)}$$

where μ = mutation rate

m = mean number of mutations

g = total number of cell generations (Sager and Ryan, 1962)

The term g is approximately equal to the total number of cells present at the time of selection, divided by the natural logarithm of 2. The mean number of mutations can be obtained from the proportion of cultures in the fluctuation analysis which contain no detectable mutants. The mutants are distributed among the cultures in a Poisson fashion. If the Poisson probability is defined mathematically as

$$P_{(x)} = \frac{m^x e^{-m}}{x!} \qquad \text{(Equation 2)}$$

when x equals 0, then the probability of obtaining no mutants is

$$P_0 = e^{-m} \qquad \text{(Equation 3)}$$

This equation can be solved for m and the mutation rate calculated from Equation 1.

The use of the data from the fluctuation analysis in this manner has several advantages over other methods for obtaining mutation rate estimates. First, the analysis provides evidence that mutation gave rise to the variant phenotype by virtue of random fluctuations in the numbers of variant cells among independent cultures. Second, the data generated can be used to estimate the rate at which the mutant phenotype occurs. Finally, the estimation of the mutation rate from the fluctuation analysis in conjunction with the Poisson, is dependent only upon the fact that mutation occurred, and knowledge of the actual number of mutational events is not needed. This last advantage is of particular importance in using lymphocyte lines, since low cloning efficiencies preclude the counting of actual mutants. The fluctuation analysis, however, is quite cumbersome technically, as large numbers of independent cultures are necessary for good, reproducible results.

Materials and Methods

A. Cell Lines

The UM-61 cell line was chosen for use in the fluctuation analyses. This lymphocyte line was established from a normal male in 1972. Although Epstein-Barr-viral antigens are detectable in the cells of this line, UM-61 was established in the absence of an Epstein-Barr-virus containing lysate. The line has been in

continuous culture since establishment, and it has maintained a normal 46 XY chromosome complement. The uncloned parental population doubles at approximately 24 hour intervals, with cell densities approaching 2×10^6 cells per ml, and viabilities of 90% or more during logarithmic growth. HGPRT activity in UM-61 is approximately 419×10^{-10} moles/mg protein/hour, and the population is completely inhibited by 5 micrograms of 6-TG per ml of culture medium (Figure 5). One 6-TG resistant, $HGPRT^-$ subline has been derived from the parental line by culturing the cells in 10 micrograms of 6-TG (Nakamura, 1974, personal communication). Thus this line shows the presence of spontaneously occuring $HGPRT^-$ cells. The UM-61 line was chosen for study because of the above characteristics.

B. Cloning on Soft Agarose

In order to insure population homogeneity with respect to the HGPRT locus, the UM-61 line was cloned on soft agarose according to the method of Sato et al. (1972). Approximately 100 cells in a volume of 0.1 ml of growth medium were mixed with 0.24% agarose. The mixture was then dispersed into 60 mm petri dishes that contained a feeder layer of human embryonic lung fibroblasts and a separating layer of 0.24% agarose. Dishes were incubated at 37°C in a humidified CO_2 and air atmosphere, and fed weekly with 0.5 ml of fresh RPMI-1640 culture medium. Approximately

four weeks later colonies of varying size were picked up with gentle suction using an 18 gauge needle to which a 3 ml syringe containing growth medium was fitted. Cells were aspirated into individual wells of microtest plates (Falcon Plastics). Cultures were maintained in the wells for varying periods of time until growth was sufficient for transfer to larger culture vessels. The final transfer was to T-25 flasks (Falcon Plastics) approximately four weeks after isolation from the agarose plates. The surviving clonal populations were used in the fluctuation analyses.

C. Fluctuation Analyses

The clonal sublines to be used were counted and the number of cells needed for a final concentration of 1000 or 5000 cells per ml were placed in a sterile flask containing medium and a magnetic stirrer. The flask was placed over a stirring apparatus to insure equal distribution of cells in the 1 mm aliquot removed for seeding the individual cultures. The regular growth medium was supplemented with conditioned medium from the parent lines in a 1:1 ratio to enhance cell survival at the low population density.

Because of technical difficulties in maintaining cell growth at low densities in a single large flask, four smaller cultures were initiated at the same cell density as the series of identical cultures. These four smaller flasks were then pooled into a single culture when cell growth was established. Fresh medium was added weekly to all cultures. When cell growth in the individual culture

was established they were also transferred to larger culture flasks, and culturing was continued until a single culture flask contained 2-3 x 10^6 cells distributed in a 3 ml culture volume.

When the cell population in the single large culture approached 10^6 cells per ml, the growth medium was removed by centrifugation. The cells were resuspended at a density of 0.5 x 10^6 cells per ml in 0.24% agarose containing 10 micrograms of 6-TG per ml. Samples of 2 ml each were then dispersed into individual petri dishes. The dishes were returned to the incubator to allow for a colony formation. Fresh medium with 6-TG was added weekly.

Five flasks from the series of identical cultures were selected at random and counted for an estimate of the total cell population. Initially, each culture was plated in 0.24% agarose with 6-TG at a density of 10^6 cells per plate to obtain colony formation. However, repeated experiments failed to yield mutant growth in the plating experiments, despite the detection of mutant cells in suspension cultures. Thus the selection for 6-TG resistant cell was carried out in suspension cultures throughout the work reported here. Fresh 6-TG medium was added to the flasks in a 1 ml volume to give a final concentration of 10 micrograms per ml. Each culture was mixed well, cells were dispersed by pipetting, and the cultures were returned to the incubator. Seventy-two hours later, 1 ml of 6-TG medium was added. One week after the first addition of 6-TG medium the culture volume was reduced, because of low cell density,

by removal of approximately 50% of the total volume and replacing this with only 0.5 ml of 6-TG medium. The medium added also contained only 5 micrograms of 6-TG per ml. Culturing was continued in reduced volume in the presence of 6-TG for two more weeks. Growth was then continued through the remainder of the experiments in the absence of 6-TG. Approximately four weeks after the addition of 6-TG selection, the medium volume was reduced to approximately 2 ml, and a sample aliquot was removed and tested with a 1% solution of trypan blue vital stain. Those cultures in which viable cells were observed were returned to the incubator to allow for continued growth of cells for enzyme assays.

D. Enzyme Assays

HGPRT assays for mutant and control cultures were performed according to the method of Sato et al. (1972). Each was run in duplicate on 1 to 2 x 10^7 cells. Soluble proteins were determined according to the Lowry method (Lowry et al. 1951).

E. Chemical Mutagenesis

Ethyl methanesulfonate (EMS, Eastman Organic Chemicals) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Aldrich Chemical Co.) were prepared in phosphate buffered saline (pH 7.2) and acetate buffer (pH 5.0) respectively, according to the method of Sato et al. Solutions were prepared fresh for each experiment. Cultures were initiated at 1 x 10^6 cells per ml from each line to be tested in culture flasks containing 10 or 30 ml of RPMI-1640 growth medium. EMS or MNNG was then added to give the final desired concentration

of mutagen per ml. Cells were exposed to EMS for 24 hours at concentrations of 50, 100, or 200 micrograms/ml. MNNG concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 or 3.0 micrograms/ml. were added for a 2 hour period.

Two mutagen treatment and selection protocols were used. Initially, cells were treated with mutagen for the designated time, washed three times with Hank's Balanced Salt Solution (HBSS), resuspended in RPMI medium, and returned to the incubator for a 72 hour recovery period. Cell counts on each culture were made to estimate the survival, and either 10^5 cells were immediately cultured in 2 ml of 6-TG selective medium in tissue culture plates (Linbro Chemical Co., New Haven, Conn.) or 10^6 cells were seeded onto 60mm plates following the general cloning procedures previously described. The fibroblast feeder layers for the cloning plates were derived from a Lesch-Nyhan patient (The fibroblasts were kindly supplied by Dr. Barbara Migeon, Baltimore, Md.). In some instances where growth of the fibroblasts was insufficient to provide feeder layers for the required number of plates, the agarose was prepared with medium composed of a 1:1 mixture of conditioned:fresh medium. Cultures were then incubated at 37°C for several weeks. Non-mutagen treated control cultures were prepared simultaneously.

In protocol II, cells were treated with mutagen as described above, washed, resuspended in 10 ml of fresh medium, and reincubated for 72 hours. Counts were then made to estimate survival. Cultures were washed again and returned to the incubator for an additional

7 days of recovery. Cell washings were made at 48 hour intervals during this period, and in those cultures receiving high doses of mutagen, culture volume was reduced to maintain a surviving cell population of 0.1 to 0.5 x 10^6 cells per ml. Ten days after mutagen treatment, cell counts were made, and cells were seeded into the 60mm cloning plates. No 6-TG was added to the growth medium, and the feeder layer of fibroblasts were derived from a normal donor (supplied by C. C. Chang, University of Michigan). This protocol differs from the preceding one in that a longer recovery period was allowed after mutagen exposure, and colony formation was allowed to occur in the absence of 6-TG selection.

Results

A. Cloning on Soft Agarose

The cloning efficiency of the UM-61 parent line on soft agarose was approximately 12%. Although 44 colonies were isolated from the agarose plates after four weeks of cloning, only three clones survived consecutive transfers and grew to cell densities suitable for use in fluctuation analyses.

In no instance was growth of cells from the UM-61 clones obtained when cells were plated on soft agarose containing 6-TG medium. Since recloning of the UM-61 clones 2, 14, and 24 in the absence of 6-TG was unsuccessful, the uncloned parental line was used to test the plating efficiency. Control cultures gave colony formation in the absence of 6-TG, but no colonies formed when

10^5 or 10^6 cells from the parental line were plated in 6-TG medium. It is not understood why 6-TG resistant cells could not be detected. However, the initial cloning efficiency of the UM-61 parent line is low in comparison to that reported by others (Sato et al., 1972) and may have precluded selection in 6-TG medium. In addition only one clone of cells from a Lesch-Nyhan patient were available for use as feeder layers. These cells may have been unable to provide the necessary functions of a feeder layer at a level to sustain clonal growth.

B. Fluctuation Analyses

A total of twenty-two different series of identical cultures were initiated, each with its appropriate single large flask. In the first eight, an initial starting population of 1000 cells was used. Although a 1000 cell inoculum was sufficient to obtain large populations in preliminary experiments with other cell lines, generally the clonal lines from UM-61 failed to grow from this low cell density, and only a few cultures survived. The ramining fourteen analyses were then initiated from 5000 cells. While this five-fold increase in the starting population improved cell survival and allowed for growth to high densities, five analyses were still unsuccessful because too few cultures contained cell populations of 2×10^6 cell which were used in selection with 6-TG. In addition not all cultures initiated in successful experiments gave rise to cell populations suitable for selection and were discarded. Thus

only nine of the fourteen analyses initiated with 5000 cells per culture generated enough cells for mutation rate estimates. The results are seen in Table 5.

C. Calculation of the Mutation Rate Estimate

Using the data from fluctuation analysis No. 9 (Table 5) as an example, the mutation rate was calculated as follows:

(1) Number of cell generations--There were 56 cultures in analysis No. 9, each of which was initiated at 5000 cells. Therefore, the initial cell population (N_i) was 56 x 5000 or 2.8×10^5. At the time 6-TG was added, five cultures selected at random showed an average of 7.76×10^6 cells per culture. If there were 56 cultures, each on the average containing 7.76×10^6 cells, the total cell population selected with 6-TG (N_s) was $56 \times 7.76 \times 10^6$ or 4.34×10^8. The number of cell generations was calculated from the expression

$$g = \frac{N_s - N_i}{\ln 2}$$ (DeMars & Held, 1972).

Therefore, $\frac{g = 4.34 \times 10^8 - 0.0028 \times 10^8}{\ln 2} = 6.28 \times 10^8$.

Thus, a total of 6.28 cell generations occurred in the 56 cultures during the period of non-selective growth.

(2) Calculation of P_0--At the end of selection with 6-TG, a total of thirty cultures yielded no viable cells. Therefore, the fraction of cultures yielding no mutants (P_0) equals $\frac{30}{56}$ or 0.53. If a recovery efficiency of 80% is considered, based on the results of

TABLE V

FLUCTATION TESTS ESTIMATING THE SPONTANEOUS RATE OF MUTATION TO 6-THIOGUANINE RESISTANCE *

ANALYSIS	TEST NUMBER 9	TEST NUMBER 10
Cell Line	UM-61-C1-24	UM-61-C1-24
Number of Cultures	56	99
Initial Population (Cells/Culture)	5×10^3	5×10^3
Cells/Culture at Time of 6-TG Selection	7.76×10^6	3.72×10^6
Total Cell Population Selected with 6-TG	4.34×10^8	3.68×10^8
Number Cell Generations	6.26×10^8	5.31×10^8
Number Cultures with No Mutants	30(24)*	67(54)
Fraction of Total Cultures With No Mutants (P_0)	0.53(0.43)	0.67(o.54)
Mean Number Mutants per Culture (m)	0.62(9.84)	0.39(0.61)
Variance of m	0.015(0.023)	0.005(0.008)
Total Number of Mutations	34.72(47.04)	38.61(60.39)
Mutation Rate per Cell Generation	0.557×10^{-7} ($o.751 \times 10^{-7}$)	0.727×10^{-7} (1.13×10^{-7})

*Values in parentheses represent corrections for 80% recovery efficiency. (See text.)

TABLE V, Continued

FLUCTATION TESTS ESTIMATING THE SPONTANEOUS RATE OF MUTATION TO 6-THIOGUANINE RESISTANCE *

ANALYSIS	TEST NUMBER 14	TEST NUMBER 16
Cell Line	IM-61-parent	UM-61-parent
Number of Cultures	80	104
Initial Population (Cells/Culture)	5×10^3	5×10^3
Cells/Culture at Time of 6-TG Selection	5.14×10^6	2.52×10^6
Total Cell Population Selected with 6-TG	4.11×10^8	2.62×10^8
Number Cell Generations	5.93×10^8	3.78×10^8
Number Cultures with No Mutants	51(41)	12(10)
Fraction of Total Cultures With No Mutants (P_0)	0.64(0.51)	0.115(0.093)
Mean Number Mutants per Culture (m)	0.45(0.68)	2.16(2.37)
Variance of m	0.007(0.012)	0.0737(0.093)
Total Number of Mutations	36.0(54.4)	224.64(246.48)
Mutation Rate per Cell Generation	0.607×10^{-7} (0.917×10^{-7})	5.94×10^{-7} 6.44×10^{-7})

*Values in parentheses represent corrections for 80% recovery efficiency. (See text.)

TABLE V, Continued

FLUCTATION TESTS ESTIMATING THE SPONTANEOUS RATE OF MUTATION TO 6-THIOGUANINE RESISTANCE *

ANALYSIS	TEST NUMBER 17	TEST NUMBER 18
Cell Line	UM-61-C1-24	UM-61-C1-24
Number of Cultures	79	64
Initial Population (Cells/Culture)	5×10^3	5×10^3
Cells/Culture at Time of 6-TG Selection	2.34×10^6	1.99×10^6
Total Cell Population Selected with 6-TG	1.87×10^8	1.28×10^8
Number Cell Generations	2.66×10^8	1.85×10^8
Number Cultures with No Mutants	47(36)	42(34)
Fraction of Total Cultures With No Mutants (P_0)	0.59(0.456)	0.656(0.53)
Mean Number Mutants per Culture (m)	0.52(0.78)	0.42(0.64)
Variance of m	0.009(0.015)	0.008(0.014)
Total Number of Mutations	41.08(61.62)	26.88(40.96)
Mutation Rate per Cell Generation	1.54×10^{-7} (2.23×10^{-7})	1.456×10^{-7} (2.22×10^{-7})

*Values in parentheses represent corrections for 80% recovery efficiency. (See text.)

TABLE V, Continued

FLUCTATION TESTS ESTIMATING THE SPONTANEOUS RATE OF MUTATION TO 6-THIOGUANINE RESISTANCE *

ANALYSIS	TEST NUMBER 19	TEST NUMBER 21
Cell Line	UM-61-C1-24	UM-61-C1-24
Number of Cultures	88	61
Initial Population (Cells/Culture)	5×10^3	5×10^3
Cells/Culture at Time of 6-TG Selection	3.32×10^5	2.37×10^6
Total Cell Population Selected with 6-TG	2.93×10^3	1.44×10^8
Number Cell Generations	4.23×10^3	2.08×10^8
Number Cultures with No Mutants	36(29)	23(22)
Fraction of Total Cultures With No Mutants (P_0)	0.409(0.34)	0.459(0.361)
Mean Number Mutants per Culture (m)	0.89(1.08)	0.78(1.02)
Variance of m	0.016(0.222)	0.003(0.029)
Total Number of Mutations	78.32(95.04)	47.58(62.22)
Mutation Rate per Cell Generation	1.85×10^{-7} (2.25×10^{-7})	2.29×10^{-7} (2.29×10^{-7})

*Values in parentheses represent corrections for 80% recovery efficiency. (See text.)

TABLE V, Continued

FLUCTATION TESTS ESTIMATING THE SPONTANEOUS RATE OF MUTATION TO 6-THIOGUANINE RESISTANCE *

ANALYSIS	TEST NUMBER 22	TEST NUMBER
Cell Line	UM-61-Cl-24	
Number of Cultures	69	
Initial Population (Cells/Culture)	5×10^3	
Cells/Culture at Time of 6-TG Selection	2.27×10^6	
Total Cell Population Selected with 6-TG	1.56×10^8	
Number Cell Generations	2.26×10^8	
Number Cultures with No Mutants	41(33)	
Fraction of Total Cultures With No Mutants (P_0)	0.594(0.478)	
Mean Number Mutants per Culture (m)	0.52(0.74)	
Variance of m	0.011(0.016)	
Total Number of Mutations	35.88(51.06)	
Mutation Rate per Cell Generation	1.58×10^{-7} (2.26×10^{-7})	

*Values in parentheses represent corrections for 80% recovery efficiency. (See text.)

the reconstruction experiments, then the corrected fraction of cultures showing no mutants (Pc) is approximately equal to $\frac{24}{56}$ or 0.43.

(3) Mean number of mutants per culture--If $P_o = e^{-m}$ (Equation 3), then

$$P_o = e^{-m} = 0.53, \text{ and}$$

$$P_c = 0.43.$$

Solving for m in both cases, the value for m is 0.62, and the corrected value is 0.84.

(4) Mean number of mutations in the 56 cultures (Lea and Coulson, 1949)--Since the mutation rate is based on the number of mutations occurring in 56 cultures rather than in a single culture, then the mean number of mutants in the 56 cultures is equal to the mean number of mutants per culture times the number of cultures, i.e., 56 x 0.62 or 34.72. The correction for recovery efficiency becomes 56 x 0.84 or 47.04.

(5) Calculation of the mutation rate--From Equation 1,

$$\mu = \frac{m}{g} \quad .$$

If m = 34.72, and g = 6.26×10^8, then

$$\mu = \frac{47.04}{6.26} \times 10^8 \quad = \quad 0.554 \times 10^{-7}.$$

The corrected value becomes

$$\mu = \frac{47.04}{6.26} \times 10^8 \quad = \quad 0.751 \times 10^{-7}.$$

(6) The variance of the estimate of m is given by the expression

$$0_m^2 = \frac{e^m - 1}{N}$$

where N = the number of identical cultures (Lea and Coulson, 1949). For analysis No. 9,

$$0_m^2 = \frac{e^{0.62} - 1}{56} = 0.015$$

The corrected value of $0_m^2 = \frac{e^{0.84} - 1}{56} = 0.023$.

Calculations for the remaining analyses were performed similarly.

The average rate of mutation to 6-TG resistance calculated from the data of 7 independent fluctuation analyses using UM-61-C1-24 was estimated to be 1.428×10^{-7} per cell generation. The estimates showed a range of 0.554×10^{-7} to 2.29×10^{-7} per cell generation. When corrections were made for an 80% recovery efficiency, the estimates ranged from 0.751×10^{-7} to 2.99×10^{-7} with a mean of 2.0×10^{-7} per cell generation. When the uncloned UM-61 parent line was used, the uncorrected mutation rates from two analyses were calculated to b3 0.607×10^{-7} and 5.94×10^{-7} per cell generation. The corrected rates were estimated to be 0.917×10^{-7} and 6.44×10^{-7} per cell generation. Analysis No. 16, in which the highest rate of mutation was found, was performed with the uncloned parental line. Pre-existing 6-TG resistant cells may have inflated the estimate of m as seen in Table 5. Otherwise, all values for the remaining 8 analyses were consistent. The results are summarized in Table VI.

TABLE VI

SUMMARY OF MUTATION RATE ESTIMATES DERIVED FROM FLUCTUATION ANALYSES

Analysis No.	Cell Line	Mutation Rate Per Cell Generation
9	UM-61-C1-24	$0.56 \pm 0.07 \times 10^{-7}$
10	UM-61-C1-24	$0.73 \pm 0.31 \times 10^{-7}$
14	UM-61-Parent	$0.61 \pm 0.05 \times 10^{-7}$
16	UM-61-Parent	$5.94 \pm 1.38 \times 10^{-7}$
17	UM-61-C1-24	$1.54 \pm 0.12 \times 10^{-7}$
18	UM-61-C1-24	$1.45 \pm 0.57 \times 10^{-7}$
19	UM-61-C1-24	$1.85 \pm 0.78 \times 10^{-7}$
21	UM-61-C1-24	$2.29 \pm 0.54 \quad 10^{-7}$
22	UM-61-C1-24	$1.58 \pm 0.39 \times 10^{-7}$

D. Enzyme Assays

Although many cultures showed the presence of viable cells post-6-TG selection, growth was slow, and surviving cultures were difficult to maintain. 6-TG medium was removed from these cultures after centrifugation and replaced with small volumes of conditioned medium to enhance cell survival. These efforts proved fruitless as viable cell concentrations calculated in some cultures at this time were less than 10^4 cells per culture. Several cultures were also lost as a result of premature subculturing and contamination. Only three cultures survived and, when pooled, provided the 20 million cells necessary for the HGPRT assay. Because of the low survival, cloning of the cells of these three cultures was not attempted, and the assays were performed on the uncloned selected populations. All three mutant cultures were from the UM-61-C1-24 subline. Mutant 13 survived from analysis No. 10, and mutants 33 and 51 were obtained from fluctuation analysis No. 9.

As seen in Table VII, these three cultures showed that HGPRT was deficient when compared to the wild-type unselected cells of the UM-61-C1-24 clonal subline. Mutants 13, 33, and 51 showed cells with 56.0%, 22.7%, and 8.5%, respectively, of the HGPRT activity of the normal line. Assays on cells from cultures of UM-61-C1-24 were performed simultaneously as a positive control, and UM-10 was used as the negative control. All other values were taken from previous assays.

VII

HGPRT ASSAYS OF NORMAL AND 6-TG RESISTANT LYMPHOCYTE LINES

Cell Line	Donor	Nanomoles ^{14}C-hypoxanthine used/ mg protein/hour	% Normal
UM-61	Normal male	41.96	
UM-61-Cl-24	Normal male	49.54	
TG-resistant Mutant-13	UM-61-Cl-24	27.59	55.69
TG-resistant Mutant-33	UM-61-Cl-24	11.25	22.71
TG-resistant	UM-61-Cl-24	4.22	8.51
UM-39	Normal female	63.00	
UM-39-TG-1	UM-39	0.07	0.11
UM-39-TG-2	UM-39	10.80	17.14
UM-1-TG	UM-1 (Normal male)	0.68	2.8
UM-10	Lesch-Nyhan male	0.20	0.5

E. Induction of Mutation with EMS and MNNG

No mutants were obtained from those cultures treated with either mutagen. Protocol 1 was used in seven experiments, and Protocol 2 was used in six. The effects of varying concentrations of EMS and MNNG on growth of the UM-61 cells are seen in Figures 7 and 8. Both mutagens affect the logarithmic growth of treated cultures when compared with the untreated control cultures. This effect on survival becomes important as no induced mutants were obtained, and raised questions as to whether both MNNG and EMS were active in the culture medium. Although some cells may have been lost during the washings of cells, logarithmic growth was not observed in any of the treated cultures, despite a viable cell population of 3×10^5 cells or better in some cultures. Cultures treated with 50 micrograms of EMS were plated in soft agarose at cell densities of 1000 cells per plate. Those incubated with 100 or 200 micrograms per ml of EMS were plated at 5000 cells per plate. Only those cultures receiving the 0.05 and 0.10 concentrations of MNNG were plated at 1000 and 5000 cells, respectively. Because of poor viabilities at the remaining concentrations of both mutagens, no cells were plated. Non-treated control cultures were plated at 200 cells per dish. No colonies formed in any mutagen-treated cultures, while untreated control cultures contained visible colonies by three weeks.

Figure 7.--The effect of varying concentrations of ethylmethanesulfonate (EMS) on the logarithmic growth of a lymphocyte line (UM-61) derived from a normal male. 24 hours ____ 0 µg/ml, ----- µg/ml, o-o-o 100 µg/ml, x-x-x 200 µg/ml, ... 300 µg/ml.

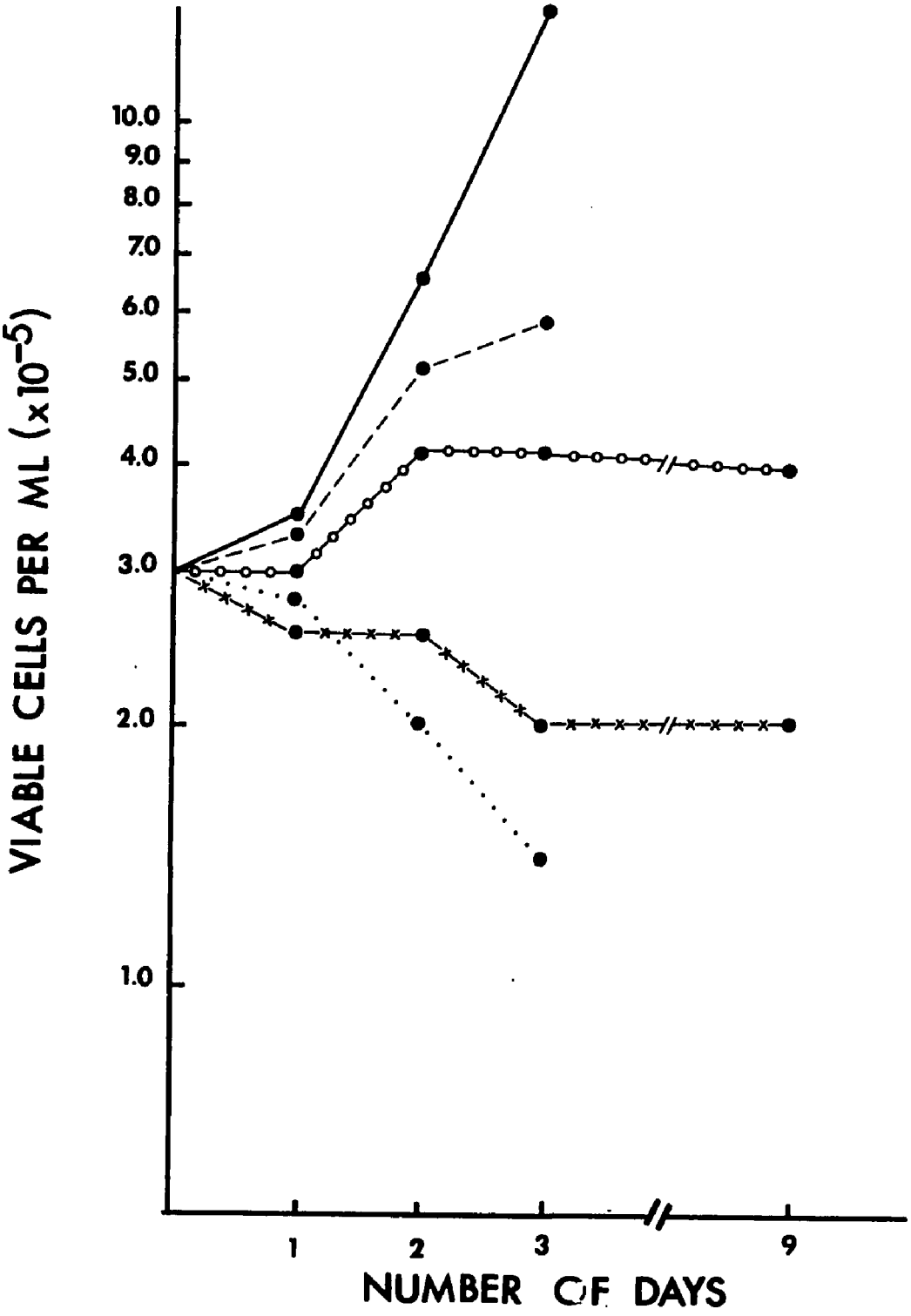

VIABLE CELLS PER ML (x10⁻⁵)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1
2
3
9
NUMBER OF DAYS

Figure 7

Figure 8.--The effect of varying concentrations of nitrosoguanidine (MNNG) on the logarithmic growth of a lymphocyte line (UM-61) derived from a normal male. 24 hours _____ 0 µg/ml, ----- 0.05 µg/ml, o-o-o 0.10 µg/ml, x-x-x 0.50 µg/ml, ... 1.0 µg/ml, _*_*_* 3.0 µg/ml.

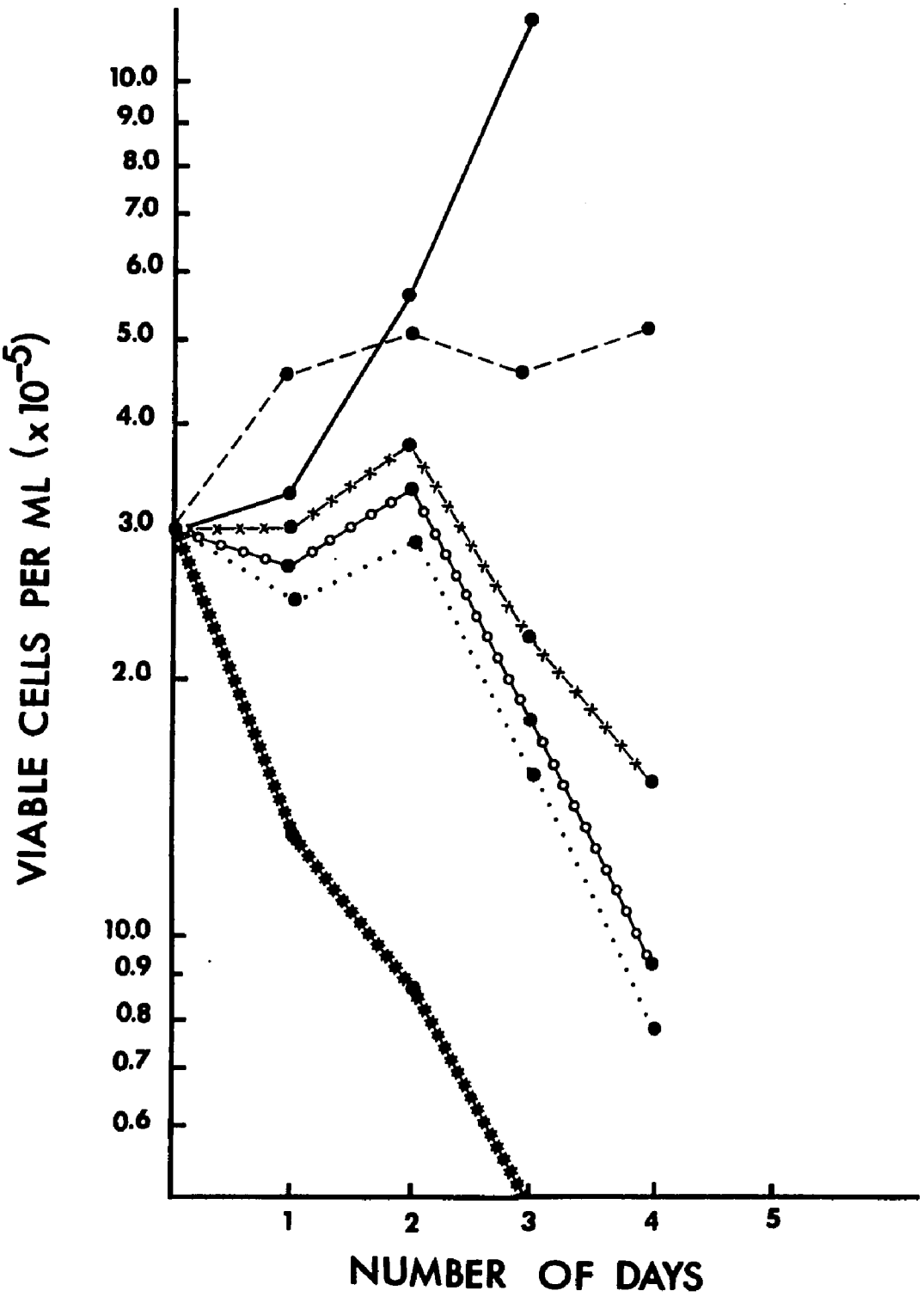

VIABLE CELLS PER ML (x10⁻⁵)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
10.0
0.9
0.8
0.7
0.6
1
2
3
4
5
NUMBER OF DAYS

Figure 8

Discussion

The technical difficulties encountered with the growth and survival of the UM-61 lymphocyte line and its clonal sublines have not permitted the collection of data in the form necessary to make the conclusions inherent in successful fluctuation analyses valid for this dissertation. The failure to obtain actual counts of 6-TG resistant cell numbers and mutational events precluded any knowledge of the distribution of the mutant phenotype in replicate samples from a single culture or in the series of identical cultures. However, it has been demonstrated that 6-TG resistant HGPRT deficient cells were present and could be isolated from a series of identical cultures (Table VII). The underlying distribution of these variant cells in the cultures is not known as actual counts of the numbers of mutational events giving rise to those cells could not be made. In view of these shortcomings, the use of the data collected to calculate the mutation rate estimates as described in the introduction to this chapter requires an assumption about the destribution of mutant cells in the identical cultures. This assumptiion is that the numbers of mutation events giving rise to the observed 6-TG resistant cells in the identical cultures are distributed in a random fashion, i.e., that no events other than mutation are responsible for the appearance of 6-TG resistance. If this assumption is valid, then the mutational events should be distributed among the identical cultures in a Poisson fashion, and

the Poisson distribution may be used in the manner described to calculate the mean number of mutational events.

Based on the above assumption, it was estimated that the rate of mutation to 6-TG resistance in the human lymphocyte line used in these experiments was on the order of 10^{-7} per cell generation.

The presumed mutation rate estimated from a fluctuation analysis is dependent upon the recognition of a particular phenotype, i.e., cell growth in the presence of 6-TG. If mutation at a locus or loci other than the HGPRT locus confers resistance to 6-TG, or if any mechanism other than mutation (Harris, 1971) will allow survival in 6-TG medium with no change in HGPRT activity, then the mean number of mutants per culture (m) as estimated in Equation 3, would represent a sum of 6-TG resistant cells. These cells may have arisen from mutation in the HGPRT locus, from a different locus (loci) conferring 6-TG resistance, or from epigenetic alterations. The fluctuation analysis does not allow one to distinguish between these alternatives, and the mutation rate then represents the rate of occurrence of the 6-TG phenotype and not solely the rate of mutation at the HGPRT locus. Thus the question arises as to whether or not the 6-TG resistance phenotype can be attriuted to loci other than the HGPRT locus, or can epigenetic mechanisms confer the 6-TG resistant phenotype.

No conclusive evidence exists to prove that 6-TG resistance is determined solely by mutation at the HGPRT locus. However, as

far as can be determined, cell growth in the presence of 6-TG does not occur in the absence of HGPRT deficiency. Miegon (1970) studied five unrelated families in which there was a Lesch-Nyhan male. On the basis of fibroblast cultures, four of these boys had HGPRT levels of less than 1% of normal. The fifth boy showed 5-15% of control levels. Clonal fibroblast populations from parents and female relatives were studied simultaneously. When fibroblasts from the four clearly $HGPRT^-$ males were cultured in 1 mg% 6-TG, the cells divided in the presence of the drug at the same rate as they did in non-selective medium. Migeon observed that only a few cells from the boy with partial HGPRT deficiency survived at the 1 mg% 6-TG concentration. When the cells from the partially HGPRT deficient male were cultured in a reduced 6-TG concentration of 0.50 mg%, more cells survived, but they divided at exceedingly slow rates. No cell survival was found in 6-TG treated cultures from the fathers of these families were detected on the basis of $HGPRT^-$ clones that survived the 6-TG selection. In no instance where HGPRT was present in normal levels did Migeon observe survival in 6-TG medium. Thus in naturally occurring HGPRT deficiency, cells show survival in 6-TG medium. Furthermore, in partial HGPRT deficiency, cell survival was found, but more cells survived after reduction of the 6-TG concentration. This suggested that a relationship may exist between the level of HGPRT activity and survival at different concentrations of 6-TG.

In sixteen 6-TG resistant lymphocyte clones studied by Sato et al. (1972) HGPRT activity varied from 0 to 1.2% of the levels found in normal cells. Chasin (1974) used Chinese hamster cultures in his studies, and in sixteen clonal sublines of diploid or tetraploid cells he found that all were deficient in HGPRT activity when compared to wild-type lines. Similarly, in the present experiments all 6-TG resistant lymphocytes were HGPRT deficient (Table VII). Mutant 13, however, was considerably higher in HGPRT activity than the other lines reported, and than those of the 6-TG resistant sublines derived from lymphocytes lines used in this study. Despite the high level of HGPRT activity, the cells of mutant 13 survived a three week period of 6-TG selection, and at least some cells are HGPRT deficient. Since the HGPRT assays were performed on uncloned populations of cells it cannot be stated with certainty whether or not the cells in mutant 13 all uniformly contain 55% normal levels of HGPRT activity, or whether there are varying levels which give an overall estimate of 55% normal activity. It should be mentioned that this culture was maintained in non-selective medium for three months in order to obtain 20 million cells for the enzyme assay. Several subcultures were necessary, and they were all combined for the enzyme assay. This period of non-selective growth may have been sufficient either for selective growth of cells with higher levels of HGPRT or for the occurrence of reversion to HGPRT activity in some subcultures, either of which could account for the observed levels of HGPRT. In addition, 6-TG selection may

have been insufficient and allowed for survival of normal cells. The cells were not returned to 6-TG medium between the end of selective growth and the time the HGPRT assays were done.

DeMars and Held (1972), however, have reported evidence that more than one locus may determine 8-AG resistance. They found one family in which an X-linked mutation conferred 8-AG resistance in association with undiminished HGPRT activity. Thus 8-AG resistance may not solely depend on HGPRT deficiency, and the same may hold for 6-TG resistance.

Presently, it is not possible to exclude the existence of loci other than the HGPRT locus that can confer 6-TG resistance. Several 6-TG resistant mutants must be examined to determine whether or not the 6-TG resistant phenotype can exist in the absence of reduced levels of HGPRT. Regardless, HGPRT deficiency can account for 6-TG resistance in all experiments so far reported.

Several lines of circumstantial evidence support the mutational origin of 6-TG resistance. First, the 6-TG resistant phenotype is stable over several generations in the absence of selection. The 6-TG resistant cells derived from the normal female line UM-39 (Table VII) have been maintained in culture under nonselective conditions for more than two years, and neither culture has lost the ability to grow in 6-TG medium nor acquired normal levels of HGPRT. This stability suggests mutation rather than adaptation in response to 6-TG.

Second, the occurrence of the 6-TG resistant phenotype is random and independent of the selective agent (Chasin, 1974).

Third, the frequency of the 6-TG resistant phenotype is increased by the use of mutagenic agents. Sato et al. (1972) found that EMS caused a 109-fold increase in the frequency of 6-TG resistant cells in their lymphocyte line. MNNG induced a 19-fold increase in y-TG resistant cells. Chasin (1974) found that the spontaneous frequency of 6-TG resistant cells in diploid and tetraploid Chinese hamster fibroblasts was 9×10^{-7} and less than 2×10^{-7}, respectively. After EMS treatment Chasin found 25 6-TG resistant colonies per 10^5 plated cells in the diploid fibroblasts, and 0.93 per 10^5 plated tetraploid cells.

Finally a comparison between the 6-TG resistant cells and normal cells in these experiments show a deficiency in a specific gene product, HGPRT. This enzyme has been shown to be coded by a locus on the X-chromosome, and is deficient in naturally occurring mutations at this locus. The lymphocytes and fibroblasts with HGPRT deficiencies show levels of enzyme activity comparable to those in cell lines derived from Lesch-Nyhan males, and parallel the growth responses of these lines in 6-TG.

Any of the above properties of the 6-TG resistant phenotype taken alone would not provide enough support for the mutational origin of the resistant cells. All five taken together, however, weigh heavily in favor of gene mutation as the origin of 6-TG resistance.

It would be interesting in future studies to perform several experiments to study the types of mutations at the HGPRT locus responsible for the observed deficiencies. The simplest study that could be done presently would be to obtain revertants by growth of $HGPRT^-$ cells in HAT medium, to look at the physical properties of the revertant enzyme. Altered thermal stability, electrophoretic mobility, and Michaelis-Menten kinetics would provide evidence of molecular changes consistent with gene mutations. Several attempts have been made to obtain revertants in some of the HGPRT deficient lymphocyte lines derived in the course of these studies. However, the adaptation of HGPRT normal cells to growth in HAT medium has proved difficult in our laboratory. The HGPRT normal cells remain viable, but population doubling times are longer, and growth levels off at lower cell densities. However, once good growth in HAT medium can be maintained for long periods of time, these reversion studies should be feasible.

Beaudet et al. (1973) prepared an antibody to HGPRT after injection of New Zealand white rabbits with hamster HGPRT. This antibody detected the presence of cross-reacting material (CRM) in some of their 8-AG resistant Chinese hamster fibroblasts. Human HGPRT exhibits marked instability with increasing purity (Kelley and Arnold, 1973), and specific antibody may be difficult to obtain. Nevertheless, a search for CRM would be possible provided an appropriate human antibody could be obtained.

Because of the presumed presence of the Epstein-Barr virus (EBV) in all lymphocyte lines, it was not known whether the transformed state of the cells in these lines might predispose them to genetic instability. If frequent gene mutations occurred in these lines as a result of their viral transformation, then they would be unsuitable for mutation rate studies similar to those performed with fibroblast cultures.

Several lines of evidence suggest that EBV is present in most established lymphocyte cell lines. First, the capacity of various established lines to transform normal human lymphocytes into permanent cell cultures is dependent upon the detection of EBV specific antigens in the cells of the line (Miller, Lisco, Kohn, and Stitt, 1971). Lines with no detectable or low EBV antigens failed to initiate permanent cell lines. Second, EBV has been found to be the causative agent of infectious mononucleosis (Henle, Henle, and Diehl, 1968). Lymphocytes from donors with no history of IM and with no detectable antibodies to EBV in their serum fail to transform into permanent cell lines in the absence of EBV (Nilsson, Klein, Henle, and Henle, 1971). Third, the transforming ability of established cell lines is neutralized by serum containing antibody to EBV. Miller et al. (1971) observed that six sera from patients with EBV antibody titers prevented transformation of normal adult lymphocytes when they were mixed with the transforming

cell lines or their lysates or extracts. Five sera from donors with no detectable EBV antibodies failed to prevent transformation of normal lymphocytes.

Finally, most lymphocyte lines established to date have been shown to contain EBV. Some lines produce viral particles as determined by electron microscopy (Epstein, 1970). Others have not been shown to produce viral particles, but show the presence of EBV antigens in cultured cells (Klein, Dombos, and Grothoskar, 1972). Although some lines show neither release of viral particles nor the presence of EBV associated antigens, they have been shown by molecular hybridization techniques to contain EBV genome equivalents (zur Hausen, Diehl, Wolf, Schulte-Holthausen, and Schneider, 1972). Nonoyama and Pagano (1971) have estimated that EBV DNA comprises between 0.06 and 1.6% or the total DNA of Burkitt's lymphoma cells and established lymphocyte cell lines, even though presence of the virus could not be detected by immunofluorescent tests. In addition, Gerber (1972) has demonstrated that 5-BUdR can activate EBV in those lines in which the virus cannot be detected by immunofluorescence.

The evidence is in favor of the hypothesis that EBV is present in most established lymphocyte lines. Because of the assumptions necessary for the calculation of the mutation rate derived in the present work, comparisons between it and those rates found by other investigators (Table 1) are not justified. The fact that the

rate of mutation calculated in the present study is within the range observed for the HGPRT locus by other investigators may be fortuitous. However, the findings of Sato et al. (1972) and Lever et al. (1974) suggest that the frequency of HGPRT$^-$ cells in lymphocyte lines is comparable to that in fibroblasts lines. Verification of the estimate of mutation to 6-TG resistance derived here will require extensive studies with other lines in which the proper data can be generated. Nevertheless, the findings here in conjunction with those of Sato et al. and Lever et al. tentatively suggest that transformation of lymphocytes by EBV may not significantly effect the frequency and rate of mutation at the HGPRT locus.

The failure to obtain induced HGPRT$^-$ cells with EMS and MNNG was unexpected, given the successful results with fibroblast lines and with lymphocyte lines as reported by Sato et al. (1972). Several different factors may have contributed to these failures in the present study. First, in Protocol 1, where 6-TG selection was imposed 72 hours post mutagen treatment, the recovery period may have been insufficient. Chu and Malling (1968) found that maximum mutant recovery was obtained in Chinese hamster cells when at least three cell divisions occurred between mutagen treatment and the addition of 8-AG selective medium. If selection was imposed immediately after mutagen exposure, no 8-AG resistant cells were recovered. The time period between mutagen treatment and addition of selection, the so-called mutation expression time, presumably

allows for the reduction of pre-formed HGPRT levels either by degradation or dilution during mitotic divisions. Preformed HGPRT would render the cells sensitive to immediate exposure to 8-AG even though they may have experienced mutation in the HGPRT locus. In addition, cell division may have allowed error-replication to occur.

Under normal culture conditions the UM-61 lymphocyte line used in the present study has a population doubling time of approximately twenty-four hours. The depression in mitotic activity observed after mutagen treatment (Figures 7 and 8) may have precluded sufficient cell divisions to reduce preformed HGPRT and therefore, prevented mutant recovery.

On the other hand Protocol 2 should have avoided any problems with delayed mutagen expression since 6-TG was neither added at any time during the mutagen expression time nor during the agarose cloning period. Thus premature addition of 6-TG selection is not solely responsible for the failure to recover $HGPRT^-$ cells.

Second, a low cloning efficiency of the lymphocytes in soft agarose may have contributed to the failure to obtain 6-TG resistant cells. The UM-61 lymphocyte line has a cloning efficiency of only 12 to 14% under non-selective conditions. Mutagen treatment may have significantly reduced this control value by either decreasing the mitotic rate or cell survival such that single cells were incapable of colony formation. MNNG in particular has been shown to induce an extended depression of plating efficiency in

baby hamster kidney cells (Orkin and Littlefield, 1971). In addition, Sato et al. (1972) experienced difficulty in isolating lymphocyte clones after treatment with MNNG. One study in our laboratory showed that the mitotic index was greatly reduced by treatment of lymphocyte cultures with MMNG (Kurlandsky, 1972, personal communication). As can be seen in Figure 7, EMS also reduces culture growth post-mutagen treatment. Reduced survival and/or mitotic activity induced by EMS or MNNG exposure may have contributed to failure of colony formation.

If low cloning efficiency played a major role in the failure of these experiements, then, increasing the basic cloning efficiency may insure recovery of induced HGPRT$^-$ cells. This may be achieved by the testing of several lines for ones with high cloning potential and using these lines for mutation induction. Alternatively, improvements in the low cloning efficiency of the UM-61 and similar lines are being attempted. The approaches being taken include: (a) repeated cloning of the same clonal isolate to select for cells with high cloning ability; (b) use of several fibroblast feeder layers from different sources as the cloning efficiency may vary with fibroblast donor (Littlefield, 1973, personal communication); (c) use of different agarose compounds since preparations may vary among sources, (d) supplementing the growth medium with nutrients such as adenine and folic acid which enhance the survival of HGPRT$^-$ fibroblasts (Felix and DeMars, 1969); and

(3) pretreatment of lymphocytes with mitomycin C to stimulate cell division (Nakamura, 1974, personal communication).

Finally, the lymphocytes of the UM-61 cell line have an unusual insensitivity to the mutagenic effects of both compounds. Sato et al. (1972) observed a biphasic survival curve for EMS and perhaps for MNNG in the lymphocyte line used in their studies. They interpreted this finding as an indication of the presence of two populations of lymphocytes with different sensitivities to the mutagens, and estimated that approximately 3% of their cell population has an increased resistance to EMS. Furthermore, one lymphocyte line used by Sato et al. was insensitive to induction of 6-TG resistance with EMS, although this line was shown to contain rare spontaneous $HGPRT^-$ cells. Thus it seems that some lymphocyte lines may not be responsive to the mutagenic action of some compounds. If this is true, then the UM-61 line used in these experiments may be another example of this insensitivity. Therefore, it may be necessary to test several lines in a similar manner in order to find a line which is both capable of forming 6-TG resistant cells spontaneously, and one which is sensitive to induction by EMS or MNNG in order to obtain induced mutations at the HGPRT locus. However, the present studies have not shown that the UM-61 line is insensitive to either EMS or MNNG, and perhaps improvements in techniques may show that mutagen induction of 6-TG resistance in UM-61 can be accomplished.

Whatever the case may be with respect to the failure of mutant induction in the present experiments, it is known that some lymphocyte lines contain HGPRT$^-$ cells and are susceptible to mutation induction with MNNG and EMS. Our experiments with the mutagens involved only the one line with a low cloning efficiency and were highly preliminary. This work with mutagens and lymphoid lines will be expanded by us at a later time.

In summary, this study indicates that spontaneously occurring 6-TG resistant cells can be selected from cultures of human lymphocyte lines. Enzyme assays on the resistant cells showed that HGPRT deficiency can account for the observed 6-TG resistant cells, and suggest that these cells have undergone mutation in the HGPRT locus or in some gene(s) governing its expression. These experiments did not include studies to determine whether or not the observed HGPRT deficiency resulted from structurally altered enzymes or mutations in synthesis and regulation. Although the rate of occurrence of the 6-TG resistant phenotype was estimated to be on the order of 10^{-7} per cell generation, technical difficulties prevented the collection of data to support the assumption that the mutational events giving rise to the phenotype are random and distributed in the identical cultures in a Poisson fashion.

The failures encountered during the course of these experiments, however, should not be interpreted to indicate that human lymphocyte lines cannot be used to study spontaneous and induced

mutation. Several other investigators have successfully derived data that favor the use of these lines in such studies. The findings reported here suggest that not all lymphocyte lines may be adapted to mutation studies and that careful selection of lines for such properties as cloning efficiency on soft agarose and growth to large cell densities from small starting numbers as well as the occurrence of spontaneous $HGPRT^-$ cells should be carefully studied before such research is undertaken.

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